hybridizing region and a 5'-non-hybridizing (tail) region with the propromoter sequence. In some aspects, the unique sequence (A/A') may be a double stranded promoter, as further described below. As described herein, the PTO may comprise RNA, DNA, or combinations thereof.

[0097] The use of a first primer (first strand cDNA primer) comprising a 3'-hybridizable region and a 5'-tail region comprising a single strand sequence of a promoter for DNA-dependent RNA polymerase has been described before in US Pat Nos. 5545522, 5716785, and Proceedings of the National Academy of Sciences (1990), 87: 1663-1667, each hereby incorporated in its entirety by reference. When first strand synthesis is carried out in the presence of a DNA-dependent DNA polymerase inhibitor, only copies of the target RNA will be generated. The product of the first and second primer extension products generated by this method is a double stranded DNA with a double stranded promoter sequence at one end, which is the substrate for the DNA dependent RNA polymerase, such as for example T7 RNA polymerase, SP6 RNA polymerase and the like.

[0098] For example, in one aspect involving transcription illustrated in Figure 3, the invention provides methods of generating multiple copies of (amplifying) an RNA sequence of interest. The first primer is the single stranded PTO comprising a 3'-hybridizing sequence (B) and 5'-non-hybridizing sequence (A) which serves as a propromoter for DNA-dependent RNA polymerase. In the presence of actinomycin, the first strand cDNA is generated by reverse transcriptase and the resulting first strand cDNA contains the sequence (Y) and 5'-sequence (A). Following inactivation or removal of actinomycin and removal of the target RNA from the complex, a second primer, which contains sequence (C), hybridizes to the 3'-end of the first strand cDNA and is extended using DNA-dependent DNA polymerase. The second strand cDNA that is generated is in a complex with the first strand cDNA and contains the double stranded promoter sequence (A/A'), thus enabling RNA synthesis through transcription via RNA polymerase. Multiple copies of the sequence complementary to the target RNA with sequence (C') and (Y) are generated in this aspect.

[0099] As previously described, the second primer may also be endogeneous in the form of a fragment or fragments of the target RNA sequence which is cleaved following first strand synthesis using methods described herein. In this case, the second primer is not hybridized to a specific sequence on the cDNA but rather to various regions of the sequence (Y) on the first strand cDNA since the fragments are generated from randomly cleaved portions and therefore are hybridized to random locations on the cDNA. In this aspect, multiple copies generated comprise the various sequences (Y_1, Y_2, $Y_{3....}$) that are homologous to various regions of the target RNA.

[00100] In another aspect, a PTO is used as the second primer to form a double stranded complex composed of a promoter sequence for transcription. One embodiment of a PTO comprises a second primer with a 3' hybridizing end and a 5' non-hybridizing end, which contains a propromoter sequence. The 3'-end (e.g. sequence (C) hybridizes to the 3'-end of the first primer extension product (first strand cDNA), which contains a 5' sequence (A) and was formed from the steps described above. Extension of the PTO by DNA polymerase produces a double stranded complex containing a sequence (A/A') at one end, and a propromoter sequence incorporated into the second strand. Following the denature of the double stranded cDNA, a primer with sequence (A) is hybridized to the second extension product and is extended to form a

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double stranded product having a double stranded promoter sequence from which transcription occurs using RNA polymerase at one end, and the sequence (A/A') at the other end. The double stranded promoter region can be used to promote transcription by an RNA polymerase. The result of the transcription can be an amplification step that generates multiple copies of a RNA sequence of interest.

[00101] In yet another aspect involving transcription, the invention provides methods of generating multiple copies of (amplifying) a sequence complementary to an RNA sequence of interest comprising: (a) hybridizing a PTO to the 3'-end of a second strand cDNA, wherein the second strand cDNA comprises a 5'-sequence (C) and a 3'-sequence (A') and is generated using any of the methods described herein and wherein the PTO comprises a 3'-hybridizing end with sequence (A) that is generally, but not necessarily, blocked so that it cannot be extended by the DNA polymerase, and a 5'-nonhybridizing propromoter sequence (P); (b) extending the second strand cDNA by DNA polymerase to produce a double stranded promoter, enabling RNA amplification through transcription via DNA-dependent RNA polymerase. Multiple copies of a sequence complementary to the target RNA are generated in this aspect.

Amplification via PCR

[00102] In some aspects of the second part of the invention, amplification methods such as linear or exponential PCR, are utilized to perform the amplification of marked cDNA.

[00103] In some aspects of the invention, exponential amplification of nucleic acids is used in the second step to amplify the marked DNA. These methods often depend on the product catalyzed formation of multiple copies of a nucleic acid or its complement. The amplification products are sometimes referred to as "amplicons." One such method for the enzymatic amplification of specific double stranded sequences of DNA is known as the polymerase chain reaction (PCR) (see U.S. Pat. No. 4,683,195 and U.S. Pat. No. 4,683,202). The DNA amplification is based on the activity of the enzyme DNA polymerase, which elongates primer molecules that bind to the template DNA by adding dNTPs and thereby copies the template sequence (Saiki et. al, Science (1988), 239: 487-491). The forward primer binds to the 3'-end of the sense strand of the template, whereas the reverse primer binds to the 3'-end of the reverse strand, hereby defining the starting points of the polymerase reaction and eventually determining the length of the amplified product.

[00104] PCR is an amplification procedure is based on repeated cycles of denaturation, oligonucleotide primer annealing, and primer extension, usually by thermophilic template dependent polynucleotide polymerase, resulting in the exponential increase in copies of the desired sequence of the polynucleotide analyte flanked by the primers. The two different PCR primers, which anneal to opposite strands of the DNA, are positioned so that the polymerase catalyzed extension product of one primer can serve as a template strand for the other, leading to the accumulation of a discrete double stranded fragment whose length is defined by the distance between the 5'-ends of the oligonucleotide primers.

[00105] Linear amplification generally refers to a method that involve the formation of one or more copies of the complement of only one strand of a nucleic acid or polynucleotide molecule, usually a nucleic acid or polynucleotide analyte. Thus, the primary difference between linear amplification and exponential

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amplification is that in the latter the product serves as a template for the formation of more products, whereas in the former process the starting sequence serves as a template for the formation of product but the product is not itself replicated. In linear amplification the amount of product formed increases as a linear function of time as opposed to exponential amplification where the amount of product formed is an exponential function of time.

[00106] In one aspect, the invention provides methods of generating multiple copies of a polynucleotide sequence complementary to an RNA sequence of interest or an RNA sequence of interest in the presence of DNA, said method comprising the steps of: (a) extending a first primer hybridized to a target RNA with an RNA-dependent DNA polymerase in the presence of a DNA-dependent DNA polymerase inhibitor, wherein the first primer is a composite primer comprising a 5'-non-hybridizing region (tailed primer), whereby a complex comprising a first primer extension product and the target RNA is produced; (b) cleaving the target RNA in the complex of step (a) with an enzyme that cleaves RNA and from an RNA/DNA hybrid; (c) inactivating and/or removing the DNA-dependent DNA polymerase inhibitor as described herein; (d) extending a second primer hybridized to the first primer extension product with a DNA-dependent DNA polymerase, whereby a second primer extension product is produced to form a complex of first and second primer extension products; (e) amplifying each of the first and second strand cDNA using either or both the first and second primers via linear or exponential amplification using techniques described in sections above, such as PCR; whereby multiple copies of a polynucleotide sequence homologous or complementary to the RNA sequence of interest are generated. In some aspects, step (c) may be performed before or in combination with step (b).

[00107] In one embodiment, as illustrated in Figure 4a, the amplification methods of the invention results in generation of DNA products comprising sequences complementary to an RNA sequence(s) of interest is as follows: (a) A first primer having a 5'-sequence (A) that is non-complementary to the target RNA binds to an RNA species in a sample by hybridization of the 3'-sequence portion (B) to form a complex in the presence of a DNA-dependent DNA polymerase inhibitor such as actinomycin; (b) A reverse transcriptase (RT) extends the hybridized first primer along the target RNA strand to which it is hybridized, to form an RNA/DNA heteroduplex. The DNA-dependent DNA polymerase inhibitor prevents extension of product from DNA in the sample. The 5'-end of the first primer extension product has the first primer sequence (A); (c) The target RNA strand of the heteroduplex may be degraded, by methods described herein, to generate a single-stranded first strand cDNA; (d) The DNA-dependent DNA polymerase inhibitor is removed, inhibited, inactivated or sequestered; (d) The second primer with sequence (C) binds to first strand cDNA by hybridization to form a complex.

[00108] Linear or exponential PCR can then be used to amplify only the marked cDNA which is formed. PCR involves, for example, linear PCR (Figure 4b) or exponential PCR (Figure 4c), wherein the cDNA generated in the first step is amplified. The unique non-complementary sequence of the first primer is used in subsequent amplification steps to generate only the desired product.

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[00109] In Figure 4b, illustrating linear PCR, the second strand cDNA with the 3'-sequence (A') is hybridized to the primer with sequence (A), which is extended by DNA polymerase to generate multiple copies of DNA that are complementary to the target RNA.

[00110] Exponential PCR is illustrated in Figure 4c, wherein both the first and second strand cDNA are exponentially amplified using exponential PCR. The two cDNA strands are denatured and the two primers with sequence (A) and sequence (C) are used simultaneously to amplify both cDNA strands. A primer with sequence (C) is annealed to the first strand cDNA comprising sequences (C') and (A) and is extended to produce strands containing sequence (C). A primer with sequence (A) is annealed to the second strand cDNA comprising sequences (C) and (A') and is extended to produce strands containing sequence (C'). Exponential PCR can also be carried out with one primer comprising a sequence (A) and the other primer comprising a sequence (Y'), complementary to sequence (Y). It will be understood that the PCR primers need not comprise all of sequence (C) or sequence (Y'), but may only comprise a portion of such sequences. In some cases, multiple PCR primer pairs can be employed in order to obtain multiplex PCR and amplification of multiple sequences of interest.

Other methods of amplification

[00111] The second part of the invention may also amplify the marked DNA sequence using other amplification methods known in the art. The following are descriptions of some examples of amplification methods. It is understood that the invention is not limited to the amplification methods described herein. Any amplification method that can amplify the marked polynucleotides produced in the first part of the invention may be used. In some cases, amplification methods can comprise clonal amplification. Clonal amplification is the generation of one or more unique sets of amplified DNA such that each set is separated physically, or chemically from other sets. Methods of clonal amplification include amplifying DNA on a solid surface such as a bead or beads, an array, or a set of wells. In some cases, clonal amplification may be performed by isolating template polynucleotides in individual microreactors such as for example individual aqueous droplets of a water in oil emulsion. Each amplified template will then be isolated from the other templates to generate clonal populations of amplified product in each droplet. In some cases, the droplets further contain a solid surface such as a bead. In some cases, initially, each bead will carry a single DNA molecule. After amplification (e.g. PCR, or SPIA), each bead will contain thousands to millions of identical copies of the DNA molecule. Other methods of clonal amplification are provided herein.

[00112] One example for achieving a nucleic acid amplification is the nucleic acid sequence based amplification (NASBA). This method is a promoter-directed, enzymatic process that induces in vitro continuous, homogeneous and isothermal amplification of a specific nucleic acid to provide RNA copies of the nucleic acid. The reagents for conducting NASBA include a first DNA primer with a 5'-tail comprising a promoter, a second DNA primer, reverse transcriptase, RNaseH, T7 RNA polymerase, NTPs and dNTPs.

[00113] Another method for amplifying a specific group of nucleic acids is the Q-beta-replicase method, which relies on the ability of Q-beta-replicase to amplify its RNA substrate exponentially. The reagents for

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conducting such an amplification include "midi-variant RNA" (amplifiable hybridization probe), NTPs, and Q-beta-replicase.

[00114] Another method for amplifying nucleic acids is known as 3SR and is similar to NASBA except that the RNase H activity is present in the reverse transcriptase. Amplification by 3SR is an RNA specific target method whereby RNA is amplified in an isothermal process combining promoter directed RNA polymerase, reverse transcriptase, and RNase H with target RNA.

[00115] Another method for amplifying nucleic acids is Transcription Mediated Amplification (TMA) used by Gen-Probe. The method is similar to NASBA in utilizing two enzymes in a self-sustained sequence replication. (See Fahy, et al., Patent Application WO 91-US8488 911113).

[00116] Another method for amplification of nucleic acids is Strand Displacement Amplification (SDA) (Westin et al., Nature Biotechnology (2000), 18: 199-202; Walker et al., Nucleic Acids Research (1992), 20(7): 1691-1696), which is an isothermal amplification technique based upon the ability of a restriction endonuclease such as HincII or BsoBI to nick the unmodified strand of a hemiphosphorothioate form of its recognition site, and the ability of an exonuclease deficient DNA polymerase such as Klenow exo minus polymerase, or Bst polymerase, to extend the 3'-end at the nick and displace the downstream DNA strand. Exponential amplification results from coupling sense and antisense reactions in which strands displaced from a sense reaction serve as targets for an antisense reaction and vice versa.

[00117] Another method for amplification of nucleic acids is Rolling Circle Amplification (RCA) (Lizardi et al., Nature Genetics (1998), 19:225-232). RCA can be used to amplify single stranded molecules in the form of circles of nucleic acids. In its simplest form, RCA involves the hybridization of a single primer to a circular nucleic acid. Extension of the primer by a DNA polymerase with strand displacement activity results in the production of multiple copies of the circular nucleic acid concatenated into a single DNA strand. The method of generating polonies is one method of clonal amplification.

[00118] In some embodiments, amplification methods can be solid-phase amplification, polony amplification, colony amplification, emulsion PCR, bead RCA, surface RCA, surface SDA, etc., as will be recognized by one of skill in the art. In some embodiments, amplification methods that results in amplification of free DNA molecules in solution or tethered to a suitable matrix by only one end of the DNA molecule can be used. Methods that rely on bridge PCR, where both PCR primers are attached to a surface (see, e.g., WO/18957 and Adessi et al., Nucleic Acids Research (2000), 28(20): E87) can be used. In some cases the methods of the invention can create a "polymerase colony technology", or "polony", referring to a multiplex amplification that maintains spatial clustering of identical amplicons (see Harvard Molecular Technology Group and Lipper Center for Computational Genetics website). These include, for example, in situ polonies (Mitra and Church, Nucleic Acid Research 27, e34, Dec. 15, 1999), in situ rolling circle amplification (RCA) (Lizardi et al., Nature Genetics (1998), 19:225-232), bridge PCR (U.S. Pat. No. 5,641,658), picotiter PCR (Leamon et al., Electrophoresis (2003), 24: 3769), and emulsion PCR (Dressman et al., PNAS (2003), 100: 8817). The methods of the invention provide new methods for generating and using polonies.

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[00119] A polony is a contraction of "polymerase colony," a small colony of DNA. Polonies are discrete clonal amplifications of a single DNA molecule, grown in a gel matrix or on a surface. This approach greatly improves the signal-to-noise ratio. Polonies can be generated using several techniques that include for example solid-phase PCR in polyacrylamide gels, bridge PCR on a solid phase, or SPIA amplification on a solid surface. In some cases, growth of clonal copies of DNA on bead surfaces may be considered a "polony" method. The method of generating polonies is one method of clonal amplification.

General techniques

[00120] 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).

[00121] Primers, oligonucleotides, and polynucleotides employed in the invention can be generated using standard techniques known in the art.

Components and Reaction Conditions used in the Methods of the Invention

Template Nucleic Acid

[00122] The RNA target is generally a polymeric nucleotide, which in the intact natural state can have about 30 to 5,000,000 or more nucleotides and in an isolated state can have about 20 to 50,000 or more nucleotides, usually about 100 to 20,000 nucleotides, more frequently 500 to 10,000 nucleotides. The RNA target to be amplified includes RNAs from any source and or/species, including human, animals, plants, and microorganisms such as bacteria, yeasts, viruses, viroids, molds, fungi, plants, and fragments thereof. Exemplary RNA targets can be obtained and purified using standard techniques in the art and includes RNAs in purified or unpurified form, which include, but are not limited to, mRNAs, tRNAs, snRNAs, rRNAs, retroviruses, small non-coding RNAs, microRNAs, polysomal RNAs, pre-mRNAs, intronic RNA, viral RNA, DNA-RNA heteroduplexes and fragments thereof. Amplification of a DNA target (including genomic DNA target) would require initial transcription of the DNA target into RNA form, which can be achieved using methods disclosed in Kurn, U.S. Pat. No. 6,251,639 B1, and by other techniques (such as expression systems) known in the art. Amplification of a RNA/DNA heteroduplex would require denaturation of the duplex to obtain a ssRNA, or denaturation followed by transcription of the DNA strand to obtain an RNA. The target RNA may be only a minor fraction of a complex mixture such as a biological sample and may be obtained from various biological materials by procedures well known in the art.

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[00123] The target RNA can be known or unknown and may contain more than one desired or suspected 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. If the target nucleic acid is double stranded (e.g., RNA/DNA heteroduplex) the initial step could be target denaturation. Denaturation may also 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

[00124] The first primer is marked in a manner that when the first primer is used to form a first primer extension product, the first primer extension product (and in some cases subsequent extension products such as the second primer extension product) are marked. Various embodiments of the first primer are used in the methods of the invention are described herein. For the methods described herein, one or more first primers may be used.

[00125] Generally, the first primer comprises a 5'-unique sequence and is generally a tailed primer comprising a non-hybridizing 5'-sequence. The 5'-unique sequence allows the first strand cDNA to be uniquely marked in a sample containing other nucleic acids. In one embodiment the first primer is a primer comprising only DNA. In another embodiment, the first primer is a primer comprising only RNA. In another embodiment, the first primer is a composite primer comprising a combination of DNA and RNA. Extension of the first primer results in a complex of first and second strand cDNAs that comprise a portion with a particular characteristic (e.g., cleavable by an enzyme) or sequence content (for example comprising a promoter for DNA dependent RNA polymerase). In another embodiment, the first primer may comprise a 5'-unique end that can partially hybridize to the target. In another embodiment, the first primer may comprise all hybridizable portions but the 5'-end is uniquely marked with groups that distinguish it from the other nucleic acid samples.

[00126] 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 first primer may comprise a combination of RNA and DNA, 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 when hybridized to a polynucleotide template. 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, first primers may 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 first primer comprises a 5'-RNA portion and a 3'-DNA portion, wherein the RNA portion is adjacent to the 3'-DNA portion. In another embodiment, the first primer comprises 5'- and 3'-DNA portions with at least one intervening RNA portion

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(i.e., an RNA portion between the two DNA portions). In yet another embodiment, the first primer of the invention comprises a 3'-DNA portion and at least one intervening RNA portion (i.e., an RNA portion between DNA portions).

[00127] The first primer extension product comprises a specific sequence complementary to an RNA sequence and a 5′ portion, preferably a 5′ end, which comprises RNA, DNA, or combinations thereof. For example, in some embodiments, the RNA portion of a first 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 some embodiments, a first primer comprises a 5′ portion (for example, a 5′ RNA portion) that is either defined or universal and non-hybridizable to a target RNA under conditions which the composite primer hybridizes to target RNA. The 3'-DNA portion may be sequence specific and hybridizable to the target sequence or contain multiple sequences, random sequences, partially random sequences, and/or a poly-dT sequences for hybridizing to the Poly-A sequence on the mRNA target.

[00128] The 5'-region of the first primer of the present invention may include a propromoter sequence recognized by an RNA polymerase such as for example T7 RNA polymerase, SP6 RNA polymerase and the like.

[00129] In other embodiments, the first primers comprises a sequence entirely of DNA with a 3'-DNA portion that generally is designed to be hybridizable to a target RNA(s). This 3'-DNA portion may be sequence specific and hybridizable to a specific sequence of the target sequence, random sequences, partially random sequences, and/or a poly-dT sequences for hybridizing to the Poly-A sequence on the mRNA target. One or multiple first primers may be used for the amplification methods of the invention. The remaining portion(s) (such as the 5'-DNA portion) of the first primer generally, but not necessarily, comprises a defined or universal sequence that is not hybridizable to a target RNA (which would constitute a tail when the primer is bound to a target RNA). Thus, and as the description herein makes clear, reference to a primer that hybridizes to a sequence (or hybridization template) encompasses embodiments in which at least a portion of the primer is hybridized, as well as those embodiments in which an entire primer is hybridized.

[00130] In still other embodiments, a plurality of first primers are used for hybridizing to the target RNA. In some embodiments, the first primer that hybridizes to target RNA and the composite amplification primer that hybridizes to second primer extension product are the same. In other embodiments, the first primer that hybridizes to target RNA and the composite amplification primer that hybridizes to second primer extension product are different.

[00131] Insofar as many mRNAs have a unique poly-A 3'-end, amplification initiated from the 3'-end sequence of mRNAs is commonly performed 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. Similarly, the methods of the invention are suited for preparation of libraries of whole transcriptome amplification. It is understood that the methods of the invention are useful not only for producing large amounts of one specific nucleic acid sequence, but also for amplifying simultaneously more than one different specific

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nucleic acid sequence located on the same or different target nucleic acid molecules. For example, the methods of the invention are useful for amplifying all mRNA in a sample, or for amplifying a multiplicity of specific RNA species (in which case a multiplicity of the first primer each comprising a 3'-portion hybridizable to specific sequences of specific RNA species could be used) or family of RNA species in a sample.

[00132] In some embodiments, the 5'-DNA portion of a first primer includes the 5'-most nucleotide of the primer. In some embodiments, the 5'-RNA portion of a first primer includes the 5'-most nucleotide of the primer. In other embodiments, the 3'-DNA portion of a first primer includes the 3'-most nucleotide of the primer. In other embodiments, the 3'-RNA portion of a first 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).

Second Primer

[00133] The first primer extension product is used to generate additional DNA or RNA strands containing sequence related, either through homology or complementarity, to the original target RNA sequence. The primer extension product may be used as a single strand template. Alternatively, the primer extension product may be used as a double stranded template by creation of a second strand via use of a DNA-dependent DNA polymerase. In order to amplify the primer extension product, secondary primers may be used that bind to the primer extension product for use in the synthesis of the second cDNA strand, complementary to the cDNA. The second primer is complementary to the cDNA and comprises DNA, RNA fragments, tailed RNA/DNA, tailed DNA/DNA, or mixtures thereof. The second primer further comprises specific sequences, multiple sequences, or random sequences. Various embodiments of the second primer used in the methods of the invention are described herein. For the methods described herein, one or more second primers can be used.

[00134] In one embodiment, the second primer is an oligonucleotide that is supplied externally. The second primer in the methods of the invention (which primes generation of the second strand cDNA) 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 strand cDNA at a site on the first strand cDNA such that the second strand cDNA would be homologous to the RNA sequence of interest. In another embodiment, the second primer is a primer comprising DNA (in some embodiments, consisting of DNA).

[00135] To achieve hybridization to a first strand cDNA (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 second primer that is hybridizable to the first strand cDNA 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 strand cDNA. Generation of primers suitable for extension by polymerization is well known in the art.

[00136] In other embodiments, the second primer comprises one or more fragments of target RNA hybridized to the first primer extension product, said one or more fragments generated by cleaving RNA in

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the complex of target RNA and first primer extension product with an enzyme that cleaves RNA from an RNA/DNA heteroduplex. In some embodiments, this second primer comprises one or more fragments of the target RNA from cleavage due to heat, chemicals, and enzymes such as RNase. For example, the target RNA in the initial complex comprising the target RNA and first primer extension product is cleaved with an enzyme (such as RNaseH), or by heating the reaction mixture to cleave the RNA target, such that at least one fragment of the template RNA remains hybridized to the composite primer extension product. In this embodiment of the invention, one (or more) template RNA fragment(s) serves as a second primer in the manner described above, to generate a fragment extension product which has the same function as the second primer extension product in the amplification methods described above.

[00137] Suitable second primers and second primer RNA fragments in the methods of the invention are long enough such that it does not dissociate from the first strand cDNA, preferably from about 3 to about 30, more preferably from about 5 or 6 to about 25, even more preferably from about 10 to about 20, and most preferably from about 12 to about 20, nucleotides in length.

[00138] In yet another embodiment, the second primer is a random primer. In other embodiments, the second primer comprises a portion (for example, a 3′ portion) that comprises a random sequence (i.e., a sequence designed to be hybridizable (under a given set of conditions) to one or more sequences in the sample). In some embodiments, the second primer comprises a 5′ portion that is non-hybridizable to a first primer extension product (tailed primer). In some embodiments, the 5'-region of the second primer further comprises defined or universal sequences. In some embodiments, the second primer comprises only a sequence that is hybridizable to a first primer extension product (primer with no tail).

[00139] In embodiments involving transcription, the second primer may comprise propromoter sequence (also referred to herein as "propromoter polynucleotide") that is capable of hybridizing to displaced first strand cDNA. Hybridization of the propromoter polynucleotide to a displaced primer extension product and extension of the 3'-end of the displaced first primer extension product (if there is an overhang) results in a double stranded promoter region that drives transcription (via DNA-dependent RNA polymerase) to produce sense RNA products. This transcription approach is described herein and in Kurn et al., U.S. Pat. No. 6,251,639.

[00140] Promoter oligonucleotides of the present invention can also be modified to prevent the synthesis of DNA there from. In one embodiment, the promoter oligonucleotides comprise a blocking moiety attached at their 3'-termini to prevent primer extension in the presence of a polymerase. Indeed, according to the present invention, at least about 80% of the oligonucleotides present in the amplification reaction which comprise a promoter further comprise a 3'-blocking moiety. In further embodiments, at least about 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% of the oligonucleotides provided to the amplification reaction which comprise a promoter are further modified to comprise a 3'-blocking moiety. In a specific embodiment, any oligonucleotide used in an amplification reaction of the present invention which comprises a promoter sequence must further comprise a 3'-terminus blocking moiety.

[00141] 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

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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 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.

[00142] 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.

Composite Amplification Primers

[00143] Composite amplification primers (or amplification composite primers) are RNA/DNA composite primers that can be used to create multiple copies of (amplify) a polynucleotide sequence isothermally using RNA cleavage, and DNA polymerase activity with strand displacement. Amplification with such primers is described, for example in U. S. Patents 6,251,639, 6,692,918, and 6,946,251. The composite amplification primer comprises sequences capable of hybridizing to a portion of a DNA template, and most often comprises sequences hybridizable to a defined 3'-portion of the DNA.

[00144] A composite amplification primer comprises at least one RNA portion that is capable of (a) binding (hybridizing) to a sequence on a DNA template independent of hybridization of the DNA portion(s) to a sequence on the same extension product; and being cleaved with a ribonuclease when hybridized to the DNA template. The composite amplification primers bind to the DNA template 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 DNA template remains intact, thus enabling annealing of another composite primer.

[00145] The composite amplification primers also comprise a 3'-DNA portion that is capable of hybridization to a sequence on the DNA template such that its hybridization to the DNA is favored over that of the nucleic acid strand that is displaced from the DNA template 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.

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DNA-dependent DNA polymerase inhibitors

[00146] The methods of the current invention inhibit formation of unwanted amplification products through the use of DNA-dependent DNA polymerase inhibitors during generation of the first primer extension product. DNA-dependent DNA polymerase inhibitors prevent enzymatic activity that generates primer extension products along DNA present in the sample with the target RNA. This method is a novel approach to amplification of polynucleotides of or DNA complementary to the target RNA in samples comprising, or which may comprise, DNA targets. Traditionally, target RNA would be purified from the DNA in the starting material (e.g. cell lysates), resulting in decreased quantity and quality of the RNA due to artifacts and losses introduced during the purification steps. Failure to isolate the RNA would result in random amplification of nucleic acid segments found in DNA as well as the RNA target or targets. The methods of the present invention allow for amplification of select RNA species, or the total RNA species, in the sample by combining the process of specifically reverse transcribing an RNA sequence in the presence of a DNA-dependent DNA polymerase inhibitor, removing the inhibitor, and allowing amplification of the uniquely marked cDNA, resulting in specific amplification of the target RNA sequences or of the sequences complementary to the target RNA sequence or sequences.

[00147] Furthermore, certain DNA-dependent DNA polymerase inhibitors are known to be effective in preventing other replication artifacts such as premature synthesis of the second strand cDNA and therefore allow amplification of only the first primer extension product. Actinomycin is an example of this type of DNA-dependent DNA polymerase inhibitor and is useful in the synthesis of single stranded first strand cDNA while inhibiting second strand synthesis.

[00148] There are several known DNA-dependent DNA polymerase inhibitors known in the art. For example, Actinomycin-D acts as a DNA-dependent DNA polymerase inhibitor by binding to DNA and preventing initiation of replication (Guy and Taylor (1978) PNAS 75:6088-92.) Other examples of DNA-dependent DNA polymerase inhibitors include, but are not limited to, actinomycin (dactinomycin), alpha-amanitin, aphidicolin (Cozad and Warner (1982) Gamete Research 6:155-60; Gonzcol and Plotkin (1985) Arch Virology 84:129-34; Haraguchi et al. (1983) Nucleic Acids Research 11:1197-1209), BP5, novobiocin (Schneck and Staudenbauer (1977) Nuc Acids Res 4:2057-64), rifampicin, rifamycin (Frolova et al. (1977) Nuc Acids Res 4:523-8), sulfoquinovosylmonoacylglycerol, sulfoquinovosyldiacylglycerol (Ohta et al. (2000) Mutat Res 467:139-52; Ohta et al. (1999) Biol Pharm Bull 22:111-16), ursane, oleanane triterpenoids, ursolic acid, oleanolic acid (Deng et al. (1999) J Nat Prod 62:1624-6), mikanolide, dihydromikanolide (U.S. Pat. No. 6,767,561), dehydroaltenusin (Mizushina et al. (2000) J Biol Chem 275:33957-61), catapol (Pungitore et al. (2004) J Nat Prod 67:357-61), taxinine, cephalomanninine (Oshige et al. (2004) Bioorganic and Medicinal Chem 12:2597-601), dipeptide alcohols (Kato et al. (2005) Int J Mol Med 16:653-9), corylifolin; bakuchiol; resveratrol; Neobavaisoflavone; daidzein; bakuchicin (Sun et al. (1998) J Nat Prod 61:362-6), levodopa, dopamine (Wick (1980) Cancer Research 40:1414-8), anacardic acid and oleic acid (Chen et al. (1998) Chem Comm 24:2769-70).

[00149] The DNA-dependent DNA polymerase inhibitors of the present invention are added in an amount effective to inhibit DNA-dependent DNA polymerase activity. As a result, replication of DNA, when

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present in the sample in combination with RNA target or targets, is inhibited. The amount of a DNA-dependent DNA polymerase inhibitor that should be added to achieve the desired inhibition is well known and understood in the art. For example, the DNA-dependent DNA polymerase inhibitor Actinomycin D may be added in a concentration from about 0.1 μg/ml to about 100 μg/ml. In a preferred embodiment, 50% of DNA replication is inhibited by addition of a DNA-dependent DNA polymerase inhibitor. In another preferred embodiment, 60% of DNA replication is inhibited by addition of a DNA-dependent DNA polymerase inhibitor. In yet other preferred embodiments, 70%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% of DNA replication is inhibited by addition of a DNA-dependent DNA polymerase inhibitor.

[00150] Combinations of DNA-dependent DNA polymerase inhibitors may be used to achieve the desired inhibition. Use of combinations of DNA-dependent DNA polymerase inhibitors may be desirable to minimize unwanted effects on the reaction conditions by decreasing the concentration of each inhibitor in the reaction mixture. Alternatively, the combination of inhibitors may be able to achieve a greater amount of inhibition, such as by inhibiting DNA-dependent DNA polymerase activity at various stages.

[00151] It is known that several of these DNA-dependent DNA polymerase inhibitors also act to inhibit DNA-dependent RNA polymerase. Thus, by selecting the appropriate inhibitors, the methods of the present invention may also be useful for inhibiting transcription. This is especially useful in reaction mixtures that contain DNA-dependent RNA polymerases, such as extracts from whole cell lysates.

[00152] Following generation of a marked DNA sequence complementary to the target RNA, the DNA-dependent DNA polymerase inhibitor is removed from the reaction conditions or inhibited, inactivated, or sequestered from functioning to allow for the synthesis of a second strand cDNA and amplification of the primer extension product. Techniques for removal of a DNA-dependent DNA polymerase inhibitor from a reaction condition are well known in the art. In some cases, the RNA-dependent DNA polymerase is also removed. These methods include binding of the nucleic acid components in the reaction mixture to a solid surface such as beads, separating the solid surfaces and removing the soluble portion of the reaction mixture which comprises the DNA-dependent DNA polymerase inhibitor, resuspending the beads to recover the bound nucleic acid components, and proceeding with the cleaned reaction mixture as described herein. Bead separation may be carried out by, for example, centrifugation, magnetic separation of magnetic beads, and the like. Other solid surfaces which bind nucleic acid are also useful for carrying out the methods of the invention. DNA-dependent DNA polymerase inhibitors may be inhibited from functioning through binding of an inhibitor (e.g. an antibody directed to the DNA-dependent DNA polymerase inhibitor). In addition, inactivation of the inhibitor may be carried out by an enzyme. For example, enzymatic inactivation of actinomycin D has been described previously (see Hou and Perlman, JBC (1970), 245: 1289-1295).

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Polynucleotide Comprising a Propromoter and a Region which Hybridizes to a Primer Extension Product

[00153] Some embodiments involving amplification of an RNA sequence of interest, 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

[00154] In some embodiments, RNA amplification methods employ a promoter sequence, provided by a propromoter template oligonucleotide (PTO), for in-vitro transcription by DNA-dependent RNA polymerase (see US Pat Nos. 5545522, 5716785, and PNAS (1990), 87: 1663-1667, each hereby incorporated in its entirety by reference).

[00155] 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 an embodiment of the invention, the portion capable of hybridizing to the 3'-end of a primer extension product comprises a sequence which is hybridizable to a defined end sequence of the second primer extension product (and thus, subsequently the 3'-end of the single stranded DNA products). In another embodiment, the portion capable of hybridizing to the 3'-end of a primer extension product comprises a random sequence. In another embodiment, the portion capable of hybridizing to the 3'-end of a primer extension product comprises a sequence, which is hybridizable to sequences found at the 3'-end of a multiplicity of first strand cDNAs.

[00156] 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.

[00157] 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

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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, and can also be empirically determined.

[00158] 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.

[00159] 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, an Agent Capable of Cleaving an RNA-DNA heteroduplex, and RNA Polymerase for SPIA

[00160] The isothermal amplification methods of the invention employ the following enzymes: an RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase, and an agent capable of cleaving a RNA strand of a RNA/DNA heteroduplex (for example, a ribonuclease such as RNase H). Some examples of the enzymes which may be used in the methods and compositions are described herein. 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. Similarly, a DNA polymerase may comprise both RNA-dependent and DNA-dependent DNA polymerase enzyme activities, or separate enzymes may be used. 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. Another RNase useful for carrying out the methods of the invention is RNase I, which cleaves single-stranded RNA.

[00161] Reverse transcriptases useful for this method may or may not have RNase H activity. The selective RNase may be provided as an RNase H activity of a reverse transcriptase, or may be provided as

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a separate enzyme, e.g., as an E. coli RNase H or a T. thermophilus RNase H, or derivatives thereof. Other enzymes which selectively degrade RNA present in an RNA:DNA duplex may also be used. Many reverse transcriptases, such as those from avian myeloblastosis 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 or has reduced levels RNase H activity. Reverse transcriptases devoid of RNase H or with reduced levels 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. The RNA-dependent DNA polymerase activity and DNA-dependent DNA polymerase activity may be provided by the same enzyme (for example, Bst polymerase), or these activities may be provided in separate enzymes. DNA polymerases with strand displacement activity are also useful.

[00162] Enzymes for use in the compositions, methods and kits of the present invention include any enzyme having reverse transcriptase activity. Such enzymes include, but are not limited to, retroviral reverse transcriptase, retrotransposon reverse transcriptase, hepatitis B reverse transcriptase, cauliflower mosaic virus reverse transcriptase, bacterial reverse transcriptase, E. coli DNA polymerase and klenow fragment, Tth DNA polymerase, Taq DNA polymerase (Saiki, R. K., et al., Science 239:487-491 (1988); U.S. Pat. Nos. 4,889,818 and 4,965,188), Tne DNA polymerase (WO 96/10640), Tma DNA polymerase (U.S. Pat. No. 5,374,553), C. Therm DNA polymerase from Carboxydothermus hydrogenoformans (EP0921196A1, Roche, Pleasanton, Calif., Cat. No. 2016338), ThermoScript (Invitrogen, Carsbad, Calif. Cat. No. 11731-015) and mutants, fragments, variants or derivatives thereof. As will be understood by one of ordinary skill in the art, modified reverse transcriptases may be obtained by recombinant or genetic engineering techniques that are routine and well-known in the art. Mutant reverse transcriptases can, for example, be obtained by mutating the gene or genes encoding the reverse transcriptase of interest by site-directed or random mutagenesis. Such mutations may include point mutations, deletion mutations and insertional mutations. Preferably, one or more point mutations (e.g., substitution of one or more amino acids with one or more different amino acids) are used to construct mutant reverse transcriptases of the invention. Fragments of reverse transcriptases may be obtained by deletion mutation by recombinant techniques that are routine and well-known in the art, or by enzymatic digestion of the reverse transcriptase(s) of interest using any of a number of well-known proteolytic enzymes. Mutant DNA polymerase containing reverse transcriptase activity can also be used as described in U.S. patent application Ser. No. 10/435,766, incorporated by reference in its entirety.

[00163] One aspect of the invention is the formation of a complex comprising an RNA/DNA partial heteroduplex. This process generally utilizes the enzymatic activities of an RNA-dependent DNA polymerase, and a DNA-dependent DNA polymerase. Generally, RNA in the RNA/DNA partial heteroduplex is cleaved by an agent (such as an enzyme, such as a ribonuclease) capable of cleaving RNA from an RNA/DNA hybrid, generating a 3'-single stranded portion with sequences that are complementary

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to RNA in a composite primer (and thus forming a binding site for a composite primer). The DNA primer extension product may also be separated (at least partially) from the RNA template using an enzyme which degrades the RNA template. Suitable enzymes, i.e., "selective RNAses," are those which act on the RNA strand of an RNA:DNA complex, and include enzymes which comprise an RNase H activity.

[00164] 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 DNA 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 and, for example, a DNA polymerase that possesses both DNA-dependent and RNA-dependent DNA polymerase activity, such as Bst DNA polymerase. The RNA-dependent DNA polymerase may comprise a RNase H enzyme activity, or separate enzymes may be used.

[00165] DNA-dependent DNA polymerases for use in the methods and compositions of the invention are capable of effecting extension of the composite primer according to the methods of the 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 formation of the complex comprising the RNA/DNA partial heteroduplex can be carried out by a DNA polymerase which comprises both RNA-dependent DNA polymerase and DNA-dependent DNA polymerase activities (such as Bst DNA polymerase, or a reverse transcriptase). In one embodiment ,amplification of an RNA sequence according to methods of the invention involves the use of a DNA polymerase that is 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 nucleic acid strand. Preferably, the DNA polymerase does not possess substantial nicking activity. Generally, the DNA polymerase preferably has little or no 5' to 3' exonuclease activity so as to minimize degradation of primer, or primer extension products. 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' to 3' exonuclease activity has been deleted, are known in the art and are suitable for the amplification methods described herein. Mutant DNA polymerases which lack both 5' to 3' nuclease and 3' to 5' nuclease activities have also been described, for example, exo $^{-/-}$-Klenow DNA polymerase. 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. 5,744,312 (and references cited therein). Generally, the DNA polymerase has little to no proofreading activity

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[00166] Suitable DNA polymerases for use in the methods and compositions of the invention include those disclosed in U.S. Pat. Nos. 5,648,211 and 5,744,312, which include exo - Vent (New England Biolabs), exo - Deep Vent (New England Biolabs), Bst (BioRad), exo - Pfu (Stratagene), Bca (Panvera), sequencing grade Taq (Promega), exo $^{-/-}$Klenow DNA polymerase, and thermostable DNA polymerases from thermoanaerobacter thermohydrosulfuricus.

[00167] One of the ribonuclease for use in the methods and compositions of the invention is capable of cleaving ribonucleotides in an RNA/DNA hybrid or heteroduplex. Preferably, the ribonuclease cleaves ribonucleotides in an RNA/DNA heteroduplex 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 invention are well known in the art, including ribonuclease H (RNase H), e.g., Hybridase.

[00168] As is well known in the art, DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity, and the ability to cleave RNA from a RNA/DNA heteroduplex may be present in different enzymes, or two or more activities may be present in the same enzyme. Accordingly, in some embodiments, the same enzyme comprises RNA-dependent DNA polymerase activity and cleaves RNA from an RNA/DNA heteroduplex. In some embodiments, the same enzyme comprises DNA-dependent DNA polymerase activity and cleaves RNA from an RNA/DNA heteroduplex. In some embodiments, the same enzyme comprises DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity and cleaves RNA from an RNA/DNA heteroduplex. In some embodiments, different enzymes comprise RNA-dependent DNA polymerase activity and DNA-dependent DNA polymerase activity. In some embodiments, different enzymes comprise RNA -dependent DNA polymerase activity and cleave RNA from an RNA/DNA heteroduplex. In some embodiments, different enzymes comprise DNA-dependent DNA polymerase activity and cleave RNA from an RNA/DNA heteroduplex. In other embodiments the RNA targets are degraded by the use of other RNases such as RNase 1, for example, for removing the target RNA following extension of all DNA first primer along the RNA targets by transcription, or a combination of RNase H and other RNases.

[00169] The DNA-dependent RNA polymerases 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 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.

[00170] In general, the enzymes used 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

[00171] 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

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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. WO 99/42618. 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. Such additives include proteins such as BSA, single strand binding proteins (for e.g., 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 other than the first and second strand cDNA synthesis steps, and strand displacement) are performed isothermally, which 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 primer extension products, 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. 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.

[00172] 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 μ M. 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

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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.

[00173] 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 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.

[00174] In the methods of the invention, the steps may be carried out in the order listed or, in some cases, may be carried out in a different order. In some methods a later step depends on the formation of a product from an earlier step, in which case such steps must be carried out in the order listed. One of ordinary skill in the art will understand which steps should be carried out in the order listed, and which steps can be carried out in a different order.

[00175] In some 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 time points during the amplification process, as required and/or permitted by the amplification reaction. Such time points, 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 (binding by another primer, primer extension and strand displacement). In these embodiments, the reaction conditions and components may be varied between the different reactions.

[00176] In some embodiments, the amplification process can be stopped at various time points, and resumed at a later time. Said time points can be readily identified by a person of skill in the art. One time point is at the end of first strand cDNA synthesis. Another time point 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 the resumption of the reactions. For example, it may be necessary to replenish the composite primer prior to beginning the linear amplification reaction if the same composite primer is being used. Alternatively, the reaction can be allowed to proceed (i.e., from start to finish) without interruption.

[00177] In some embodiments the reaction can be allowed to proceed without purification of intermediate complexes, for example, to remove primer. Products can be purified at various time points, which can be

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readily identified by a person of skill in the art. One time point is at the end of first strand cDNA synthesis. Another time point is at the end of second strand cDNA synthesis. In some embodiments, the removal of primers and/or target at the end of a defined step by enzymes with appropriate nuclease activities are also useful, for example, cleavage of the RNA portion of free composite tailed primer prior to the isothermal amplification step by treatment with RNase 1. In some embodiment, routine purification of the complex of first and second cDNA results in slightly higher amplification efficiency in subsequent linear amplification steps.

[00178] 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. The global amplification by the methods of the invention and/or the amplification of selected targets, when present in the sample, are useful for various methods which enable highly parallel nucleic acid interrogations.

Uses of the invention

[00179] The invention provides methods and compositions to selectively amplify RNA in the presence of DNA. The amplification products from this invention can be used for a variety of purposes including, but not limited to, those described herein. For example, purposes using the amplification products of the invention include methods of sequencing, quantitating, or detecting a specific transcript or group of transcripts, genotyping (such as nucleic acid mutation detection, determining the presence or absence of a sequence of interest, analyzing splice variants), preparing an immobilized nucleic acid (which can be a nucleic acid immobilized on a microarray, a surface, a bead, or one or more wells in a plate), and characterizing nucleic acids using methods such as probe hybridization (Southern and Northern blotting, hybridization to probe arrays). The analysis of cellular RNA can be used in methods including gene expression profiling, Q-PCR, digital PCR, SAGE, 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). Amplification products generated may also be further manipulated to generate labeled targets (with or without fragmentation) for analysis using microarrays and the like. Amplification products generated may also be used for analysis by sequencing. In some cases, sequencing may be performed by massively parallel sequencing methods including but not limited to nanopore sequencing, and massively parallel sequencing by various commercial platforms such as a Genome Sequencer from 454/Roche, the SOLiD system from Applied Biosystems, the Polonator by Dover

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Systems, the Helicos Genetic Analysis Platform by Helicos Biosciences, the Genome Analyzer by Illumina, and Single-Molecule Realtime (SMRT) technology from Pacific Biosystems.

[00180] The invention allows selective RNA amplification from a sample comprising total nucleic acids (i.e. DNA and RNA), thereby providing a means for amplification and analysis, detection, and quantification of target RNA sequences directly from cell or sample lysate without purification of the RNA targets. The sample may be from the same cells or tissues or from a combination of cells and other nucleic acid components, or other biological samples such as environmental samples. For example, samples may include viral RNA in a host sample, viral RNA in an environmental sample, or cell-free nucleic acids such as in blood or other bodily fluids. The samples may be single cell samples, samples of a few cells, or samples with small numbers of cells, for example 10s to hundreds of cells. The methods could be used, for example for performing gene expression analysis on single to small numbers of cells using the cell lysate directly as the sample with little or no further purification. In some cases the cell lysate could be treated to remove or denature all enzymes and/or proteins in the sample resulting in a total nucleic acid sample that can be used directly for the methods described herein.

[00181] The amplification methods of the invention are useful, for example, for pre-amplification and target preparation for analysis such as for example sequencing, Q-PCR, digital PCR, SAGE, or massively parallel analysis such as massively parallel sequencing, high density PCR arrays, microarray hybridization, and massively parallel Q-PCR of an RNA sequence or set of RNA sequences 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). 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. For sequencing methods based on methods described herein wherein the amplified product is RNA, the appropriate rNTPs, or analogs thereof, which may be labeled or unlabeled, may be used. Analysis of the reaction products for elucidation of sequence information can be carried out using any of various methods known in the art for sequencing.

[00182] In some embodiments, the methods are useful for pre-amplification and/or preparing target polynucleotide(s) for sequencing by synthesis using the methods commercialized by 454/Roche Lifesciences including but not limited to the methods and apparatus described in *Margulies et al., Nature (2005) 437:376-380 (2005)*; and US Patent Nos. 7,244,559; 7,335,762; 7,211,390; 7,244,567; 7,264,929; 7,323,305. In general, target polynucleotides may be amplified by the methods of the present invention, immobilized onto beads, and compartmentalized in a water-in-oil PCR emulsion. In some cases, alternative amplification methods may be employed in the water-in-oil emulsion such as any of the methods provided herein. When the emulsion is broken, amplified fragments remain bound to the beads. The beads may be enriched and loaded into wells of a fiber optic slide so that there is approximately 1 bead in each well. Nucleotides are flowed across and into the wells in a fixed order in the presence of polymerase, sulfhydrolase, and luciferase. Addition of nucleotides complementary to the target strand

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results in a chemiluminescent signal that is recorded such as by a camera. The combination of signal intensity and positional information generated across the plate allows software to determine the DNA sequence.

[00183] In some embodiments, the methods are useful for pre-amplification and/or preparing target polynucleotide(s) for sequencing by the methods commercialized by Helicos BioSciences Corporation (Cambridge, MA) as described in U.S. application Ser. No. 11/167046, and US Patent Nos. 7501245; 7491498; 7,276,720; and in U.S. Patent Application Publication Nos. US20090061439; US20080087826; US20060286566; US20060024711; US20060024678; US20080213770; and US20080103058. In general, target polynucleotides may be amplified by the methods of the present invention, and then immobilized onto a flow-cell surface. Polymerase and labeled nucleotides are then flowed over the immobilized DNA. After fluorescently labeled nucleotides are incorporated into the DNA strands by a DNA polymerase, the surface is illuminated with a laser, and an image is captured and processed to record single molecule incorporation events to produce sequence data.

[00184] In some embodiments, the methods are useful for pre-amplification and/or preparing target polynucleotide(s) for sequencing by the sequencing by ligation methods commercialized by Applied Biosystems (e.g. SOLiD sequencing). In general, target polynucleotides may be amplified by the methods of the present invention, and then incorporated into a water in oil emulsion along with polystyrene beads and amplified by for example PCR. In some cases, alternative amplification methods may be employed in the water-in-oil emulsion such as any of the methods provided herein. The amplified product in each water microdroplet formed by the emulsion interact, bind, or hybridize with the one or more beads present in that microdroplet leading to beads with a plurality of amplified products of substantially one sequence. When the emulsion is broken, the beads float to the top of the sample and are placed onto an array. Sequencing primers are then added along with a mixture of four different fluorescently labeled oligonucleotide probes. The probes bind specifically to the two bases in the polynucleotide to be sequenced immediately adjacent and 3' of the sequencing primer to determine which of the four bases are at those positions. After washing and reading the fluorescence signal form the first incorporated probe, a ligase is added. The ligase cleaves the oligonucleotide probe between the fifth and sixth bases, removing the fluorescent dye from the polynucleotide to be sequenced. The whole process is repeated using a different sequence primer, until all of the intervening positions in the sequence are imaged. The process allows the simultaneous reading of millions of DNA fragments in a 'massively parallel' manner. This 'sequence-by-ligation' technique uses probes that encode for two bases rather than just one allowing error recognition by signal mismatching, leading to increased base determination accuracy.

[00185] In some embodiments, the methods are useful for pre-amplification and/or preparing target polynucleotide(s) for sequencing by the sequencing by ligation methods commercialized by Dover Systems. Generally, target polynucleotides may be pre-amplified and/or prepared by the methods of the present invention. The target polynucleotides may then amplified in an emulsion in the presence of magnetic beads. Any amplification methods may be employed in the water-in-oil emulsion such as any of the methods provided herein. The resulting beads with immobilized clonal polynucleotide polonies are

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then purified by magnetic separation, capped, amine functionalized, and covalently immobilized in a series of flow cells. Then, a series of anchor primers are flowed through the cells, where they hybridize to the synthetic oligonucleotide sequences at the 3' or 5' end of proximal or distal genomic DNA tags. Once an anchor primer is hybridized, a mixture of fully degenerate nonanucleotides ('nonamers') and T4 DNA ligase is flowed into the cell; each of the nonamer mixture's four components being labeled with one of four fluorophores, which correspond to the base type at the query position. The fluorophore-tagged nonamers selectively ligate onto the anchor primer, providing a fluorescent signal that identifies the corresponding base on the genomic DNA tag. Once the probes are ligated, fluorescently labeling the beads, the array is imaged in four colors. Each bead on the array will fluoresce in only one of the four images, indicating whether there is an A, C, G, or T at the position being queried. After imaging, the array of annealed primer-fluorescent probe complex, as well as residual enzyme, are chemically striped using guanidine HCl and sodium hydroxide. After each cycle of base reads at a given position have been completed, and the primer-fluorescent probe complex has been stripped, the anchor primer is replaced, and a new mixture of fluorescently tagged nonamers is introduced, for which the query position is shifted one base further into the genomic DNA tag. Seven bases are queried in this fashion, with the sequence performed from the 5' end of the proximal tag, followed by six base reads with a different anchor primer from the 3' end of the proximal tag, for a total of 13 base pair reads for this tag. This sequence is then repeated for the 5' and 3' ends of the distal tag, resulting in another 13 base pair reads. The ultimate result is a read length of 26 bases (thirteen from each of the paired tags).

[00186] In some embodiments, the methods are useful for pre-amplification and/or preparing target polynucleotide(s) for sequencing by the methods commercialized by Illumina as described US Patent Nos. 5,750,341; 6,306,597; and 5,969,119. In general, target polynucleotides may be pre-amplified and/or prepared by the methods of the present invention to produce amplified nucleic acid sequences tagged at one (e.g. (A)/(A') or both ends (e.g. (A)/(A') and (C)/(C')). In some cases, single stranded nucleic acid tagged at one or both ends is amplified by the methods of the present invention (e.g. by SPIA or linear PCR). The resulting nucleic acid is then denatured and the single stranded amplified polynucleotides are randomly attached to the inside surface of flow-cell channels. Unlabeled nucleotides are added to initiate solid-phase bridge amplification to produce dense clusters of double-stranded DNA. The initiate the first base sequencing cycle, four labeled reversible terminators, primers, and DNA polymerase are added. After laser excitation, florescence form each cluster on the flow cell is imaged. The identity of the first base for each cluster is then recorded. Cycles of sequencing are performed to determine the fragment sequence one base at a time. For paired-end sequencing, such as for example, when the polynucleotides are labeled at both ends by the methods of the present invention, sequencing templates can be regenerated in-situ so that the opposite end of the fragment can also be sequenced.

[00187] In some embodiments, the methods are useful for pre-amplification and/or preparing target polynucleotide(s) for sequencing by the methods commercialized by Pacific Biosciences as described in US Patent Nos. 7462452; 7476504; 7405281; 7170050; 7462468; 7476503; 7315019; 7302146; 7313308; and US Application Publication Nos. US20090029385; US20090068655; US20090024331; and

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US20080206764. In general, target polynucleotides may be pre-amplified and/or prepared by the methods of the present invention. The amplified polynucleotides may then be immobilized in zero mode waveguide arrays. Polymerase and labeled nucleotides are added in a reaction mixture, and nucleotide incorporations are visualized via fluorescent labels attached to the terminal phosphate groups of the nucleotides. The fluorescent labels are clipped off as part of the nucleotide incorporation. In some cases, circular templates are utilized to enable multiple reads on a single molecule.

[00188] The DNA or RNA amplification products generated according to the methods of the invention are also suitable for analysis for the detection of any alteration (interchangeably called "mutations") 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, alterations due to post transcriptional alterations, and/or mRNA processing, including splice variants. Sequence alterations include deletion, substitution, insertion and/or transversion of one or more nucleotide.

[00189] The invention provides methods to characterize (for example, detect presence or absence of and/or quantify) an RNA sequence of interest by generating DNA or RNA 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 or combinations thereof. These amplified products may be labeled or unlabeled. The detection and/or quantitation of specific nucleic acid sequences is an important technique for identifying and classifying microorganisms, diagnosing infectious diseases, detecting and characterizing genetic abnormalities, identifying genetic changes associated with cancer, studying genetic susceptibility to disease, and measuring response to various types of treatment. Such procedures are also useful in detecting and quantitating microorganisms in foodstuffs, water, industrial and environmental samples, seed stocks, and other types of material where the presence of specific microorganisms may need to be monitored. Other applications are found in the forensic sciences, anthropology, archaeology, and biology where measurement of the relatedness of nucleic acid sequences has been used to identify criminal suspects, resolve paternity disputes, construct genealogical and phylogenetic trees, and aid in classifying a variety of life forms.

[00190] A number of methods to detect and/or quantitate nucleic acid sequences are well known in the art. These include hybridization to a labeled probe, and various permutations of the polymerase chain reaction (PCR), coupled with hybridization to a labeled probe (For example, see U.S. Pat. Nos. 4,683,195; 4,683,202; 4,800,159; Mullis et al., Meth. Enzymol. (1987) 155: 335-350; and Murakawa et al., DNA (1988) 7:287-295).

[00191] 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, enable the detection and/or quantification of the expression of a multitude of genes in a given specimen.

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[00192] The amplification methods of the invention are also suitable for use in determining the levels of gene expression in a sample since the methods described herein are capable of amplifying one or a plurality of 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 target RNA 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.

[00193] The single-stranded 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 provide preparing a library from the single stranded DNA or RNA products of the invention. In another aspect, the invention provides methods of preparing a library from the double stranded cDNA produced by the methods of the invention comprising two composite primers. Methods for preparing libraries from double stranded cDNA are well known in the art. In still another aspect, the invention provides methods for making a library, said method comprising the preparation of subtractive hybridization probes using any of the methods described herein.

[00194] The methods of the invention offer the ability to efficiently amplify mRNA in the presence of DNA under conditions that provide for high specificity of target amplification and which is generally reflective of the distribution in the input RNA. Thus, the utility of the detection/quantification methods which can be used with the amplification products of the invention, such as those based on the powerful array technology, real time PCR, quantitative TaqMan, quantitative PCR using molecular beacons and the like, should be greatly enhanced.

Compositions and Kits of the Invention

[00195] The invention also provides compositions and kits used in the methods described herein and may be incorporated as parts of kits, e.g., diagnostic kits for clinical or criminal laboratories, or nucleic amplification kits for general laboratory use. The present invention thus includes kits which include some or all of the reagents necessary to carry out the methods of the present invention, e.g., sample preparation reagents, oligonucleotides, binding molecules, stock solutions, nucleotides, polymerase inhibitors, enzymes, positive and negative control target sequences, test tubes or plates, labeling reagents, fragmentation reagents, detection reagents, purification matrices, and an instruction manual.

[00196] The compositions of the invention may be any or any combination of component(s), reaction mixture, and/or intermediate described herein. For example, the invention provides a composition comprising a first primer, reverse transcriptase, and one or more types of DNA-dependent DNA polymerase inhibitors, and combinations thereof. The invention also provides compositions comprising the amplification products described herein. Accordingly, the invention provides a population of DNA or

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RNA molecules which are copies or the complement 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.

[00197] In another aspect, the invention provides a population of sense polynucleotide (e.g., DNA) molecules and antisense polynucleotide (e.g., DNA) molecules which are copies and complements of a target sequence, which are produced by any of the methods described herein. The invention also includes compositions and various configurations (such as arrays) of these populations, which may be homogeneous (same sequence) or heterogeneous (different sequence). These populations may be any assembly of sequences obtained from the methods described herein, including those based on mRNA, as well as certain species or classes of mRNA, other RNA species, or viral RNA.

[00198] 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 for samples comprising total nucleic acids: amplifying an RNA sequence; sequencing of an RNA sequence of interest; detection of sequence mutation based on amplifying an RNA sequence (e.g., genotyping or nucleic acid mutation detection); detection of an RNA sequence alteration including splice variants or fused transcripts; determining presence or absence of a sequence of interest; methods of gene expression analysis and profiling; methods of detection and quantification of RNA targets in samples comprising total nucleic acids; methods of whole transcriptome analysis for the detection, discovery, and/or quantification of sense or antisense transcripts, or coding and non-coding transcripts; methods of preparing amplification products for massively parallel analysis including massively parallel sequencing (e.g. pyrosequencing, sequencing by synthesis, sequencing by hybridization, single molecule sequencing, nanopore sequencing, and sequencing by ligation), high density PCR arrays, microarray hybridization, SAGE, digital PCR, and massively parallel Q-PCR; methods of subtractive hybridization; methods of preparing a subtractive hybridization probe; methods of differential amplification; methods of preparation of libraries (including cDNA and differential expression libraries); methods of preparation of an immobilized nucleic acid (which can be a nucleic acid immobilized on a microarray), and methods of characterizing amplified nucleic acid products generated by the methods of the invention.

[00199] The kits of the invention comprise one or more containers comprising any combination of the components described herein. Kits may include, but are not limited, to the following examples.

[00200] A kit may comprise any of the composite primers described herein with any of the listed DNA-dependent DNA polymerase inhibitors herein. In some embodiments, a kit comprises two or more composite primers, reverse transcriptase, and DNA-dependent DNA polymerase inhibitors, which may or may not be separately packaged. A kit may comprise a composite primer and a polynucleotide comprising a propromoter sequence, which may be a PTO, with DNA-dependent DNA polymerase inhibitors. A kit may further comprise a second primer, which can comprise a random primer. The composite primer may be labeled or unlabeled.

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[00201] Kits may also optionally further include any of one or more of the enzymes described herein (for example, RNA-dependent DNA polymerase such as reverse transcriptase, and ribonuclease such as RNase H), as well as deoxynucleoside triphosphates (labeled or unlabeled) and/or ribonucleoside triphosphates (labeled or unlabeled) and/or nucleotide analogs and/or non-canonical nucleotides. 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 or RNA transcript effect termination of nucleotide polymerization. The kits may comprise tailed deoxyoligonucleotides, wherein the tailed 5'-sequence is non-hybridizable to the target RNA and may further comprise a single-stranded DNA sequence of a promoter for DNA-dependent RNA polymerase.

[00202] 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, kits of the invention can comprise: a composite primer (which can comprise a poly-dT portion and/or can be a random primer), a DNA-dependent DNA polymerase inhibitor(s), and instructions for using the primer with a DNA-dependent DNA polymerase inhibitor to amplify RNA according to methods of the invention. In another example, any of these kits further comprises one or more controls (which can be, for example, RNA template, composite primers, and/or double stranded cDNA complex (comprising first and second strand cDNA) comprising a 3'-single-stranded DNA portion).

[00203] 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 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 that involve transcription, the RNA polymerase (if included) is preferably provided separately from the components used in the steps prior to the transcription steps.

[00204] 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.

[00205] The following Examples are provided to illustrate, but not limit, the invention.

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EXAMPLES

Example 1: Amplification of sequences from a target RNA in the presence of DNA using actinomycin to inhibit DNA-dependent DNA polymerase activity

[00206] A RNA target is amplified using a method involving a first primer, comprising composite RNA/DNA portions, wherein the 3'-DNA end of the primers comprises either poly-dT sequence or a random sequence. First strand cDNA synthesis is performed with this first primer in the presence of Actinomycin D, an inhibitor of DNA-dependent DNA polymerase. Removal of Actinomycin D and RNA target from the reaction mixture is performed by binding the nucleic acids to magnetic beads. Second strand cDNA synthesis is then carried out and is followed by amplification, which is performed using a composite amplification primer, DNA polymerase with strand displacement activity, and RNase H. The global RNA amplification was carried out using the WT-Ovation Pico amplification system (NuGEN Technologies Inc. http://www.nugeninc.com/html/03_products5.html) with additional reagents and protocol modifications described below. As described herein, the results of the example demonstrate selective amplification of RNA target from samples comprising mixtures of RNA and DNA.

Materials

[00207] All reagents for the WT-Ovation Pico RNA Amplification System (NuGEN Technologies Inc.) were used in this example. HeLa (S3) total RNA was purchased from Ambion. Human genomic DNA was purchased from Clontech. Random hexamer (N6 DNA oligonucleotide) was purchased from Operon. Actinomycin D was purchased from USB.

General comments

[00208] The WT-Ovation Pico amplification system was used to perform global RNA amplification. For selective amplification of RNA targets from samples comprising genomic DNA, the procedure was modified to include Actinomycin D in the step involving first strand cDNA synthesis. Additional clean up following first strand cDNA synthesis was added to the manufacturer protocol, which result in the removal of Actinomycin D prior to second strand cDNA synthesis. In addition, second strand cDNA synthesis was carried out in the presence of random hexamers (DNA oligonucleotides). All other steps were the same as for the WT-Ovation Pico RNA Amplification System.

First strand cDNA synthesis

[00209] First stand cDNA synthesis is initiated randomly across the length of RNA transcripts using composite first primers containing a 3'-random sequence DNA portion (for example, a random hexamer sequence) and a 5'-non-complementary and non-hybridizable RNA portion. In addition to the composite first primer, another composite first primer, comprising a 3'-poly-DT sequence DNA portion and the same 5'-non-hybridizing RNA portion as the previous primer, is used. In both the WT-Ovation Pico RNA amplification protocol and the modified procedure of the current invention, 1 to 2 µl of total RNA sample

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comprising 0.5 to 50 ng total RNA purified from a sample to be analyzed, with or without DNA, are mixed with 1 to 2 µl of a mixture containing the two first primers. The resulting mixture was transferred to a heating block or a thermal cycler and incubated at 65°C for 2 minutes. The mixture is then removed from the heating block or thermocycler and placed on ice.

[00210] A second mixture containing reverse transcriptase and a buffer were added to the first mixture above to a total volume of 10 µl. In samples subjected to the selective RNA amplification, Actinomycin D was added to the mixture to a final concentration of 50 ng/µl. The reactions were carried out by incubation at the following temperatures and durations: 4°C for 1min, 25°C for 10 min, 42°C for 10 min, 70°C for 15 min., 4°C and hold. The reaction mixture is then placed on ice.

Removal of Actinomycin D

[00211] Prior to second strand cDNA synthesis, removal of the DNA-dependent DNA polymerase and Actinomycin D is performed using magnetic beads under conditions that allow binding of the nucleic acids in the mixture to the beads. This clean up step allows separation of the reaction medium containing Actinomycin D from the nucleic acids and was conducted following the manufacturer instructions. To the reaction mixture above, 16 µl of bead suspensions (RNAClean, Agencourt) were added and the mixture was incubated at room temperature for 10 min. The beads were separated using a magnetic plate and the supernatant was discarded. The beads with the bound nucleic acids from the above reaction mixture were washed three times with 70% ethanol and air dried for 10 min at room temperature. This clean up step was performed for samples subjected to the selective RNA amplification according to the method of the invention and is a modified protocol of the WT-Ovation Pico RNA amplification.

Second strand cDNA synthesis

[00212] A mixture of DNA polymerase and buffer was added to the first strand synthesis reaction mixture for synthesis of the second strand cDNA. In the case of the modified protocol for selective RNA amplification according the method of the current invention, the reaction mixture also contained random hexamers (N6 DNA oligonucleotides) at 100 ng/µl. Second strand cDNA synthesis was carried out in a total volume of 20µl. For selective RNA amplification according to the method of the current invention, the beads used for the removal of Actinomycin D, as described above, were left in the second strand cDNA synthesis reaction mixture. Second strand cDNA synthesis reactions were carried out by incubation at the following temperatures for the following durations: 4°C for 1 min, 25°C for 10 min, 50°C for 30 min, 70°C for 5 min, and cooled to 4°C.

Purification of the double stranded cDNA

[00213] To each second strand cDNA reaction mixture, 32 µl of bead suspension (RNAClean® Agencourt) were added and the mixture was incubated for 10 min at room temperature. The beads were separate on a magnetic plate and further processed as described herein.

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Amplification

[00214] Amplification was carried out according to the manufacturer's instruction with modification of the total reaction volume from 160 µl to 80 µl (www.nugeninc.com).

[00215] Beads were suspended in the amplification mixture containing 20 µl of composite amplification primer, 40 µl of reaction buffer, and 20 µl enzyme mixture. The amplification reactions were carried out at following temperatures and durations: 40°C for 1 min., 47°C for 60 min., and 95°C for 5 min. The reaction were cooled to 4°C and held at this temperature until purification of the amplification products.

Results

[00216] The selective amplification of RNA from samples comprising low levels of input total HeLa RNA, with or without excess genomic human DNA, employed a modified protocol of the WT-Ovation Pico RNA amplification system, which included Actinomycin D in the first strand cDNA synthesis (to inhibit DNA-dependent DNA polymerase) and removal of Actinomycin D prior to second strand cDNA synthesis. The results from selective RNA amplification from a sample comprising DNA, are shown in Table 1, and includes the yield of amplified cDNA products generated by the non-modified protocol and the modified protocol, which is designed according to the method of the current invention. In control reactions, the selective amplification of RNA is demonstrated by the marked reduction in the yield of amplification products from samples comprising only genomic DNA and no RNA.

Table 1

Input		Yield µg SPIA™ amplified cDNA	
RNA	DNA	WT-Ovation Pico	Selective RNA amplification
1 ng		5.92	5.86
		5.56	5.34
1ng	1ng	5.80	5.02
		4.46	5.65
1 ng	9 ng	5.83	5.76
		5.53	5.68
No RNA	9 ng	4.82	2.66
		5.24	2.31
No RNA	No DNA	1.00	0.74
		0.85	0.76

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[00217] The amplified cDNA was further quantified by analysis of various transcripts in the amplification products using real time PCR. Aliquots of non-purified amplification products were diluted 1:80 in Tris-EDTA and 2 µl of the diluted amplification products were added to PCR mixture with SYBR Green and the specified PCR primers (sequence information given in Table 3). Relative quantification is given by Ct values for the various measured transcripts (amplified cDNA) in Table 2.

Table 2: Real time PCR quantification of specific amplified cDNA products (non-purified)

Input		PGK-1 (Ct)		GAPE 12 (Ct)		CD44-1 (Ct)		CD44-3 (Ct)	
RNA	DNA	WT-Ovation Pico	Selective amplification	WT-Ovation Pico	Selective amplification	WT-Ovation Pico	Selective amplification	WT-Ovation Pico	Selective amplification
1 ng	No DNA	19.1	18.8	17.2	18.1	23.3	20.4	19.2	21.4
		18.9	19.7	17.9	16.5	22.5	20.7	18.8	22.2
1ng	1ng	19.0	18.9	17.8	17.5	23.4	21.6	18.2	22.0
		19.1	19.2	17.8	17.5	22.8	21.4	18.0	22.0
1 ng	9 ng	19.9	19.3	18.2	18.0	23.3	20.8	20.0	20.3
		19.3	19.5	18.1	18.2	23.1	21.7	19.1	21.8
No RNA	9 ng	33.3	None	29.2	34.3	None	None	35.1	28.4
		23.2	35.9	31.5	33.0	None	None	33.2	34.7
No RNA	No DNA	None	None	39.5	None	None	None	None	None
		None	38.5	35.6	36.4	None	None	None	None

Table 3: PCR primer pairs used for quantification by real time PCR

Assay	PCR Primer sequences	Gene
GAPE-12	tccactggcgtcttcacc ggcagagatgatgacccttttt	glyceraldehyde-3-phosphate dehydrogenase
CD44-3	ttgttactttgacttttcagagcac gcaatatacatatcatgctttcctca	CD44 antigen (homing function and Indian blood group system)
CD44-1	gacaccatggacaagttttgg cggcaggttatattcaaatcg	CD44 antigen (homing function and Indian blood group system)
PGK1	ctgtggcttctggcatacct cttgctgctttcaggacca	phosphoglycerate kinase 1

Example 2: Whole transcriptome analysis of sequences from total RNA in the presence of DNA using actinomycin to inhibit DNA-dependent DNA polymerase activity

[00218] A RNA target is amplified using a method involving a first primer, comprising composite RNA/DNA portions, wherein the 3'-DNA end of the primers comprises either poly-dT sequence or a random sequence. First strand cDNA synthesis is performed with this first primer in the presence of Actinomycin D, an inhibitor of DNA-dependent DNA polymerase. Removal of Actinomycin D and RNA target from the reaction mixture is performed by binding the nucleic acids to magnetic beads. Second strand cDNA synthesis is then carried out and is followed by amplification, which is performed using a composite amplification primer, DNA polymerase with strand displacement activity, and RNase H. The global RNA amplification is carried out using the WT-Ovation Pico amplification system (NuGEN

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Technologies Inc. http://www.nugeninc.com/html/03_products5.html) with additional reagents and protocol modifications described below. The amplification products are then analyzed by sequencing with a Genome Sequencer platform (454/Roche Lifesciences). As described herein, the results of the example demonstrate selective amplification and analysis of RNA target from samples comprising mixtures of RNA and DNA.

Materials

[00219] All reagents for the WT-Ovation Pico RNA Amplification System (NuGEN Technologies Inc.) are used in this example. HeLa (S3) total RNA is purchased from Ambion. Human genomic DNA is purchased from Clontech. Random hexamer (N6 DNA oligonucleotide) is purchased from Operon. Actinomycin D is purchased from USB.

General comments

[00220] The WT-Ovation Pico amplification system is used to perform global RNA amplification. For selective amplification of RNA targets from samples comprising genomic DNA, the procedure is modified to include Actinomycin D in the step involving first strand cDNA synthesis. Additional clean up following first strand cDNA synthesis is added to the manufacturer protocol, which results in the removal of Actinomycin D prior to second strand cDNA synthesis. In addition, second strand cDNA synthesis is carried out in the presence of random hexamers (DNA oligonucleotides). All other amplification steps are the same as for the WT-Ovation Pico RNA Amplification System. The sequencing analysis is performed according to the manufacturers instructions.

First strand cDNA synthesis

[00221] First stand cDNA synthesis is initiated randomly across the length of RNA transcripts using composite first primers containing a 3'-random sequence DNA portion (for example, a random hexamer sequence) and a 5'-non-complementary and non-hybridizable RNA portion. In addition to the composite first primer, another composite first primer, comprising a 3'-poly-DT sequence DNA portion and the same 5'-non-hybridizing RNA portion as the previous primer, is used. In both the WT-Ovation Pico RNA amplification protocol and the modified procedure of the current invention, 1 to 2 µl of total RNA sample comprising 0.5 to 50 ng total RNA purified from a sample to be analyzed, with or without DNA, are mixed with 1 to 2 µl of a mixture containing the two first primers. The resulting mixture is transferred to a heating block or a thermal cycler and incubated at 65°C for 2 minutes. The mixture is then removed from the heating block or thermocycler and placed on ice.

[00222] A second mixture containing reverse transcriptase and a buffer is added to the first mixture above to a total volume of 10 µl. In samples subjected to the selective RNA amplification, Actinomycin D is added to the mixture to a final concentration of 50 ng/µl. The reactions are carried out by incubation at the following temperatures and durations: 4°C for 1min, 25°C for 10 min, 42°C for 10 min, 70°C for 15 min., 4°C and hold. The reaction mixture is then placed on ice.

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Removal of Actinomycin D

[00223] Prior to second strand cDNA synthesis, removal of the DNA-dependent DNA polymerase and Actinomycin D is performed using magnetic beads under conditions that allow binding of the nucleic acids in the mixture to the beads. This clean up step allows separation of the reaction medium containing Actinomycin D from the nucleic acids and is conducted following the manufacturer instructions. To the reaction mixture above, 16 μl of bead suspensions (RNAClean, Agencourt) are added and the mixture is incubated at room temperature for 10 min. The beads are separated using a magnetic plate and the supernatant is discarded. The beads with the bound nucleic acids from the above reaction mixture are washed three times with 70% ethanol and air dried for 10 min at room temperature. This clean up step is performed for samples subjected to the selective RNA amplification according to the method of the invention and is a modified protocol of the WT-Ovation Pico RNA amplification.

Second strand cDNA synthesis

[00224] A mixture of DNA polymerase and buffer is added to the first strand synthesis reaction mixture for synthesis of the second strand cDNA. In the case of the modified protocol for selective RNA amplification according the method of the current invention, the reaction mixture also contains random hexamers (N6 DNA oligonucleotides) at 100 ng/μl. Second strand cDNA synthesis is carried out in a total volume of 20μl. For selective RNA amplification according to the method of the current invention, the beads used for the removal of Actinomycin D, as described above, are left in the second strand cDNA synthesis reaction mixture. Second strand cDNA synthesis reactions are carried out by incubation at the following temperatures for the following durations: 4°C for 1 min, 25°C for 10 min, 50°C for 30 min, 70°C for 5 min, and cooled to 4°C.

Purification of the double stranded cDNA

[00225] To each second strand cDNA reaction mixture, 32 μl of bead suspension (RNAClean® Agencourt) are added and the mixture is incubated for 10 min at room temperature. The beads are separated on a magnetic plate and further processed as described herein.

Amplification

[00226] Amplification is carried out according to the manufacturer's instruction with modification of the total reaction volume from 160 μl to 80 μl (www.nugeninc.com).

[00227] Beads are suspended in the amplification mixture containing 20 μl of composite amplification primer, 40 μl of reaction buffer, and 20 μl enzyme mixture. The amplification reactions are carried out at following temperatures and durations: 40°C for 1 min., 47°C for 60 min., and 95°C for 5 min. The reactions are cooled to 4°C.

IDT Exhibit 1017.0550

Analysis

The amplified polynucleotides are diluted 1,000-fold, immobilized onto beads, and compartmentalized in a water-in-oil emulsion in the presence of PCR primers and reagents including buffer, enzyme and dNTP's. The immobilized polynucleotides are amplified by thermocycling in the water-in-oil emulsion for 20 cycles. When the emulsion is broken, amplified fragments remain bound to the beads. The beads are enriched to select for optimally sized amplicons and loaded into wells of a fiber optic slide so that there is approximately 1 bead in each well. Nucleotides are flowed across and into the wells in a fixed order in the presence of polymerase, sulfhydrolase, and luciferase. Addition of nucleotides complementary to the target strand results in a chemiluminescent signal that is recorded by a camera. The combination of signal intensity and positional information generated across the plate allows software to determine the DNA sequence.

Results

[00228] The selective amplification of RNA from samples comprising low levels of input total HeLa RNA, with or without excess genomic human DNA, employs a modified protocol of the WT-Ovation Pico RNA amplification system, which includes Actinomycin D in the first strand cDNA synthesis (to inhibit DNA-dependent DNA polymerase) and removal of Actinomycin D prior to second strand cDNA synthesis. The resulting selectively amplified polynucleotide targets are then used to perform whole-transcriptome analysis by massively parallel sequencing. The massively parallel sequencing identifies: (i) transcript abundance at both the exon and transcript level; (ii) transcriptional start and stop sites; (iii) exon-exon linkages in mature transcripts; (iv) single-base changes corresponding to SNPs and mutations; and (v) novel exons belonging to already characterized genes. The massively parallel sequencing also provides numerous examples of novel splicing events when the reads that could not be mapped directly to the genome were searched against a database of all possible exon-exon junctions within and between genes. A large number of reads appear to represent bona fide transcripts of novel expressed elements. The sequence data also allows the identification of single-base variations corresponding to novel SNPs and mutations, with many of these genes either cell cycle–or E2F transcription–related..

[00229] 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.

[00230] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will be apparent to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

IDT Exhibit 1017.0551

CLAIMS

WHAT IS CLAIMED IS:

1. A method of selectively generating multiple copies of a polynucleotide sequence of or complementary to target RNA which is in a sample comprising DNA, said method comprising the steps of:

(a) hybridizing to the target RNA a first primer comprising a sequence (A) that is not complementary to the target RNA, and a sequence (B) at the 3'-end which hybridizes to the target RNA;

(b) extending the first primer with at least one enzyme comprising RNA-dependent DNA polymerase activity in the presence of at least one compound comprising DNA-dependent DNA polymerase inhibitor activity, whereby a complex comprising a primer extension product and the target RNA is produced, whereby the first primer extension product comprises a sequence (Y) that is complementary to the target RNA and comprises sequence (A);

(c) disabling or removing at least one compound comprising DNA-dependent DNA polymerase inhibitor activity; and

(d) producing multiple copies of a polynucleotide sequence complementary to the target RNA and/or complementary to sequence (Y) using sequence (A).

2. A method of selectively generating multiple copies of a polynucleotide sequence complementary to a target RNA which is in a sample comprising DNA, said method comprising the steps of:

(a) hybridizing to the target RNA a first primer comprising a sequence (A) that does not hybridize to the target RNA, and a sequence (B) at the 3'-end, which hybridizes to the target RNA;

(b) extending the first primer with at least one enzyme comprising RNA-dependent DNA polymerase activity in the presence of at least one compound comprising DNA-dependent DNA polymerase inhibitor activity, whereby a complex comprising a first primer extension product and the target RNA is produced, whereby the first primer extension product comprises a sequence (Y) that is complementary to the target RNA and comprises sequence (A);

(c) disabling or removing at least one compound comprising DNA-dependent DNA polymerase inhibitor activity;

(d) cleaving the target RNA in the complex of step (b).

(e) extending a second primer along the first primer extension product with at least one enzyme comprising DNA-dependent DNA polymerase activity, wherein the second primer comprises sequence (C) that is complementary to a sequence (C') on the first primer extension product to produce a complex comprising the first primer extension product and a second primer extension product, whereby the second primer extension product comprises the sequence (C), a sequence (Y') complementary to sequence (Y) and sequence (A') complementary to sequence (A).

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(f) producing multiple copies of a polynucleotide sequence complementary to the target RNA using a primer with a sequence (A) and/or sequence (A').

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

4. The method of claims 1or 2, wherein the method further comprises clonally producing multiple copies of a polynucleotide sequence complementary to the target RNA using a primer with a sequence (A) and/or sequence (A') on a solid substrate or in an emulsion.

5. The method of claim 3, wherein the sequence (A') is used as a priming site for amplification.

6. The method of claim 3, wherein the producing of multiple copies in step (f) are produced using amplification methods comprising polymerase chain reaction (PCR).

7. The method of claim 6, wherein a PCR primer comprises sequence (A) of the first primer.

8. The method of claim 7, wherein a PCR primer comprises sequence (C) of the second primer.

9. The method of claim 7, wherein the producing of multiple copies in step (f) comprises performing PCR with primer pairs wherein the first PCR primer is complementary to sequence (A') and the second PCR primer is complementary to all or a portion of sequence (C') or all or a portion of sequence (Y).

10. The method of claim 6 wherein the producing of multiple copies in step (f) comprises performing single primer, linear PCR using a PCR primer complementary to sequence (A').

11. The method of claim 3 wherein the producing of multiple copies in step (f) comprises an amplification method comprising the use of an RNA/DNA composite primer, RNase H, and a DNA dependent DNA polymerase with strand displacement activity.

12. The method of claims 1or 2, wherein the first primer comprises an RNA and a DNA sequence wherein a 5'-RNA sequence comprises sequence (A) and a 3'-DNA sequence comprises sequence (B); whereby the complex formed in step (e) comprises an RNA/DNA heteroduplex at one end; and wherein the producing of multiple copies in step (f) comprises the steps of (i) cleaving the RNA from the heteroduplex, (ii) hybridizing to sequence (A') an amplification primer comprising a 5' RNA sequence and a 3' DNA sequence, (iii) extending the amplification primer with at least one enzyme comprising DNA dependent DNA polymerase activity and comprising strand displacement activity, (iv) cleaving the RNA from the extended amplification primer in the heteroduplex with at least one enzyme comprising a specificity for cleaving RNA in a DNA-RNA heteroduplex; and (v) repeating steps (ii) through (iv) to

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produce multiple copies of amplification product comprising sequence (Y) that is complementary to a portion of the RNA template.

13. The method of claims 1or 2, wherein the producing of multiple copies in step (f) comprises in-vitro-transcription (IVT).

14. The method of claim 13 wherein sequence (A) comprises a pro-promoter, whereby the complex formed in step (e) comprises a double stranded promoter for a DNA dependent RNA polymerase comprising sequences (A) and (A'), wherein (A) is a DNA sequence.

15. The method of claim 2, wherein the second primer comprises a fragment from the cleaved RNA target.

16. The method of claims 1 or 2, wherein the DNA-dependent DNA polymerase inhibitor activity is derived from a compound comprising at least one of the following: actinomycin, alpha-amanitin, aphidicolin, BP5, novobiocin, rifampicin, rifamycin, sulfoquinovosylmonoacylglycerol, sulfoquinovosyldiacylglycerol, ursane, oleanane triterpenoids, ursolic acid, oleanolic acid, mikanolide, dihydromikanolide, dehydroaltenusin, catapol, taxinine, cephalomanninine, dipeptide alcohols, corylifolin; bakuchiol; resveratrol; Neobavaisoflavone; daidzein; bakuchicin, levodopa, dopamine, anacardic acid and oleic acid.

17. The method of claims 1 or 2, wherein the DNA-dependent DNA polymerase inhibitor activity is derived from a compound comprising actinomycin.

18. The method of claims 1 or 2, wherein the sample comprises total nucleic acid in a biological fluid.

19. The method of claim 18, wherein the biological fluid is selected from the group consisting of: plasma, serum, urine, saliva.

20. The method of claims 1 or 2, wherein the sample comprises viral RNA.

21. The method of claims 1 or 2, wherein the sample comprises cell or tissue lysates.

22. The method of claims 1 or 2, wherein the second primer comprises a tailed primer with a 5'-sequence which does not hybridize to the first primer extension product and which comprises a propromoter sequence such that RNA transcripts are produced comprising a sequence homologous to the target RNA; whereby multiple copies of the homologous sequence of the RNA sequence of interest are generated.

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23. The method of claims 1 or 2, wherein the producing of multiple copies in step (f) comprises hybridizing a PTO, comprising a propromoter sequence and a 3'-hybridizing region, to the 3'-end of a first primer extension product under conditions which allow transcription to occur by RNA polymerase, such that RNA transcripts are produced comprising sequences homologous to the target RNA; whereby multiple copies of the RNA sequence of interest are generated.

24. The method of claims 1 or 2, wherein the producing of multiple copies in step (f) comprises hybridizing a PTO, comprising a propromoter sequence and a 3'-hybridizing region, to the 3'-end of a second primer extension 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.

25. The method of claims 1 or 2, wherein the target RNA is mRNA.

26. The method of claims 1 or 2, wherein the first primer comprises a sequence complementary to poly-A on RNA.

27. The method of claims 1 or 2, wherein the sequence (B) which hybridizes to the target RNA comprises a random sequence.

28. The method of claims 1 or 2, wherein the first primer comprises a mixture of first primers comprising a first primer comprising a sequence complementary to poly-A on RNA, and further comprising a first primer comprising a sequence which hybridizes to the target RNA and comprises a random sequence.

29. The method of claim 15, wherein said fragment generated by cleaving RNA in the complex of step (b) with an enzyme that cleaves RNA from an RNA/DNA heteroduplex, by heat, or by chemical means.

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

31. The method of claim 30, wherein said method comprises at least two different first primers.

32. The method of claim 2, wherein cleaving the RNA in a heteroduplex of the RNA target and the first primer extension product is performed by RNase H.

33. A method of sequencing an RNA sequence of interest, said method comprising analyzing amplification products to determine sequence, said amplification products produced by the method of any of claims 1 or 2 in the presence of a mixture of NTPs and NTP analogs such that transcription is terminated upon incorporation of an NTP analog

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34. The method of claim 33, wherein the target RNA is mRNA, a whole transcriptome or substantial fraction thereof, or a set of whole transcriptomes or substantial fractions thereof.

35. A method of detecting a mutation in a target RNA, comprising analyzing sequences of amplification products for the presence of a mutation as compared to a reference polynucleotide sequence, said amplification products produced by the method of any of claims 1 or 2.

36. The method of claim 35, wherein the target RNA is mRNA.

37. A method of producing a nucleic acid immobilized to a substrate comprising immobilizing amplification products on a substrate, said amplification products produced by the method of any of claims 1 or 2.

38. The method of claim 37, wherein the target RNA is mRNA.

39. The method of claim 37, wherein the substrate is a microarray.

40. A method for performing expression analysis of one or more target RNA sequences comprising:

(a) collecting a sample of nucleic acid comprising RNA and DNA;

(b) optionally enriching the nucleic acid in the sample;

(c) contacting the nucleic acid with an inhibitor of DNA-dependent DNA polymerase activity;

(d) hybridizing to the target RNA a first primer comprising a sequence (A) that is not complementary to the target RNA, and a sequence (B) at the 3'-end which hybridizes to the target RNA;

(e) extending the first primer with at least one enzyme comprising RNA-dependent DNA polymerase activity in the presence of at least one compound comprising DNA-dependent DNA polymerase inhibitor activity, whereby a complex comprising a primer extension product and the target RNA is produced, whereby the first primer extension product comprises a sequence (Y) that is complementary to the target RNA and comprises sequence (A);

(f) disabling or removing at least one compound comprising DNA-dependent DNA polymerase inhibitor activity;

(g) producing multiple copies of a polynucleotide sequence complementary to the target RNA and/or complementary to sequence (Y) using sequence (A); and

(h) further analyzing the amplified products of step (g).

41. The method of claim 40, wherein step (h) further analyzing the products of step (g) includes whole transcriptome analysis, whole transcriptome profiling, microarray analysis, quantitative PCR, pyrosequencing, sequencing by ligation, dye-terminator sequencing, whole transcriptome sequencing, RNA sequencing by massively parallel sequencing, or digital PCR.

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42. The method of claim 40, wherein the DNA-dependent DNA polymerase inhibitor activity is derived from a compound comprising at least one of the following: actinomycin, a derivative of actinomycin, alpha-amanitin, aphidicolin, BP5, novobiocin, rifampicin, rifamycin, sulfoquinovosylmonoacylglycerol, sulfoquinovosyldiacylglycerol, ursane, oleanane triterpenoids, ursolic acid, oleanolic acid, mikanolide, dihydromikanolide, dehydroaltenusin, catapol, taxinine, cephalomanninine, dipeptide alcohols, corylifolin; bakuchiol; resveratrol; Neobavaisoflavone; daidzein; bakuchicin, levodopa, dopamine, anacardic acid and oleic acid.

43. The method of claim 40, wherein step (g) comprises PCR, linear PCR, in vitro transcription, or single primer isothermal amplification.

44. A method of generating at least one double stranded DNA product corresponding to a sequence of a target RNA or a portion thereof comprising:

(a) adding to a reaction mixture comprising target RNA and further comprising DNA:

(i) at least one first primer comprising a 3' annealing sequence and a 5' tail sequence;

(ii) a DNA-dependent DNA polymerase inhibitor; and

(iii) an RNA-dependent DNA polymerase;

(b) disabling or removing the at least one DNA-dependent DNA polymerase inhibitor from the reaction mixture;

(c) annealing and extending at least one all DNA second primer comprising an annealing sequence with a DNA-dependent DNA polymerase, wherein said double stranded product is labeled at least one end; and

(d) clonally amplifying one or both strands of the double-stranded product.

45. The method of claim 44, wherein the clonal amplification is performed on a solid substrate, in an emulsion, or both.

46. The method of claim 44, wherein the clonal amplification generates polonies.

47. A kit for amplifying a target RNA comprising: a tailed first primer, a reverse transcriptase enzyme, a DNA-dependent DNA polymerase inhibitor, and optionally a second primer.

48. The kit of claim 47 wherein the first tailed primer comprises a 3' random portion, and the second primer comprises a random primer.

49. The kit of claim 47 further comprising a composite amplification primer, and wherein the tailed first primer is a composite primer comprising a 3' DNA portion and a 5' RNA portion.

50. The kit of claim 47, wherein the kit further comprises instructions for the use of the kit.

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51. The kit of claim 47, wherein the kit further comprises a DNA-dependent DNA polymerase.

52. The kit of claim 47, wherein the kit further comprises reagents for DNA purification.

IDT Exhibit 1017.0558

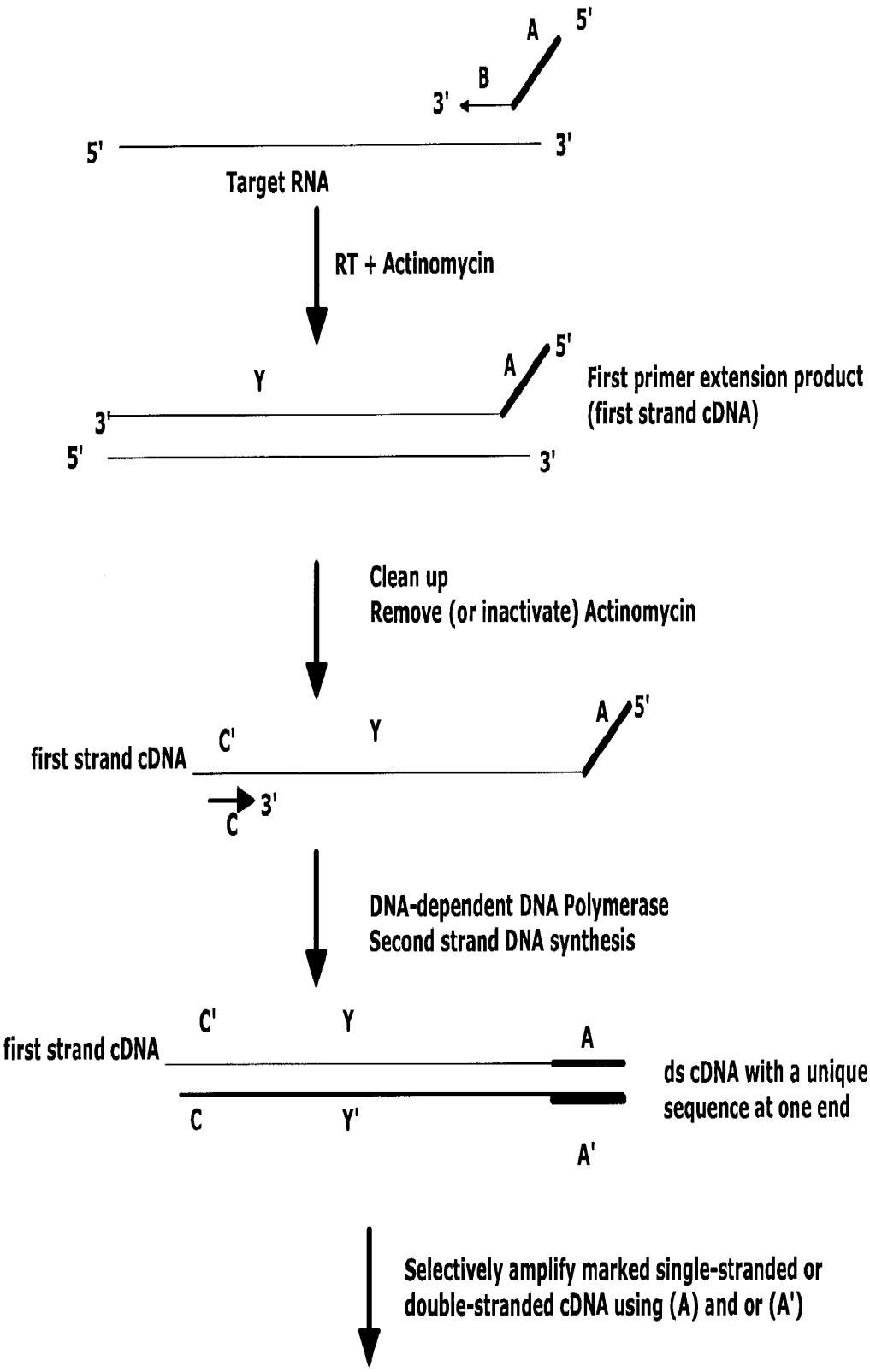

A
5'
B
3'
5'
3'
Target RNA
RT + Actinomycin
Y
A
5'
3'
5'
3'
First primer extension product
(first strand cDNA)
Clean up
Remove (or inactivate) Actinomycin
first strand cDNA
C'
Y
A
5'
C
3'
DNA-dependent DNA Polymerase
Second strand DNA synthesis
first strand cDNA
C'
Y
A
C
Y'
A'
ds cDNA with a unique
sequence at one end
Selectively amplify marked single-stranded or
double-stranded cDNA using (A) and or (A')

Figure 1

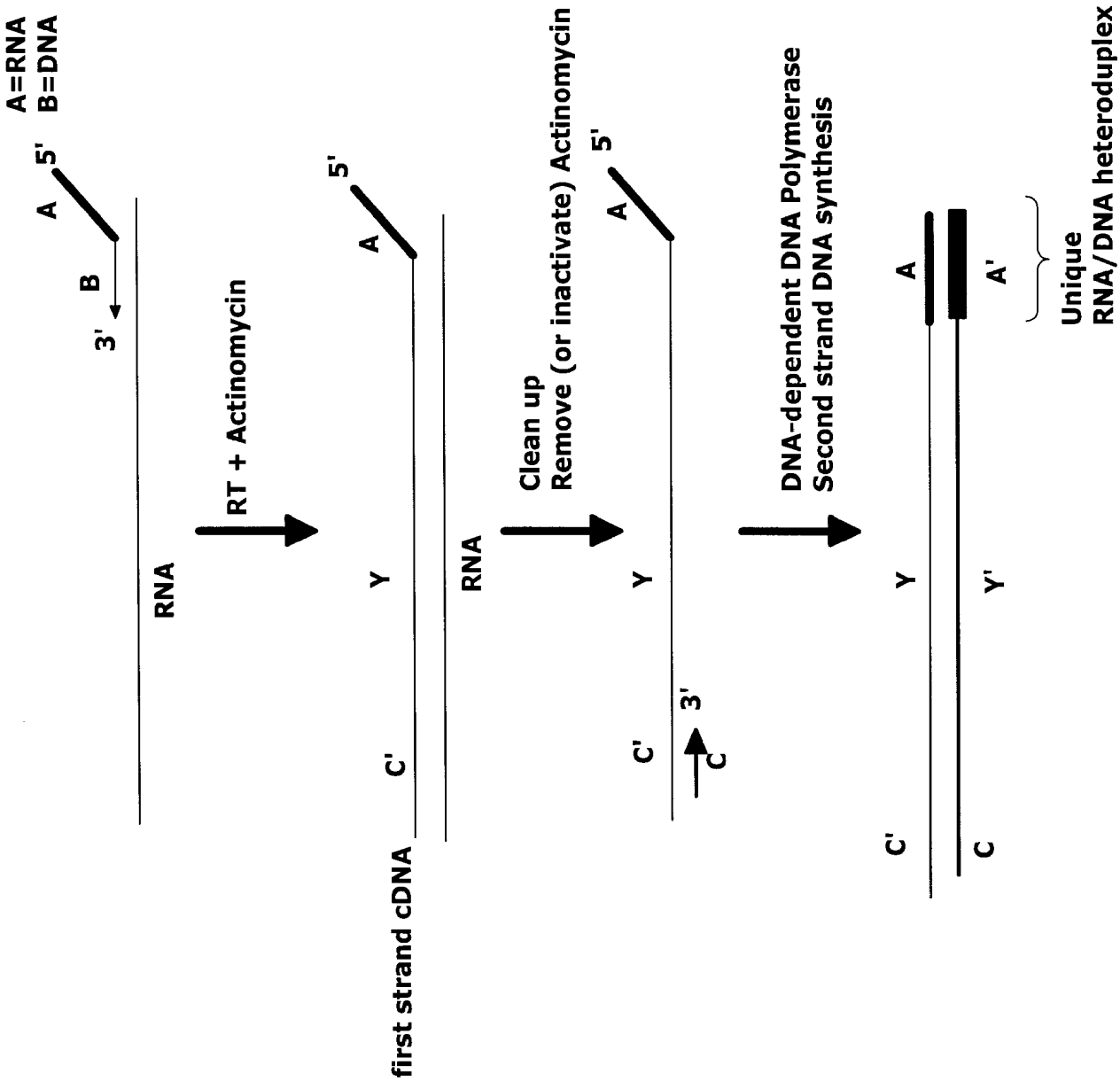

A=RNA
B=DNA
5'
A
B
3'
RNA
RT + Actinomycin
5'
A
Y
C'
first strand cDNA
RNA
Clean up
Remove (or inactivate) Actinomycin
5'
A
Y
C'
3'
C
DNA-dependent DNA Polymerase
Second strand DNA synthesis
A
A'
Y
Y'
C'
C
Unique
RNA/DNA heteroduplex

Figure 2a

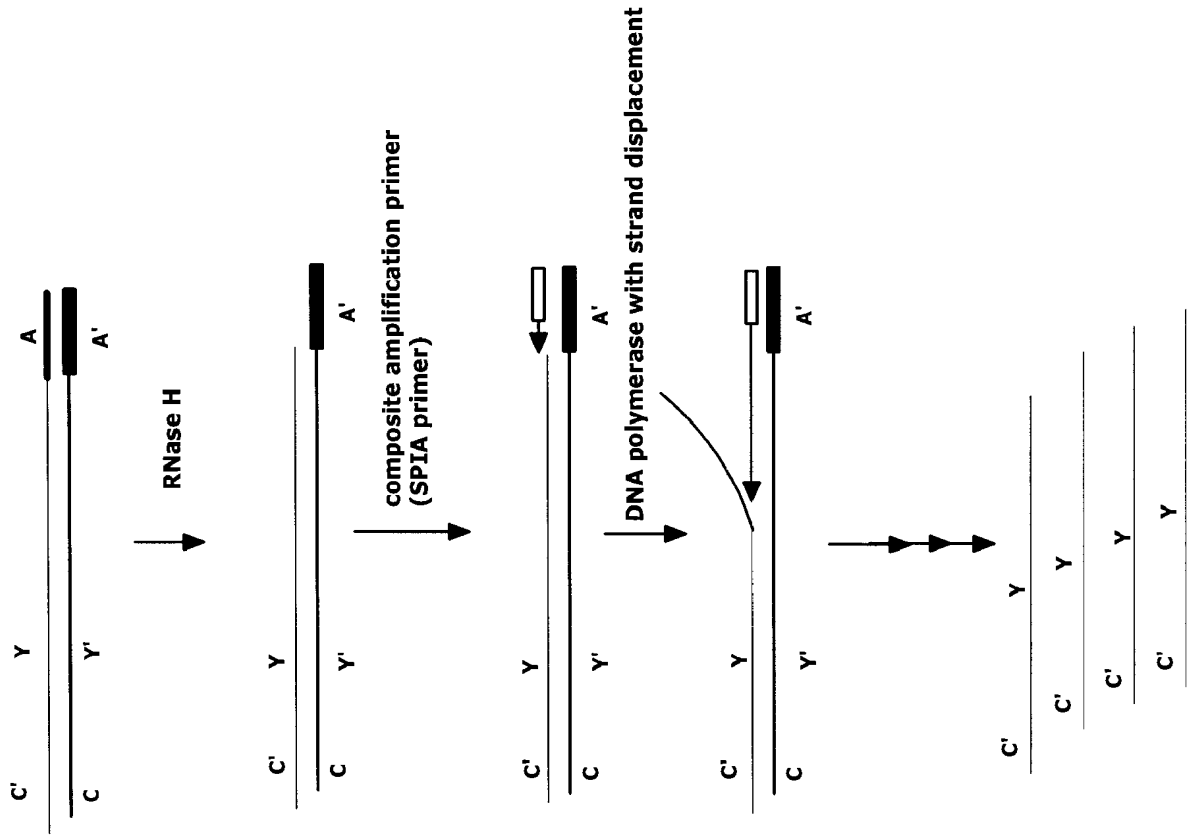

A
A'
Y
Y'
C'
C
RNase H
composite amplification primer
(SPIA primer)
DNA polymerase with strand displacement

Figure 2b

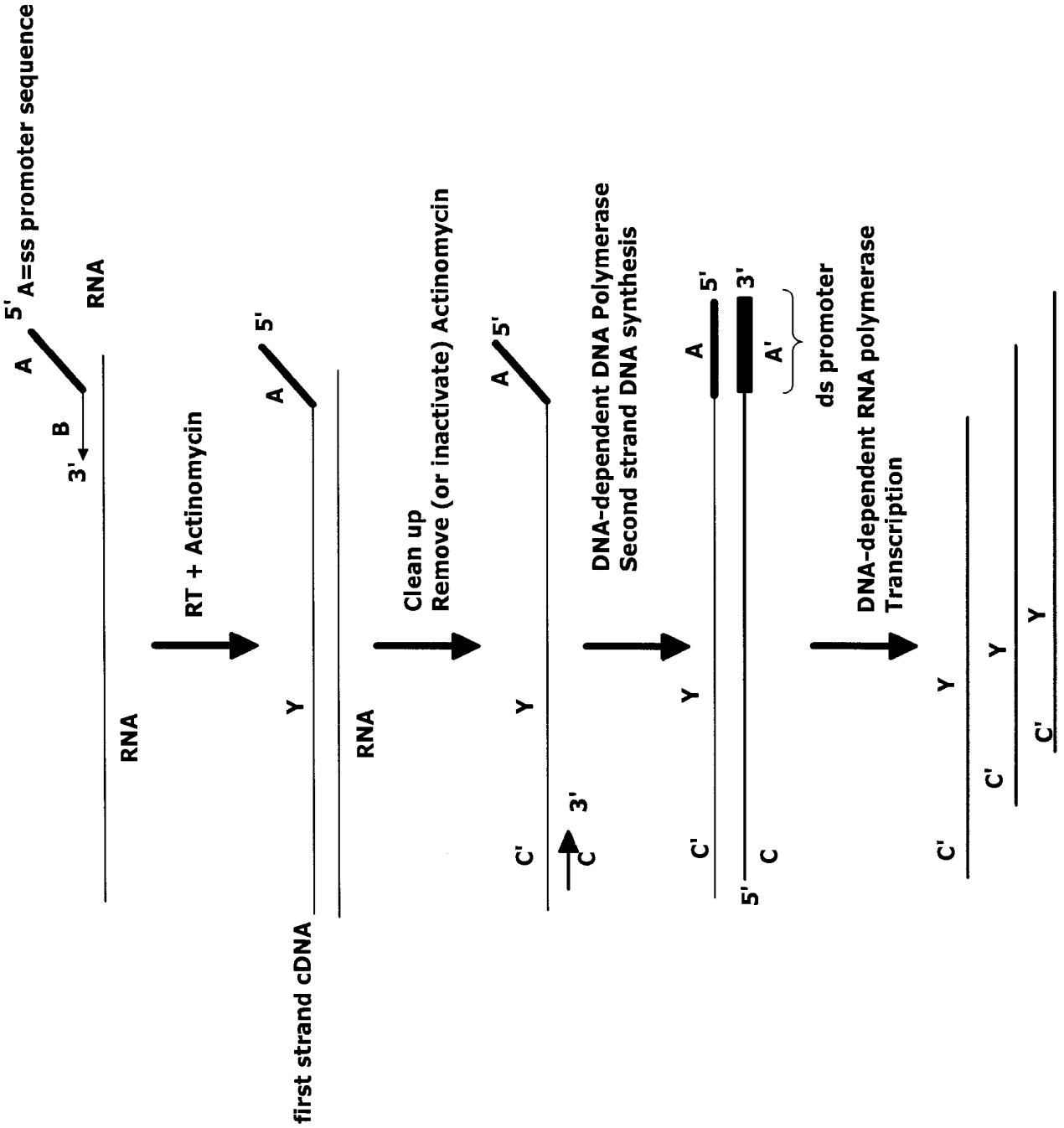

5' A=ss promoter sequence
A
B
3'
RNA
RT + Actinomycin
5'
A
Y
RNA
first strand cDNA
Clean up
Remove (or inactivate) Actinomycin
5'
A
Y
C'
3'
C
DNA-dependent DNA Polymerase
Second strand DNA synthesis
A
5'
3'
A'
Y
C'
5'
C
ds promoter
DNA-dependent RNA polymerase
Transcription
Y
C'
Y
C'
Y
C'

Figure 3

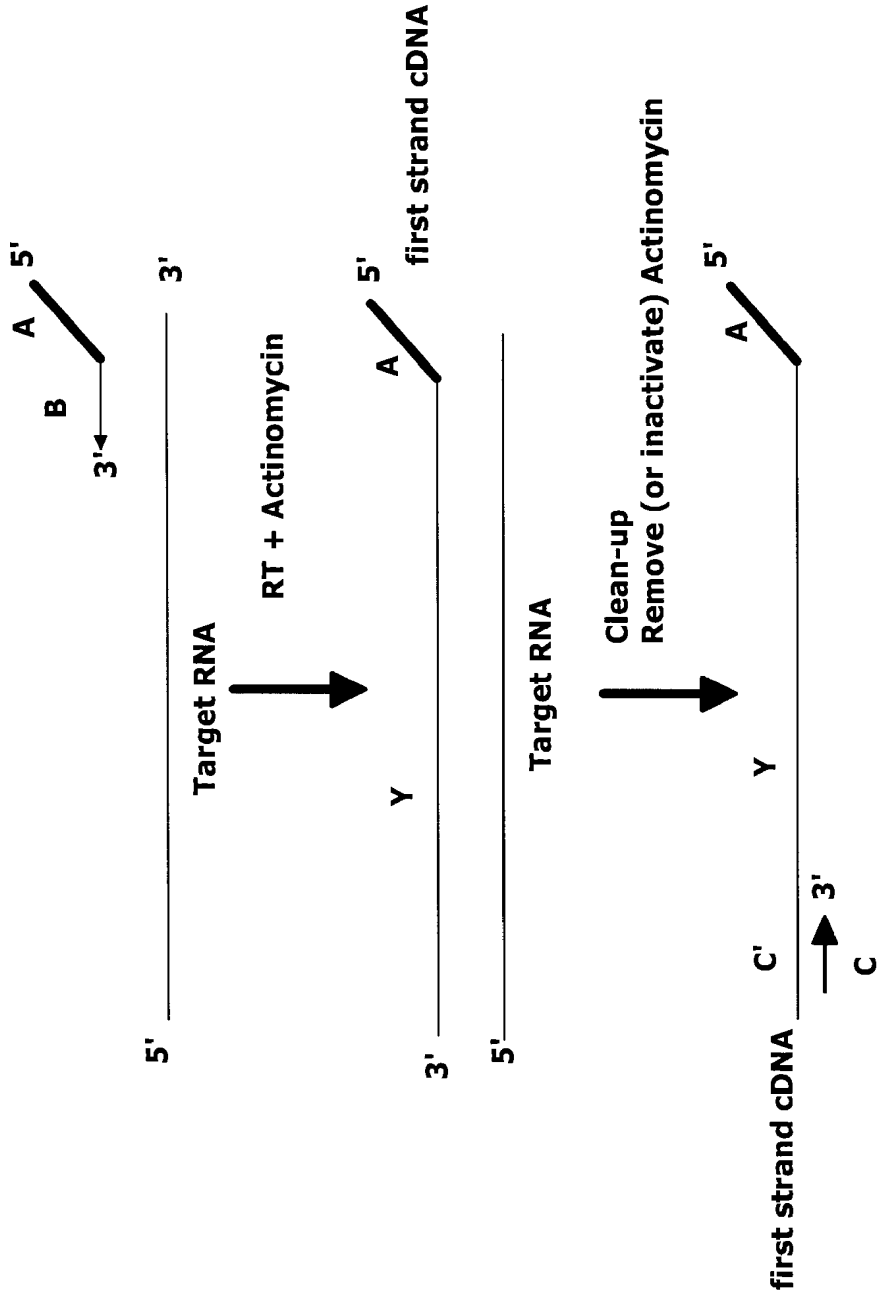

5'
A
B
3'
Target RNA
3'
5'
RT + Actinomycin
5'
A
Y
3'
first strand cDNA
Target RNA
5'
Clean-up
Remove (or inactivate) Actinomycin
5'
A
Y
C'
C
3'
first strand cDNA

Figure 4 a

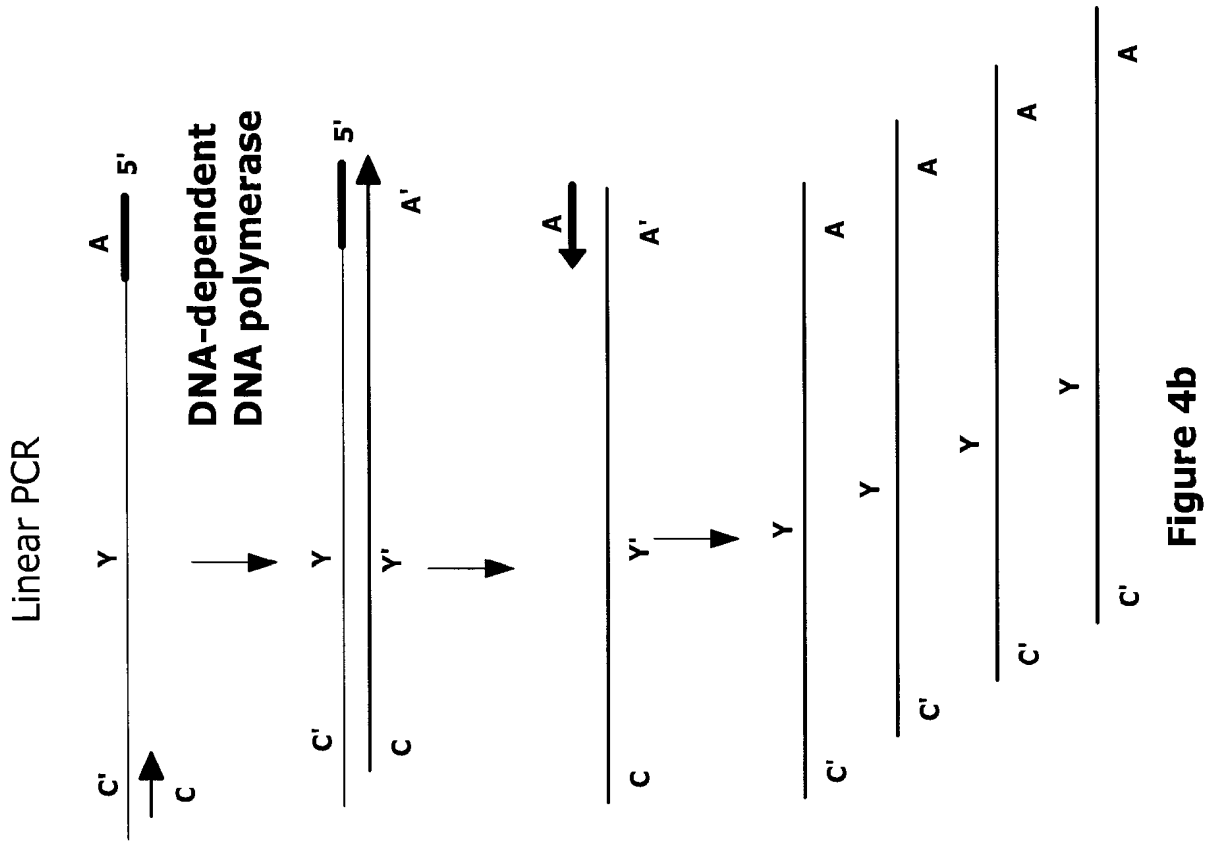


Figure 4b

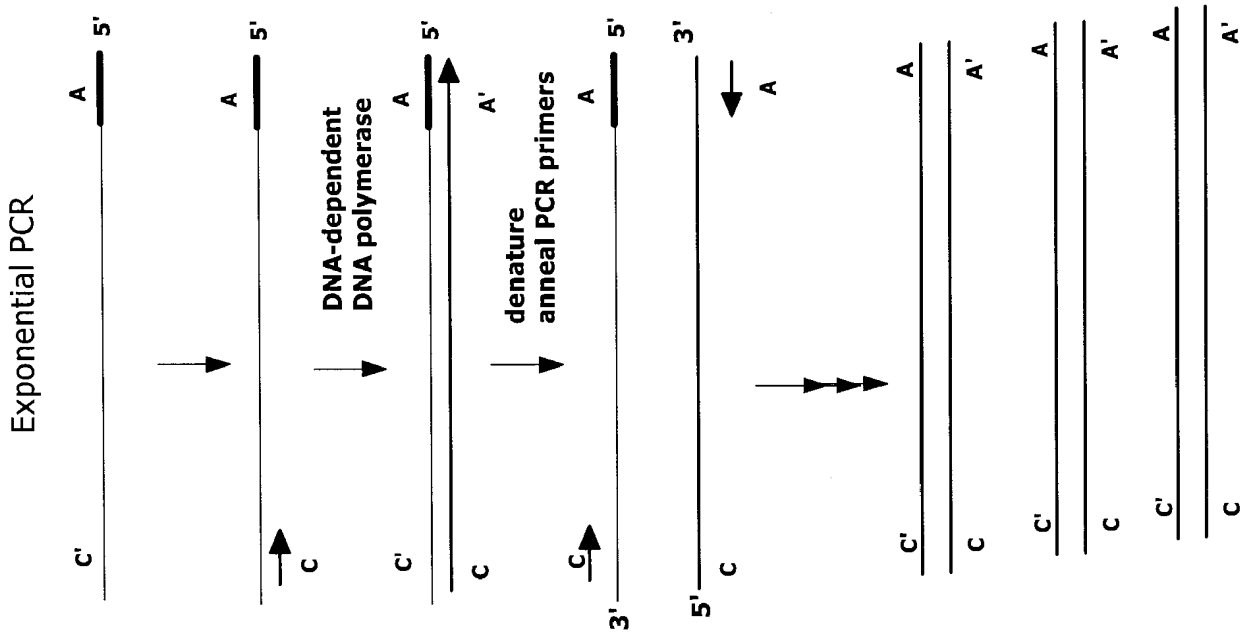

Exponential PCR
DNA-dependent
DNA polymerase
denature
anneal PCR primers
A
A'
C
C'
5'
3'

Figure 4c

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— *with sequence listing part of description (Rule 5.2(a))*

WO 2012/013932 A1

(54) **Title**: IMPROVED RACE

(57) **Abstract**: The present invention relates to a method of generating a cDNA molecule or pool of cDNA molecules which comprise adapter sequences 5' and 3' of the sequence corresponding to the 5' or 3' ends of the original mRNA molecule. There is further provided a method of amplifying a desired 5' or 3' end of said modified cDNA or pool of cDNA molecules whilst minimising the generation of false positives and/or non-specific amplification. There is also provided a kit for carrying out the method of the present invention.

IDT Exhibit 1017.0566

Improved RACE

Field of the Invention

The present invention relates to a method of generating a cDNA molecule or pool of cDNA molecules which comprise adapter sequences 5' and 3' of the sequence corresponding to the 5' or 3' ends of the original mRNA molecule. There is further provided a method of amplifying a desired 5' or 3' end of said modified cDNA or pool of cDNA molecules whilst minimising the generation of false positives and/or non-specific amplification. There is also provided a kit for carrying out the method of the present invention.

INTRODUCTION

The genomes of an increasing number of organisms have been sequenced, however the exact structure of many transcripts remain unresolved, particularly their 5' untranslated regions (UTRs). Many open reading frames are predicted and need to be supported by experimental data to obtain an accurate annotation of the genome. For example, Salehi-Ashtiani et al (1) performed large scale rapid amplification of cDNA ends (RACE) on *C. elegans*, identifying UTRs, new exons and redefined previously annotated exons. The lack of experimental data supporting gene structure is in part due to the methodologies employed in cloning gene sequences. Cloning of novel cDNAs typically results in sequences that only partially represent the mRNA species, with the 3' ends usually well represented and the 5' end of the sequence often incomplete. This tendency can be due to RNA degradation, or is a result of the cDNA synthesis reaction not reaching completion. When working with novel organisms,

where the genome has not been sequenced, obtaining the 5' end of the cDNA, although in theory quite simple, can often prove to be technically challenging.

The unknown 5' sequence from the ends of cDNA molecules can be cloned using a PCR technique know as rapid amplification of cDNA ends (RACE) (2). In Classic RACE, homopolymer tailing of the cDNA is used to append a linker sequence to the cDNA terminus. Extension of unknown regions of the cDNA is then achieved through PCR using a gene specific primer and a primer that can bind and prime DNA synthesis from the linker sequence.

Different versions of RACE have been developed, making significant improvements to the original methodology, including New RACE (3), and Cap finder RACE (5, 6). In New RACE, an anchor primer is ligated to the 5' end of the mRNA molecule prior to reverse transcription, which subsequently synthesised cDNA molecules incorporate into their sequence (3). Therefore only full length cDNA molecules are amplified by PCR using the anchor primer and gene specific primer. The Cap-switch RACE (Cap finder) protocol exploits the Murine moloney leukaemia virus (MMLV) reverse transcriptase ability to add an extra 2-4 cytosine residues to the 3' ends of cDNA molecules after reaching the cap structure at the 5' end of the mRNA template. When a primer with multiple guanine residues at its 3' end is present in the reaction mix, annealing of the poly G to poly C sequences occurs, allowing for copying of the annealed primer sequence to the cDNA, adding an adapter sequence to the end of the cDNA (5, 6). These methods, have been developed into commercially available RACE kits including Clontech (Cap switching Smart RACE), Invitrogen (5'RACE system) and Ambion (First Choice RLM-RACE kit).

Many of the commercially available kits offer protocols that allow the amplification of 5' cDNA ends of many genes from a single cDNA synthesis reaction. Despite claims of specific amplification of the desired 5' region of a target gene, a common problem that occurs in RACE reactions is obtaining non-specific amplification products (6, 7, 8). The use of a second nested PCR reaction has been shown to increase the specificity of the reaction, however, non-specific amplification and false positive products are still often obtained. When cDNA synthesis is primed using an oligo-dT primer, all mRNA species are reverse transcribed, and the 3' primer site will subsequently be incorporated into the 3' end of all newly transcribed cDNAs. When cDNA templates such as these are used for PCR, non-specific amplification is likely due to the incorporation of the primer sequence in every cDNA molecule in the universal pool of full length cDNAs.

Prevention of PCR carryover contamination by incorporation of dUTP during PCR and uracil-DNA glycosylase treatment is a widely used technique (9) (covered by patents US5035996, US 5683896 and US5945313). dUTP is incorporated into all amplicons by substituting dUTP for dTTP in PCR reactions. Treatment of PCR products with UDG prior to thermal cycling cleaves DNA at uracil bases thereby inhibiting amplification of carryover contamination from previous PCR reactions (10). This function can be exploited to remove unwanted DNA sequences from reaction mixtures. For example, the use of dUTP and UDG to increase the specificity of 3' RACE reactions has been reported (11, see also US5334515), however, no methods using dUTP/UDG for 5' RACE are currently available.

It is amongst the objects of the present invention to obviate and/or mitigate one or more of the aforementioned disadvantages.

Summary of the Invention

The present invention is based on the development of a modified version of the RACE protocol which enables the amplification reaction to be simplified by degrading non-target modified cDNA sequences using uracil DNA glycosylase (UDG). Essentially, a double stranded cDNA is synthesised to incorporate dUTP, and this then serves as template for subsequent amplification of cDNA ends of any target gene. During synthesis of the modified double stranded cDNA, PCR primer sites (where dUTP replaces dTTP) are incorporated at the 3' and 5' ends of each cDNA molecule. These primer sites are incorporated at the 3' and 5' ends by way of adapter sequences. The modified double stranded cDNA, incorporating dUTP, now serves as a universal pool for PCR amplification of cDNA ends for any gene of interest.

Thus, in a first aspect there is provided a method for generating a modified cDNA molecule comprising sequence corresponding to the 5' and 3' ends of an mRNA molecule comprising the steps of:

a) annealing a first adapter oligonucleotide to an mRNA molecule, said first adapter comprising a 3' portion which is substantially complimentary to the 3' poly A tail of the mRNA molecule and a 5' portion which comprises one or more uracil residues;

b) extending from said first adapter, utilizing dNTPs which include dUTP, so as to generate a first modified cDNA strand, which comprises one or more, typically a plurality of uracil residues in its sequence;

c) incorporating a second adapter, which is different to the first adapter, at the 3' end of the first cDNA strand, wherein the second adapter comprises at least a 3' portion which is not specific to the mRNA molecule and which comprises one or more uracil residues in the sequence;

d) copying said first modified cDNA strand by extending from a first primer which comprises one or more uracils and comprises at least a 3' portion which is substantially complementary to a portion of the second adapter sequence, using dNTPs which include dUTP, thereby generating a modified double stranded cDNA molecule, which includes one or more, typically a plurality of uracil residues in the first and second strands and adapter sequences 5' and 3' of sequence corresponding to the 5'and 3' ends of the mRNA molecule.

Conveniently the method may be conducted on a pool of mRNA molecules, thereby generating a single pool of cDNA molecules, each cDNA molecule from the pool comprising the same 5' and 3' adapter sequences respectively

It is to be understood that the term "modified cDNA molecule" relates to a cDNA molecule which comprises one or more, typically many uracil molecules throughout its sequence. Thus, the modified cDNA molecules of the present invention comprise uracils in place of many thymidine residues. However, not all thymidine residues need to be replaced by a uracil and indeed the present inventors have observed that it can be desirable not to replace all thymidine residues with a uracil. Thus, typically at least 1 in 1, to 1 in 20, such as 1 in 1 to 1, in 4, especially 1 in 1.05 to 1 in 2 thymidine residues are replaced by a uracil residue.

The first adapter oligonucleotide typically comprises a 3' portion which comprises a stretch of T residues, so as to be able to complimentary base pair with the poly A tail of the mRNA molecule. Typically the stretch will be 10-40 Ts in length, such as 15-30 Ts in length. The 3' end of the first adapter may be a T or may incorporate other residues to ensure proper annealing and extension from the first adapter. Indeed a pool of first adapters may be provided which include possible variations at the 3' end so as to ensure proper annealing and extension from the adapter. The 5' end of the first adapter sequence is designed to be not complimentary to eukaryotic sequences based on blast homology searches and generally not to be specific to the mRNA sequence.

The term "substantially complimentary" as used throughout the description, is to be understood to mean that complimentary is such that specific binding at high stringency, (see for example ref 3) for what is generally understood as high stringency, but the skilled addressee is well aware that relevant sequences need not be 100% complimentary. Thus, a small degree of variation may be permitted. Typically any nucleotide sequence as described herein will comprise a complimentary portion which is at least 95%, such as 97-99% identical with the sequence to which it is to bind.

After denaturation, typically by heat, of the RNA, the poly dT sequence of the first adapter base pairs to the polyA sequence of the mRNA.

The extension from the first adapter is carried out using dNTPs which comprise dUTP, but as mentioned above, conveniently dTTP may also be present, albeit in much lower (e.g. <10%) amounts than dUTP, in the dNTP mix. The extension reaction may be catalysed using a variety of reverse transcriptases, such as avian myeloblastosis virus (AMV) or Moloney murine leukaemia virus (M-MLV). However, in one embodiment, the reverse transcriptase is M-MLV reverse transciptase. As mentioned previously M-

MLV reverse transcriptase adds 2-4 cytosine residues to the 3' ends of cDNA molecules and by using the cap switching techniques previously described (5, 6), it is possible to incorporate a second adapter (containing uracil) at the 3' end of the first cDNA strand.

Other reverse transcriptases may be employed and the second adapter incorporated at 3' end of the first modified cDNA strand using enzymes such as terminal transferase (2). An alternative approach to incorporate a uracil containing adapter in the 3' end of the newly synthesised first strand cDNA is to use the RNA ligase method previously described (3), however to make this compatible with the technique described herein, dUTP should replace dTTP in the dNTP mix used for first strand cDNA synthesis.

Before copying the first cDNA strand, RNA of the RNA/cDNA hybrid may be removed by way of RNAse H treatment to degrade RNA of the RNA/cDNA hybrid and the cDNA purified from degraded RNA and unincorporated adapter molecules by way of affinity purification (e.g. silica-based purification) using a suitable column purification method, such as by using QIA quick (Qiagen Inc., Chatsworth, CA, USA).

The second cDNA strand may be easily generated by copying the first cDNA strand using a first primer (where uracil replaces thymidine) which is able to specifically bind to the second adapter sequence at 3' end of the first cDNA strand, using a suitable DNA polymerase, such as Taq polymerase and a dNTP mix comprising dUTP.

The resulting double stranded cDNA can be purified by way of affinity purification (e.g. silica-based purification) using a suitable column purification method, such as by using QIA quick (Qiagen Inc., Chatsworth, CA, USA) and then used as a template for the specific amplification of the ends of target genes. However, the modified resulting

double stranded cDNA molecule(s) can be amplified further by using said first primer and a second primer which is able to specifically bind to the sequence at the 3' end of the second strand cDNA strand and which second primer comprises one or more uracil residues, and the dNTP mix contains dUTP, so as to generate an amplified cDNA molecule(s), which comprises a plurality of uracil residues within the first and second strands of the amplified cDNA molecule.

Further rounds of amplification may be carried out, such as 20-25 cycles of PCR, so as to increase the cDNA copies.

To specifically amplify the ends of target genes, an asymmetric amplification reaction using a single gene specific primer and standard dNTPs (without dUTP) is performed. The reaction mixture is then treated with UDG to simplify the PCR reaction mixture by degrading non-target cDNAs. A subsequent amplification reaction using a nested gene specific primer in combination with a primer which is complimentary to an adapter sequence which has been incorporated into the 5' or 3' end of the cDNA, during cDNA synthesis, results in specific amplification of an end of the cDNA. This technique provides a novel and reliable method to specifically amplify the 5' and/or 3' ends of cDNAs from a single pool of cDNA.

Thus, in a further aspect, the present invention provides a method of amplifying a 5' or 3' end of an mRNA molecule for enabling identification of sequence at a respective 5' or 3' end of the mRNA molecule, the method comprising:

a) providing the double stranded cDNA molecule or pool of cDNA molecules according to the first aspect, or amplified copies thereof;

b) asymmetrically amplifying the 5' or 3' end of said cDNA molecule or one of said cDNA molecules in the pool using a gene specific primer which is substantially complementary to an internal portion of the cDNA sequence, using dNTPs which do not include dUTP, so as to generate an amplified gene specific single stranded DNA molecule;

c) adding UDG so as to degrade sequences which comprise uracil thereby leaving only an amplified gene specific single stranded DNA molecule;

d) optionally generating an amplified double stranded DNA gene specific product comprising a sequence corresponding to the 5' or 3' end of a specific mRNA molecule using a gene nested specific primer and a primer which comprises a 3' portion which is substantially complementary to the 5' or 3' end of the amplified gene specific single stranded DNA molecule.

Step d) is preferable in order to increase specificity and yield of the amplified 5' or 3' end to be detected

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The gene specific primers are generally comprised only of DNA bases (i.e. A, C, G and T) and may be designed to hybridise specifically to a desired mRNA sequence. The gene specific primers may be designed and synthesised based on a known mRNA sequence, or may be based on a consensus sequence ascertained from previously sequenced gene sequences from other organisms.

The skilled addressee is very familiar with asymmetric amplification or PCR and a number of cycles (e.g. 10-40) may be performed to linearly amplify the sequence of

interest and hence the 5' or 3' end of the cDNA. It is to be appreciated that the gene specific primer can be chosen to anneal to either particular strand of the modified cDNA molecule and hence generate amplified copies of the 5' or 3' end of a particular mRNA, as desired.

UDG is added so as to degrade the modified cDNA molecule(s) present once the asymmetric amplification step has been completed. Thus only the amplified single stranded DNA molecule which includes a complimentary DNA copy representative of the desired 5' or 3' end of the mRNA molecule remains as a viable target for a second exponential PCR amplification.

Exponential PCR amplification of the desired amplified single stranded DNA molecule is achieved using a second nested gene specific primer and a primer which is able to specifically bind to the sequence at the 5' or 3' end of the amplified single stranded DNA molecule. Further rounds of amplification may be carried out as necessary.

Preferably the 5' end of the mRNA molecule is amplified.

Once an amplified gene specific product comprising a sequence corresponding to the 5' or 3' end of an mRNA molecule has been generated, the appropriately amplified 5' or 3' end sequence can easily be ascertained by conventional cloning and gene sequencing techniques known to the skilled addressee, utilizing a suitable primer.

As well as the methods described hereinabove, the present invention also provides a kit for use in generating the modified cDNA molecule(s) and/or the amplified DNA molecule which includes the amplified 5' or 3' end. A kit for generating suitable modified cDNA molecules may comprise first and second adapter molecules, a dNTP

mix which includes dUTP and first and second primer molecules for amplifying the modified cDNA molecule. A kit for generating an amplified DNA molecule which comprises a sequence corresponding to a 5' or 3' end of a mRNA molecule may comprise UDG and a primer for use in amplifying the 5' or 3' end. Such kits may also comprise other suitable reagents such as buffers, salts solutions and the like, as well as instructions for carrying out the methods as described hereinabove.

Detailed Description

The present invention will now be further described with reference to the figures which show:

Figure 1 shows a diagrammatic representation of a double stranded template (T-RACE cDNA) synthesis protocol according to the present invention. First strand cDNA synthesis using a dNTP mix which includes dUTP is primed using an oligo-dT adapter-A primer. After reaching the 5' end of the mRNA (dashed line), oligo(dC) is added to the end of the cDNA by the M-MLV reverse transcriptase. Base pairing between the oligo(dC) and the oligo(dG) of adapter-B occurs, and through the process of template switching, the reverse compliment of adapter-B (adapter B') is incorporated into the 3' end of the newly synthesised cDNA as previously described (5, 6). Adapter A and B both contain dUTP instead of dTTP. Adapter A and B serve as priming sites for PCR amplification using primers A and B, which also have dTTP replaced by dUTP. PCR is performed using a dNTP mix which contains dUTP, so the resulting double stranded cDNA (T-RACE ready cDNA) contains dUTP residues throughout and dUTP containing adapters at the 3' and 5' ends.

Figure 2 shows a diagrammatic representation of the 5' specific amplification of cDNA ends using T-RACE. T-RACE ready cDNA serves as template for an asymmetric PCR reaction using a single gene specific primer (GSP1) and standard dNTPs (without dUTP). This reaction produces single stranded target cDNAs which include the adapter sequence comprised of standard dNTPs. UDG treatment degrades all non-target cDNAs and adapters containing dUTP. Subsequent PCR using a nested gene specific primer 2 (GSP2) with the 5' T-RACE primer results in the specific amplification of target cDNA ends.

Figure 3 shows (A) Specific amplification of 5' cDNA ends from Atlantic salmon (Ss) and Zebrafish (Dr) fast muscle. Lane 1 STAC3 (Ss), Lane 2 IGF-I (Ss) Lane 3 CMYA5 (Dr), Lane 4 MAFbx (Ss), Lane 5 STAC3 (Ss). DNA size marker of 5000, 2000, 850, 400 and 200 bp is shown in Lane M. In all cases, a single amplicon was produced, except for STAC3 (Lane 1) where two alternative splice variants were obtained. 5' T-RACE successfully amplified IGF-I (Lane 2) which is expressed at low levels in Atlantic salmon muscle, and a long transcript, CMYA5 (Lane 3), which is over 6kb in length. To demonstrate that long amplicons can be amplified using the T-RACE method, we amplified ~1.9 kb from STAC3 (Lane 5). (B) To demonstrate that the single pool of cDNA can be used for both 5' and 3' amplification of cDNA ends, we amplified the 3' ends of Zebrafish CMYA5 (Lane 1) and Atlantic salmon MAFbx (Lane 2) and STAC3 (Lane 3). DNA size marker of 5000, 2000, 850, 400 and 200 bp is shown in Lane M.

Figure 4 shows for background purposes non-specific amplification of products without UDG treatment. To demonstrate the increased specificity obtained by UDG treatment, we performed the same reactions to those in figure 1A but without UDG treatment prior to thermal cycling.

MATERIALS AND METHODS

RNA extraction

Total RNA was extracted from 100mg of Atlantic salmon (*Salmo salar L.*) or zebrafish (*Danio rerio*) fast skeletal muscle tissue using Lysing matrix D (Qbiogene, Irvine, CA) with 1ml Tri Reagent (Sigma, Gillingham,Dorset, UK) and homogenised using a Fast Prep instrument (Qbiogene, Irvine, CA). Total RNA was quantified based on absorbance at 260nm. Genomic DNA contamination was removed by treatment with Turbo DNA-free (Ambion, Austin, Texas, USA), and the integrity of purified RNA confirmed by agarose gel electrophoresis.

T-RACE ready cDNA synthesis

The T-RACE ready protocol is depicted in figure 1 and the primers used listed in table 1.

First strand cDNA synthesis incorporating dUTP and 5' and 3' adapter sequences

First strand cDNA synthesis was performed using a modified dNTP mixture which contained dUTP and a MMLV reverse transcriptase. cDNA synthesis was primed with Adapter A (table 1) using 1µg total RNA. The RNA, Adapter A and dNTPs were incubated at 65 °C for 5 minutes to denature the RNA and then chilled on ice before addition of the remaining components in the following reaction mixture (20 µl): 2.5 uM Adapter A, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1.0 mM dUTP, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.1mM dTTP, 40 U RNAse inhibitor (RNase OUT, Invitrogen, Carlsbad, CA, USA), 200 U Superscript II (Invitrogen, Carlsbad, CA, USA) and incubated for 1 h at 42 °C. After incubation, 0.4 µl of 100 mM $MnCl_2$, and 1 µl of Adapter B (10 µM) was added to the reaction which was further

incubated for 15 min at 42°C. The reaction was then heat inactivated at 70 °C for 10 minutes. Degradation of the RNA in the RNA/cDNA hybrid was achieved by addition of 1µl RNAse H (Invitrogen, Carlsbad, CA, USA) and incubation at 37 °C for 15 minutes. The reaction was purified through a QIAquick PCR purification column and eluted in 30 µl elution buffer as per manufacturer's guidelines (Qiagen Inc., Chatsworth, CA, USA).

Second strand synthesis incorporating dUTP by PCR

Second strand synthesis was achieved by PCR using the priming sites incorporated at the 3' (Adapter B) and 5' (Adapter A) ends of the first strand cDNA. 2 µl of the purified first strand cDNA was used as template, and amplified in the following reaction (50 µl): 2.5 mM MgCl2, 1.0 mM dUTP, 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.1mM dTTP, 0.5 µM primer A, 0.5 µM primer B (table 1). The reaction mixture was mixed and heated to 95 °C before addition of 5 units of Taq DNA polymerase (Bioline, London, UK). The reaction mixture was then incubated at 95 °C for a further 2 minutes, followed by 20 cycles of 95 °C denaturing for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C 12 minutes. The reaction was then chilled on ice and purified through a QIAquick PCR purification column (Qiagen Inc., Chatsworth, CA, USA) and eluted in 100ul elution buffer as per manufacturer's guidelines. The purified double stranded cDNA, incorporating dUTP and 5' and 3' adapter sequences (T-RACE ready cDNA) serves as template for the following PCR reactions.

T-RACE PCR

The PCR reactions for the targeted amplification of cDNA ends are shown in figure 2, and the primers used listed in table 1.

Asymmetric PCR using standard dNTPs

Asymmetric PCR was performed using a gene specific primer (GSP1) and standard dNTPs (dATP, dTTP, dGTP, dCTP) in the following hot start 20 µl reaction: 0.5 µM GSP1, 2µl T-RACE ready cDNA, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl, 3 mM $MgCl_2$, 0.25 mM dNTPs. The reaction mixture was heated to 95 °C for 2.5 min before addition of 1 unit of Taq DNA polymerase (Bioline, London, UK), and then cycled 20 times with the following conditions: 95 °C 30 s, 60 °C 30 s, 72 °C 2 min. The reaction was chilled on ice, purified through a MinElute PCR purification column (Qiagen Inc., Chatsworth, CA, USA) and eluted in 11µl water. The length of time for the 72 °C extension step of the PCR should be varied according to expected amplicon length.

Uracil DNA glycosylase degradation of non target dUTP containing cDNAs

The entire asymmetric reaction is used in the following steps using a nested gene specific primer (GSP2), and the primers used for PCR do not contain dUTP. cDNAs incorporating dUTP were removed by UDG treatment to leave only those cDNAs synthesised with standard dNTPs during the asymmetric PCR. The following 17.8 µl reaction was performed: 10 µl asymmetric PCR product, 2 µl (2 units) Uracil DNA glycosylase, 2 µl 10 x PCR buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl), 1.2 µl 50 mM $MgCl_2$, 1 µl nested GSP2, 1µl of 10 µM T-RACE primer (table 1), 0.6 µl water, and incubated at 37 °C for 30 minutes. Depending on whether 3' or 5' cDNA ends are targeted, the 3' or 5' T-RACE primer is used for amplification.

Targeted amplification of cDNA ends (T-RACE PCR)

The UDG treated cDNA mixture was heated to 95 °C before addition of 2 µl 2.5 mM standard dNTPs and 0.2 µl (1unit) of Taq DNA polymerase (Bioline, London, UK). The reaction was denatured at 95 °C for 5 min followed by 35 cycles of 95 °C 30 s, 60 °C 30s, 72 °C 2 min, followed by a final extension of 7 min at 72 °C. The length of time for

the 72 °C extension step of the PCR should be varied according to expected amplicon length.

Cloning

PCR products were separated on a 1.2% (m/v) agarose gel, and the bands excised and purified using a QIAquick gel extraction kit (Qiagen Inc., Chatsworth, CA, USA). The PCR products were cloned into a T/A pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA) and transformation of chemically competent TOP10 *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA). Individual colonies were grown and plasmids purified using a QIAprep Spin Miniprep Kit (Qiagen Inc., Chatsworth, CA, USA). Sequencing was performed using T3 and T7 sequencing primers with Big Dye terminator v3.1 sequencing chemistry (Applied Biosystems, Foster City, CA, USA) at the University of Dundee Sequencing Facility.

Table 1. Primer and adapter sequences used for first and second strand cDNA synthesis and specific amplification of cDNA ends.

Primer	Sequence (5' – 3')	Application
Adapter A	AUAUCUCGAGUUCGCGCCGGAUCC$(T)_{25}$VN	cDNA synthesis and adapter-A incorporation
Adapter B	AUAUGCACUGCCGCGUCUGAGGGGGGGG	Cap finder adapter-B incorporation
Primer A	AUAUCUCGAGUUCGCGCCGGAU	2nd strand cDNA synthesis
Primer B	AUAUGCACUGCCGCGUCUGA	2nd strand cDNA synthesis
5' T-RACE primer	ATATGCACTGCCGCGTCTGA	5' Specific Amplification of cDNA ends

3' T-RACE primer	ATATCTCGAGTTCGCGCCGGAT	3' Specific amplification of cDNA ends

RESULTS AND DISCUSSION

We report the development of a novel method for acquiring the ends especially the 5' ends of cDNA molecules using a modification of previously published methods. Using this approach, we have identified the 5' ends of cDNA molecules expressed in muscle of zebrafish and Atlantic salmon (Fig. 3A). The improved method involves the production of double stranded cDNA incorporating dUTP, and the addition of 5' and 3' adapters which also contain dUTP (Fig. 1). By producing the second strand of cDNA, a priming site for a gene specific primer for 5' RACE is introduced, allowing for an asymmetric PCR reaction to be performed using standard dNTP mix. This asymmetric PCR using only a gene specific primer produces a cDNA fragment which includes the 5' adapter sequence, and is comprised of standard dNTPs (Fig. 2). Degradation of dUTP containing cDNA molecules by Uracil DNA glycosylase (UDG) treatment leaves only the molecules comprised of standard dNTPs primed by the gene specific primer (Fig. 2). This now serves as the template for PCR amplification using a nested gene specific primer and a 5' T-RACE primer, thus specifically amplifying the 5'end of the desired cDNA sequence.

To demonstrate the effectiveness of our approach, we amplified the 5' ends of several genes of differing lengths whose mRNAs are present in varying abundance (Fig. 3A). For example, we amplified the 5' ends of zebrafish *myospryn* (*CMYA5*), a gene over 6kb in length, Atlantic salmon *MAFbx*, and *insulin-like growth factor-I* (*IGF-I*) which is expressed at low levels in muscle (Fig 3A). We also demonstrated that long amplicons

can be generated using this method by amplifying the ~1.9 kb 5' end of *STAC3* (Fig. 3A). Using GSPs and the 5' T-RACE primer, in all cases we were able to obtain a single amplicon (except for *STAC3* where we identified two alternatively spliced transcripts) of the correct size (Fig. 3A), the identity of which was confirmed by sequencing. We also used the same cDNA pool to amplify the 3' ends for a number of genes (Fig. 3B), demonstrating that a single cDNA pool can be used for both 5' and 3' amplification of cDNA ends.

The current method has several advantages over existing methodologies. Standard RACE methods often result in non specific amplification giving false positive results (6, 7, 8). When cDNA is synthesised using oligo dT, the subsequent incorporation of the adapter sequence for 5' RACE (through terminal transferase tailing or cap finder methods) occurs in every cDNA molecule. Due to this, mispriming events during PCR will often lead to exponential amplification, and smears, rather than distinct bands are often obtained. The use of a nested PCR reaction should increase the specificity of the RACE reaction, however non-specific products are frequently obtained. The T-RACE method described herein degrades all of the non-target cDNA molecules, except for those that were primed by the gene specific primer and synthesised using standard dNTPs, thereby significantly simplifying the templates present in the PCR reaction. As the gene specific primer is used in an asymmetric PCR, where higher annealing temperatures are permitted, rather than during cDNA synthesis, the specificity of the reaction is increased. By using a nested primer in the second PCR reaction, specific amplification of the desired molecule is achieved.

During the development of this method, we made several changes to our original protocol which improved the efficiency of the reactions. Taq polymerase is less efficient at incorporating dUTP than dTTP (10) which could impact on the double

stranded cDNA synthesis. It was observed that a ratio of dUTP:dTTP of 8:1, improved the efficiency of the T-RACE reaction, but still resulted in sufficient degradation of non-target cDNAs to give specific amplification. We also observed an improvement in the specificity of the PCR reactions by adding the $MnCl_2$ and adapter-A after cDNA synthesis has completed. Presumably this modification decreases the chances of adapter-A priming cDNA synthesis during the first strand cDNA reaction which could lead to spurious amplification products in subsequent reactions. Performing all reactions as a hot-start (adding Taq DNA polymerase after reactions have reached 95°C) improved specificity and this could also be achieved using a commercially available hot-start enzyme. Originally our method did not use a PCR (exponential) amplification step during double stranded cDNA synthesis, and we were unable to amplify rare transcripts such as IGF-I from this template. This is likely to be due to the fact that the asymmetric PCR performed using standard dNTPs, which is critical to the success of this method, does not result in exponential amplification of products. However, performing 20 cycles of PCR for the second strand synthesis improved the outcomes for amplifying rare transcripts, most likely by greatly increasing the number of targets available for the asymmetric PCR. The use of Uracil DNA glycosylase and dUTP has been widely used to control PCR product carryover contamination (9, 10), and its effectiveness in simplifying 5'RACE reactions is clearly demonstrated here (Fig. 3A and 4). Nested PCR reactions, which were not treated with UDG prior to thermal cycling resulted in smears and non specific amplification products (Fig. 3), whereas treatment with UDG resulted in specific amplification (Fig. 3A).

The method described herein offers several advantages over other protocols that are currently in use. Using UDG treated PCR products as template results in specific amplification, which in most cases results in a single amplicon of the correct size. We

have now used this technique to amplify the 5' ends of several cDNAs, each one from the same T-RACE ready cDNA pool using total RNA as starting material.

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Claims

1. a method for generating a modified cDNA molecule comprising sequence corresponding to the 5' and 3' ends of an mRNA molecule comprising the steps of:

e) annealing a first adapter oligonucleotide to an mRNA molecule, said first adapter comprising a 3' portion which is substantially complimentary to the 3' poly A tail of the mRNA molecule and a 5' portion which comprises one or more uracil residues;

f) extending from said first adapter, utilizing dNTPs which include dUTP, so as to generate a first modified cDNA strand, which comprises one or more, typically a plurality of uracil residues in its sequence;

g) incorporating a second adapter, which is different to the first adapter, at the 3' end of the first cDNA strand, wherein the second adapter comprises at least a 3' portion which is not specific to the mRNA molecule and which comprises one or more uracil residues in the sequence;

h) copying said first modified cDNA strand by extending from a first primer which comprises one or more uracils and comprises at least a 3' portion which is substantially complementary to a portion of the second adapter sequence, using dNTPs which include dUTP, thereby generating a modified double stranded cDNA molecule, which includes one or more, typically a plurality of uracil residues in the first and second strands and adapter sequences 5' and 3' of sequence corresponding to the 5'and 3' ends of the mRNA molecule.

2. The method according to claim 1 wherein the method may be conducted on a pool of mRNA molecules, thereby generating a single pool of cDNA molecules, each cDNA molecule from the pool comprising the same 5' and 3' adapter sequences respectively.

3. The method according to either of claims 1 or 2 wherein at least 1 in 1, to 1 in 20, such as 1 in 1 to 1, in 4, especially 1 in 1.05 to 1 in 2 thymidine residues of the cDNA are replaced by a uracil residue.

4. The method according to any preceding claim wherein the first adapter oligonucleotide comprises a 3' portion which comprises a stretch of T residues, so as to be able to complimentary base pair with the poly A tail of the mRNA molecule.

5. The method according to any preceding claim wherein the extension reaction is catalysed by a reverse transcriptase, such as avian myeloblastosis virus (AMV) or Moloney murine leukaemia virus (M-MLV).

6. The method according to claim 5 wherein the reverse transcriptase is MMLV and the second adapter is added using a cap switching technique.

7. The method according to claim 5 wherein the second adapter is added using terminal transferase or RNA ligase.

8. The method according to any preceding claim wherein the second cDNA strand is generated by copying the first cDNA strand using a first primer (where uracil replaces thymidine) which is able to specifically bind to the second adapter sequence at 3' end of the first cDNA strand, using a suitable DNA polymerase, such as Taq polymerase and a dNTP mix comprising dUTP.

9. A method of amplifying a 5' or 3' end of an mRNA molecule for enabling identification of sequence at a respective 5' or 3' end of the mRNA molecule, the method comprising:

e) providing the double stranded cDNA molecule or pool of cDNA molecules according to any preceding claim, or amplified copies thereof;

f) asymmetrically amplifying the 5' or 3' end of said cDNA molecule or one of said cDNA molecules in the pool using a gene specific primer which is substantially complementary to an internal portion of the cDNA sequence, using dNTPs which do not include dUTP, so as to generate an amplified gene specific single stranded DNA molecule;

g) adding UDG so as to degrade sequences which comprise uracil thereby leaving only an amplified gene specific single stranded DNA molecule;

h) optionally generating an amplified double stranded DNA gene specific product comprising a sequence corresponding to the 5' or 3' end of a specific mRNA molecule using a gene nested specific primer and a primer which comprises a 3' portion which is substantially complementary to the 5' or 3' end of the amplified gene specific single stranded DNA molecule.

10. The method according to claim 9 wherein **[step?]** (d) is included.

11. The method according to claims 9 or 10 wherein exponential amplification of the desired amplified single stranded DNA molecule is achieved using a second nested

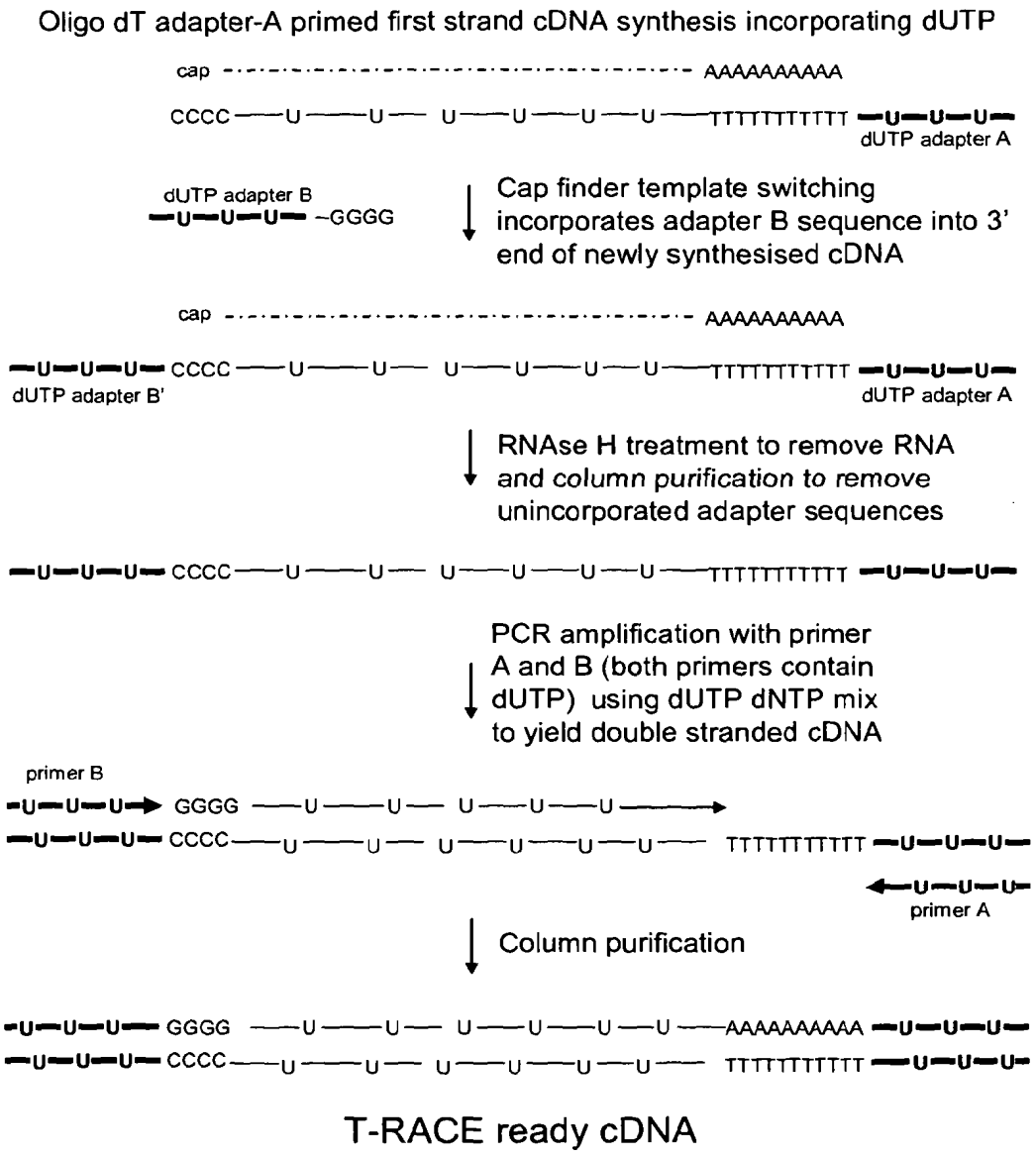

Oligo dT adapter-A primed first strand cDNA synthesis incorporating dUTP
cap
AAAAAAAAAA
CCCC
TTTTTTTTTTT
dUTP adapter A
dUTP adapter B
–GGGG
Cap finder template switching incorporates adapter B sequence into 3' end of newly synthesised cDNA
dUTP adapter B'
RNAse H treatment to remove RNA and column purification to remove unincorporated adapter sequences
PCR amplification with primer A and B (both primers contain dUTP) using dUTP dNTP mix to yield double stranded cDNA
primer B
GGGG
primer A
Column purification
T-RACE ready cDNA

Figure 1

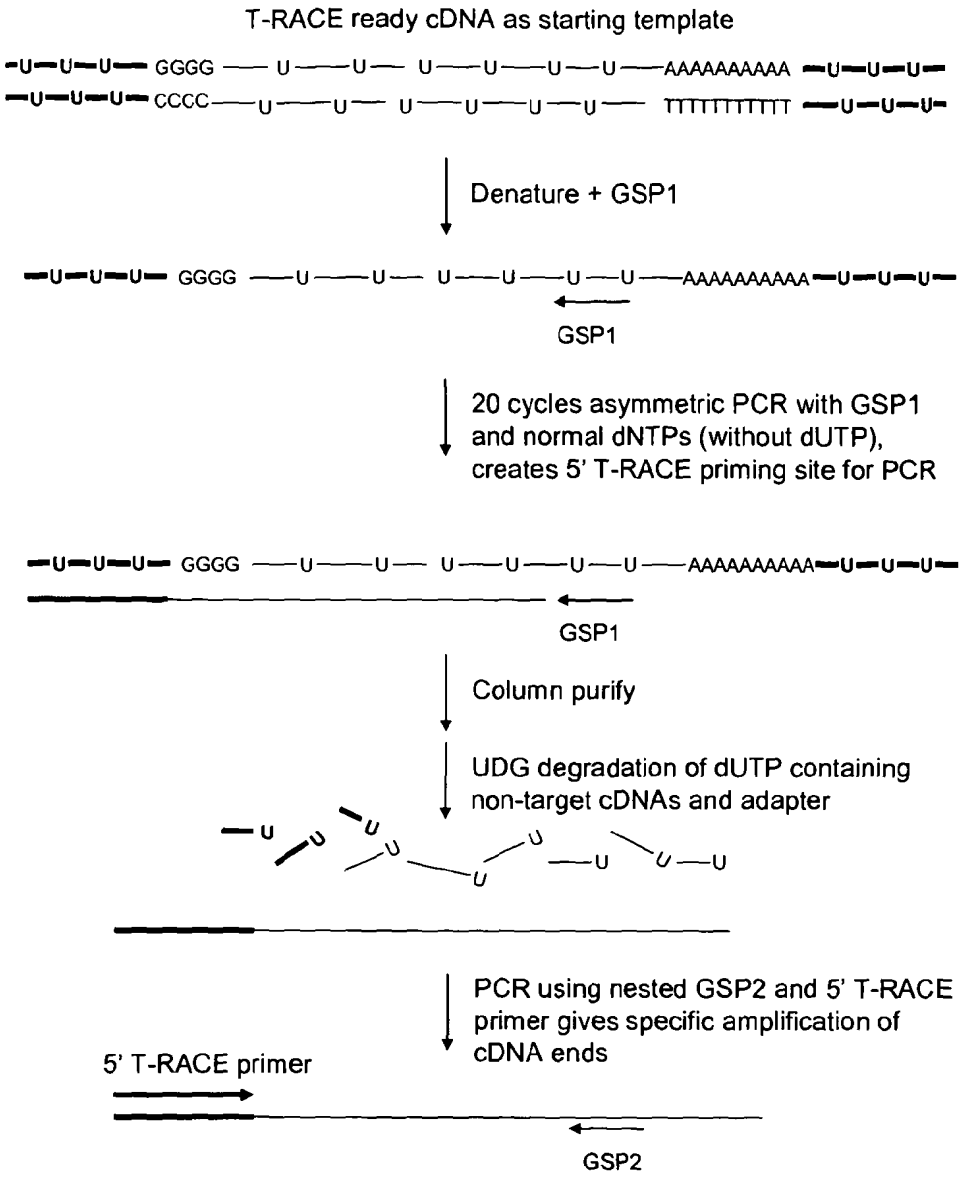

T-RACE ready cDNA as starting template
GGGG
AAAAAAAAAA
CCCC
TTTTTTTTTT
Denature + GSP1
GSP1
20 cycles asymmetric PCR with GSP1 and normal dNTPs (without dUTP), creates 5' T-RACE priming site for PCR
GSP1
Column purify
UDG degradation of dUTP containing non-target cDNAs and adapter
PCR using nested GSP2 and 5' T-RACE primer gives specific amplification of cDNA ends
5' T-RACE primer
GSP2

Figure 2

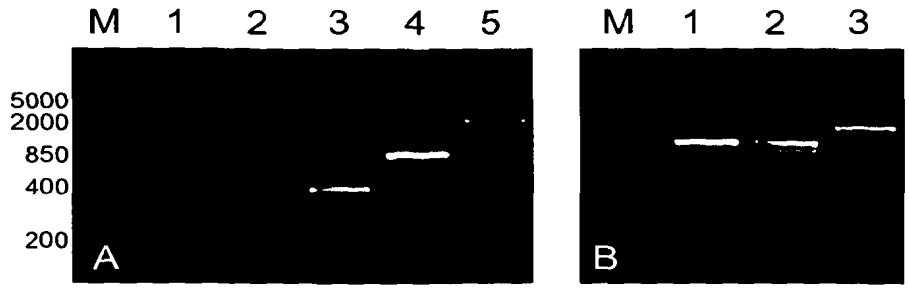


Figure 3

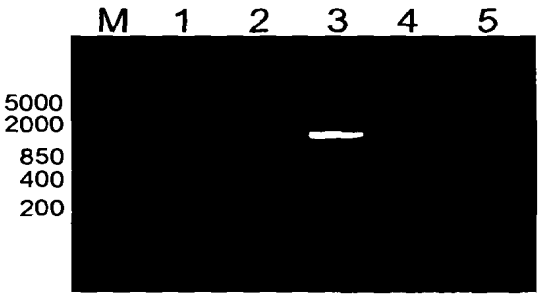


Figure 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2011/001131

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/10
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBASE, BIOSIS, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHRAMM G ET AL: "A simple and reliable 5'-RACE approach.", NUCLEIC ACIDS RESEARCH, vol. 28, no. 22, E96, 15 November 2000 (2000-11-15), pages 1-4, XP002661255, ISSN: 1362-4962 cited in the application figure 1 ----- -/--	1-10

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

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 October 2011	15/11/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Weinberg, Suzanna

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Form PCT/ISA/210 (second sheet) (April 2005)

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2011/001131

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 11
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 11

Claim 11 is incomplete and hence lacks clarity to the extent that no additional feature can be determined, which feature can be searched.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2) declaration be overcome.

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LONGO M C ET AL: "Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions", GENE, vol. 93, no. 1, 1 September 1990 (1990-09-01), pages 125-128, XP023545615, ELSEVIER, AMSTERDAM, NL ISSN: 0378-1119, DOI: 10.1016/0378-1119(90)90145-H [retrieved on 1990-09-01] cited in the application page 126, left-hand column	1-10
X,P	BOWER NEIL I ET AL: "Targeted rapid amplification of cDNA ends (T-RACE)--an improved RACE reaction through degradation of non-target sequences.", NUCLEIC ACIDS RESEARCH, vol. 38, no. 21, E194, November 2010 (2010-11), pages 1-7, XP002661009, ISSN: 1362-4962, DOI: 10.1093/nar/gkq816 the whole document	1-10
A	BUCHMAN G W ET AL: "Selective RNA amplification: a novel method using dUMP-containing primers and uracil DNA glycosylase.", PCR METHODS AND APPLICATIONS, vol. 3, no. 1, August 1993 (1993-08), pages 28-31, XP002661010, ISSN: 1054-9803 cited in the application figure 1	1-10
A	WO 92/18521 A1 (LIFE TECHNOLOGIES INC [US]) 29 October 1992 (1992-10-29) page 22 - page 23; figure 4	1-10
A	SCHMIDT W M ET AL: "CapSelect: a highly sensitive method for 5' CAP-dependent enrichment of full-length cDNA in PCR-mediated analysis of mRNAs.", NUCLEIC ACIDS RESEARCH, vol. 27, no. 21, E31, 1 November 1999 (1999-11-01), pages I-IV, XP002661269, ISSN: 1362-4962 cited in the application abstract; figure 1	1-10

4

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2011/001131

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9218521 A1	29-10-1992	US 5334515 A	02-08-1994

Form PCT/ISA/210 (patent family annex) (April 2005)

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PCT/US2015/066949

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(26) Publication Language: English

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62/198,097 28 July 2015 (28.07.2015) US

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(74) Agents: **ROY, Madhuri** et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

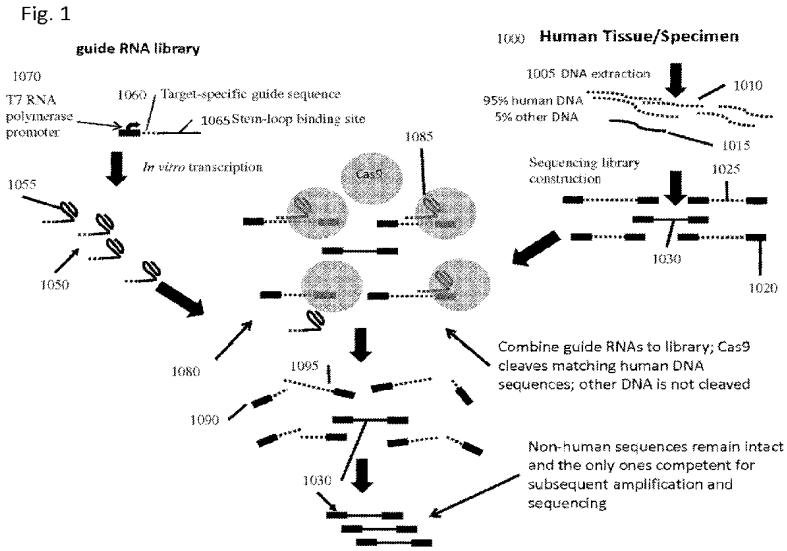
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

(54) **Title**: COMPOSITIONS AND METHODS FOR TARGETED DEPLETION, ENRICHMENT, AND PARTITIONING OF NUCLEIC ACIDS USING CRISPR/CAS SYSTEM PROTEINS

Fig. 1



(57) **Abstract**: Provided herein are methods and compositions for depleting targeted nucleic acid sequences from a sample, enriching for sequences of interest from a sample, and/or partitioning of sequences from a sample. The methods and compositions are applicable to biological, clinical, forensic, and environmental samples.

WO 2016/100955 A2

COMPOSITIONS AND METHODS FOR TARGETED DEPLETION, ENRICHMENT, AND PARTITIONING OF NUCLEIC ACIDS USING CRISPR/CAS SYSTEM PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. Provisional Application Serial No. 62/094,980, filed on December 20, 2014, and of U.S. Provisional Application Serial No. 62/198,097, filed on July 28, 2015. The contents of these applications are hereby incorporated by reference herein in their entireties.

BACKGROUND OF THE INVENTION

[0002] Many human clinical DNA samples, or sample libraries such as cDNA libraries derived from RNA, or extracted DNA samples taken from tissue, fluids, or other host material samples contain highly abundant sequences that have little informative value and increase the cost of sequencing. While methods have been developed to deplete these sequences (e.g., via hybridization capture), these methods are often time-consuming and can be inefficient. Moreover hybridization capture often looks to capture the DNA sequences of interest while discarding the remaining sequences. As a result depletion by hybridization capture is not a viable option when the DNA sequences of interest are not known in advance, e.g. when screening a sample to study all microbial or non-host DNA sequences.

[0003] While shotgun sequencing of human samples to study microbial DNA can be done, low levels of microbial DNA in many samples has precluded the shotgun sequencing of many complex and/or interesting samples, due to cost. This is true of, for example, a metagenomic analysis of a sample, where the sample contains more than one species of organism (eukaryotic, prokaryotic, or viral organisms). For example, DNA libraries derived from whole human blood often contain >99% human DNA. Therefore, to detect an infectious agent circulating in human blood from shotgun sequencing, one would need to sequence to very high coverage in order to ensure sufficient coverage. Thus much of the cost associated with sequencing high human DNA samples provides relatively little metagenomic data. As a result many human tissue DNA samples are considered unsuitable for metagenomic sequencing merely because the data yield is low compared to the resources required. Thus there is a need in the art to increase microbial DNA yield in high host DNA samples and

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specifically to increase the percent of microbial DNA being sequenced when sequencing high host endogenous (HHE) DNA samples.

[0004] Recent developments in DNA extraction have provided some sequencing techniques to the point that the field of metagenomics has transitioned from focusing on PCR-amplified 16S ribosomal RNA markers to shotgun sequencing of the whole metagenome. However, shotgun sequencing can yield less than desirable results when sequencing HHE DNA samples due to the low percentage of microbial DNA in the overall sample material. Moreover, shotgun sequencing often fails to provide enough information to make an accurate resolution in metagenomic analysis especially when the selected molecules (e.g., 16S ribosomal RNA) represent only a single lineage. Furthermore, 16S ribosomal RNA lineages cannot often differentiate pathogenic from non-pathogenic strains of closely related bacteria, a key goal of clinical metagenomic analysis.

[0005] Instead the use of whole genome DNA and RNA sequences is preferred for metagenetic analysis because it provides information from the entire metagenome. Thus there is a need in the art to provide a DNA and RNA sequencing technique for metagenomic analysis in order to derive improved resolution. For example, whole genome analysis of metagenomes from the fecal material of obese and normal weight patients has revealed highly reproducible differences in microbial community structure. These materials tend to have very high microbial DNA content (>99% microbe and <1% human).

[0006] In contrast, sequencing libraries derived from many other tissues including human blood, vagina, nasal mucosal membrane, and lung typically contain >90% human and <10% microbial DNA. While samples with <10% microbial DNA can still, with sufficient sequencing, yield enough information for metagenomic analyses, the required amount of sequencing of specimens with less target DNA is costly and thus untenable for many researchers.

[0007] Thus there exists a need in the art to achieve a low-cost, efficient method and compositions for metagenomic analyses. Such methods and compositions are provided herein.

[0008] All patents, patent applications, publications, documents, web links, and articles cited herein are incorporated herein by reference in their entireties.

BRIEF SUMMARY OF THE INVENTION

[0009] Provided herein are methods and compositions for depleting targeted nucleic acid sequences from a sample, enriching for sequences of interest from a sample, and/or

partitioning of sequences from a sample. The methods and compositions are applicable to biological, clinical, forensic, and environmental samples.

[0010] In one aspect, provided herein is a method of enriching a sample for sequences of interest, comprising: (a) providing a sample comprising sequences of interest and targeted sequences for depletion, wherein the sequences of interest comprise less than 30% of the sample; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to the targeted sequences, and whereby the targeted sequences are cleaved. In any of the embodiments disclosed herein, the method further comprises extracting the sequences of interest and the targeted sequences for depletion from the sample. In any of the embodiments disclosed herein, the method further comprises fragmenting the extracted sequences. In any of the embodiments disclosed herein, the method further comprises adapter ligating the 5' and 3' ends of the fragmented extracted sequences. In any of the embodiments disclosed herein, the cleaved targeted sequences are removed by size-exclusion. In any of the embodiments disclosed herein, the cleaved targeted sequences are removed with the use of biotin. In any of the embodiments disclosed herein, the method further comprises amplifying the sequences of interest. In any of the embodiments disclosed herein, the targeted sequences for depletion are followed by a Protospacer Adjacent Motif or (PAM) sequence. In any of the embodiments disclosed herein, the method comprises contacting the sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes. In any of the embodiments disclosed herein, the sample is any one of a biological sample, a clinical sample, a forensic sample or an environmental sample. In any of the embodiments disclosed herein, the sample comprises host nucleic acid sequences targeted for depletion and non-host nucleic acid sequences of interest. In any of the embodiments disclosed herein, the non-host nucleic acid sequences comprise microbial nucleic acid sequences. In any of the embodiments disclosed herein, the microbial nucleic acid sequences are bacterial, viral or eukaryotic parasitic nucleic acid sequences. In any of the embodiments disclosed herein, the sample is contacted with CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to DNA corresponding to ribosomal RNA sequences. In any of the embodiments disclosed herein, the sample is contacted with CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to mitochondrial DNA. In any of the embodiments disclosed herein, the sample is contacted with CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to sequences encoding globin proteins, sequences encoding a transposon, sequences encoding retroviral sequences, sequences comprising telomere sequences,

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sequences comprising sub-telomeric repeats, sequences comprising centromeric sequences, sequences comprising intron sequences, sequences comprising Alu repeats, sequences comprising SINE repeats, sequences comprising LINE repeats, sequences comprising dinucleic acid repeats, sequences comprising trinucleic acid repeats, sequences comprising tetranucleic acid repeats, sequences comprising poly-A repeats, sequences comprising poly-T repeats, sequences comprising poly-C repeats, sequences comprising poly-G repeats, sequences comprising AT -rich sequences, or sequences comprising GC-rich sequences. In any of the embodiments disclosed herein, the extracted nucleic acids incudes any one of single stranded DNA, double stranded DNA, single stranded RNA, double stranded RNA, cDNA, synthetic DNA, artificial DNA, and DNA/RNA hybrids. In any of the embodiments disclosed herein, the sequences of interest comprise less than 10% of the extracted nucleic acids.In any of the embodiments disclosed herein, the sequences of interest comprise less than 5% of the extracted nucleic acids. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is Cas9, Cpf1, Cas3, Cas8a-c, Cas10, Cse1, Csy1, Csn2, Cas4, Csm2, or Cm5. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is Cas9. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is catalytically dead, for example the catalytically dead CRISPR/Cas system protein is dCas9. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is a CRISPR/Cas system protein nickase, for example Cas9 nickase. In any of the embodiments disclosed herein, the CRISPR/Cas System protein is thermostable. In any of the embodiments disclosed herein, method further comprises amplifying the product of step (b) using adapter-specific PCR. In any of the embodiments disclosed herein, method further comprises treating the product of step (b) with an enzyme that has exonuclease activity. In any of the embodiments disclosed herein, the enzyme is Exonuclease III or BAL-31. In any of the embodiments disclosed herein, method further comprises including the use of positive control target sequences. In any of the embodiments disclosed herein, method further comprises including the use of negative control gRNAs. In any of the embodiments disclosed herein, the sample is selected from whole blood, plasma, serum, tears, saliva, mucous, cerebrospinal fluid, teeth, bone, fingernails, feces, urine, tissue, and a biopsy.

[0011] In another aspect, provided herein is a method of enriching a sample comprising: (a) providing a sample comprising mitochondrial DNA and non-mitochondrial DNA, wherein the mitochondrial DNA and non-mitochondrial DNA are adapter-ligated, and wherein the adapters are ligated to the 5' and 3' ends of the mitochondrial DNA and non-mitochondrial DNA; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA

complexes, wherein the gRNAs are complementary to the mitochondrial DNA, thereby generating mitochondrial DNA adapter ligated only on one end and non-mitochondrial DNA adapter ligated on both the 5' and 3' ends; and (c) enriching the sample for non-mitochondrial DNA. In any one of the embodiments disclosed herein, step (c) comprises amplifying the product of step (b) using adapter-specific PCR. In any of the embodiments disclosed herein, step (c) comprises treating the product of step (b) with an enzyme that has exonuclease activity. In any of the embodiments disclosed herein, the enzyme is Exonuclease III or BAL-31. In any of the embodiments disclosed herein, the method is carried out following a method for analyzing genomic DNA comprising treating DNA isolated from a population of cells with an insertional enzyme to produce a plurality of tagged fragments of non-mitochondrial genomic DNA, whereby also generating a residual amount of tagged mitochondrial DNA. In any of the embodiments disclosed herein, the method further comprises extracting the mitochondrial DNA and non-mitochondrial DNA from the sample. In any of the embodiments disclosed herein, the method further comprises fragmenting the extracted sequences. In any of the embodiments disclosed herein, the mitochondrial DNA is removed by size-exclusion. In any of the embodiments disclosed herein, the mitochondrial DNA is removed with the use of biotin. In any of the embodiments disclosed herein, the enriching the sample comprises amplifying the non-mitochondrial DNA. In any of the embodiments disclosed herein, the method comprises contacting the sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is Cas9, Cpf1, Cas3, Cas8a-c, Cas10, Cse1, Csy1, Csn2, Cas4, Csm2, or Cm5. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is Cas9. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is catalytically dead, for example dCas9. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is a CRISPR/Cas system protein nickase, for example Cas9 nickase. In any of the embodiments disclosed herein, the CRISPR/Cas System protein is thermostable. In any of the embodiments disclosed herein, the enriching comprises amplifying the product of step (b) using adapter-specific PCR. In any of the embodiments disclosed herein, the enriching comprises treating the product of step (b) with an enzyme that has exonuclease activity. In any of the embodiments disclosed herein, the enzyme is Exonuclease III or BAL-31.In any of the embodiments disclosed herein, the method further comprises including the use of positive control target sequences. In any of the embodiments disclosed herein, the method further comprises including the use of negative control gRNAs. In any of the embodiments disclosed herein, the sample is selected from whole blood,

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plasma, serum, tears, saliva, mucous, cerebrospinal fluid, teeth, bone, fingernails, feces, urine, tissue, and a biopsy.

[0012] In another aspect, provided herein is a method of enriching a sample comprising: (a) providing a sample comprising nucleic acids from a first genome and nucleic acids from a second genome, wherein the nucleic acids from the first genome are adapter-ligated on their 5' and 3' ends and wherein the nucleic acids from the second genome are adapter-ligated on their 5' and 3' ends; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to sites targeted in the nucleic acids from the first genome, thereby generating nucleic acids from the first genome adapter ligated only on one end and nucleic acids from the second genome adapter ligated on both the 5' and 3' ends; and (c)enriching the sample for nucleic acids from the second genome. In any of the embodiments disclosed herein, the sample further comprises nucleic acids from additional genomes. In any of the embodiments disclosed herein, the first genome is a host genome, and the second genome is a non-host genome. In any of the embodiments disclosed herein, the method comprises contacting the sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes. In any of the embodiments disclosed herein, the nucleic acids are selected from the group consisting of single stranded DNA, single stranded RNA, double stranded DNA, double stranded RNA, DNA/RNA hybrids, cDNA, synthetic DNA, and artificial DNA. In any of the embodiments disclosed herein, the nucleic acids are double stranded DNA. In any of the embodiments disclosed herein, the nucleic acids are DNA, and wherein the contacting of step (b) generates first genome- DNA adapter ligated on the 5' end but not the 3' end and second genome DNA adapter ligated on both the 5' and 3' ends. In any of the embodiments disclosed herein, the nucleic acids are DNA. In any of the embodiments disclosed herein, the DNA is genomic DNA. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is Cas9, Cpf1, Cas3, Cas8a-c, Cas10, Cse1, Csy1, Csn2, Cas4, Csm2, or Cm5. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is Cas9. In any of the embodiments disclosed herein, the targeted sites in the first genome nucleic acids are followed by a Protospacer Adjacent Motif or (PAM) sequence that can be bound by Cas9. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is catalytically dead, for example dCas9. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is a CRISPR/Cas system protein nickase, for example Cas9 nickase. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is thermostable. In any of the embodiments disclosed herein, the first genome is from an organism selected from the group consisting of a human, cow, horse,

sheep, pig, monkey, dog, cat, gerbil, bird, mouse, and rat. In any of the embodiments disclosed herein, the second genome is from a prokaryotic organism. In any of the embodiments disclosed herein, the second genome is from an eukaryotic organism. In any of the embodiments disclosed herein, the second genome is from a parasite. In any of the embodiments disclosed herein, the second genome is from a virus, bacterial, fungus, or protozoa. In any of the embodiments disclosed herein, the adapter-ligated first genome nucleic acids and adapter-ligated second genome nucleic acids range from 50-1000bp. In any of the embodiments disclosed herein, the second genome nucleic acids comprise less than 50% of the total nucleic acids in the sample. In any of the embodiments disclosed herein, the second genome nucleic acids comprise less than 5% of the total nucleic acids in the sample. In any of the embodiments disclosed herein, the sample is any one of a biological sample, a clinical sample, a forensic sample or an environmental sample. In any of the embodiments disclosed herein, step (c) comprises amplifying the product of step (b) using adapter-specific PCR. In any of the embodiments disclosed herein, step (c) comprises treating the product of step (b) with an enzyme that has exonuclease activity. In any of the embodiments disclosed herein, the enzyme is Exonuclease III or BAL-31. In any of the embodiments disclosed herein, step (c) comprises removing the first genome nucleic acids by size-exclusion. In any of the embodiments disclosed herein, step (c) comprises removing the first genome nucleic acids with the use of biotin. In any of the embodiments disclosed herein, the method further comprises amplifying the product of step (b) using adapter-specific PCR. In any of the embodiments disclosed herein, the method further comprises treating the product of step (b) with an enzyme that has exonuclease activity. In any of the embodiments disclosed herein, the enzyme is Exonuclease III or BAL-31. In any of the embodiments disclosed herein, the method further comprises including the use of positive control target sequences. In any of the embodiments disclosed herein, the method further comprises including the use of negative control gRNAs. In any of the embodiments disclosed herein, the sample is selected from whole blood, plasma, serum, tears, saliva, mucous, cerebrospinal fluid, teeth, bone, fingernails, feces, urine, tissue, and a biopsy.

[0013] In another aspect, provided herein is a method of enriching a sample comprising: (a) providing a sample comprising host nucleic acids and non-host nucleic acids, wherein the host nucleic acids and non-host nucleic acids are adapter-ligated, and wherein the adapters are ligated to the 5' and 3' ends of the host nucleic acids and the non-host nucleic acids; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to targeted sites in the host nucleic acids, thereby

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generating host- nucleic acids adapter ligated only on one end and non-host nucleic acids adapter ligated on both the 5' and 3' ends; and (c) enriching the sample for non-host nucleic acids. In any of the embodiments disclosed herein, the method comprises contacting the sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes. In any of the embodiments disclosed herein, the nucleic acids are selected from the group consisting of single stranded DNA, single stranded RNA, double stranded DNA, double stranded RNA, DNA/RNA hybrids, cDNA, synthetic DNA, and artificial DNA. In any of the embodiments disclosed herein, the nucleic acids are double stranded DNA. In any of the embodiments disclosed herein, the nucleic acids are DNA, and wherein the contacting of step (b) generates host- DNA adapter ligated on the 5' end but not the 3' end and non-host DNA adapter ligated on both the 5' and 3' ends. In any of the embodiments disclosed herein, the nucleic acids are DNA. In any of the embodiments disclosed herein, the DNA is genomic DNA. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is Cas9, Cpf1, Cas3, Cas8a-c, Cas10, Cse1, Csy1, Csn2, Cas4, Csm2, or Cm5. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is Cas9. In any of the embodiments disclosed herein, the targeted sites in the host nucleic acids are followed by a Protospacer Adjacent Motif or (PAM) sequence that can be bound by Cas9. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is catalytically dead, for example dCas9. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is a CRISPR/Cas system protein nickase, for example Cas9 nickase. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is thermostable. In any of the embodiments disclosed herein, the host is selected from the group consisting of a human, cow, horse, sheep, pig, monkey, dog, cat, gerbil, bird, mouse, and rat. In any of the embodiments disclosed herein, the non-host is a prokaryotic organism. In any of the embodiments disclosed herein, the non-host is a eukaryotic organism. In any of the embodiments disclosed herein, the non-host is a parasite. In any of the embodiments disclosed herein, the non-host is selected from the group consisting of a virus, bacterial, fungus, and protozoa. In any of the embodiments disclosed herein, the adapter-ligated host nucleic acids and non-host nucleic acids range from 50-1000bp. In any of the embodiments disclosed herein, the non-host nucleic acids comprise less than 50% of the total nucleic acids in the sample. In any of the embodiments disclosed herein, the non-host nucleic acids comprise less than 5% of the total nucleic acids in the sample. In any of the embodiments disclosed herein, the sample is any one of a biological sample, a clinical sample, a forensic sample or an environmental sample. In any of the embodiments disclosed herein, step (c) comprises amplifying the product of step (b) using adapter-specific

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PCR. In any of the embodiments disclosed herein, step (c) comprises treating the product of step (b) with an enzyme that has exonuclease activity. In any of the embodiments disclosed herein, the enzyme is Exonuclease III or BAL-31. In any of the embodiments disclosed herein, step (c) comprises removing the host nucleic acids by size-exclusion. In any of the embodiments disclosed herein, step (c) comprises removing the host nucleic acids with the use of biotin. In any of the embodiments disclosed herein, the method further comprises amplifying the product of step (b) using adapter-specific PCR. In any of the embodiments disclosed herein, the method further comprises treating the product of step (b) with an enzyme that has exonuclease activity. In any of the embodiments disclosed herein, the enzyme is Exonuclease III or BAL-31. In any of the embodiments disclosed herein, the method further comprises including the use of positive control target sequences. In any of the embodiments disclosed herein, the method further comprises including the use of negative control gRNAs. In any of the embodiments disclosed herein, the sample is selected from whole blood, plasma, serum, tears, saliva, mucous, cerebrospinal fluid, teeth, bone, fingernails, feces, urine, tissue, and a biopsy.

[0014] In another aspect, provided herein is a method of depleting targeted sequences in a sample comprising:(a) providing a sample comprising sequences of interest and targeted sequences for depletion; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to the targeted sequences, thereby generating cleaved targeted sequences; and (c) contacting the product of step (b) with an enzyme that has exonuclease activity. In any of the embodiments disclosed herein, the enzyme is Exonuclease III or BAL-31. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is Cas9. In any of the embodiments disclosed herein, the targeted sequences for depletion comprise sequences encoding globin proteins, sequences encoding a transposon, sequences encoding retroviral sequences, sequences comprising telomere sequences, sequences comprising sub-telomeric repeats, sequences comprising centromeric sequences, sequences comprising intron sequences, sequences comprising Alu repeats, sequences comprising SINE repeats, sequences comprising LINE repeats, sequences comprising dinucleic acid repeats, sequences comprising trinucleic acid repeats, sequences comprising tetranucleic acid repeats, sequences comprising poly-A repeats, sequences comprising poly- T repeats, sequences comprising poly-C repeats, sequences comprising poly-G repeats, sequences comprising AT -rich sequences, or sequences comprising GC-rich sequences. In any of the embodiments disclosed herein, the method comprises contacting the sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes. In any of the

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embodiments disclosed herein, the sample is selected from whole blood, plasma, serum, tears, saliva, mucous, cerebrospinal fluid, teeth, bone, fingernails, feces, urine, tissue, and a biopsy.

[0015] In another aspect, provided herein is a method of generating cleaved targeted sequences in a sample comprising:(a) providing a sample comprising sequences of interest and targeted sequences for cleavage; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to the targeted sequences, thereby generating cleaved targeted sequences; (c) dissociating the CRISPR/Cas system protein from the cleaved targeted sequences; (d) generating additional cleaved targeted sequences; and (e) recovering the uncut sequences of interest. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is thermostable. In any of the embodiments disclosed herein, the thermostable CRISPR/Cas system protein is thermostable Cas9. In any of the embodiments disclosed herein, the dissociating of the CRISPR/Cas system protein from the cleaved targeted sequences is achieved by elevating the temperature of the mixture of step (b) to at least 75°. In any of the embodiments disclosed herein, the generating of additional cleaved targeted sequences is achieved by lowering the temperature of the mixture of step (b) to at least 50°. In any of the embodiments disclosed herein, step (e) comprises amplifying the product of step (d) using adapter-specific PCR. In any of the embodiments disclosed herein, step (e) comprises treating the product of step (d) with an enzyme that has exonuclease activity. In any of the embodiments disclosed herein, the enzyme is Exonuclease III or BAL-31. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is Cas9. In any of the embodiments disclosed herein, the targeted sequences for cleavage comprise sequences encoding globin proteins, sequences encoding a transposon, sequences encoding retroviral sequences, sequences comprising telomere sequences, sequences comprising sub-telomeric repeats, sequences comprising centromeric sequences, sequences comprising intron sequences, sequences comprising Alu repeats, sequences comprising SINE repeats, sequences comprising LINE repeats, sequences comprising dinucleic acid repeats, sequences comprising trinucleic acid repeats, sequences comprising tetranucleic acid repeats, sequences comprising poly-A repeats, sequences comprising poly- T repeats, sequences comprising poly-C repeats, sequences comprising poly-G repeats, sequences comprising AT -rich sequences, or sequences comprising GC-rich sequences. In any of the embodiments disclosed herein, the method comprises contacting the sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes. In any of the

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embodiments disclosed herein, the sample is selected from whole blood, plasma, serum, tears, saliva, mucous, cerebrospinal fluid, teeth, bone, fingernails, feces, urine, tissue, and a biopsy.

[0016] In another aspect, provided herein is a method of depleting targeted sequences in a sample comprising:(a)providing a sample comprising sequences of interest and targeted sequences for cleavage; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to the targeted sequences, thereby generating cleaved targeted sequences, and wherein the CRISPR/Cas system protein is thermostable; (c) elevating the temperature of the mixture of step (b) to at least 75°; (d) lowering the temperature of the mixture of step (b) at least 50°; (e) repeating steps (c) and (d) at least once; and (f) recovering the uncut sequences of interest. In any of the embodiments disclosed herein, step (f) comprises amplifying the product of step (e) using adapter-specific PCR. In any of the embodiments disclosed herein, step (f) comprises treating the product of step (e) with an enzyme that has exonuclease activity. In any of the embodiments disclosed herein, the enzyme is Exonuclease III or BAL-31. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is Cas9. In any of the embodiments disclosed herein, the targeted sequences for cleavage comprise sequences encoding globin proteins, sequences encoding a transposon, sequences encoding retroviral sequences, sequences comprising telomere sequences, sequences comprising sub-telomeric repeats, sequences comprising centromeric sequences, sequences comprising intron sequences, sequences comprising Alu repeats, sequences comprising SINE repeats, sequences comprising LINE repeats, sequences comprising dinucleic acid repeats, sequences comprising trinucleic acid repeats, sequences comprising tetranucleic acid repeats, sequences comprising poly-A repeats, sequences comprising poly- T repeats, sequences comprising poly-C repeats, sequences comprising poly-G repeats, sequences comprising AT -rich sequences, or sequences comprising GC-rich sequences. In any of the embodiments disclosed herein, the method comprises contacting the sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes. In any of the embodiments disclosed herein, the sample is selected from whole blood, plasma, serum, tears, saliva, mucous, cerebrospinal fluid, teeth, bone, fingernails, feces, urine, tissue, and a biopsy.

[0017] In another aspect, provided herein is a method for serially depleting targeted nucleic acids in a sample comprising:(a) providing a sample comprising host nucleic acids and non-host nucleic acids, wherein the non-host nucleic acids comprise nucleic acids from at least

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one known non-host organism and nucleic acids from at least one unknown non-host organism; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are configured to hybridize to targeted sequences in the host nucleic acids, whereby a portion of the host nucleic acids are cleaved; (c) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are configured to hybridize to targeted sequences in the at least one known non-host nucleic acids, whereby a portion of the at least one known non-host nucleic acids are cleaved; and (d) isolating the nucleic acids from the unknown non-host organism. In any of the embodiments disclosed herein, the method comprises contacting the sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes configured to hybridize to targeted sequences in the host nucleic acids. In any of the embodiments disclosed herein, the method comprises contacting the sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes configured to hybridize to targeted sequences in the at least one known non-host nucleic acids. In any of the embodiments disclosed herein, the sample is selected from whole blood, plasma, serum, tears, saliva, mucous, cerebrospinal fluid, teeth, bone, fingernails, feces, urine, tissue, and a biopsy. In any of the embodiments disclosed herein, the nucleic acids from an unknown non-host organism comprise less than 5% of the total nucleic acids in the sample. In any of the embodiments disclosed herein, the host is a human. In any of the embodiments disclosed herein, the at least one known non-host organism is a species of Streptococcus.

[0018] In another aspect, provided herein is a method for analyzing genomic DNA, comprising:(a) treating DNA isolated from a population of cells from a sample with an insertional enzyme to produce a plurality of tagged fragments of non-mitochondrial genomic DNA, whereby also generating a residual amount of tagged mitochondrial DNA; (b) enriching the product of step (a) for non-mitochondrial DNA according to any of the relevant methods provided herein. For example, such a method of enriching a sample can comprise: (a) providing a sample comprising mitochondrial DNA and non-mitochondrial DNA, wherein the mitochondrial DNA and non-mitochondrial DNA are adapter-ligated, and wherein the adapters are ligated to the 5' and 3' ends of the mitochondrial DNA and non-mitochondrial DNA; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to the mitochondrial DNA, thereby generating mitochondrial DNA adapter ligated only on one end and non-mitochondrial DNA adapter ligated on both the 5' and 3' ends; and (c) enriching the sample for non-mitochondrial DNA. In any of the embodiments disclosed herein, the method further

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comprises (a) sequencing at least some of the tagged fragments to produce a plurality of sequence reads; and (b) making an epigenetic map of a region of the genome of said cells by mapping information obtained from the sequence reads to the region. In any of the embodiments disclosed herein, the sample is selected from whole blood, plasma, serum, tears, saliva, mucous, cerebrospinal fluid, teeth, bone, fingernails, feces, urine, tissue, and a biopsy.

[0019] In another aspect, provided herein is a composition comprising a mixture of DNA from a first genome and DNA from a second genome, wherein the first genome DNA and the second genome DNA are adapter-ligated, and wherein the first genome DNA is complexed to a gRNA-CRISPR/Cas system protein complex. In any of the embodiments disclosed herein, the first genome is from a host organism and the second genome is from a non-host organism. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is Cas9. In any of the embodiments disclosed herein, the CRISPR/Cas system protein is thermostable.

[0020] In another aspect, provided herein are kits. In another aspect, provided herein is a kit comprising: (a) CRISPR/Cas system protein; and (b) gRNAs, wherein the gRNAs are complementary to mitochondrial DNA. In another aspect, provided herein is a kit comprising (a) a CRISPR/Cas system protein; (b) gRNAs, wherein the gRNAs are complementary to a target of interest; and (c) an enzyme having exonuclease activity. In another aspect, provided herein is a kit comprising (a) a CRISPR/Cas system protein, wherein the CRISPR/Cas system protein is thermostable; and (b) gRNAs, wherein the gRNAs are complementary to a target of interest. In another aspect, provided herein is a kit comprising (a) a CRISPR/Cas system protein; and (b) a first set of gRNAs, wherein the gRNAs are complementary to target sequences of interest; and (c) a control set of reagents. In another aspect, provided herein is a kit comprising: (a) reagents for isolating DNA from a population of cells; (b) an insertional enzyme; (c) a CRISPR/Cas system protein; and (d) a plurality of gRNAs, wherein the gRNAs are complementary to mitochondrial DNA. In any of the kits disclosed herein, the kit further comprises a collection of Y-shaped adapters or poly-G adapters. In any of the kits disclosed herein, the CRISPR/Cas system protein is Cas9. In any of the kits disclosed herein, the kits further comprise a positive control set of reagents, for example the positive control set of reagents comprise a collection of nucleic acid fragments, wherein the fragments comprise the target sequences of interest, to which the gRNAs are at least 85% complementary. In any of the kits disclosed herein, the kits further comprise a negative control set of reagents, for example, the negative control set of reagents comprise a second set of gRNAs, wherein the second set of gRNAs exhibit reduced binding to the target sequences of interest, as compared

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to the first set of gRNAs; or for example the negative control set of reagents can comprise a collection of nucleic acid fragments, wherein the fragments are no more than 90% complementary to the first set of gRNAs. In any one of the kits disclosed herein, the kit further comprises at least 10^2 unique gRNAs.

[0021] In another aspect the invention provides a method of depleting targeted sequences in a sample comprising: providing a sample comprising sequences of interest and targeted sequences for depletion; and contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to the targeted sequences, thereby generating cleaved targeted sequences. In a related aspect, the invention provides a method for one of depletion and partitioning of targeted sequences in a sample comprising: providing nucleic acids extracted from a sample, wherein the extracted nucleic acid sequences comprise sequences of interest and targeted sequences for one of depletion and partitioning; providing a plurality of guide RNAs (gRNA)- CRISPR/Cas system protein complexes, wherein the gRNAs are configured to hybridize to different targeted sequences; mixing the nucleic acids with the gRNA- CRISPR/Cas system protein complexes, wherein at least a portion of the plurality of gRNA- CRISPR/Cas system protein complexes hybridizes to the targeted sequences. In any one of the embodiments disclosed herein, the method further comprises extracting the nucleic acid sequences from the sample. In any one of the embodiments disclosed herein the CRISPR/Cas system protein is Cas9. In any one of the embodiments disclosed herein, the method further comprises fragmenting the extracted nucleic acids. In any one of the embodiments disclosed herein, the method further comprises adapter ligating the 5' and 3' ends of the fragmented extracted nucleic acids. In any one of the embodiments disclosed herein, the method further comprises incubating the mixture to cleave the targeted sequences. In any one of the embodiments disclosed herein, each sequence targeted for depletion is followed by a Protospacer Adjacent Motif or (PAM) sequence that can be bound by a Cas9 protein derived from a bacterial species but wherein the sequences of interest (for enrichment) are not targeted by the gRNAs. In any one of the embodiments disclosed herein, the Cas9 protein comprises a catalytically dead Cas9 that includes an affinity tag previously attached thereto, further comprising partitioning the mixture into a first portion that includes the complementary target specific nucleic sequence present in the mixture that include a guide RNA/Cas9 complex and a second portion that includes fragmented extracted nucleic acid sequences that are not bound by a guide RNA/Cas9 complex, wherein the partitioning is performed using the affinity tag. In any one of the embodiments disclosed herein, the cleaved extracted nucleic acid sequences are

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smaller than the fragmented extracted nucleic acid sequences that have not been cleaved, the method further comprising, removing the cleaved extracted nucleic acid sequences from the mixture by size selective exclusion. In any one of the embodiments disclosed herein, the method further comprises amplifying the fragmented extracted nucleic acid sequences that have not been cleaved. In any one of the embodiments disclosed herein, the method further comprises analyzing each of the first and second portions by amplification and sequencing. In any one of the embodiments disclosed herein, the sample is any one of a biological sample, a clinical sample, a forensic sample or an environmental sample. In any one of the embodiments disclosed herein, the extracted nucleic acids comprise host nucleic acid sequences and non-host nucleic acid sequences. In any one of the embodiments disclosed herein, the non-host nucleic acid sequences comprise microbial nucleic acid sequences. In any one of the embodiments disclosed herein, the microbial nucleic acid sequences include any one of bacterial, viral and eukaryotic parasitic nucleic acid sequences. In any one of the embodiments disclosed herein, at least a portion of the host nucleic acid sequences are included in the sequences being targeted for one of depletion and partitioning.In any one of the embodiments disclosed herein, substantially all of the host nucleic acid sequences are included in the sequences being targeted for one of depletion and partitioning. In any one of the embodiments disclosed herein, the extracted nucleic acids comprise a plurality of different ribosomal RNA sequences and the plurality of different target specific gRNAs comprise at least a portion of the target nucleic acid sequences configured to hybridize to a portion of the plurality of different ribosomal RNA sequences. In any one of the embodiments disclosed herein, the extracted nucleic acid sequences comprise a plurality of different mitochondrial nucleic acid sequences and the plurality of different target specific gRNAs comprise one or more target nucleic acid sequence configured to hybridize to a portion of the plurality of different mitochondrial nucleic acid sequences. In any one of the embodiments disclosed herein, the extracted nucleic acid sequences comprise a plurality of different repetitive nucleic acid sequences in the nucleic acid sequences being targeted for depletion and the plurality of different target specific gRNAs comprise at least one target nucleic acid sequence that can hybridize to a portion of the plurality of different repetitive nucleic acid sequences. In any one of the embodiments disclosed herein, the extracted nucleic acids incudes any one of single stranded DNA, double stranded DNA, single stranded RNA, double stranded RNA, cDNA, synthetic DNA, artificial DNA, and DNA/RNA hybrids. In any one of the embodiments disclosed herein, the sequences of interest comprise less than

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50% of the extracted nucleic acids. In any one of the embodiments disclosed herein, the sequences of interest comprise less than 5% of the extracted nucleic acids.

[0022] In another aspect the invention provides a guide RNA library comprising a plurality of target-specific guide RNAs, each configured to hybridize to a selected target nucleic acid sequence, wherein each guide RNA comprises a sequence that can be bound by a Cas9 protein. In any one of the embodiments disclosed herein, each selected target nucleic acid sequence is immediately followed by a protospacer adjacent motif. In any one of the embodiments disclosed herein, target specific gRNAs include gRNAs suitable for hybridizing with selected target nucleic acid sequences in the human genome. In any one of the embodiments disclosed herein, the human genome includes nucleic acid sequences comprising a plurality of different repetitive nucleic acid sequences and the target-specific gRNAs includes guide elements suitable for hybridizing with each of the plurality of different repetitive nucleic acid sequences.

[0023] In another aspect, the technology disclosed herein relates to methods and systems for selective or targeted depletion and/or selective or targeted partitioning of nucleic acid samples using a Cas9-enzyme- mediated method.

[0024] In another aspect, the technology disclosed herein relates to forming a library of gRNAs wherein each gRNA is suitable to hybridize to a nucleic acid sequence being targeted for removal from the overall nucleic acid sample wherein the sequence being targeted is followed by a Protospacer Adjacent Motif (PAM) sequence. By mixing the gRNAs with nucleic acid samples and a Cas9 protein derived from a bacterial species the gRNAs hybridize to the nucleic acid sequence being targeted for removal and the Cas9 enzyme forms a guide RNA/Cas9 complex that bonds to the nucleic acid sequence being targeted for removal. Thereafter the guide RNA/Cas9 complex can be incubated to cut the targeted nucleic acid sequences so that cut sequences can be separated from uncut nucleic acid sequences or so that targeted sequences that are bonded by a guide RNA/Cas9 complex can be partitioned from DNA sequences that are not bonded to by a guide RNA/Cas9 complex.

[0025] In another aspect, the technology disclosed herein is also useful for depleting host molecules from nucleic acid samples that contain low levels (e.g. <50%) of non-host nucleic acids.

[0026] In another aspect, the technology disclosed herein comprises: 1) combining a genomic DNA sample or sequencing library with a mixture of Cas9-gRNA complexes, wherein the Cas9-gRNA complexes comprise a Cas9 protein and Cas9-associated gRNAs that are complementary to a pre-defined site in the genome; and b) incubating the reaction

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mixture to cut only the target regions. In the embodiment in which a sequencing library is targeted, Cas9 cutting would separate the two sequencing adapters and hence make these fragments unable to be amplified. In the embodiment in which genomic DNA is targeted, Cas9 cutting would make these fragments small enough to be removed by size selection.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] FIG. 1 illustrates a general schematic of the depletion method using Cas9 to selectively cleave target sequences in a library.

[0028] FIG. 2 illustrates a general schematic of the depletion method using catalytically dead Cas9 to partition libraries into target and non-target sequences.

[0029] FIG. 3 illustrates an exemplary strategy to deplete mitochondrial DNA from a sequencing library, leaving only non-mitochondrial DNA for sequencing.

[0030] FIG. 4 illustrates the results of a depletion experiment in which mitochondrial DNA was depleted by ~44% (measured by qPCR).

[0031] FIG. 5 illustrates the results of a second experiment in which a higher number of guide RNAs was used and mitochondrial DNA was depleted by ~70% (measured by qPCR).

[0032] FIG. 6A illustrates the results of sequencing a library before and after depletion of mitochondrial DNA. FIG. 6B illustrates depletion of mitochondrial DNA using different numbers of gRNAs.

[0033] FIG. 7 illustrates Cas9 Nickase-mediated depletion of DNA.

[0034] FIG. 8 illustrates Cas9-mediated depletion followed by Exonuclease III treatment.

[0035] FIG. 9 illustrates Cas9-mediated depletion followed by Exonuclease Bal-31 treatment.

[0036] FIG. 10 illustrates biotin labeling during Cas-9 mediated depletion

[0037] FIG. 11 illustrates using a thermostable Cas9 to increase the efficiency of Cas9-mediated depletion.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0038] Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those

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described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

[0039] The headings provided herein are not limitations of the various aspects or embodiments of the invention. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

[0040] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, et al., DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Markham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, N.Y. (1991) provide one of skill with the general meaning of many of the terms used herein. Still, certain terms are defined below for the sake of clarity and ease of reference.

[0041] Numeric ranges are inclusive of the numbers defining the range.

[0042] The term "sample" as used herein relates to a material or mixture of materials, typically, although not necessarily, in liquid form, containing one or more analytes of interest.

[0043] The term "nucleic acid sample," as used herein denotes a sample containing nucleic acids. Nucleic acid samples used herein may be complex in that they contain multiple different molecules that contain sequences. Genomic DNA from a mammal (e.g., mouse or human) are types of complex samples. Complex samples may have more then 10^4, 10^5, 10^6 or 10^7 different nucleic acid molecules. A DNA target may originate from any source such as genomic DNA, cDNA, or an artificial DNA construct. Any sample containing nucleic acid, e.g., genomic DNA made from tissue culture cells or a sample of tissue may be employed herein.

[0044] The term "nucleotide" is intended to include those moieties that contain not only the known purine and pyrimidine bases, but also other heterocyclic bases that have been modified. Such modifications include methylated purines or pyrimidines, acylated purines or pyrimidines, alkylated riboses or other heterocycles. In addition, the term "nucleotide" includes those moieties that contain hapten or fluorescent labels and may contain not only conventional ribose and deoxyribose sugars, but other sugars as well. Modified nucleosides or nucleotides also include modifications on the sugar moiety, e.g., wherein one or more of the hydroxyl groups are replaced with halogen atoms or aliphatic groups, or are functionalized as ethers, amines, or the like.

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[0045] The term "nucleic acids" and "polynucleotides" are used interchangeably herein. Polynucleotide is used to describe a nucleic acid polymer of any length, e.g., greater than about 2 bases, greater than about 10 bases, greater than about 100 bases, greater than about 500 bases, greater than 1000 bases, up to about 10,000 or more bases composed of nucleotides, e.g., deoxyribonucleotides or ribonucleotides, and may be produced enzymatically or synthetically (e.g., PNA as described in U.S. Patent No. 5,948,902 and the references cited therein) which can hybridize with naturally occurring nucleic acids in a sequence specific manner analogous to that of two naturally occurring nucleic acids, e.g., can participate in Watson-Crick base pairing interactions. Naturally-occurring nucleotides include guanine, cytosine, adenine and thymine (G, C, A and T, respectively). DNA and RNA have a deoxyribose and ribose sugar backbone, respectively, whereas PNA's backbone is composed of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. In PNA various purine and pyrimidine bases are linked to the backbone by methylene carbonyl bonds. A locked nucleic acid (LNA), often referred to as inaccessible RNA, is a modified RNA nucleotide. The ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2' oxygen and 4' carbon. The bridge "locks" the ribose in the 3'-endo (North) conformation, which is often found in the A-form duplexes. LNA nucleotides can be mixed with DNA or RNA residues in the oligonucleotide whenever desired. The term "unstructured nucleic acid," or "UNA," is a nucleic acid containing non-natural nucleotides that bind to each other with reduced stability. For example, an unstructured nucleic acid may contain a G' residue and a C' residue, where these residues correspond to non-naturally occurring forms, i.e., analogs, of G and C that base pair with each other with reduced stability, but retain an ability to base pair with naturally occurring C and G residues, respectively. Unstructured nucleic acid is described in US20050233340, which is incorporated by reference herein for disclosure of UNA.

[0046] The term "oligonucleotide" as used herein denotes a single-stranded multimer of nucleotides.

[0047] Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

[0048] The term "cleaving," as used herein, refers to a reaction that breaks the phosphodiester bonds between two adjacent nucleotides in both strands of a double-stranded DNA molecule, thereby resulting in a double-stranded break in the DNA molecule.

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[0049] The term "cleavage site, as used herein, refers to the site at which a double-stranded DNA molecule has been cleaved.

[0050] The term "hybridization" refers to the process by which a strand of nucleic acid joins with a complementary strand through base pairing as known in the art. A nucleic acid is considered to be "selectively hybridizable" to a reference nucleic acid sequence if the two sequences specifically hybridize to one another under moderate to high stringency hybridization and wash conditions. Moderate and high stringency hybridization conditions are known (see, e.g., Ausubel, et al., Short Protocols in Molecular Biology, 3rd ed., Wiley & Sons 1995 and Sambrook et al., Molecular Cloning: A Laboratory Manual, Third Edition, 2001 Cold Spring Harbor, N.Y.). One example of high stringency conditions includes hybridization at about 42 °C in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.5% SDS and 100 μg/ml denatured carrier DNA followed by washing two times in 2X SSC and 0.5% SDS at room temperature and two additional times in 0.1 X SSC and 0.5% SDS at 42 °C.

[0051] The term "duplex," or "duplexed," as used herein, describes two complementary polynucleotides that are base-paired, i.e., hybridized together.

[0052] The term "amplifying" as used herein refers to generating one or more copies of a target nucleic acid, using the target nucleic acid as a template.

[0053] The term "depleting," with respect to a genome, refers to the removal of one part of the genome from the remainder of the genome to produce a product that is isolated from the remainder of the genome. The term "depleting" also encompasses removal of DNA from one species while retaining DNA from another species.

[0054] The term "genomic region," as used herein, refers to a region of a genome, e.g., an animal or plant genome such as the genome of a human, monkey, rat, fish or insect or plant. In certain cases, an oligonucleotide used in the method described herein may be designed using a reference genomic region, i.e., a genomic region of known nucleotide sequence, *e.g.*, a chromosomal region whose sequence is deposited at NCBI's Genbank database or other databases, for example.

[0055] The term "genomic sequence," as used herein, refers to a sequence that occurs in a genome. Because RNAs are transcribed from a genome, this term encompasses sequence that exist in the nuclear genome of an organism, as well as sequences that are present in a cDNA copy of an RNA (e.g., an mRNA) transcribed from such a genome.

[0056] The term "genomic fragment," as used herein, refers to a region of a genome, e.g., an animal or plant genome such as the genome of a human, monkey, rat, fish or insect or plant. A genomic fragment may be an entire chromosome, or a fragment of a chromosome. A

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genomic fragment may be adapter ligated (in which case it has an adapter ligated to one or both ends of the fragment, or to at least the 5' end of a molecule), or may not be adapter ligated.

[0057] In certain cases, an oligonucleotide used in the method described herein may be designed using a reference genomic region, i.e., a genomic region of known nucleotide sequence, *e.g.*, a chromosomal region whose sequence is deposited at NCBI's Genbank database or other databases, for example. Such an oligonucleotide may be employed in an assay that uses a sample containing a test genome, where the test genome contains a binding site for the oligonucleotide.

[0058] The term "ligating," as used herein, refers to the enzymatically catalyzed joining of the terminal nucleotide at the 5' end of a first DNA molecule to the terminal nucleotide at the 3' end of a second DNA molecule.

[0059] If two nucleic acids are "complementary," each base of one of the nucleic acids base pairs with corresponding nucleotides in the other nucleic acid. The term "complementary" and "perfectly complementary" are used synonymously herein.

[0060] The term "separating," as used herein, refers to physical separation of two elements (e.g., by size or affinity, etc.) as well as degradation of one element, leaving the other intact.

[0061] In a cell, DNA usually exists in a double-stranded form, and as such, has two complementary strands of nucleic acid referred to herein as the "top" and "bottom" strands. In certain cases, complementary strands of a chromosomal region may be referred to as "plus" and "minus" strands, the "first" and "second" strands, the "coding" and "noncoding" strands, the "Watson" and "Crick" strands or the "sense" and "antisense" strands. The assignment of a strand as being a top or bottom strand is arbitrary and does not imply any particular orientation, function or structure. Until they become covalently linked, the first and second strands are distinct molecules. For ease of description, the "top" and "bottom" strands of a double-stranded nucleic acid in which the top and bottom strands have been covalently linked will still be described as the "top" and "bottom" strands. In other words, for the purposes of this disclosure, the top and bottom strands of a double-stranded DNA do not need to be separated molecules. The nucleotide sequences of the first strand of several exemplary mammalian chromosomal regions (e.g., BACs, assemblies, chromosomes, etc.) is known, and may be found in NCBI's Genbank database, for example.

[0062] The term "top strand," as used herein, refers to either strand of a nucleic acid but not both strands of a nucleic acid. When an oligonucleotide or a primer binds or anneals "only to a top strand," it binds to only one strand but not the other. The term "bottom strand," as used

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herein, refers to the strand that is complementary to the "top strand." When an oligonucleotide binds or anneals "only to one strand," it binds to only one strand, e.g., the first or second strand, but not the other strand. If an oligonucleotide binds or anneals to both strands of a double-stranded DNA, the oligonucleotide may have two regions, a first region that hybridizes with the top strand of the double-stranded DNA, and a second region that hybridizes with the bottom strand of the double-stranded DNA.

[0063] The term "double-stranded DNA molecule" refers to both double-stranded DNA molecules in which the top and bottom strands are not covalently linked, as well as double-stranded DNA molecules in which the top and bottom stands are covalently linked. The top and bottom strands of a double-stranded DNA are base paired with one other by Watson-Crick interactions.

[0064] The term "denaturing," as used herein, refers to the separation of at least a portion of the base pairs of a nucleic acid duplex by placing the duplex in suitable denaturing conditions. Denaturing conditions are well known in the art. In one embodiment, in order to denature a nucleic acid duplex, the duplex may be exposed to a temperature that is above the T_m of the duplex, thereby releasing one strand of the duplex from the other. In certain embodiments, a nucleic acid may be denatured by exposing it to a temperature of at least 90 ^{o}C for a suitable amount of time (e.g., at least 30 seconds, up to 30 mins). In certain embodiments, fully denaturing conditions may be used to completely separate the base pairs of the duplex. In other embodiments, partially denaturing conditions (e.g., with a lower temperature than fully denaturing conditions) may be used to separate the base pairs of certain parts of the duplex (e.g., regions enriched for A-T base pairs may separate while regions enriched for G-C base pairs may remain paired). Nucleic acid may also be denatured chemically (e.g., using urea or NaOH).

[0065] The term "genotyping," as used herein, refers to any type of analysis of a nucleic acid sequence, and includes sequencing, polymorphism (SNP) analysis, and analysis to identify rearrangements.

[0066] The term "sequencing," as used herein, refers to a method by which the identity of consecutive nucleotides of a polynucleotide are obtained.

[0067] The term "next-generation sequencing" refers to the so-called parallelized sequencing-by-synthesis or sequencing-by-ligation platforms, for example, those currently employed by Illumina, Life Technologies, and Roche, etc. Next-generation sequencing methods may also include nanopore sequencing methods or electronic-detection based methods such as Ion Torrent technology commercialized by Life Technologies.

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[0068] The term "host nucleic acids" refers to nucleic acids that originate from the multicellular eukaryotic subject from which the sample was obtained. Host nucleic acids can be, e.g., plant or animal, including mammals particularly humans. The term "host nucleic acids" includes nuclear nucleic acids as well as nucleic acids present in other organelles, e.g., the mitochondria and chloroplast (if the host is a plant), but not the nucleic acids from microbes that often grow on or in the subject.

[0069] The term "non-host nucleic acids" refers to nucleic acids not belonging to the host from which the sample was obtained. Non-host nucleic acids can be viral, bacterial, or other microbial DNA.

[0070] The term "microbial nucleic acids" refers to nucleic acids that is microbial (e.g., bacterial or viral or from a eukaryotic parasite) in origin that is present in a sample.

[0071] The term "host DNA" refers to DNA that originates from the multicellular eukaryotic subject from which the sample was obtained. Host DNA can be, e.g., plant or animal, including mammals particularly humans. The term "host DNA" includes nuclear DNA as well as DNA present in other organelles, e.g., the mitochondria and chloroplast (if the host is a plant), but not the DNA from microbes that often grow on or in the subject.

[0072] The term "non-host DNA" refers to DNA not belonging to the host from which the sample was obtained. Non-host DNA can be viral, bacterial, or other microbial DNA.

[0073] The term "microbial DNA" refers to genomic DNA that is microbial (e.g., bacterial or viral or from a eukaryotic parasite) in origin that is present in a sample.

[0074] The term "complementary DNA" or cDNA refers to a double-stranded DNA sample that was produced from an RNA sample by reverse transcription of RNA (using primers such as random hexamers or oligo-dT primers) followed by second-strand synthesis by digestion of the RNA with RNaseH and synthesis by DNA polymerase.

[0075] The term "RNA promoter adapter" is an adapter that contains a promoter for a bacteriophage RNA polymerase, e.g., the RNA polymerase from bacteriophage T3, T7, SP6 or the like.

[0076] The term "metagenomics sample" refers to a sample that contains more than one species of organism (eukaryotic, prokaryotic, or viral organisms).

[0077] The term "metagenomics analysis" refers to the analysis of a metagenomics sample.

[0078] Other definitions of terms may appear throughout the specification.

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Objects of the Invention

[0079] In view of the problems associated with conventional methods and apparatus set forth above it is an object of the present invention to deplete selected nucleic acids from a nucleic acid sample using an enzyme mediated cutting mechanism.

[0080] It is another object of the present invention to provide a method that depletes selected host nucleic acids from sequencing libraries that include host and non-host nucleic acids in order to increase the proportion of non-host nucleic acid fragments for the purposes of pursuing downstream applications, including, but not limited to amplification, sequencing, cloning, etc.

[0081] It is another object of the present invention to provide a metagenomic analysis method that is unbiased relative to the fragments from across the genome of a particular species by selectively depleting some or all of the particular species' genome from a metagenomics sample.

[0082] It is further object of the present invention to gain better resolution in metagenomic analysis by increasing the number of sample reads that map to the a species' metagenome without increasing and in some instances while decreasing the amount of sequencing.

[0083] It is a further object of the present invention to provide methods and compositions to deplete mitochondrial DNA from a sample comprising mitochondrial DNA and non-mitochondrial DNA.

Nucleic Acids, Samples

[0084] Nucleic acids of the invention (targeted for enrichment, partitioning, or depletion) can be single stranded DNA, single stranded RNA, double stranded DNA, double stranded RNA, artificial DNA, artificial RNA, synthetic DNA, synthetic RNA, and RNA/DNA hybrids.

[0085] The nucleic acids of the invention can be a genomic fragment, comprising a region of the genome, or the whole genome itself. In one embodiment, the genome is a DNA genome. In another embodiment, the genome is a RNA genome.

[0086] Nucleic acids of the invention can be obtained from a eukaryotic or prokaryotic organism; from a mammalian organism or a non-mammalian organism; from an animal or a plant; from a bacteria or virus; from an animal parasite; or from a pathogen.

[0087] Nucleic acids of the invention can be obtained from any mammalian organism. In one embodiment the mammal is a human. In another embodiment the mammal is a livestock animal, for example a horse, a sheep, a cow, a pig, or a donkey. In another embodiment, a

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mammalian organism is a domestic pet, for example a cat, a dog, a gerbil, a mouse, a rat. In another embodiment the mammal is a type of a monkey.

[0088] Nucleic acids of the invention can be obtained from any bird or avian organism. An avian organism includes, but is not limited, to a chicken, turkey, duck and goose.

[0089] Nucleic acids of the invention can be obtained from a plant. In one embodiment, the plant is rice, maize, wheat, rose, grape, coffee, fruit, tomato, potato, or cotton.

[0090] In some embodiments, nucleic acids of the invention are obtained from a species of bacteria. In one embodiment, the bacteria are tuberculosis-causing bacteria.

[0091] In some embodiments, nucleic acids of the invention are obtained from a virus.

[0092] In some embodiments, nucleic acids of the invention are obtained from a species of fungi.

[0093] In some embodiments, nucleic acids of the invention are obtained from a species of algae.

[0094] In some embodiments, nucleic acids of the invention are obtained from any mammalian parasite.

[0095] In some embodiments, nucleic acids of the invention are obtained from any mammalian parasite. In one embodiment, the parasite is a worm. In another embodiment, the parasite is a malaria-causing parasite. In another embodiment, the parasite is a Leishmaniasis-causing parasite. In another embodiment, the parasite is an amoeba.

[0096] In one embodiment, the nucleic acids of the invention include nucleic acids that are targets of gRNAs (also referred to interchangeably herein as gRNA elements) and nucleic acids that are not the targets of the gRNAs, in the same sample.

[0097] In one embodiment, the nucleic acids in a sample include target nucleic acids/targeted sequences (targets of gRNAs) and nucleic acids of interest/sequences of interest (not targeted by gRNAs).

[0098] In one embodiment, the nucleic acids all belong to the same organism, but a subset is targeted for depletion or partitioning. For example nucleic acids of little informative value may be targeted. Examples of nucleic acids of little informative value include, but are not limited to: mitochondrial DNA, mitochondrial RNA, mitochondrial rRNA, repetitive sequence, multi-copy sequence, sequence encoding globin proteins, sequence encoding a transposon, sequence encoding retroviral sequence, sequence comprising telomere sequence, sequence comprising sub-telomeric repeats, sequence comprising centromeric sequence, sequence comprising intron sequence, sequence comprising Alu repeats, SINE repeats, LINE repeats, dinucleic acid repeats, trinucleic acid repeats, tetranucleic acid repeats, poly-A

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repeats, poly- T repeats, poly-C repeats, poly-G repeats, AT -rich sequence, or GC-rich sequence.

[0100] In one embodiment, a sample is contacted with CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to target sequences for depletion, the target sequences encoding globin proteins, sequences encoding a transposon, sequences encoding retroviral sequences, sequences comprising telomere sequences, sequences comprising sub-telomeric repeats, sequences comprising centromeric sequences, sequences comprising intron sequences, sequences comprising Alu repeats, sequences comprising SINE repeats, sequences comprising LINE repeats, sequences comprising dinucleic acid repeats, sequences comprising trinucleic acid repeats, sequences comprising tetranucleic acid repeats, sequences comprising poly-A repeats, sequences comprising poly- T repeats, sequences comprising poly-C repeats, sequences comprising poly-G repeats, sequences comprising AT -rich sequences, or sequences comprising GC-rich sequences.

[0101] In one exemplary embodiment, ribosomal RNA may be targeted for depletion or partitioning. In another exemplary embodiment repetitive DNA may be targeted for depletion or partitioning. In such an embodiment, the present method is usable to deplete the ribosomal or repetitive DNA or any other DNA that has little informative value in order to reduce the cost of sequencing these elements of the DNA extraction and improve data yield.

[0102] In one embodiment, the target DNA may be non-mitochondrial DNA (e.g. genomic DNA), and the DNA of interest may be the mitochondrial DNA, and the mitochondrial DNA is enriched by targeting and depleting the non-mitochondrial human DNA.

[0103] In one embodiment, the target DNA for depletion may be mitochondrial DNA, and the DNA of interest may be the non-mitochondrial DNA, and the non-mitochondrial DNA is enriched by targeting and depleting the mitochondrial human DNA. In an exemplary embodiment, an ATAC-Seq procedure (for example see WO2014/189957 is carried out resulting in unwanted residual mitochondrial DNA; the methods of the invention can be used to deplete unwanted mitochondrial DNA from the sample.

[0104] In one embodiment, the nucleic acids to be depleted or partitioned out may be a non-mappable region of a genome; and the nucleic acids to be retained for further analysis/sequencing/cloning may be mappable regions of a genome. In one embodiment, the nucleic acids to be depleted or partitioned out may be a mappable region of a genome; and the nucleic acids to be retained for further analysis/sequencing/cloning may be non-mappable regions of a genome. Examples of non mappable regions include telomeres, centromeres, repetitive regions, or other genomic regions that contain features harder to map.

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[0105] In some embodiments, the methods of the invention are carried out on samples comprising host nucleic acids and non-host nucleic acids. In one embodiment, the host nucleic acids are mammalian, and the non-host nucleic acids are not mammalian (e.g., bacterial, viral, fungal, protozoan). In one embodiment, the host nucleic acids are human and the non-host nucleic acids are bacterial. In one embodiment, the host nucleic acids are human and the non-host nucleic acids are viral. In one embodiment, the host nucleic acids are human and the non-host nucleic acids are fungal. In one embodiment, the host nucleic acids are human and the non-host nucleic acids are protozoan. In one embodiment, the host nucleic acids are from a type of livestock. In one embodiment, the host nucleic acids are from a monkey. In one specific embodiment, the host nucleic acids are human and the non-host nucleic acids are from an unidentified pathogen, for example a known or unknown virus, or a known or unknown bacteria, or a known or unknown animal parasite. In one specific embodiment, the host nucleic acids are from a cow and the non-host nucleic acids are viral, bacterial, fungal, or protozoan.

[0106] In samples that contain both host and non-host nucleic acids, the host and non-host may have, for example, a host-pathogen relationship or a symbiotic relationship. In some embodiments, the non-host fraction of a total nucleic acid sample is obtained from a host may be derived from the microbiome that is associated with the host.

[0107] In one embodiment, a sample obtained from a host organism (e.g. a human host) contains nucleic acids from more than one non-host organism, e.g. from a plurality of non-host organisms comprising at least one unknown non-host organism to be identified. The compositions and methods of the invention can be utilized to serially process the nucleic acids in the sample to first deplete/partition out the host-DNA, and then subsequently deplete/partition out other known non-host nucleic acids that are not of interest, to arrive at a remaining pool of nucleic acids that represent a particular non-host of interest. For example, such an embodiment would be applicable in a situation where it a host would be expected to be harboring more than one known bacteria, for example existing symbiotically. For example, such an embodiment would be applicable to detect an unknown pathogen in a saliva sample from an individual, the saliva being known to harbor several species of bacteria, for example species of Streptococcus. In another example, the sample may be a fecal sample from a mammalian host, comprising known and unknown non-host nucleic acids.

[0108] In one embodiment, a sample obtained from a known host organism (e.g., a human host) contains non-host nucleic acids but the non-host is not known (e.g.an unknown pathogenic bacteria, virus, or other pathogen in the human blood) until the host nucleic acids

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are depleted or partitioned, such that the non-host nucleic acids are enriched and be subjected to further downstream analysis.

[0109] In an exemplary embodiment, where the DNA includes host-DNA and non-host DNA the present method is usable to deplete substantially all of the host DNA in order to build a library of non-host DNA for further analysis. In cases where the DNA includes nucleic and mitochondrial DNA the present method is usable to deplete the mitochondrial DNA in order to build a library of nuclear DNA for further analysis; or to deplete the nuclear DNA in order to build a library of mitochondrial DNA for further analysis. In cases where the DNA includes abundant sequences of ribosomal RNA or repetitive DNA that may have little informative value, the present method is usable to deplete the ribosomal or repetitive DNA or any other DNA that has little informative value in order to reduce the cost of sequencing these elements of the DNA extraction and improve data yield. In each application, the resulting DNA has significantly fewer DNA fragments to sequence thereby reducing sequencing cost and complexity. Moreover according to the methods of the present invention the depleted sample still provides all of the DNA material present in the biological sample that was not actively depleted which provides the advantage of reducing the sequencing costs as well as improving the data yield by sequencing smaller less diverse DNA samples.

[0110] In one embodiment, the nucleic acids of the invention are obtained from a biological sample. The biological sample from which the nucleic acids are obtained include but are not limited to whole blood, plasma, serum, tears, saliva, mucous, cerebrospinal fluid, teeth, bone, fingernails, feces, urine, tissue, biopsy, etc. The biological sample may include forensic samples such as teeth, bone, fingernails or the like. The biological sample may include tissue, a tissue biopsy, for example a resected lung tissue. The biological sample may include a clinical sample, which refers to a sample obtained in a clinical setting, such as a in a hospital, or clinic.

[0111] In one embodiment, the nucleic acids of the invention are obtained from an environmental sample, for example from water, soil, air, or rock.

[0112] In one embodiment, the nucleic acids of the invention are obtained from a forensic sample, for example, a sample obtained from an individual at a crime scene, from a piece of evidence, post-mortem, as a part of an ongoing investigation or the like.

[0113] In on embodiment, the nucleic acids of the invention are provided in a library.

[0114] The nucleic acids of the invention are either provided or extracted from a sample. Extraction can extract substantially all the nucleic acid sequences from a specimen. This can

be, for example, nucleic acid sequences of the host and nucleic acid sequences of any non-human or non-host organism present in the specimen.

[0115] An extraction may produce host nucleic acids and non-host nucleic acids at a ratio of anywhere between 99.999:0.001 to 0.001:99.999. An extraction may produce targeted nucleic acids and nucleic acids of interest at a ratio of anywhere between 99.999:0.001 to 0.001:99.999. An extraction may produce nucleic acids to be depleted to nucleic acids to be retained/analyzed/sequenced at a ratio of anywhere between 99.999:0.001 to 0.001:99.999. An extraction may produce nucleic acids to be partitioned to nucleic acids to be retained/analyzed/sequenced at a ratio of anywhere between 99.999:0.001 to 0.001:99.999. An extraction from a biological sample may produce host nucleic acids to be depleted to nucleic acids from more than one non-host, to be retained/analyzed/sequenced, at a ratio of anywhere between 99.999:0.001 to 0.001:99.999. In these embodiments, the ratios can be equal to or fall anywhere in between 99.999:0.001 to 0.001:99.999, for example the ratio can be 99:1, 95:5, 90:10, 85:15, 80:20, 75:25, 70: 30, 65:35, 60:40, 55:45, 50:50, 45:55, 40:60, 35:65, 30:70, 25:75, 20:80, 15:85, 10:90, 5:95, and 1:99.

[0116] After the extraction, the extracted nucleic sequences can be fragmented to reduce the lengths of each extracted nucleic acids to a more manageable length for amplifying, sequencing or the like.

[0117] As provided herein, depletion of at least 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% of the starting nucleic acid material can be depleted or partitioned. This depletion or partitioning can be achieved in no greater than 10 minutes, 15 minutes, 20 minutes, 30 minutes, 45 minutes, 60 minutes, 75 minutes, 90 minutes, 105 minutes, 120 minutes, 150 minutes, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 11 hours or 12 hours.

[0118] In one embodiment, the nucleic acids of the invention are adapter-ligated. Nucleic acids of the invention to be adapter-ligated can range from 10bp to 1000bp. For example the nucleic acid to be adapter-ligated may be at least 10, 15, 20, 25, 50, 75, 100, 125, 150, 175, 200, 25, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, or 1000 bp. In one specific embodiment, the nucleic acid to be adapter ligated is 100bp. In one specific embodiment, the nucleic acid to be adapter ligated is 200bp. In one specific embodiment, the nucleic acid to be adapter ligated is 300bp. In one specific embodiment, the nucleic acid to be adapter ligated is 400bp. In one specific embodiment, the nucleic acid to be adapter ligated is 500bp.

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[0119] In some embodiments a sample comprises a library of nucleic acid fragments, wherein the nucleic acid fragments are adapter-ligated on their 5' and 3' ends. In such embodiments an adapter is ligated to each end of each of the nucleic acid fragments, at the 5' and 3' ends. In other embodiments an adapter may be ligated to only one end of each of the fragments. In some embodiments, adapters further comprise intervening sequence between the 5' terminal end and/or the 3' terminal end. For example an adapter can further comprise a barcode sequence.

[0120] In some embodiments the adapter is a nucleic acid that is ligatable to both strands of a double-stranded DNA molecule.

[0121] In some embodiments, adapters are ligated prior to depletion/enrichment. In other embodiments, adapters are ligated at a later step.

[0122] In some embodiments the adapters are linear. In some embodiments the adapters are linear Y-shaped. In some embodiments the adapters are linear circular. In some embodiments the adapters are hairpin adapters.

[0123] In various embodiments the adapter may be a hairpin adapter i.e., one molecule that base pairs with itself to form a structure that has a double-stranded stem and a loop, where the 3' and 5' ends of the molecule ligate to the 5' and 3' ends of the double-stranded DNA molecule of the fragment, respectively.

[0124] Alternately, the adapter may be a Y-adapter ligated to one end or to both ends of a fragment, also called a universal adapter. Alternately, the adapter may itself be composed of two distinct oligonucleotide molecules that are base paired with one another. Additionally a ligatable end of the adapter may be designed to be compatible with overhangs made by cleavage by a restriction enzyme, or it may have blunt ends or a 5' T overhang.

[0125] The adapter may include double-stranded as well as single-stranded molecules. Thus the adapter can be DNA or RNA, or a mixture of the two. Adapters containing RNA may be cleavable by RNase treatment or by alkaline hydrolysis.

[0126] Adapters can be 10 to 100bp in length although adapters outside of this range are usable without deviating from the present invention. In specific embodiments, the adapter is at least 10bp, at least 15bp, at least 20bp, at least 25bp, at least 30bp, at least 35bp, at least 40bp, at least 45bp, at least 50bp, at least 55bp, at least 60bp, at least 65bp, at least 70bp, at least 75bp, at least 80bp, at least 85bp, at least 90bp, or at least 95bp in length.

[0127] In certain cases, an adapter may comprise an oligonucleotide designed to match a nucleotide sequence of a particular region of the host genome, *e.g.*, a chromosomal region whose sequence is deposited at NCBI's Genbank database or other databases. Such an

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oligonucleotide may be employed in an assay that uses a sample containing a test genome, where the test genome contains a binding site for the oligonucleotide. In further examples the fragmented nucleic acid sequences may be derived from one or more DNA sequencing libraries. An adapter may be configured for a next generation sequencing platform, for example for use on an Illumina sequencing platform or for use on an IonTorrents platform, or for use with Nanopore technology.

Guide RNAs

[0128] Provided herein are guide RNAs (gRNAs), wherein the gRNAs are complementary to (selective for, can hybridize with) targeted sites or targeted sequences in the nucleic acids, for example in genomic DNA from a host. In one embodiment, the present invention provides a guide RNA library which comprises a collection of gRNAs, configured to hybridize with a nucleic acid sequence targeted for depletion or partitioning.

[0129] In one embodiment, the gRNAs are selective for target nucleic acids (or targeted sequences) in a sample, but are not selective for sequences of interest in the sample.

[0130] In one embodiment, the gRNAs are selective for host nucleic acids in a biological sample from a host, but are not selective for non-host nucleic acids in the sample from a host. In one embodiment, the gRNAs are selective for non-host nucleic acids from a biological sample from a host but are not selective for the host nucleic acids in the sample. In one embodiment, the gRNAs are selective for both host nucleic acids and a subset of the non-host nucleic acids in a biological sample from a host. For example, where a complex biological sample comprises host nucleic acids and nucleic acids from more than one non-host organisms, the gRNAs may be selective for more than one of the non-host species. In such embodiments, the gRNAs are used to serially deplete or partition the sequences that are not of interest. For example, saliva from a human contains human DNA, as well as the DNA of more than one bacterial species, but may also contain the genomic material of an unknown pathogenic organism. In such an embodiment, gRNAs directed at the human DNA and the known bacteria can be used to serially deplete the human DNA, and the DNA of the known bacterial, thus resulting in a sample comprising the genomic material of the unknown pathogenic organism.

[0131] In an exemplary embodiment, the gRNAs are selective for human host DNA obtained from a biological sample from the host, but do not hybridize with DNA from an unknown organism (e.g. pathogen(s)) also in the sample.

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[0132] In some embodiments, the gRNAs are selective for a target nucleic acid sequences which are followed by Protospacer Adjacent Motif (PAM) sequences that can be bound by a Cas9. In some embodiments, the sequence of the gRNAs is determined by the CRISPR/Cas system protein type. For example, in various embodiments the gRNAs may be tailored to different Cas9 types as the PAM sequence can vary by the species of the bacteria from which Cas9 is derived.

[0133] gRNAs can range in size for example, from 50-250 base pairs. For example a gRNA can be at least 50bp, 55bp, 60bp, 65bp, 70bp, 75bp, 80bp, 85bp, 90bp, 95bp, 100bp, 110bp, 120bp, 125bp, 130bp, 140bp, 150bp, 160bp, 170bp, 175bp, 180bp, 190bp, or 195 bp. In specific embodiments, the gRNA is 80bp, 90bp, 100bp, or 110bp. Each target-specific gRNA comprises a base pair sequence that is complementary to a pre-defined site in a target nucleic acid that is followed by a Protospacer Adjacent Motif or (PAM) sequence that can be bound by a CRISPR/Cas system protein, for example a Cas9 protein, derived from a bacterial species. In specific embodiments, the base pair sequence of the gRNA that is complementary to a pre-defined site in a target nucleic acid is 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, or 50 base pairs.

[0134] The present invention also provides for gRNA libraries. A gRNA library can comprise a number of different species- specific gRNAs each, configured to hybridize with (be selective for) a nucleic acid sequence being targeted for depletion or partitioning. Each gRNA includes a target-specific guide sequence and a stem loop binding site that is formed to bind with a particular CRISPR/Cas system protein, for example with a Cas9 protein. The library can comprise a plurality of different guide RNAs, each having a different 15 to 20 base pair sequence that is complementary to a different pre-defined site in the nucleic acid being targeted, that is followed by an appropriate PAM sequence that can be bound by a CRISPR/Cas system protein. For each guide RNA the PAM sequence is present in the pre-defined DNA target sequence of the nucleic acid of interest but is not present in the corresponding target specific guide sequence.

[0135] Generally according to the present invention, any nucleic acid sequence in a genome of interest, with a pre-defined target sequence followed by the appropriate PAM sequence can be hybridized by a corresponding guide RNA provided in the guide RNA library and bound by CRISPR/Cas system protein, for example Cas9. In various embodiments the gRNA library may be tailored to different CRISPR/Cas system proteins, for example different Cas9 types since the PAM sequence can vary by the species of the bacteria from which protein is derived.

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[0136] Different target specific sequences in the gRNAs can be generated. This can be done by using a promoter for a bacteriophage RNA polymerase, e.g., the RNA polymerase from bacteriophage T3, T7, SP6 or the like. Accordingly each different T7 RNA polymerase promoter provides a different target specific sequence suitable for hybridizing to a different target nucleic acid sequence. A non-limiting exemplary set of forward primers usable for both annealing and subsequent PCR reactions is listed in Tables 1 and 2 of Example 1 provided below.

[0137] Without being limited to theory, the distance between gRNAs to arrive at >95% cleavage of the target nucleic acid can be computed, if the gRNAs display ~100% efficacy: this can be computed by measuring the distribution of library size and determining the mean, N and the standard deviation SD; N-2SD = minimum size for >95% of the library, ensuring that there is one guide RNA per fragment of this size to ensure >95% depletion. This can also be described as the Maximum distance between guide RNAs = Mean of library size - 2x (standard deviation of library size).

[0138] In the embodiments provided herein a gRNA library can be amplified to include a large number of copies of each different gRNAas well as a large number of different gRNAs as may be suitable to for the desired depletion results. The number of unique gRNAs in a given guide RNA library may range from 1 unique gRNAs to as many as 10^{10} unique gRNAs or approximately 1 unique guide RNA sequence for every 2 base pairs in the human genome. In some embodiments, the library comprises, at least 10^2 unique gRNAs. In some embodiments library comprises at least 10^2, at least 10^3, at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10} unique gRNAs. In one exemplary embodiment, the library comprises about 10^3 unique gRNAs.

[0139] In the embodiments provided herein, the methods comprise contacting a sample comprising nucleic acids with a plurality of CRISPR/Cas9 system protein-gRNA complexes, wherein the gRNAs are complementary to target sequences, such sequences targeted for depletion. In some embodiments, the method comprises using gRNAs which can base-pair with the targeted sites, wherein the sample is contacted with at least 10^2 unique gRNAs. In some embodiments the sample is contacted with at least 10^2, at least 10^3, at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10} unique gRNAs. In one exemplary embodiment, the sample is contacted with about 10^3 unique gRNAs.

[0140] In the embodiments provided herein, the methods comprise contacting a sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes, where the unique nature of the complex is determined by the unique nature of the gRNA itself. For example, 2

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unique CRISPR/Cas system protein-gRNA complexes may share the same CRISPR/Cas system protein, but the gRNAs differ, even if by only 1 nucleotide. Thus, in some embodiments, the method comprises contacting a sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes. In some embodiments the sample is contacted with at least 10^2, at least 10^3, at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10} unique CRISPR/Cas system protein-gRNA complexes. In one exemplary embodiment, the sample is contacted with about 10^3 unique CRISPR/Cas system protein-gRNA complexes.

[0141] In the embodiments provided herein, the methods comprise contacting a sample comprising a genomic DNA with a plurality of CRISPR/Cas9 system protein-gRNA complexes, wherein the gRNAs are complementary to sites targeted in the genome for depletion. In some embodiments, the method comprises using gRNAs which can base-pair with the targeted DNA, wherein the target site of interest is spaced at least every 1 bp, at least every 2 bp, at least every 3 bp, at least every 4 bp, at least every 5 bp, at least every 6 bp, at least every 7 bp, at least every 8 bp, at least every 9 bp, at least every 10 bp, at least every 11 bp, at least every 12 bp, at least every 13 bp, at least every 14 bp, at least every 15 bp, at least every 16 bp, at least every 17 bp, at least every 18 bp, at least every 19 bp, 20 bp, at least every 25 bp, at least every 30 bp, at least every 40 bp, at least every 50 bp, at least every 100 bp, at least every 200 bp, at least every 300 bp, at least every 400 bp, at least every 500 bp, at least every 600 bp, at least every 700 bp, at least every 800 bp, at least every 900 bp, at least every 1000 bp, at least every 2500 bp, at least every 5000 bp, at least every 10,000 bp, at least every 15,000 bp, at least every 20,000 bp, at least every 25,000 bp, at least every 50,000 bp, at least every 100,000 bp, at least every 250,000 bp, at least every 500,000 bp, at least every 750,000bp, or even at least every 1,000,000 bp across a genome of interest.

[0142] In the embodiments provided herein, the methods comprise contacting a sample comprising nucleic acids targeted for depletion with a plurality of CRISPR/Cas9 system protein-gRNA complexes, wherein the gRNAs are complementary to the nucleic acids targeted for depletion. In some embodiments the molar ratio of the gRNA:nucleic acids targeted for depletion is 1:1, 5:1, 10:1, 50:1, 100:1, 150:1, 250:1,500:1,750:1,1000:1, 1500:1, 2000:1, 2500:1, 5000:1, 7500:1, or even 10,000:1. In an exemplary embodiment the molar ratio of the gRNA:nucleic acids targeted for depletion is 500:1.

[0143] In the embodiments provided herein, the methods comprise contacting a sample comprising nucleic acids targeted for depletion with a plurality of CRISPR/Cas9 system protein-gRNA complexes, wherein the gRNAs are complementary to the nucleic acids

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targeted for depletion. In some embodiments the weight to weight ratio of the gRNA:nucleic acids targeted for depletion is 1:1, 5:1, 10:1, 20:1, 30:1, 40:1, 50:1, 60:1, 70:1, 80:1, 90:1, 100:1, 150:1, 250:1,500:1,750:1,1000:1, 1500:1, 2000:1, 2500:1, 5000:1, 7500:1, or even 10,000:1. In an exemplary embodiment the weight to weight ratio of the gRNA:nucleic acids targeted for depletion is ranges between 50:1 and 100:1.

[0144] The following description refers to Figure 1 as an exemplary embodiment. In Figure 1, a guide RNA library (1050) comprises a large number of different human specific gRNAs (1055) each configured to hybridize with a human DNA or nucleic acid sequence being targeted for depletion from the collection of DNA extraction fragments (1025) or any other DNA fragments that can be depleted because they are not of interest. In this embodiment, the nucleic acid sequences being targeted for depletion are not sequences that are present in the non-human genetic fragments (1030) that are of interest for further analyzed. Each guide RNA (1055) includes a target specific guide sequence (1060) and a stem loop binding site (1065) that is formed to bind with a Cas9 protein. Each target specific guide sequence (1060) is a 15 to 20 base pair sequence that is complementary to a pre-defined site in the human genome that is followed by a Protospacer Adjacent Motif or (PAM) sequence that can be bound by a Cas9 protein derived from a bacterial species. Other base pair lengths are usable ranging from about 8 to 100 base pairs without deviating from the present invention.

[0145] In Figure 1, the different target specific sequence (1060) is generated by a different T7 RNA polymerase promoter sequences (1070) that contains a promoter for a bacteriophage RNA polymerase, e.g., the RNA polymerase from bacteriophage T3, T7, SP6 or the like. Accordingly each different T7 RNA polymerase promoter (1070) provides a different target specific sequence (1060) suitable for hybridizing to a different human DNA sequence.

[0146] In Figure 1, the guide RNA library (1050) is amplified to include a large number of copies of each different gRNA(1055) as well as a large number of different gRNAs e.g. (1055a) (1055b) (1055n) as may be suitable to for the desired depletion results.

CRISPR/Cas System Proteins

[0147] Provided herein compositions and methods for the depletion of unwanted nucleic acids, and/or enrichment of sequences of interest in a sample. These compositions and methods utilize a CRISPR/Cas system protein.

[0148] In some embodiments, CRISPR/Cas system proteins include proteins from CRISPR Type I systems, CRISPR Type II systems, and CRISPR Type III systems.

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[0149] In some embodiments, CRISPR/Cas system proteins can be from any bacterial or archaeal species.

[0150] In some embodiments, the CRIPR/Cas system proteins are from, or are derived from CRISPR/Cas system proteins from Streptococcus pyogenes, Staphylococcus aureus, Neisseria meningitidis, Streptococcus thermophiles, Treponema denticola, Francisella tularensis, Pasteurella multocida, Campylobacter jejuni, Campylobacter lari, Mycoplasma gallisepticum, Nitratifractor salsuginis, Parvibaculum lavamentivorans, Roseburia intestinalis, Neisseria cinerea, Gluconacetobacter diazotrophicus, Azospirillum, Sphaerochaeta globus, Flavobacterium columnare, Fluviicola taffensis, Bacteroides coprophilus, Mycoplasma mobile, Lactobacillus farciminis, Streptococcus pasteurianus, Lactobacillus johnsonii, Staphylococcus pseudintermedius, Filifactor alocis, Legionella pneumophila, Suterella wadsworthensis, or Corynebacter diphtheria.

[0151] In some embodiments, examples of CRISPR/Cas system proteins can be naturally occurring or engineered versions.

[0152] In some embodiments, naturally occurring CRISPR/Cas system proteins include Cas9, Cpf1, Cas3, Cas8a-c, Cas10, Cse1, Csy1, Csn2, Cas4, Csm2, and Cm5. In an exemplary embodiment, the CRISPR/Cas system protein comprises Cas9.

[0153] A “CRISPR/Cas system protein-gRNA complex" refers to a complex comprising a CRISPR/Cas system protein and a guide RNA. The guide RNA may be composed of two molecules, i.e., one RNA ("crRNA") which hybridizes to a target and provides sequence specificity, and one RNA, the "tracrRNA", which is capable of hybridizing to the crRNA. Alternatively, the guide RNA may be a single molecule (i.e., a gRNA) that contains crRNA and tracrRNA sequences. A CRISPR/Cas system protein may be at least 60% identical (e.g., at least 70%, at least 80%, or 90% identical, at least 95% identical or at least 98% identical or at least 99% identical) to a wild type CRISPR/Cas system protein. The CRISPR/Cas system protein may have all the functions of a wild type CRISPR/Cas system protein, or only one or some of the functions, including binding activity, nuclease activity, and nuclease activity.

[0154] The term "CRISPR/Cas system protein-associated guide RNA" refers to a guide RNA as described above (comprising a crRNA molecule and a tracrRNA molecule, or comprising a single RNA molecule that includes both crRNA and tracrRNA sequences). The CRISPR/Cas system protein -associated guide RNA may exist as isolated RNA, or as part of a CRISPR/Cas system protein -gRNA complex.

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Cas9

[0155] In some embodiments, the CRISPR/Cas system protein comprises Cas9. The Cas9 of the present invention can be isolated, recombinantly produced, or synthetic.

[0156] Cas9 proteins that can be used in the embodiments herein can be found in http://www.nature.com/nature/journal/v520/n7546/full/nature14299.html.

[0157] In some embodiments, the Cas9 is a Type II CRISPR system derived from Streptococcus pyogenes, Staphylococcus aureus, Neisseria meningitidis, Streptococcus thermophiles, Treponema denticola, Francisella tularensis, Pasteurella multocida, Campylobacter jejuni, Campylobacter lari, Mycoplasma gallisepticum, Nitratifractor salsuginis, Parvibaculum lavamentivorans, Roseburia intestinalis, Neisseria cinerea, Gluconacetobacter diazotrophicus, Azospirillum, Sphaerochaeta globus, Flavobacterium columnare, Fluviicola taffensis, Bacteroides coprophilus, Mycoplasma mobile, Lactobacillus farciminis, Streptococcus pasteurianus, Lactobacillus johnsonii, Staphylococcus pseudintermedius, Filifactor alocis, Legionella pneumophila, Suterella wadsworthensis, or Corynebacter diphtheria.

[0158] In some embodiments, the Cas9 is a Type II CRISPR system derived from S. *pyogenes* and the PAM sequence is NGG located on the immediate 3' end of the target specific guide sequence. The PAM sequences of Type II CRISPR systems from exemplary bacterial species can also include: *Streptococcus pyogenes* (NGG), *Staph aureus* (NNGRRT), *Neisseria meningitidis* (NNNNGA TT), *Streptococcus thermophilus* (NNAGAA) and *Treponema denticola* (NAAAAC) which are all usable without deviating from the present invention. ((http://www.nature.com/nature/journal/v520/n7546/full/nature14299.html)).

[0159] In one exemplary embodiment, Cas9 sequence can be obtained, for example, from the pX330 plasmid (available from Addgene), re-amplified by PCR then cloned into pET30 (from EMD biosciences) to express in bacteria and purify the recombinant 6His tagged protein.

[0160] The "Cas9-gRNA complex" refers to a complex comprising a Cas9 protein and a guide RNA. The guide RNA may be composed of two molecules, i.e., one RNA ("crRNA") which hybridizes to a target and provides sequence specificity, and one RNA, the "tracrRNA", which is capable of hybridizing to the crRNA. Alternatively, the guide RNA may be a single molecule (i.e., a gRNA) that contains crRNA and tracrRNA sequences. A Cas9 protein may be at least 60% identical (e.g., at least 70%, at least 80%, or 90% identical, at least 95% identical or at least 98% identical or at least 99% identical) to a wild type Cas9 protein, e.g., to the *Streptococcus pyogenes* Cas9 protein. The Cas9 protein may have all the

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functions of a wild type Cas9 protein, or only one or some of the functions, including binding activity, nuclease activity, and nuclease activity.

[0161] The term "Cas9-associated guide RNA" refers to a guide RNA as described above (comprising a crRNA molecule and a tracrRNA molecule, or comprising an RNA molecule that includes both crRNA and tracrRNA sequences). The Cas9-associated guide RNA may exist as isolated RNA, or as part of a Cas9-gRNA complex.

Catalytically Dead CRISPR/Cas System Proteins

[0162] In some embodiments, engineered examples of CRISPR/Cas system proteins include catalytically dead CRISPR/Cas system proteins. The term "catalytically dead" generally refers to a CRISPR/Cas system protein that has inactivated HNH and RuvC nucleases. Such a protein can bind to a target site in any nucleic acid (where the target site is determined by the guide RNA), but the protein is unable to cleave or nick the double-stranded DNA.

[0163] In some embodiments, the catalytically dead CRISPR/Cas system protein is dCas9, dCpf1, dCas3, dCas8a-c, dCas10, dCse1, dCsy1, dCsn2, dCas4, dCsm2, or dCm5.

[0164] In one embodiment the catalytically dead CRISPR/Cas system protein is a dCas9. Accordingly the dCas9 allows partition of the mixture into unbound nucleic acids and dCas9-bound fragments targeted for partitioning. In one embodiment, a dCas9/gRNA complex binds to targets determined by the gRNA sequence. The dCas9 bound can prevent cutting by Cas9 while other manipulations proceed. This is depicted in FIG. 2. In one embodiment, the dCas9/gRNA complex binds to the targets determined by the gRNA sequence, and the bound portion of the target nucleic acid sample can be removed by binding of an affinity tag (e.g., biotin) previously attached to the dCas9 protein. The bound nucleic acid sequences can be eluted from the Cas9/gRNA complex by denaturing conditions and then amplified and sequenced. Conversely, in another embodiment, those dCas9 targeted nucleic acids can be discarded, and the remaining nucleic acids can be analyzed by amplification and sequencing.

CRISPR/Cas System Protein Nickases

[0165] In some embodiments, engineered examples of CRISPR/Cas system proteins also include Cas nickases. A Cas nickase refers to a modified version of a CRISPR/Cas system protein, containing a single inactive catalytic domain.

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[0166] In some embodiments, the CRISPR/Cas system protein nickase is Cas9 nickase, Cpf1 nickase, Cas3 nickase, Cas8a-c nickase, Cas10 nickase, Cse1 nickase, Csy1 nickase, Csn2 nickase, Cas4 nickase, Csm2 nickase, or Cm5 nickase.

[0167] In one embodiment, the CRISPR/Cas system protein nickase is Cas9 nickase.

[0168] In some embodiments, a Cas9 nickase can be used to bind to target sequence. The term "Cas9 nickase" refers to a modified version of the Cas9 protein, containing a single inactive catalytic domain, for example, either the RuvC- or the HNH-domain. With only one active nuclease domain, the Cas9 nickase cuts only one strand of the target DNA, creating a single-strand break or "nick". Depending on which mutant is used, the guide RNA-hybridized strand or the non-hybridized strand may be cleaved. Cas9 nickases bound to 2 gRNAs that target opposite strands can create a double-strand break in the DNA. This "dual nickase" strategy increases the specificity of cutting because it requires that both Cas9/gRNA complexes be specifically bound at a site before a double-strand break is formed.

[0169] In an exemplary embodiment, depletion of DNA can be carried out using a Cas9 nickase. In one embodiment, the method comprises: making a DNA sequencing library comprising DNA to be removed (for example human DNA not of interest) and DNA of interest (for example an DNA from an unknown pathogen); designing guide RNAs so that all the DNA to be depleted will have two guide RNA binding sites in close proximity (for example, less than 15 bases apart) on opposite DNA strands; adding Cas9 Nickase and guide RNA to the DNA library. In this embodiment, the Cas9 Nickase recognizes its target sites on the DNA to be removed and cuts only one strand. For DNA to be depleted, two separate Cas9 Nickases can cut both strands of the DNA to be removed (e.g. human DNA) in close proximity; only the DNA to be removed (e.g. human DNA) will have two Cas9 nickase sites in close proximity which creates a double stranded break. If a Cas9 Nickase recognizes non-specifically or at low affinity a site on the DNA of interest (e.g. pathogen DNA), it will only cut one strand which would not prevent subsequent PCR amplification or downstream processing of the DNA molecule. This is pictorially depicted in FIG. 7. In this embodiment, the chances of two guide RNAs recognizing two sites non-specifically in close enough proximity is negligible (<1x10-14). This embodiment would be particularly useful if regular, Cas9-mediated cleavage cuts too much of the DNA of interest.

Dissociable and Thermostable CRISPR/Cas System Proteins

[0170] Although CRISPR/Cas System proteins can be used in combination with a library of guide RNAs to efficiently deplete a collection of target DNA, large amounts (e.g. >30

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pmoles) of CRISPR/Cas System proteins and guide RNAs may be needed. One reason for this usually >100 fold excess amount over target DNA is that, unlike classical restriction enzymes such as EcoRI, which detach completely from their target DNA after cleavage, CRISPR/Cas System proteins are not completely recycled after completion of the cutting reaction. CRISPR/Cas System proteins, for example Cas9, can remain bound to one of the two daughter DNA product molecules (see FIG. 11, open circles on the left). As a result, more CRISPR/Cas System proteins and gRNA may need to be provided in order to achieve complete depletion of unwanted DNA.

[0171] In some embodiments, to overcome this problem, dissociable CRISPR/Cas System proteins are provided herein. For example upon cleavage of targeted sequences, the CRISPR/Cas System protein can be made to dissociate from the gRNA, or from the target. In some embodiments, the dissociation is induced by elevating the temperature of the reaction. This can act to increase processivity of the enzyme, by allowing it to complex with available gRNAs, re-associate with additional target sequences and generate additional cleaved target sequences.

[0172] In some embodiments to overcome this problem, thermostable CRISPR/Cas System proteins are provided herein. In such embodiments, the reaction temperature is elevated, inducing dissociation of the protein; the reaction temperature is lowered, allowing for the generation of additional cleaved target sequences. In some embodiments, thermostable CRISPR/Cas system proteins maintain at least 50% activity, at least 55% activity, at least 60% activity, at least 65% activity, at least 70% activity, at least 75% activity, at least 80% activity, at least 85% activity, at least 90% activity, at least 95% activity, at least 96% activity, at least 97% activity, at least 98% activity, at least 99% activity, or 100% activity, when maintained for at least 75°C for at least 1 minute. In some embodiments, thermostable CRISPR/Cas system proteins maintain at least 50% activity, when maintained for at least 1 minute at least at 75°C, at least at 80°C, at least at 85°C, at least at 90°C, at least at 91°C, at least at 92°C, at least at 93°C, at least at 94°C, at least at 95°C, 96°C, at least at 97°C, at least at 98°C, at least at 99°C, or at least at 100°C. In some embodiments, thermostable CRISPR/Cas system proteins maintain at least 50% activity, when maintained at least at 75°C for at least 1 minute, 2 minutes, 3 minutes, 4 minutes, or 5 minutes. In some embodiments, a thermostable CRISPR/Cas system protein maintains at least 50% activity when the temperature is elevated, lowered to 25°C-50°C. In some embodiments, the temperature is lowered to 25°C, to 30°C, to 35°C, to 40°C, to 45°C, or to 50°C In one exemplary embodiment, a thermostable enzyme retains at least 90% activity after 1 min at 95°C.

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[0173] In some embodiments, the thermostable CRISPR/Cas system protein is thermostable Cas9, thermostable Cpf1, thermostable Cas3, thermostable Cas8a-c, thermostable Cas10, thermostable Cse1, thermostable Csy1, thermostable Csn2, thermostable Cas4, thermostable Csm2, or thermostable Cm5.

[0174] In some embodiments the thermostable CRISPR/Cas system protein is thermostable Cas9.

[0175] In one exemplary embodiment, thermostable Cas9 complexed with a guide RNAs (for example a gRNA library against human DNA) can be applied to a sequencing library of DNA mixture (containing for example 95% human DNA and 5% viral DNA). As depicted in FIG. 11 (grey circles on the right), after allowing Cas9 to digest for a period of time, the temperature of the sample mixture can be elevated, for example upto 95°C or greater, which can cause DNA denaturation, as well as dissociation of gRNA and Cas9 from the DNA targets. The binding of Cas9 to gRNAs can be increased so that the Cas9-gRNA dissociates from the DNA target as an intact complex, despite of DNA denaturation. Dimethyl sulfoxide can be added to reduce the temperature required for DNA denaturation, so that the Cas9 protein structure is not be affected. Cas9 preferentially binds to target sites that have not been cut, and a thermostable Cas9 can retain activity after boiling. Because of these features, by elevating the temperature, for example upto 100□C, and cooling down the reaction to, for example, 37°C, a thermostable Cas9 can remain capable of binding to its gRNA and cutting more of its substrate. By allowing the recycling of Cas9, the depletion efficiency can be increased, and as less Cas9 will be needed in the reaction, the off-target (non-specific) cleavage probability can also be decreased.

[0176] Thermostable CRISPR/Cas System proteins can be isolated, for example, identified by sequence homology in the genome of thermophilic bacteria Streptococcus thermophilus and Pyrococcus furiosus. CRISPR/Cas system genes can then be cloned into an expression vector. In one exemplary embodiment, a thermostable Cas9 protein is isolated.

[0177] In another embodiment, a thermostable CRISPR/Cas system protein can be obtained by in vitro evolution of a non-thermostable CRISPR/Cas system protein. The sequence of a CRISPR/Cas system protein can be mutagenized to improve its thermostability. In some embodiments, this can be achieved by site-directed mutagenesis to remove excess loop sequences, increasing the number of ionic bridges between protein domains, or by diluting into droplets and PCR to create a pool of potential mutants. In one exemplary embodiment, a thermostable Cas9 is produced by *in vitro* evolution of a non-thermostable Cas9.

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Methods of the Invention

Enrichment of Sequences of Interest/Depletion of Targeted Sequences

[0178] Provided herein are methods of enriching a sample for sequences of interest, by depleting targeted sequences that are not of interested (target sequences are sequences targeted for depletion).

[0179] In one embodiment, a method of enriching a sample for sequences of interest, comprises:

providing a sample comprising sequences of interest and targeted sequences for depletion, wherein the sequences of interest comprise less than 50%, less than 45%, less than 40%, less than 35%, less than 30%, less than 25%, less than 20%, less than 15%, less than 10%, less than 9%, less than 8%, less than 7%, less than 6%, less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, less than 0.5%, less than 0.1%, less than 0.05%, less than 0.01%, less than 0.005%, or even less than 0.001% of the sample; contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to the targeted sequences, whereby the targeted sequences are cleaved. In an exemplary embodiment the CRISPR/Cas system protein is Cas9.

[0180] In one embodiment, a method of enriching a sample for non-mitochondrial DNA comprises: (a) providing a sample comprising mitochondrial DNA and non-mitochondrial DNA, wherein the mitochondrial DNA and non-mitochondrial DNA are adapter-ligated, and wherein the adapters are ligated to the 5' and 3' ends of the mitochondrial DNA and non-mitochondrial DNA; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to the mitochondrial DNA, thereby generating mitochondrial DNA adapter ligated only on one end and non-mitochondrial DNA adapter ligated on both the 5' and 3' ends; and (c) enriching the sample for non-mitochondrial DNA. In some embodiments the sample is enriched using adapter-specific PCR. In some embodiments, the enriching comprises treating the product of step (b) with an enzyme that has exonuclease activity, for example Exonuclease III or BAL-31. For example, if Exonuclease III is utilized, the adapters may be Y-shaped. If BAL-31 is utilized, the adapters may comprise poly-G tails. In some embodiments the sample is enriched by purification of the sequences of interest.

[0181] In another embodiment, a method of enriching a sample comprises: (a) providing a sample comprising nucleic acids from a first genome and nucleic acids from a second genome, wherein the nucleic acids from the first genome are adapter-ligated on their 5' and 3' ends and wherein the nucleic acids from the second genome are adapter-ligated on their 5'

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and 3' ends; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to sites on the nucleic acids from the first genome, thereby generating nucleic acids from the first genome adapter ligated only on one end and nucleic acids from the second genome adapter ligated on both the 5' and 3' ends; and (c) enriching the sample for nucleic acids from the first genome. In some embodiments the sample is enriched using adapter-specific PCR. In some embodiments, the enriching comprises treating the product of step (b) with an enzyme that has exonuclease activity, for example Exonuclease III or BAL-31. For example, if Exonuclease III is utilized, the adapters may be Y-shaped. If BAL-31 is utilized, the adapters may comprise poly-G tails. In some embodiments the sample is enriched by purification of the sequences of interest.

[0182] In another embodiment, a method of enriching a sample for non-host nucleic acids comprises: providing a sample comprising host nucleic acids and non-host nucleic acids, wherein the host nucleic acids and non-host nucleic acids are adapter-ligated, and wherein the adapters are ligated to the 5' and 3' ends of the host nucleic acids and the non-host nucleic acids; contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to targeted sites in the host nucleic acids, thereby generating host- nucleic acids adapter ligated only on one end and non-host nucleic acids adapter ligated on both the 5' and 3' ends; and amplifying the products using adapter-specific PCR or purifying, or treatment with exonuclease .

[0183] In another embodiment, provided herein is a method for serially depleting targeted nucleic acids in a sample comprising:(a) providing a sample comprising host nucleic acids and non-host nucleic acids, wherein the non-host nucleic acids comprise nucleic acids from at least one known non-host organism and nucleic acids from at least one unknown non-host organism; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are configured to hybridize to targeted sequences in the host nucleic acids, whereby a portion of the host nucleic acids are cleaved; (c) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are configured to hybridize to targeted sequences in the at least one known non-host nucleic acids, whereby a portion of the at least one known non-host nucleic acids are cleaved; and (d) isolating the nucleic acids from the unknown non-host organism.

[0184] Figure 1 provides a first non-limiting exemplary method relates to processing a human tissue specimen (1000). In a first step (1005) a DNA extraction extracts substantially all the nucleic acid sequences from the specimen (1000) including nucleic acid sequences of the human host and nucleic acid sequences of any non-human or non-host organism present

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in the specimen (1000). The DNA extraction step (1005) may produce about 95% human DNA (1010) and about 5% non-human DNA (1015) wherein the non-human DNA (1015) largely comprises microbial DNA and in the present example method embodiment the microbial or non-human DNA (1015) is of primary interest for further DNA analysis. After the DNA extraction step (1005) the extracted nucleic sequences (1010) and (1015) are fragmented to reduce the lengths of each extracted nucleic sequence to a more manageable length for amplifying, sequencing or the like. An adapter (1020) is ligated to each end of each of the fragments. Each extracted nucleic sequence fragment or genomic fragment comprises a region of a genome, e.g., each fragment (1025) includes a nucleic acid sequence, shown as a dashed line between two adapters (1020), from a region of the human genome while each fragment (1030) includes a nucleic acid sequence, shown as a solid line between two adapters (1020), from a region of one of the non-host organisms extracted from the tissue sample. Each extracted nucleic acid or genomic fragment comprises a region of a genome, e.g., each fragment (1025) includes a nucleic acid sequence, shown as a dashed line between two adapters (1020), from a region of the human genome while each fragment (1030) includes a nucleic acid sequence, shown as a solid line between two adapters (1020), from a region of one of the non-host organisms extracted from the tissue sample. However, it will be recognized that the sequences are indistinguishable without further analysis.

[0185] An exemplary application of the compositions and methods described herein is provided in Figure 1. The figure depicts a non-limiting exemplary embodiment of the present invention that includes a depletion method for depleting a DNA extraction of a biological sample. In Figure 1, the entire fragmented DNA extraction (1025) and (1030) as well as the entire guide RNA library (1050) is mixed with a Cas9 protein in a mixture (1080). While in the mixture (1080) each target specific guide sequence (1060) hybridizes to matching nucleic acid sequences found in human DNA fragments (1025) and the Cas9 protein and the guide RNA stem binding sites (1065) form a guide RNA/Cas9 complex (1085) that binds guide RNA (1055) to the human DNA fragments (1025). If the guide RNA library (1050) is well designed, substantially all the human DNA fragments (1025) will be bound by a guide RNA/Cas9 complex. Thereafter the mixture (1080) is incubated in a manner that cases the Cas9 protein to cut or cleave both strands of each human DNA fragment (1025) that is bound by a guide RNA/Cas9 complex. After cutting, the human DNA fragment (1025) are in two pieces (1090) (1095) and any uncut DNA fragment (1030) remain intact with adapter (1020) still attached to each end and ready for amplification and further study.

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[0186] In various further processing steps occurring after the depletion targets have been cut or cleaved the mixture (1080) may be sized-selectively filtered to separate uncut fragments (1030) from cut fragments (1090) and (1095). Thereafter the uncut fragments (1030) can be amplified and sequenced. Alternately the amplification process can be used to sort the cut from the uncut segments since in some amplification systems only segments having an adapter (1020) at each end of the fragment is amplified.

[0187] Another exemplary application of the compositions and methods described herein is provided in Figure 2. Figure 2 depicts a second non-limiting exemplary method relates to processing a specimen or sequencing library (2000) that involves partitioning, and not depletion. In a first step (2005) a DNA extraction extracts substantially all the nucleic acid sequences from the specimen (2000) including nucleic acid sequences of a human host and nucleic acid sequences of any other non-host organism present in the specimen (2000). In the present example the DNA extraction step (2005) may produce about 5 to 40% human DNA and about 95 to 60% non-human DNA. As described with respect to Figure 1 above, the DNA extraction sample is fragmented and adapter ligated. A further described with respect to Figure 1 above, a guide RNA library (2050) of gRNAs is developed to hybridize with a large number of different human specific gRNAs (2055) each configured to hybridize with a human DNA or nucleic acid sequence being targeted for binding by a Cas9 complex. However instead of cutting the targeted sequences, the method shown in Figure 2 is used to partition the fragmented nucleic acid sample into two fractions which can each be amplified separately. As described above the guide library (2050) and fragmented DNA extraction sample are combined with Cas9 in a mixture (2080). However in the present example embodiment the CAs9 comprises a catalytically dead Cas9 (dCas9). The term "catalytically dead" refers to a Cas protein that has inactivated HNH and RuvC nucleases. Such a protein can bind to a target site in double-stranded DNA (where the target site is determined by the guide RNA), but the protein is unable to cleave or nick the double-stranded DNA. Accordingly the dCas9 partitions the mixture (2080) into unbound non-human DNA fragments (2030) and dCas9 bound human DNA fragments (2090). The dCas9/gRNA complex only binds to the targets determined by the gRNA sequence, and the bound portion of the target nucleic acid sample is removed by binding of an affinity tag (e.g., biotin) previously attached to the dCas9 protein. The bound nucleic acid sequences (2090) can be eluted from the Cas9/gRNA complex by denaturing conditions and then amplified and sequenced. Similarly, the unbound nucleic acid sequences (2030) can be amplified and sequenced.

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Depletion of Large Fragments of DNA Without Adapters

[0188] In some embodiments, the methods provided herein are used for depletion. However, instead of cleaving a library containing adapters, the guide RNAs are chosen to cleave multiple times in a pool of fragmented DNA of a large size (e.g., anywhere from 100 bp – 10 kb). Following cleavage with CRISPR/Cas system protein-gRNA complexes, the DNA is subjected to size selection to remove small fragments (e.g., at least 1/2 or 1/3 of the average size of intact fragments). This is assisted, for example, by treatment with Lambda exonuclease, which is a 5'phosphate specific-exonuclease that proceeds in a 5' to 3' direction and would attack any fragments that had previously been cut with the CRISPR/Cas system protein. The resulting libraries can then be subjected to sequencing by single-molecule sequencers that do not require sequencing adapters; or adapters can be ligated for downstream analyses.

Depletion Using Thermostable CRISPR/Cas System Proteins

[0189] In another embodiment, a method of generating cleaved targeted sequences in a sample comprises: (a) providing a sample comprising sequences of interest and targeted sequences for cleavage; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to the targeted sequences, thereby generating cleaved targeted sequences; (c) dissociating the CRISPR/Cas system protein from the cleaved targeted sequences; (d) generating additional cleaved targeted sequences; and (c) recovering the uncut sequences of interest. In some embodiments, the CRISPR/Cas system protein is thermostable. In some embodiments, the dissociating of the CRISPR/Cas system protein from the cleaved targeted sequences is achieved by elevating the temperature of the mixture of step (b) to at least 75°. In some embodiments, the generating of additional cleaved targeted sequences is achieved by lowering the temperature of the mixture of step (b) to at least 50°.

[0190] In another embodiment, a method of depleting targeted sequences in a sample comprises: (a) providing a sample comprising sequences of interest and targeted sequences for depletion; (b) contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to the targeted sequences, thereby generating cleaved targeted sequences, and wherein the CRISPR/Cas system protein is thermostable; (c) elevating the temperature of the mixture of step (b) to at least 75°; (d) lowering the temperature of the mixture of step (b) at least 50°; repeating steps (c) and (d) at least once; and (f) recovering the uncut sequences of interest.

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CRISPR/Cas System Protein-Mediated Depletion in the Presence of a CRISPR/Cas Protein Nickase

[0191] In some embodiments, depletion of unwanted, targeted nucleic acids can be carried out using a CRISPR/Cas system protein nickase. In one embodiment, the method comprises: making a DNA sequencing library comprising DNA to be removed (for example human DNA not of interest) and DNA of interest (for example an DNA from an unknown pathogen); designing guide RNAs so that all the DNA to be depleted will have two guide RNA binding sites in close proximity (for example, less than 15 bases apart) on opposite DNA strands; adding CRISPR/Cas system protein Nickase and guide RNAs to the DNA library. In this embodiment, the CRISPR/Cas system protein Nickase can recognize its target sites on the DNA to be removed and cuts only one strand. For DNA to be depleted, two separate CRISPR/Cas system protein Nickases can cut both strands of the DNA to be removed (e.g. human DNA) in close proximity; only the DNA to be removed (e.g. human DNA) will have two CRISPR/Cas system protein nickase sites in close proximity which creates a double stranded break. If a CRISPR/Cas system protein Nickase recognizes non-specifically or at low affinity a site on the DNA of interest (e.g. pathogen DNA), it can only cut one strand which would not prevent subsequent PCR amplification or downstream processing of the DNA molecule. This is pictorially depicted in FIG. 7. In this embodiment, the chances of two guide RNAs recognizing two sites non-specifically in close enough proximity is negligible (<1x10-14). This embodiment would be particularly useful if regular, CRISPR/Cas system protein -mediated cleavage cuts too much of the DNA of interest.

CRISPR/Cas System Protein-Mediated Depletion and Biotin Labeling

[0192] In some embodiments, the CRISPR/Cas system protein cleaved products are eliminated with the use of biotin. For example in a sample originally comprising <5% DNA of interest and >95% DNA targeted for depletion, the >95% DNA is fragmented and depleted, and the uncut (<5% DNA of interest) DNA comprises a biotin label is purified by binding to streptavidin beads. This example illustrates a method of removing unwanted DNA (depleted DNA fragmented) after CRISPR/Cas system protein -mediated cleavage without the use of an exonuclease.

[0193] An exemplary embodiment is depicted in FIG. 10. In this exemplary embodiment a DNA mixture (containing >95% unwanted human DNA, and <5% other DNA of interest) is fragmented, end-repaired and ligated to adapters following regular protocols, then amplified

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by PCR using primers of Biotin-P5, (for example 5'Biotin-AATGATACGGCGACCACCGA) and P7 (for example, 5'-CAAGCAGAAGACGGCATACGA). This ensures that the entire sequencing library possesses a 5'Biotin label only on one end of the DNA molecules. The DNA mixture is then subjected to CRISPR/Cas system protein (for example, Cas9) digestion, complexed with, for example for this case, a guide RNA library against mitochondrial DNA (the unwanted DNA). DNA molecules that have been cleaved by the CRISPR/Cas system protein (for example in this case, mitochondrial DNA) will lose the Biotin labeled adaptor, and thus cannot be sequenced or PCR amplified; while intact DNA libraries uncut by Cas9 (for example in this case, non-mitochondrial DNA of interest) still carry the Biotin label. As a result, intact DNA uncut by the CRISPR/Cas system protein can then be recovered by adding streptavidin beads. Samples can then be subject to further downstream applications, such as sequencing, cloning, for further enrichment.

Exonuclease Treatment Following Depletion

[0194] In some embodiments, it is desirable to remove cut fragments prior to PCR amplification or sequencing.

[0195] In one embodiment, CRISPR/Cas system protein-mediated depletion is followed by an exonuclease treatment. The exonuclease treatment can further degrade a CRISPR/Cas system protein cleaved nucleic acid, while leaving the uncut nucleic acid comprising sequences of interest intact.

[0196] In one embodiment, a method of depleting targeted sequences in a sample comprises: providing a sample comprising sequences of interest and targeted sequences for depletion; contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to the targeted sequences, thereby generating cleaved targeted sequences; and contacting the product of step (b) with an exonuclease. In one embodiment the exonuclease is Exonuclease III. In another embodiment, the exonuclease is BAL-31.

[0197] In one exemplary embodiment, DNA is extracted from a biological sample and fragmented and a library, for example a sequencing library, comprising the fragments of DNA, is thus created. Y-shaped adapters or circular adapters are ligated to the DNA fragments. The DNA fragments comprise DNA sequences of interest, and targeted sequences (DNA not of interest). The library is contacted with a plurality of CRISPR/Cas system protein-gRNA complexes, and the CRISPR/Cas system protein cuts the target DNA not of

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interest and leaves the other DNA sequences of interest intact. The resulting products are contacted with Exonuclease III. Exonuclease III can initiate unidirectional 3'>5' degradation of one DNA strand by using blunt end or 5' overhangs, yielding single-stranded DNA and nucleotides; it is not active on single-stranded DNA, and thus 3' overhangs, such as the Y-shaped adapter ends, are resistant to degradation. As a result, intact double-stranded DNA libraries uncut by the CRISPR/Cas system protein is not digested by Exonuclease III, while DNA molecules that have been cleaved by the CRISPR/Cas system protein are degraded by Exonuclease III with its 3'>5' activity from the blunt ends cut by Cas9 towards the adaptors. FIG. 8 illustrates an exemplary embodiment. A DNA mixture (containing >95% unwanted human DNA, and <5% other DNA of interest is fragmented, end-repaired and ligated to Y-shaped adapters (or circular adapters) following regular protocols, but not amplified by PCR. The DNA mixture is then subjected to Cas9 digestion, complexed with, for example for this case, a guide RNA library against mitochondrial DNA. Exonuclease III is added. Unwanted mitochondrial DNA is digested. The remaining intact double-stranded DNA libraries are then recovered by column-purification and/or PCR-amplified.

[0198] In another exemplary embodiment, Exonuclease BAL-31 is used degrade CRISPR/Cas system protein- cleaved DNA while leaving the uncut DNA of interest intact. In one exemplary embodiment, DNA is extracted from a biological sample and fragmented and a library, for example a sequencing library, comprising the fragments of DNA, is thus created. The DNA fragments comprise DNA sequences of interest, and targeted sequences (DNA not of interest). The 3' ends of the fragments are tailed with poly-dG using terminal transferase. The library is contacted with a plurality of CRISPR/Cas system protein-gRNA complexes, and the CRISPR/Cas system protein cuts the target DNA not of interest and leaves the other DNA sequences of interest intact. The resulting products are contacted with Exonuclease BAL-31. Exonuclease BAL-31 has two activities: double-stranded DNA exonuclease activity, and single-stranded DNA/RNA endonuclease activity. The double-stranded DNA exonuclease activity allows BAL-31 to degrade DNA from open ends on both strands, thus reducing the size of double-stranded DNA. The longer the incubation, the greater the reduction in size of the double-stranded DNA, making it useful for depleting medium to large DNA (>200 bp). It was noted that the single-stranded endonuclease activity of BAL-31 allows it to digest poly-A, -C or -T very rapidly, but is extremely low in digesting poly-G (Marrone and Ballantyne, 2008). Because of this nature, adding single-stranded poly-dG at 3' ends of the libraries serves as a protection from being degraded by BAL-31. As a result, DNA molecules that have been cleaved by a CRISPR/Cas system protein can be

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degraded by BAL-31 with its double-stranded DNA exonuclease activity from the double-stranded blunt end cut by the CRISPR/Cas system protein towards the other end carrying poly-dG, effecting depletion; while intact DNA libraries uncut by the CRISPR/Cas system protein are not digested by BAL-31 due to their 3' end poly-dG protection. FIG. 9 depicts an exemplary embodiment of this approach. Sequencing libraries (containing, for example >95% human DNA and <5% other are prepared. The libraries are tailed with poly-dG at 3' ends using Terminal Transferase. poly-dG tailed libraries are then subjected to Cas9 digestion, complexed with a guide RNA library, for example a guide RNA library against mitochondrial DNA. The products are then incubated with Exonuclease BAL-31, which initiates digestion of ends not capped by the poly-dG. The products are then subject to further PCR or purified on DNA purification columns. The remaining intact double stranded DNA libraries can then be recovered by column purification or PCR-amplified.

Controls for Monitoring Depletion

[0199] In the embodiments discussed, it is desirable to provide positive and/or negative controls. A positive control can ensure depletion of target nucleic acids proceeds with fidelity.

[0200] In some embodiments, a control set of reagents is a positive control set of reagents, positive control target sequences. In an exemplary embodiment, the positive control set of reagents comprise a collection of nucleic acid fragments, wherein the fragments comprise the target sequences, to which the gRNAs are at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% complementary. This control could be run alongside a user's reaction to ensure that all components are working properly. After depletion with a CRISPR/Cas system protein, elimination of the positive control target sequences could be measured by gel electrophoresis or qPCR.

[0201] In some embodiments, the control set of reagents is a negative control set of reagents. In some embodiments a negative control ensures that off-target cutting is minimal or nonexistent. In an exemplary embodiment, the negative control set of reagents comprise a second set of gRNAs, wherein the second set of gRNAs exhibit reduced binding to the target sequences, as compared to the first set of gRNAs. In some embodiments, the second set of gRNAs exhibit 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, or 85% reduced binding to the target sequences, as compared to the first set of RNAs. In another exemplary embodiment, the negative control set of reagents comprise a second set of gRNAs, wherein the second set of gRNAs are no more than 50%, 55%, 60%,

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65%, 70%, 75%, 80%, 85%, 90%, or 95% complementary to target sequences. The negative control could be a set of gRNAs with less than 100% identity to the gRNAs used; for example, with 1, 2, 3, 4, 5, or more mismatches to the target-specific sequence. In some embodiments, the negative control set of reagents comprise a collection of nucleic acid fragments, wherein the fragments are no more than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% complementary to the first set of gRNAs. The DNA library could have with less than 100% identity to the targeted sequences of gRNAs used in the kit; for example, with 1, 2, 3, 4, 5, or more mismatches to the complementary sequence of the gRNA. This control could be run alongside a user's reaction to ensure that all components are working properly and to measure any off-target activity of the enzyme. After depletion with CRISPR/Cas system protein, the amount of off-target depletion can be measured by gel electrophoresis or qPCR.

[0202] With respect to the positive and negative controls contemplated herein, complementarity of the gRNA to its target as provided herein can refer to the complementarity of the gRNA along the entire length of the target. In some embodiments, however the complementarity may be more impacted by mismatches of nucleic acids at the terminal end of the gRNA, at the area binding closest to the PAM sequence. Mismatches in this region may impact the capacity to bind the target in a greater manner than mismatches of nucleic acids elsewhere along the gRNA. Likewise, mismatches in the gRNA further away from the PAM sequence may impact the capacity to bind the target less than mismatches of nucleic acids elsewhere along the gRNA. In some embodiments, the negative control gRNA has a lower complimentarily near the PAM sequence, but higher complementarity farther away from the PAM sequence. For example, in some embodiments, the negative control gRNA has 50%-70% complementarity near the PAM sequence, but 70%-100% complementarity farther away from the PAM sequence. In some embodiments, the negative control gRNA has 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% complementarity along the entire length of the target.

Depleting Mitochondrial DNA Following ATAC-seq

[0203] The Assay for Transposase-Accessible Chromatin Using Sequencing (ATAC-seq) technique is used in molecular biology to study chromatin, namely its accessible sites. The method is based on tagging open chromatin regions with sequencing adaptors by transposase, such that DNA fragments from these open chromatin regions can be amplified and subjected to high-throughput sequencing (WO2014/189957; Buenrostro et al., Nature Methods, Vol.

10, No. 12, December 2013, p. 1213). Although ATAC-seq requires much less starting material compared to other assays for genome-wide chromatin accessibility, one of the main drawbacks is that the sequencing library is often contaminated with high percentage of mitochondrial DNA (which is also open DNA), and requires many more sequencing reads for standard accessibility studies of the human genome, such as DNase-seq and FAIRE-seq (M. Tsompana and MJ. Buck, 2014). Therefore there is a need in the art to remove unwanted mitochondrial DNA contamination from the sequencing library following an ATAC-Seq procedure, in order to decrease sequencing costs. The present disclosure provides a method to selectively deplete mitochondrial DNA from a sample that has undergone ATAC-Seq.

[0204] Provided herein is a method for analyzing genomic DNA, comprising: (a) treating DNA isolated from a population of cells with an insertional enzyme to produce a plurality of tagged fragments of non-mitochondrial genomic DNA, whereby also generating a residual amount of tagged mitochondrial DNA; (b) enriching the product of step (a) for non-mitochondrial DNA according to any of the enrichment or depletion methods provided herein. In some embodiments, the method further comprises sequencing at least some of the tagged fragments to produce a plurality of sequence reads; and making an epigenetic map of a region of the genome of said cells by mapping information obtained from the sequence reads to the region.

[0205] In another aspect, the present disclosure provides a method for assisting the determination of accessibility of a polynucleotide at a site, wherein the polynucleotide is from a cell sample, said method comprising: inserting a plurality of molecular tags with an insertional enzyme into the polynucleotide and using the molecular tags to determine accessibility at the site, and removing unwanted DNA contamination using the CRISPR/Cas system-based depletion methods described herein. The cell sample can be from a primary source. The cell sample may consist of a single cell. The cell sample may consist of a finite number of cells (e.g. less than about 500,000 cells).

[0206] The insertional enzyme can be any enzyme capable of inserting a nucleic acid sequence into a polynucleotide. In some cases, the insertional enzyme can insert the nucleic acid sequence into the polynucleotide in a substantially sequence-independent manner. The insertional enzyme can be prokaryotic or eukaryotic. Examples of insertional enzymes include, but are not limited to, transposases, HERMES, and HIV integrase. The transposase can be a Tn transposase (e.g. Tn3, Tn5, Tn7, Tn10, Tn552, Tn903), a MuA transposase, a Vibhar transposase (e.g. from Vibrio harveyi), Ac-Ds, Ascot-I, BsI, Cin4, Copia, En/Spm, F element, hobo, Hsmarl, Hsmar2, IN (HIV), ISI, IS2, IS3, IS4, IS5, IS6, ISIO, IS2I, IS30,

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IS50, ISSI, ISI50, IS256, IS407, IS427, IS630, IS903, IS911, IS982, IS103I, ISL2, LI, Mariner, P element, Tam3, Tel, Tc3, Tel, THE-I, Tn/O, TnA, Tn3, Tn5, Tn7, TnlO, Tn552, Tn903, Toll, Tol2, TnlO, Tyl, any prokaryotic transposase, or any transposase related to and/or derived from those listed above. In certain instances, a transposase related to and/or derived from a parent transposase can comprise a peptide fragment with at least about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91 %, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% amino acid sequence homology to a corresponding peptide fragment of the parent transposase. The peptide fragment can be at least about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45, about 50, about 60, about 70, about 80, about 90, about 100, about 150, about 200, about 250, about 300, about 400, or about 500 amino acids in length. For example, a transposase derived from Tn5 can comprise a peptide fragment that is 50 amino acids in length and about 80% homologous to a corresponding fragment in a parent Tn5 transposase. In some cases, the insertion can be facilitated and/or triggered by addition of one or more cations. The cations can be divalent cations such as, for example, Ca^{2+}, Mg^{2+} and Mn^{2+}.

[0207] The molecular tags can comprise sequencing adaptors, locked nucleic acids (LNAs), zip nucleic acids (ZNAs), RNAs, affinity reactive molecules (e.g. biotin, dig), self-complementary molecules, phosphorothioate modifications, azide or alkyne groups. In some cases, the sequencing adaptors can further comprise a barcode label. Further, the barcode labels can comprises a unique sequence. The unique sequences can be used to identify the individual insertion events. Any of the tags can further comprise fluorescence tags (e.g. fluorescein, rhodamine, Cy3, Cy5, thiazole orange, etc.).

Kits and Articles of Manufacture

[0208] The present application provides kits comprising any one or more of the compositions described herein, not limited to adapters, gRNAs, gRNA libraries, and the like.

[0209] In one embodiment, the kit comprises a collection or library of gRNAs wherein the gRNAs are targeted to human DNA sequences. In another embodiment, the kit comprises a collection or library of gRNAs wherein the gRNAs are targeted to bovine DNA sequences. In another embodiment, the kit comprises a collection or library of gRNAs wherein the gRNAs are targeted to human ribosomal RNA sequences. In another embodiment, the kit comprises a collection or library of gRNAs wherein the gRNAs are targeted to human mitochondrial DNA sequences.

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[0210] In some embodiments, the kit comprises: a CRISPR/Cas system protein and gRNAs, wherein the gRNAs are complementary to mitochondrial DNA; or wherein the gRNAs are complementary to the entire genome; or the wherein the gRNAs are complementary to a cDNA made from a whole transcriptome; or wherein the gRNAs are complementary to cDNA made form at least 10, at least 25, at least 50, at least 100, at least 250, at least 500, at least 750, at least 10^2, at least 10^3, at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10} unique transcripts; or wherein the gRNAs are complementary to cDNA made from the most abundant transcripts in a transcriptome, for example the most abundant 100, 75, 50, 25, 20, 15, or 10 transcripts in a transcriptome; or wherein the gRNAs are complementary to cDNA made from a subset of the most abundant 100, 75, 50, 25, 20, 15, or 10 transcripts in a transcriptome.

[0211] In some embodiments, the kit comprises: a CRISPR/Cas system protein and gRNAs, wherein the CRISPR/Cas system protein comprises a mixture of CRISPR/Cas system proteins from different bacteria; or wherein the CRISPR/Cas system protein is engineered; wherein the gRNAs are complementary to mitochondrial DNA; or wherein the gRNAs are complementary to genomic DNA, representing the entire genome; or wherein the gRNAs are complementary to cDNA libraries derived from the whole transcriptome; or wherein the gRNAs are complementary to cDNA libraries derived from at least 10, at least 25, at least 50, at least 100, at least 250, at least 500, at least 750, at least 10^2, at least 10^3, at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10} unique transcripts; or wherein the gRNAs are complementary to the cDNA libraries derived from the most abundant 100, 75, 50, 25, 20, 15, or 10 transcripts in a transcriptome; or wherein the gRNAs are complementary to the cDNA libraries derived from a subset of the most abundant 100, 75, 50, 25, 20, 15, or 10 transcripts in a transcriptome; or wherein the gRNAs comprise a mixture of gRNAs capable of targeting a CRISPR/Cas system protein from different bacterial species to the target sequences.

[0212] In some embodiments, the kit comprises: a CRISPR/Cas system protein; gRNAs, wherein the gRNAs are complementary to a target of interest; and an enzyme having exonuclease activity. In one embodiment, the exonuclease is Exonuclease III. In one embodiment, the exonuclease is BAL-31.

[0213] In some embodiments, the kit comprises: a CRISPR/Cas system protein, wherein the CRISPR/Cas system protein is thermostable; and gRNAs, wherein the gRNAs are complementary to a target of interest.

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[0214] In some embodiments, the kit comprises: a CRISPR/Cas system protein; and a first set of gRNAs, wherein the gRNAs are complementary to target sequences of interest; and a control set of reagents. In one embodiment, the control set of reagents is a positive control set of reagents. In an exemplary embodiment, the positive control set of reagents comprise a collection of nucleic acid fragments, positive control target sequences, wherein the fragments comprise the target sequences, to which the gRNAs are complementary. In one embodiment, the control set of reagents is a negative control set of reagents. In an exemplary embodiment, the negative control set of reagents comprise a second set of gRNAs, wherein the second set of gRNAs exhibit reduced binding to the target sequences, as compared to the first set of gRNAs. In another exemplary embodiment, the negative control set of reagents comprise a collection of nucleic acid fragments, wherein the fragments are no more than 90% complementary to the first set of gRNAs.

[0215] In some embodiments, the kit comprises: reagents for isolating DNA from a population of cells; an insertional enzyme; a CRISPR/Cas system protein; and a plurality of gRNAs, wherein the gRNAs are complementary to mitochondrial DNA; or wherein the gRNAs are complementary to the entire genome; or the wherein the gRNAs are complementary to the whole transcriptome; or wherein the gRNAs are complementary to the top 100, 75, 50, 25, 20, 15, or 10 genes in a transcriptome.

[0216] The present application also provides articles of manufacture comprising any one of the kits described herein. Examples of an article of manufacture include vials (including sealed vials).

[0217] The following examples are included for illustrative purposes and are not intend to limit the scope of the invention.

EXAMPLES

Example 1: Depletion of Mitochondrial DNA from ATAC-seq Libraries, Library 1

Overview

[0218] As depicted in Figure 4, the depletion method was tested on a human DNA library, which has been previously sequenced and shown to consist of 94% human nuclear DNA and 6% mitochondrial DNA (Library 1). Depletion was performed using recombinant Cas9 and a library of 25 guide RNAs specifically designed for mitochondrial DNA. A control depletion was performed using an unrelated guide RNA that does not target mitochondrial DNA. About

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40% of the human mitochondrial DNA was depleted. The depletion was completed in 20 minutes, followed by PCR to amplify the library.

Expression of Cas9

[0219] Cas9 (from S. pyogenes) was cloned into the pET30 expression vector (EMD biosciences) to insert the hexahistidine tag immediately upstream of the Cas9 start codon. The resulting plasmid was transformed into the Rosetta (DE3) BL21 bacterial strain (EMD biosciences) and grown in 1L of LB media with vigorous aeration until optical density of the culture (OD at 600 nm) reached 0.4. The temperature was lowered to 25°C, 0.2 mM IPTG was added and the culture grown for another four hours. Cells were then harvested by centrifugation (1,000xg for 20 min at 4°C), resuspended in 10 ml binding buffer (20 mM Tris pH8, 0.5 M NaCl, 5 mM Imidazole, 0.05% NP40) and lysed by sonication (7x10 second bursts at 30% power, Sonifier 250, Branson). Insoluble cell debris were removed by centrifugation at 10,000xg for 20 min; supernatant containing soluble protein was then mixed with 0.4 ml of NTA beads (Qiagen) and loaded onto a column. Beads were washed three times with 4 ml binding buffer, then eluted with 3 x 0.5 ml of binding buffer supplemented with 250 mM Imidazole. Eluted fractions were then concentrated and buffer exchanged with storage buffer (10 mM Tris pH8, 0.3 M NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol) using a 30,000 MWCO protein concentrator (Life Technologies), verified by SDS PAGE followed by Colloidal Blue staining (Life Technologies), quantified, then stored at -20°C for later use.

[0220] A mutant Cas9 nickase, a D10A mutant of S. pyogenes Cas9, was produced and purified using the same procedures used to produce Cas9 as above.

Preparation of Guide RNA Libraries

[0221] Twenty-five constructs were designed with each one containing the T7 RNA polymerase promoter, 20 base pairs of mitochondrial DNA specific sequence, and the guide RNA scaffold from S. pyogenes. For each construct two oligonucleotides were ordered (see Tables 1 and 2 for sequences) and combined at 1 μM final concentration in 5 μl, then heated at 98°C for 3 minutes, then cooled at a rate of 5°C/minute to 55°C, and finally incubated at 55°C for 5 minutes.

[0222] Annealed oligos were then ligated to the T7GRNE vector which contains the guide RNA scaffold by golden gate cloning; annealed oligos were combined to 500 ng of T7GRNE

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plasmid, 1 µl of BsaI restriction enzyme and 1 µl of T7 DNA ligase in a total volume of 20 µl. Reactions were incubated in a thermal cycler for 10 cycles of 37°C for 15 minutes followed by 20°C for 10 minutes, followed by a single cycle at 80°C for 15 minutes. Products were then PCR amplified using the specific forward primer (see Tables 1 and 2) and the reverse primer (5'-CAGAGCGAGGTATGTAGG-3'). Cycling conditions were as follows: 30 cycles of 95°C for 1 min, 57°C for 30 s, and 72°C for 90 s. Successful reactions were confirmed by the presence of a 1.6 kb fragment by agarose gel electrophoresis and SYBR safe staining. Successful reactions were pooled together and purified using a PCR cleanup kit (Thermo Fisher Scientific).

[0223] Guide RNAs were designed using the following rules:

[0224] Rule 1: Found the following sequence (5'>3' direction) in the genomic region of interest (for example the entire exome or mitochondrial genome) on any strand:

NNNNNNNNNNNNNNNNNNNNGG

[0225] Rule 2: Underlined sequence (20-mer) is the targeting part of guide RNA included in the oligonucleotide ordered .

a. Eliminated any 20-mer that has more than 55% GC content
b. Eliminated any 20-mer that has more than 13 stem loop forming base pairs (predicted for example, by either Mfold or Vienna softwares)
c. If first nucleotide of 20-mer is not A/G, then one G residue was added at 5' end to yield a 21-mer

d. Rule 3: Added the T7 promoter and primer binding site to yield the following primer (5'>3'):
GCCTCGAGCTAATACGACTCACTATAG(G)NNNNNNNNNNNNNNNNNNNN

(G) indicates the G to be added if the 20-mer does not have a purine at the 5'end

In vitro transcription

[0226] To transcribe the guide libraries into guide RNA, we assembled the following in vitro transcription reaction mixture: 10 µl purified library (~500 ng), 6.5 µl of H2O, of 2.25 µl of ATP, 2.25 µl of CTP, 2.25 µl of GTP, 2.25 µl of UTP, 2.25 µl 10x reaction buffer (NEB) and 2.25 µl of T7 RNA polymerase mix. The reaction was incubated at 37°C for 24 hours, then diluted 10-fold in H2O. A single reaction produced ~40 µg of RNA. The yield and size of the

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RNA (~150 base pairs) was checked by running 1 µl of the reaction on a 5% TBE/Urea gel and staining with SYBR Gold (Life Technologies).

DNA-specific Cas9-mediated depletion

[0227] Diluted guide RNA (1 µl, equivalent to 2 pmol) was combined with 3 µl 10x Cas9 reaction buffer (NEB), 20 µl H2O and 1 µl of recombinant Cas9 enzyme (NEB, 1 pmol/µl). A control reaction using a control guide RNA targeting the following sequence (5'-GGATTTATACAGCACTTTAA-3') was performed separately, using the same parameters. This sequence is absent from either the human chromosomal or mitochondrial DNA. Reactions were incubated for 15 min at 37°C, then supplemented with 5 µl diluted DNA library (50 pg/µl) and incubation at 37°C continued for 90 min. The reactions were terminated by adding RNase A (Thermo Fisher Scientific) at a 1:100 dilution, heating to 98°C for 5 min, then cooling to room temperature and adding 100 µl H2O. Reactions were then stored at -20°C until use.

Quantification of mitochondrial DNA after depletion

[0228] For each reaction (each test condition and the reaction using the control guide RNA) two separate 5 µl samples were analyzed by real time PCR using both control and test primers. For control primers reactions, samples were incubated with 2 µl H2O, 0.25 µl of 10 µM primer P5 (5'-AATGATACGGCGACCACCGA-3'), and 0.25 µl of 10 µM P7, (5'-CAAGCAGAAGACGGCATACGA-3') and 7.5 µl of 2 x Maxima SYBR Green master mix (Thermo Fisher Scientific). For test primers reactions, samples were incubated with 1.75 µl H2O, 0.5 µl of 10 µM mitochondrial primers (see tables 1 and 2), and 0.25 µl of 10 µM P7, (5'-CAAGCAGAAGACGGCATACGA-3') and 7.5 µl of 2 x Maxima SYBR Green master mix (Thermo Fisher Scientific). Reactions were incubated in an iCycler real-time PCR thermal cycler (BioRad) using the following 2-step cycling conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C for 45 s.

[0229] For the two real time PCR conditions, using control and test primers, a calibration curve was performed. The diluted DNA library (50 pg/ µl) was further diluted 1:10, 1:100,1:1000, 1:10,000 and these dilutions were analyzed by real time PCR using the same reaction conditions, instrument and cycling conditions described above.

[0230] The amount of total DNA and mitochondrial DNA was deduced from the results of the control and test primers reactions, respectively. For each DNA library depletion experiment, the ratio of mitochondrial DNA : total DNA was determined and normalized to

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the experiment using the control guide RNA, then plotted as a column graph shown in Figure 4.

Table 1: Forward primers - used in both the annealing and subsequent PCR reactions

Oligo Name	Sequence
T7-13-F	GCC TCG AGC TAA TAC GAC TCA CTA TAG GCT CTT AAA ACT AGG CGG CTA G
T7-39-F	GCC TCG AGC TAA TAC GAC TCA CTA TAG ATT TAC ACT CAC AAC ACC CTG
T7-41-F	GCC TCG AGC TAA TAC GAC TCA CTA TAG AAC AGC TAT CCA TTG GTC TTG
T7-43-F	GCC TCG AGC TAA TAC GAC TCA CTA TAG GCA GCC GGA AGC CTA TTC GCG
T7-61-F	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTA ATG AGG ATG TAA GCC CGG
T7-63-F	GCC TCG AGC TAA TAC GAC TCA CTA TAG ATA TTT ACA AGA GGA AAA CCG
T7-65-F	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTT TGA AGC TTA GGG AGA GCT G
T7-67-F	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTA TGG CTT TGA AGA AGG CGG
T7mtgRNA3	GCC TCG AGC TAA TAC GAC TCA CTA TAG TAG ATG ACG GGT TGG GCC AGG
T7mtgRNA7	GCC TCG AGC TAA TAC GAC TCA CTA TAG AGC TTT ACA GTG GGC TCT AGG
T7mtgRNA11	GCC TCG AGC TAA TAC GAC TCA CTA TAG ATG GCA GCT TCT GTG GAA CGG
T7mtgRNA15	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTG GTA AGG GCG ATG AGT GTG
T7mtgRNA31	GCC TCG AGC TAA TAC GAC TCA CTA TAG TCC ATA ACG CTC CTC ATA CTG
T7mtgRNA33	GCC TCG AGC TAA TAC GAC TCA CTA TAG TCT CCC TTC ACC ATT TCC GAG
T7mtgRNA35	GCC TCG AGC TAA TAC GAC TCA CTA TAG GCC TAT GAG TGA CTA CAA AAG
T7mtgRNA37	GCC TCG AGC TAA TAC GAC TCA CTA TAG CTT TGC CGC CTG CGA AGC AGG
T7mtgRNA57	GCC TCG AGC TAA TAC GAC TCA CTA TAG ATG TCT CCA TCT ATT GAT GAG
T7mtgRNA79	GCC TCG AGC TAA TAC GAC TCA CTA TAG GGA GGC CTG CCC CCG CTA ACG
T7mtgRNA81	GCC TCG AGC TAA TAC GAC TCA CTA TAG CGA GCC GAG CTG GGC CAG CCG
T7mtgRNA83	GCC TCG AGC TAA TAC GAC TCA CTA TAG GCG AGC AGG AGT AGG AGA GAG

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T7mtgRNA85	GCC TCG AGC TAA TAC GAC TCA CTA TAG CAA CAC TTT CTC GGC CTA TCG
T7mtgRNA87	GCC TCG AGC TAA TAC GAC TCA CTA TAG ACT TTG ACA AAG TTA TGA AAG
T7mtgRNA89	GCC TCG AGC TAA TAC GAC TCA CTA TAG TCA AAT CAA TTG GCC ACC AAG
T7mtgRNA91	GCC TCG AGC TAA TAC GAC TCA CTA TAG ACT CAT TCA ACC AAT AGC CCG
T7mtgRNA93	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTG CTC ACA CGA TAA ACC CTG

Table 2: Reverse primers - used during the anneal reaction and the real time PCR reactions

Oligo Name	Sequence
mtgRNA2	aaaacTTCTAAACGCTAATCCAAGCct
mtgRNA14	aaaacTAGCCGCCTAGTTTTAAGAGct
mtgRNA40	aaaacAGGGTGTTGTGAGTGTAAATct
mtgRNA42	aaaacAAGACCAATGGATAGCTGTTct
mtgRNA44	aaaacGCGAATAGGCTTCCGGCTGCct
mtgRNA62	aaaacCGGGCTTACATCCTCATTACct
mtgRNA64	aaaacGGTTTTCCTCTTGTAAATATct
mtgRNA66	aaaacAGCTCTCCCTAAGCTTCAAAct
mtgRNA68	aaaacCGCCTTCTTCAAAGCCATACct
mtgRNA4	aaaacCTGGCCCAACCCGTCATCTAct
mtgRNA8	aaaacCTAGAGCCCACTGTAAAGCTct
mtgRNA12	aaaacCGTTCCACAGAAGCTGCCATct
mtgRNA16	aaaacACACTCATCGCCCTTACCACct
mtgRNA32	aaaacAGTATGAGGAGCGTTATGGAct
mtgRNA34	aaaacTCGGAAATGGTGAAGGGAGAct
mtgRNA36	aaaacTTTTGTAGTCACTCATAGGCct
mtgRNA38	aaaacCTGCTTCGCAGGCGGCAAAGct
mtgRNA58	aaaacTCATCAATAGATGGAGACATct
mtgRNA80	aaaacGTTAGCGGGGGCAGGCCTCCt
mtgRNA82	aaaacGGCTGGCCCAGCTCGGCTCGct
mtgRNA84	aaaacTCTCTCCTACTCCTGCTCGCct
mtgRNA86	aaaacGATAGGCCGAGAAAGTGTTGct

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mtgRNA88	aaaacTTTCATAACTTTGTCAAAGTct
mtgRNA90	aaaacTTGGTGGCCAATTGATTTGAct
mtgRNA92	aaaacGGGCTATTGGTTGAATGAGTct
mtgRNA94	aaaacAGGGTTTATCGTGTGAGCACct

Example 2: Depletion of Mitochondrial DNA from ATAC-seq Libraries, Library 2

Overview

[0231] As depicted in Figure 5 and 6, the depletion method was tested on a human DNA library, which has been previously sequenced and shown to consist of 55% human nuclear DNA and 45% mitochondrial DNA (Library 2). Depletion was performed using recombinant Cas9 and a library of 90 guide RNAs specifically designed for mitochondrial DNA, no gaps in the mitochondrial genome were left. A control depletion was performed using an unrelated guide RNA that does not target mitochondrial DNA.

[0232] The pre- and post-capture libraries were quantified by real time PCR using two different sets of primers: P5 with P7, which amplifies all DNA library fragments or mitochondrial DNA specific primers which only amplify mitochondrial DNA. The ratio of these two separate reactions was used to quantify the depletion of mitochondrial DNA

[0233] The pre- and post-capture libraries were also sequenced on an Illumina MiSeq platform, and the resulting reads were mapped to the human genome to determine mitochondrial DNA content.

Expression of Cas9

[0234] Cas9 (from S. pyogenes) was cloned into the pET30 expression vector (EMD biosciences) to insert the hexahistidine tag immediately upstream of the Cas9 start codon. The resulting plasmid was transformed into the Rosetta (DE3) BL21 bacterial strain (EMD biosciences) and grown in 1L of LB media with vigorous aeration until optical density of the culture (OD at 600 nm) reached 0.4. The temperature was lowered to 25°C, 0.2 mM IPTG was added and the culture grown for another four hours. Cells were then harvested by centrifugation (1,000xg for 20 min at 4°C), resuspended in 10 ml binding buffer (20 mM Tris pH8, 0.5 M NaCl, 5 mM Imidazole, 0.05% NP40) and lysed by sonication (7x10 second bursts at 30% power, Sonifier 250, Branson). Insoluble cell debris were removed by centrifugation at 10,000xg for 20 min; supernatant containing soluble protein was then mixed with 0.4 ml of NTA beads (Qiagen) and loaded onto a column. Beads were washed three

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times with 4 ml binding buffer, then eluted with 3 x 0.5 ml of binding buffer supplemented with 250 mM Imidazole. Eluted fractions were then concentrated and buffer exchanged with storage buffer (10 mM Tris pH8, 0.3 M NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol) using a 30,000 MWCO protein concentrator (Life Technologies), verified by SDS PAGE followed by Colloidal Blue staining (Life Technologies), quantified, then stored at -20°C for later use.

[0235] A mutant Cas9 nickase, a D10A mutant of S. pyogenes Cas9, was produced and purified using the same procedures used to produce Cas9 as above.

Preparation of guide RNA libraries

[0236] T7-guideRNA oligonucleotides (Table 3) and a separate oligonucleotide, stlgR (sequence, GT TTT AGA GCT AGA AAT AGC AAG TTA AAA TAA GGC TAG TCC GTT ATC AAC TTG AAA AAG TGG CAC CGA GTC GGT GCT TTT TTT GGA TCC GAT GC) were ordered and synthesized (IDT).

[0237] The stlgR oligonucleotide (300 pmol) was sequentially 5' phosphorylated using T4 PNK (New England Biolabs) and then 5' adenylated sing the 5'adenylation kit (New England Biolabs), according to the manufacturer's instructions. T7-guide RNAs oligonucleotides (5 pmol) and the 5'adenylated stlgR (10 pmol) were then ligated using thermostable 5'App DNA/RNA ligase (New England Biolabs) at 65C for one hour. Ligation reactions were heat inactivated at 90°C for 5 min, then amplified by PCR (using OneTaq, New England Biolabs, 30 cycles of 95°C 30 secs, 57°C 20 secs, 72°C, 20 secs) with primers ForT7 (sequence GCC TCG AGC TAA TAC GAC TCA C) and gRU (sequence AAAAAAAGCACCGACTCGGTG). PCR products were purified using PCR cleanup kit (Life Technologies) and verified by agarose gel electrophoresis and sequencing.

[0238] Verified products were then used as templates for in vitro transcription reactions using the HiScribe T7 transcription kit (New England Biolabs). 500-1000 ng of template was incubated overnight at 37°C according to the manufacturer's instruction. The resulting guide RNA(s) was purified using the RNA cleanup kit (Life Technologies), eluted into 100 µl of RNase-free water, quantified and stored at -20°C until use.

In vitro transcription

[0239] To transcribe the guide libraries into guide RNA, we assembled the following in vitro transcription reaction mixture: 10 µl purified library (~500 ng), 6.5 µl of H2O, of 2.25 µl of

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ATP, 2.25 µl of CTP, 2.25 µl of GTP, 2.25 µl of UTP, 2.25 µl 10x reaction buffer (NEB) and 2.25 µl of T7 RNA polymerase mix. The reaction was incubated at 37°C for 24 hours, then diluted 10-fold in H2O. A single reaction produced ~40 µg of RNA. The yield and size of the RNA (~150 base pairs) was checked by running 1 µl of the reaction on a 5% TBE/Urea gel and staining with SYBR Gold (Life Technologies).

DNA-specific Cas9-mediated depletion

[0240] Multiple guide RNAs (from single or multiple in vitro transcription reactions) were selected to ensure that >95% of target DNA to be depleted contained at least one guide RNA target sequence. This can be computed by measuring the distribution of library size and determining the mean, N and the standard deviation SD; N-2SD = minimum size for >95% of the library, ensuring that there is one guide RNA per this size to ensure >95% depletion. This can also be described as the Maximum distance between guide RNAs = Mean of library size - 2x(standard deviation of library size). In the case of the mitochondrial library, the library contains DNA fragments that are 450 base pairs in average length, with a standard deviation of 100. This results in 250bp between guide RNAs at a maximum. Here, the DNA fragments in these libraries are 450 bp in average length (with an estimated standard deviation of 100 bp), <5% of DNA is smaller than 250 bp assuming a normal distribution. Assuming guide RNAs will target 100% of their assigned targets, >95% of target DNA should contain at least one guide RNA site and be depleted.

[0241] In this example, all guides constructed in example 2 in addition to those constructed in example 1 were used. These multiple guide RNAs were mixed in equimolar amounts to add to 80 pmol total, then combined with 40 pmol purified Cas9 protein in 60 µl Cas9 reaction buffer (20 mM HEPES pH 6.5, 100 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA) and incubated for 10 min at 37°C. 5 ng of DNA mixture (containing the desired DNA and the target DNA to be depleted) was added and the incubation continued for another 20 min. The reaction was stopped by adding 140 µl of TE buffer (10 mM Tris pH 8, 1 mM EDTA) containing 2 µg/ml RNase A and incubating for 20 min at 37°C. DNA was recovered using the PCR cleanup kit (Life Technologies) without any addition of isopropanol prior to column binding, and eluted in 20 µl of 10mM Tris pH 8. Eluted DNA was re-amplified using the HiFidelity master mix (New England Biolabs) according to the manufacturer's instructions, with the appropriate primers (for example P5 and P7 for re-amplifying Illumina based DNA sequencing libraries) and for 8-13 cycles of PCR.

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Sequencing and Bioinformatics

[0242] PCR products were purified using the PCR cleanup kit and used as templates for high-throughput DNA sequencing (Illumina MiSeq or NextSeq).

[0243] Fastq files were aligned to the human genome reference (hg20) using bwa or bowtie to determine proportions of reads mapping to the nuclear vs. mitochondrial genomes.

Table 3 – Additional guide RNAs used in Example 2

Oligo Name	Sequence
mtgRNA97	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTC AGT GGG GGT GAG GTA AAA
mtgRNA99	GCC TCG AGC TAA TAC GAC TCA CTA TAG GCT GAT CCT CCA AAT CAC CAC
mtgRNA101	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTT GGT ATC CTA GTG GGT GAG
mtgRNA103	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTT AGC CTA GCC ACA CCC CCA
mtgRNA105	GCC TCG AGC TAA TAC GAC TCA CTA TAG GGC CGT TAA ACA TGT GTC AC
mtgRNA107	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTT AGC GAT GGA GGT AGG AT
mtgRNA109	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTA TAA CCT CCC CCA AAA TTC
mtgRNA111	GCC TCG AGC TAA TAC GAC TCA CTA TAG GCA TTG CGA GAC GCT GGA GC
mtgRNA113	GCC TCG AGC TAA TAC GAC TCA CTA TAG AAA AGA TAA AAT TTG AAA TC
mtgRNA115	GCC TCG AGC TAA TAC GAC TCA CTA TAG GGT TTA TCG ATT ACA GAA C
mtgRNA117	GCC TCG AGC TAA TAC GAC TCA CTA TAG AAA TCT CCT AAG TGT AAG TT
mtgRNA119	GCC TCG AGC TAA TAC GAC TCA CTA TAG GGG ATT TAG AGG GTT CTG T
mtgRNA121	GCC TCG AGC TAA TAC GAC TCA CTA TAG GAA AGG TTA AAA AAA GTA AA
mtgRNA123	GCC TCG AGC TAA TAC GAC TCA CTA TAG AGG GTT TAC GAC CTC GAT GT
mtgRNA125	GCC TCG AGC TAA TAC GAC TCA CTA TAG GAG GGT GAT GGT AGA TGT GG
mtgRNA127	GCC TCG AGC TAA TAC GAC TCA CTA TAG GAT AAA TCA TAT TAT GGC CA
mtgRNA129	GCC TCG AGC TAA TAC GAC TCA CTA TAG GGA ATG CTG GAG ATT GTA A
mtgRNA131	GCC TCG AGC TAA TAC GAC TCA CTA TAG AAA TTA AGA ATG GTT ATG TT
mtgRNA133	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTT TGT GGG TTT AAG TCC CAT

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mtgRNA135	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTG ATG CCA GCA GCT AGG AC
mtgRNA137	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTT CAC CGT AGG TGG CCT GAC
mtgRNA139	GCC TCG AGC TAA TAC GAC TCA CTA TAG TGG AGG GTT CTT CTA CTA TT
mtgRNA141	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTG TTA GGA AAA GGG CAT AC
mtgRNA143	GCC TCG AGC TAA TAC GAC TCA CTA TAG ATC TGT TTT TAA GCC TAA TG
mtgRNA145	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTT GTG TTT AAT ATT TTT AGT
mtgRNA147	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTG GTT GGT GTA AAT GAG TG
mtgRNA149	GCC TCG AGC TAA TAC GAC TCA CTA TAG GAA AAC GTA GGC TTG GAT TA
mtgRNA151a	GCC TCG AGC TAA TAC GAC TCA CTA TAG GGC CAG TGC CCT CCT AAT TG
mtgRNA153a	GCC TCG AGC TAA TAC GAC TCA CTA TAG AAA TCC ACC CCT TAC GAG TG
mtgRNA155a	GCC TCG AGC TAA TAC GAC TCA CTA TAG GCA TTT ACC ATC TCA CTT CT
mtgRNA157a	GCC TCG AGC TAA TAC GAC TCA CTA TAG ATA AAA TAT GAT TAG TTC TG
mtgRNA159a	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTA GGA GAG TGA TAT TTG ATC
mtgRNA161a	GCC TCG AGC TAA TAC GAC TCA CTA TAG GGT ATG AGT TTT TTT TGT TA
mtgRNA163a	GCC TCG AGC TAA TAC GAC TCA CTA TAG GCC TCT CAG CCG ATG AAC AGT
mtgRNA165a	GCC TCG AGC TAA TAC GAC TCA CTA TAG AGT GTT AGA GTT TGG ATT AG
mtgRNA167a	GCC TCG AGC TAA TAC GAC TCA CTA TAG GTT TGT GTA TGA TAT GTT TG
mtgRNA169a	GCC TCG AGC TAA TAC GAC TCA CTA TAG GGA GTA GGG TTA GGA TGA G
mtgRNA171a	GCC TCG AGC TAA TAC GAC TCA CTA TAG GAC CTA GTT CAA TGA ATC TG
mtgRNA173a	GCC TCG AGC TAA TAC GAC TCA CTA TAG GGA CGC CTC CTA GTT TGT TA
mtgRNA175a	GCC TCG AGC TAA TAC GAC TCA CTA TAG GCA GGT GTC TCC TCT ATC TT
mtgRNA177a	GCC TCG AGC TAA TAC GAC TCA CTA TAG GAT TCA AAT TAT GTG TTT TT
mtgRNA179a	GCC TCG AGC TAA TAC GAC TCA CTA TAG TCA TCT CCG GTT TAC AAG AC

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Example 3: Depletion of all human sequences from a DNA library (transcriptome or all genomic DNA) using the CRISPR/Cas9 system

Transcriptome-wide gRNA library:

[0244] Guide RNAs are generated, which are tiled across the entire transcriptome. The design is based on the blood transcriptome. A pipeline for the computational selection of guide RNAs spaced every ~100-200 bp across each transcript is developed, based on the knowledge that there is expected to be an NGG site every ~10-20 bp in the human genome on average). In this way, given a typical RNA-seq library insert size of ~200-500 bp, each insert is expected to contain a target site. Following completion of the design, oligo pools containing the guide RNA target sequences and surrounding motifs are ordered, allowing for subsequent addition of a T7 promoter and the invariant portion of the guide RNA sequence. This approach enables the regeneration of the libraries for a period of time before needing to order new pools. Based on an array size of 90,000 elements, it is expected that guide RNAs spaced every ~200 bp across the transcriptome with two arrays (assuming a transcriptome size of ~30M bases) will be generated. These guide RNAs are then used along with Cas9 to deplete all human DNA from an RNA-seq library as described in the previous examples. Depletion and sequencing data analysis is done as described for the previous examples.

Validation:

[0245] The described approach is tested on test RNA-seq libraries. These RNA-seq libraries are made from a mixture of human and E. coli RNA in order to simulate the types of sequences being targeted. Following depletion of the library, sequencing is performed, to get ~10X coverage of the transcriptome. As above, the proportion of all sequences mapping to human before and after depletion is measured; and the Kruskal-Wallis one-way ANOVA test can be used to determine statistical significance of depletion.

[0246] It is expected that this method, with the optimal gRNA designs, adjusting levels of Cas9 in the reaction, will achieve 90% depletion of non-ribosomal human sequences from an RNA-seq library, with less than 5% of non-human sequences being depleted.

Example 4: Enriching a human blood sample for viral or other pathogen derived sequences

[0247] A human blood sample carrying a known pathogen (e.g., Ebola) or an unknown pathogen is obtained and RNA is extracted (e.g., using a PAXgene Blood RNA extraction kit, Qiagen) and converted into a sequencing library using standard methods (e.g., using a KAPA

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RNA-seq library preparation kit). After cDNA creation and adapter ligation, the library is then mixed with Cas9 and a guide RNA pool targeting every ~200 bases in the human transcriptome. The reaction is incubated at 37C for 20 minutes, allowing any fragments containing the target sequences to be cleaved by Cas9. The library is then purified and amplified using adapter-specific primers. Human sequences are effectively depleted. Only uncleaved sequences are amplified, and the resulting library is enriched for viral or pathogen-derived sequences.

Example 5: Enriching a sample from livestock to monitor for viruses and other pathogens

[0248] In this example, the method is used to monitor viruses and other pathogens in milk and livestock.

[0249] RNA from cow milk or bovine (cow) blood is extracted and converted into a sequencing library as described in the above example. After cDNA creating and adapter ligation, the library is mixed with Cas9 and a guide RNA pool targeting ~200 bases in the Bos taurus transcriptome. The reaction is incubated at 37C for 20 minutes, allowing any fragments containing the target sequences to be cleaved by Cas9. The library is then purified and amplified using adapter-specific primers. Bovine sequences are effectively depleted. Only uncleaved sequences are amplified, and the resulting library is enriched for viral or pathogen-derived sequences. In this scenario, the cow-depleted sequencing library can be sequenced and used to monitor viruses and other pathogens in milk and livestock.

Example 6: Depletion of Large Fragments of DNA Without Adapters

[0250] This example shows a method that supports depletion without the need for adapters. In this example, the method is used for depletion experiments as described in the examples above. However, instead of cleaving a library containing adapters, the guide RNAs are chosen to cleave multiple times in a pool of fragmented DNA of large size (e.g., anywhere from 100 bp – 10 kb). Following cleavage with Cas9/gRNA complexes, the DNA is subjected to size selection to remove small fragments (e.g., at least 1/2 or 1/3 of the average size of intact fragments). This could be assisted, for example, by treatment with Lambda exonuclease, which is a 5'phosphate specific-exonuclease that proceeds in a 5' to 3' direction and would attack any fragments that had previously been cut with Cas9. The resulting libraries can then be subjected to sequencing single-molecule sequencers that do not require sequencing adapters; or adapters can be ligated for downstream analyses.

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Example 7: Depletion Followed by Exonuclease III Treatment

[0251] In some embodiments, it is desirable to remove cut fragments prior to PCR amplification or sequencing. This example illustrates a method of removing unwanted DNA after Cas9-mediated cleavage, by using Exonuclease III to degrade cleaved DNA while leaving the uncut DNA of interest intact.

[0252] As depicted in FIG. 8, the DNA mixture (containing >95% human DNA targeted for depletion, and <5% other DNA of interest) is fragmented, end-repaired and ligated to Y-shaped adapters (or circular adaptors such as NEBNext®) following regular protocols, but not amplified by PCR. The DNA mixture is then subjected to Cas9 digestion, complexed with, for example for this case, a guide RNA library against mitochondrial DNA, as described earlier. The Cas9 reaction is diluted 1:3 with TE (10 mM Tris-Cl pH 8, 1 mM EDTA) supplemented with 0.5% RNase A (Thermo Fisher Scientific), and incubated for 5 min at 37°C. Proteinase K (NEB) and then added at a final concentration of 8 units/ml and incubated for 5 min at 37°C. DNA is then recovered using the GeneJET PCR Purification Kit (Thermo Fisher Scientific) and eluted in 20 µl of 1X CutSmart® Buffer (NEB). Exonuclease III (NEB) is then added (50 units) and incubated at 37°C for 20 min, followed by heat inactivation for 30 min at 65°C.

[0253] Exonuclease III can initiate unidirectional 3'>5' degradation of one DNA strand by using blunt end or 5' overhangs, yielding single-stranded DNA and nucleotides; it is not active on single-stranded DNA, and thus 3' overhangs, such as the Y-shaped adapter ends, are resistant to degradation. As a result, intact double-stranded DNA libraries uncut by Cas9 (for example in this case, non-mitochondrial DNA) is not be digested by Exonuclease III, while DNA molecules that have been cleaved by Cas9 (for example in this case, mitochondrial DNA) is degraded by Exonuclease III with its 3'>5' activity from the blunt ends cut by Cas9 towards the adaptors.

[0254] Thus unwanted DNA is digested. The remaining intact double-stranded DNA libraries are then recovered using the GeneJET PCR Purification Kit (Thermo Fisher Scientific) and eluted in 20 µl 10 mM Tris-Cl pH 8. Samples are then quantified using Qubit Fluorometer (Life Technologies) and sequenced on the MiSeq system (Illumina).

[0255] Alternatively, unwanted DNA can be digested, as above, while intact DNA is recovered, for example, by column purification or PCR-amplification.

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Example 8: Depletion Followed by Exonuclease Bal-31

[0256] In some embodiments, it is desirable to remove cut fragments prior to PCR amplification or sequencing. This example illustrates an alternative method of removing unwanted DNA after Cas9-mediated cleavage, by using Exonuclease Bal-31 to degrade cleaved DNA while leaving the uncut DNA of interest intact.

[0257] As depicted in FIG. 9, Sequencing libraries (containing, for example >95% human DNA targeted for depletion (for example mitochondrial DNA) and <5% DNA of interest) are prepared according to conventional methods, including a PCR amplification step. Libraries (500 nM) are then tailed with poly-dG at 3' ends using Terminal Transferase (NEB) and 3 mM dGTP according to manufacturer's instruction – incubating 30 min at 37 °C, then heat inactivated at 75°C for 20 min. 3' poly-dG tailed libraries are then subjected to Cas9 digestion, complexed with, for example for this case, a guide RNA library against mitochondrial DNA, as described earlier, then heat inactivated at 75°C for 20 min. Reactions were then diluted 1:5 in 1x Exonuclease Bal-31 Reaction Buffer (NEB), supplemented with 10 units of Exonuclease Bal-31 (NEB) and incubated at 37 °C for 30 min.

[0258] Exonuclease Bal-31 has two activities: double-stranded DNA exonuclease activity, and single-stranded DNA/RNA endonuclease activity. The double-stranded DNA exonuclease activity allows BAL-31 to degrade DNA from open ends on both strands, thus reducing the size of double-stranded DNA. The longer the incubation, the greater the reduction in size of the double-stranded DNA, making it useful for depleting medium to large DNA (>200 bp). It is noted that the single-stranded endonuclease activity of BAL-31 allows it to digest poly-A, -C or -T very rapidly, but is extremely low in digesting poly-G (Marrone and Ballantyne, 2008). Because of this nature, adding single-stranded poly-dG at 3' ends of the libraries can serve as a protection from being degraded by BAL-31, which we have verified using PCR products. As a result, DNA molecules that have been cleaved by Cas9 (for example in this case, mitochondrial DNA) will be degraded by BAL-31 with its double-stranded DNA exonuclease activity from the double-stranded blunt end cut by Cas9 towards the other end carrying poly-dG, effecting depletion; while intact DNA libraries uncut by Cas9 (for example in this case, non-mitochondrial DNA) will not be digested by BAL-31 due to their 3' end poly-dG protection.

[0259] After Exonuclease Bal-31 incubation, the reaction mixture is heat inactivated at 75°C for 20 min, and DNA is recovered using the GeneJET PCR Purification Kit (Thermo Fisher Scientific). Samples are then quantified using Qubit Fluorometer (Life Technologies) and sequenced on the MiSeq system (Illumina).

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[0260] Alternatively, unwanted DNA can be digested, as above, while intact DNA is recovered, for example, by column purification.

Example 9: Cas9 Depletion and Biotin Labeling

[0261] In some embodiments, the Cas9 cleaved products are eliminated (for example the >95% mitochondrial DNA is eliminated) and the uncut (<5% DNA of interest) DNA comprises a biotin label is purified by binding to streptavidin beads. This example illustrates a method of removing unwanted DNA after Cas9-mediated cleavage without using an exonuclease, and is depicted in FIG. 10.

[0262] A DNA mixture (containing >95% unwanted human DNA, and <5% other DNA of interest) was fragmented, end-repaired and ligated to adapters following regular protocols, then amplified by PCR using primers of Biotin-P5 (5'Biotin-AATGATACGGCGACCACCGA) and P7 (5'-CAAGCAGAAGACGGCATACGA). This ensures that the entire sequencing library possesses a 5'Biotin label only on one end of the DNA molecules. The DNA mixture is then subjected to Cas9 digestion, complexed with, for example for this case, a guide RNA library against mitochondrial DNA (the unwanted DNA), as described earlier. The Cas9 reaction is diluted 1:3 with TE (10 mM Tris-Cl pH 8, 1 mM EDTA) supplemented with 0.5% RNase A (Thermo Fisher Scientific), and incubated for 5 min at 37°C. Proteinase K (NEB) is then added at a final concentration of 8 units/ml and incubated for 5 min at 37°C. DNA molecules that are cleaved by Cas9 (for example in this case, mitochondrial DNA) will lose the Biotin labeled adaptor, thus cannot be sequenced or PCR amplified; while intact DNA libraries uncut by Cas9 (for example in this case, non-mitochondrial DNA of interest) will still carry the Biotin label. As a result, intact DNA uncut by Cas9 can then be recovered by adding streptavidin beads (Dynabeads MyOne Streptavidin C1, Thermo Fisher Scientific) to a final concentration of 2%. Beads are captured using a magnetic stand and washed 4 times with TE buffer. DNA is released from the beads by heating to 95°C for 3 min, followed by 6 cycles of PCR using P5 and P7 primers (non-biotinylated). DNA is then purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific). Samples are then quantified using Qubit Fluorometer (Life Technologies) and sequenced on the MiSeq system (Illumina).

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Example 10: Increasing Efficiency of Cas9-Mediated Depletion Using Thermostable Cas9

[0263] Although Cas9 can be used in combination with a library of guide RNAs to efficiently deplete a collection of target DNA, large amounts (>30 pmoles) of Cas9 and guide RNAs are needed. The reason for this usually >100 fold excess amount over target DNA is that, unlike classical restriction enzymes such as EcoRI, which detach completely from their target DNA after cleavage, Cas9 is not recycled after completion of the cutting reaction. Cas9 can remain tightly bound to one of the two daughter DNA product molecules (see FIG. 11, open circles on the left). As a result, more Cas9 and gRNA needs to be provided in order to achieve complete depletion of unwanted DNA. To overcome this problem, a thermostable Cas9 (defined as one that retains >90% activity after 1 min at 95°C) complexed with guide RNAs (for example a gRNA library against human DNA) can be applied to a sequencing library of DNA mixture (containing for example 95% human DNA and 5% viral DNA). As depicted in FIG. 11 (grey circles on the right), after allowing Cas9 to digest for a period of time, the sample mixture can be boiled, which will cause DNA denaturation, as well as dissociation of gRNA and Cas9 from the DNA targets. The binding of Cas9 to gRNAs can be increased so that the Cas9-gRNA dissociates from the DNA target as an intact complex, despite of DNA denaturation. Dimethyl sulfoxide can be added to reduce the temperature required for DNA denaturation, so that the Cas9 protein structure will not be affected. Cas9 will preferentially bind to target sites that have not been cut, and a thermostable Cas9 will retain activity after boiling. Because of these features, by boiling and cooling down the reaction to 37°C, a thermostable Cas9 will remain capable of binding to its gRNA and cutting more of its substrate. By allowing the recycling of Cas9, the depletion efficiency is increased, and as less Cas9 will be needed in the reaction, the off-target (non-specific) cleavage probability will also be decreased.

[0264] Two exemplary methods to make Thermostable Cas9 are provided.

[0265] The first method is isolation of thermostable Cas9. The gene for thermostable Cas9 and its corresponding guide RNA will be identified by sequence homology in the genome of thermophilic bacteria *Streptococcus thermophilus* and *Pyrococcus furiosus*. Cas9 genes will then be cloned into an expression vector pET30 (Novagen) to express Cas9 as described in earlier sections. Guide RNA sequences will be assembled into oligonucleotides and synthetized (IDT). Active combinations of Cas9 and guide RNA will be assessed by digestion of sequenced DNA templates at 37°C (control) and at 37°C after treatment at 95°C for 3X5 min.

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The second method is *in vitro* evolution of *S. pyogenes* Cas9. The sequence of Cas9 will be mutagenized to improve its thermostability. Briefly, this will be done by site-directed mutagenesis to remove excess loop sequences, increasing the number of ionic bridges between protein domains, or by diluting into droplets and PCR to create a pool of potential mutants. All mutants will be assessed for activity and thermostability as described above.

Example 11: Controls to Monitor Depletion of Unwanted DNA and Off-Target Events

[0266] In the embodiments discussed, it is desirable to provide positive and/or negative controls. A positive control would ensure depletion of unwanted DNA is proceeding with fidelity and efficiency. A negative control would ensure that off-target cutting was minimal or nonexistent.

[0267] The positive control could consist of a sequencing library with inserts containing either all or most of the target sequences of the gRNAs included in the kit. This control would be run alongside a user's reaction to ensure that all components are working properly. After depletion with Cas9, elimination of the target sequences could be measured by gel electrophoresis or qPCR.

[0268] The negative control could consist of either 1) a set of gRNAs with less than 100% identity to the gRNAs used in the kit; for example, with 1, 2, 3, 4, 5, or more mismatches to the target-specific sequence; or 2) a DNA library having inserts with less than 100% identity to the targeted sequences of gRNAs used in the kit; for example, with 1, 2, 3, 4, 5, or more mismatches to the complementary sequence of the gRNA. This control would be run alongside a user's reaction to ensure that all components are working properly and to measure any off-target activity of the enzyme. After depletion with Cas9, the amount of off-target depletion could be measured by gel electrophoresis or qPCR .

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CLAIMS

What is claimed is:

1. A method of enriching a sample for sequences of interest, comprising:

a. providing a sample comprising sequences of interest and targeted sequences for depletion, wherein the sequences of interest comprise less than 30% of the sample;

b. contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to the targeted sequences, and whereby the targeted sequences are cleaved.

2. The method of claim 1, further comprising extracting the sequences of interest and the targeted sequences for depletion from the sample.

3. The method of claim 2, further comprising fragmenting the extracted sequences.

4. The method of claim 3, further comprising adapter ligating the 5' and 3' ends of the fragmented extracted sequences.

5. The method of claim 1, wherein the cleaved targeted sequences are removed by size-exclusion or with the use of biotin.

6. The method of claim 1, further comprising amplifying the sequences of interest.

7. The method of claim 1, wherein the method comprises contacting the sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes.

8. The method of claim 1, wherein the sample is any one of a biological sample, a clinical sample, a forensic sample or an environmental sample.

9. The method of claim 1, wherein the sample comprises host nucleic acid sequences targeted for depletion and non-host nucleic acid sequences of interest.

10. The method of claim 9, wherein the non-host nucleic acid sequences comprise

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microbial nucleic acid sequences.

11. The method of claim 10, wherein the microbial nucleic acid sequences are bacterial, viral or eukaryotic parasitic nucleic acid sequences.

12. The method of claim 1, wherein the sample is contacted with CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to mitochondrial DNA.

13. The method of claim 1, wherein the sample is contacted with CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to DNA corresponding to ribosomal RNA sequences, sequences encoding globin proteins, sequences encoding a transposon, sequences encoding retroviral sequences, sequences comprising telomere sequences, sequences comprising sub-telomeric repeats, sequences comprising centromeric sequences, sequences comprising intron sequences, sequences comprising Alu repeats, sequences comprising SINE repeats, sequences comprising LINE repeats, sequences comprising dinucleic acid repeats, sequences comprising trinucleic acid repeats, sequences comprising tetranucleic acid repeats, sequences comprising poly-A repeats, sequences comprising poly- T repeats, sequences comprising poly-C repeats, sequences comprising poly-G repeats, sequences comprising AT -rich sequences, or sequences comprising GC-rich sequences.

14. The method of claim 2, wherein the extracted nucleic acids incudes any one of single stranded DNA, double stranded DNA, single stranded RNA, double stranded RNA, cDNA, synthetic DNA, artificial DNA, and DNA/RNA hybrids.

15. The method of claim 1, wherein the sequences of interest comprise less than 10% of the extracted nucleic acids.

16. The method of claim 1, wherein the CRISPR/Cas system protein is Cas9.

17. The method of claim 1, wherein the CRISPR/Cas system protein is catalytically dead.

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18. The method of claim 17, wherein the catalytically dead CRISPR/Cas system protein is dCas9.

19. The method of claim 1, wherein the CRISPR/Cas system protein is a CRISPR/Cas system protein nickase.

20. The method of claim 19, wherein the CRISPR/Cas system protein nickase is a Cas9 nickase.

21. The method of claim 1, wherein the CRISPR/Cas System protein is thermostable.

22. The method of claim 1, further comprising amplifying the product of step (b) using adapter-specific PCR.

23. The method of claim 1, further comprising treating the product of step (b) with an enzyme that has exonuclease activity.

24. The method of 23, wherein the enzyme is Exonuclease III or BAL-31.

25. The method of claim 1, wherein the sample is selected from whole blood, plasma, serum, tears, saliva, mucous, cerebrospinal fluid, teeth, bone, fingernails, feces, urine, tissue, and a biopsy.

26. A method of enriching a sample comprising:

a. providing a sample comprising host nucleic acids and non-host nucleic acids, wherein the host nucleic acids and non-host nucleic acids are adapter-ligated, and wherein the adapters are ligated to the 5' and 3' ends of the host nucleic acids and the non-host nucleic acids;
b. contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are complementary to targeted sites in the host nucleic acids, thereby generating host- nucleic acids adapter ligated only on one end and non-host nucleic acids adapter ligated on both the 5' and 3' ends; and
c. enriching the sample for non-host nucleic acids.

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27. The method of claim 26, wherein the method comprises contacting the sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes.

28. The method of claim 26, wherein the nucleic acids are DNA, and wherein the contacting of step (b) generates host- DNA adapter ligated on the 5' end but not the 3' end and non-host DNA adapter ligated on both the 5' and 3' ends.

29. The method of claim 26, wherein the CRISPR/Cas system protein is Cas9, Cpf1, Cas3, Cas8a-c, Cas10, Cse1, Csy1, Csn2, Cas4, Csm2, or Cm5.

30. The method of claim 29, wherein the CRISPR/Cas system protein is Cas9.

31. The method of claim 26, wherein the CRISPR/Cas system protein is dCas9.

32. The method of claim 26, wherein the CRISPR/Cas system protein is Cas9 nickase.

33. The method of claim 26, wherein the CRISPR/Cas system protein is thermostable.

34. The method of claim 26, wherein the host is selected from the group consisting of a human, cow, horse, sheep, pig, monkey, dog, cat, gerbil, bird, mouse, and rat.

35. The method of claim 26, wherein the non-host is a prokaryotic organism.

36. The method of claim 26, wherein the non-host is selected from the group consisting of a eukaryote, virus, bacterial, fungus, and protozoa.

37. The method of claim 26, wherein the adapter-ligated host nucleic acids and non-host nucleic acids range from 50-1000bp.

38. The method of claim 26, wherein the non-host nucleic acids comprise less than 50% of the total nucleic acids in the sample.

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39. The method of claim 26, wherein the sample is any one of a biological sample, a clinical sample, a forensic sample or an environmental sample.

40. The method of claim 26, wherein step (c) comprises amplifying the product of step (b) using adapter-specific PCR.

41. The method of claim 26, wherein step (c) comprises treating the product of step (b) with an enzyme that has exonuclease activity.

42. The method of claim 41 wherein the enzyme is Exonuclease III or BAL-31.

43. The method of claim 26, wherein step (c) comprises removing the host nucleic acids by size-exclusion.

44. The method of claim 26, wherein step (c) comprises removing the host nucleic acids with the use of biotin.

45. The method of claim 26, further comprising amplifying the product of step (b) using adapter-specific PCR.

46. The method of claim 26, further comprising treating the product of step (b) with an enzyme that has exonuclease activity.

47. The method of claim 46, wherein the enzyme is Exonuclease III or BAL-31.

48. The method of claim 26, further comprising including the use of positive control target sequences.

49. The method of claim 26, further comprising including the use of negative control gRNAs.

50. The method of claim 26, wherein the sample is selected from whole blood, plasma, serum, tears, saliva, mucous, cerebrospinal fluid, teeth, bone, fingernails, feces, urine, tissue, and a biopsy.

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51. A method for serially depleting targeted nucleic acids in a sample comprising:

a. providing a sample comprising host nucleic acids and non-host nucleic acids, wherein the non-host nucleic acids comprise nucleic acids from at least one known non-host organism and nucleic acids from at least one unknown non-host organism;

b. contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are configured to hybridize to targeted sequences in the host nucleic acids, whereby a portion of the host nucleic acids are cleaved;

c. contacting the sample with a plurality of CRISPR/Cas system protein-gRNA complexes, wherein the gRNAs are configured to hybridize to targeted sequences in the at least one known non-host nucleic acids, whereby a portion of the at least one known non-host nucleic acids are cleaved; and

d. isolating the nucleic acids from the unknown non-host organism.

52. The method of claim 51, wherein the method comprises contacting the sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes configured to hybridize to targeted sequences in the host nucleic acids.

53. The method of claim 51, wherein the method comprises contacting the sample with at least 10^2 unique CRISPR/Cas system protein-gRNA complexes configured to hybridize to targeted sequences in the at least one known non-host nucleic acids.

54. The method of claim 51, wherein the sample is selected from whole blood, plasma, serum, tears, saliva, mucous, cerebrospinal fluid, teeth, bone, fingernails, feces, urine, tissue, and a biopsy.

55. The method of claim 51, wherein the nucleic acids from an unknown non-host organism comprise less than 5% of the total nucleic acids in the sample.

56. The method of claim 51, wherein the host is a human.

57. A composition comprising a mixture of DNA from a first genome and DNA from a second genome, wherein the first genome DNA and the second genome DNA are

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adapter-ligated, and wherein the first genome DNA is complexed to a gRNA-CRISPR/Cas system protein complex.

58. The composition of claim 57, wherein the first genome is from a host organism and the second genome is from a non-host organism.

59. The composition of claim 57, wherein the CRISPR/Cas system protein is Cas9.

60. The composition of claim 57, wherein the CRISPR/Cas system protein is thermostable.

61. A kit comprising:

a. CRISPR/Cas system protein; and
b. gRNAs, wherein the gRNAs are complementary to mitochondrial DNA.

62. A kit comprising:

a. a CRISPR/Cas system protein;
b. gRNAs, wherein the gRNAs are complementary to a target of interest; and
c. an enzyme having exonuclease activity.

63. A kit comprising:

a. a CRISPR/Cas system protein, wherein the CRISPR/Cas system protein is thermostable; and
b. gRNAs, wherein the gRNAs are complementary to a target of interest.

64. A kit comprising:

a. a CRISPR/Cas system protein; and
b. a first set of gRNAs, wherein the gRNAs are complementary to target sequences of interest; and
c. a control set of reagents.

65. The kit of claim 64 further comprising a positive control set of reagents.

66. The kit of claim 64 further comprising a negative control set of reagents.

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67. A kit comprising:

a. reagents for isolating DNA from a population of cells;

b. an insertional enzyme;

c. a CRISPR/Cas system protein; and

d. a plurality of gRNAs, wherein the gRNAs are complementary to mitochondrial DNA.

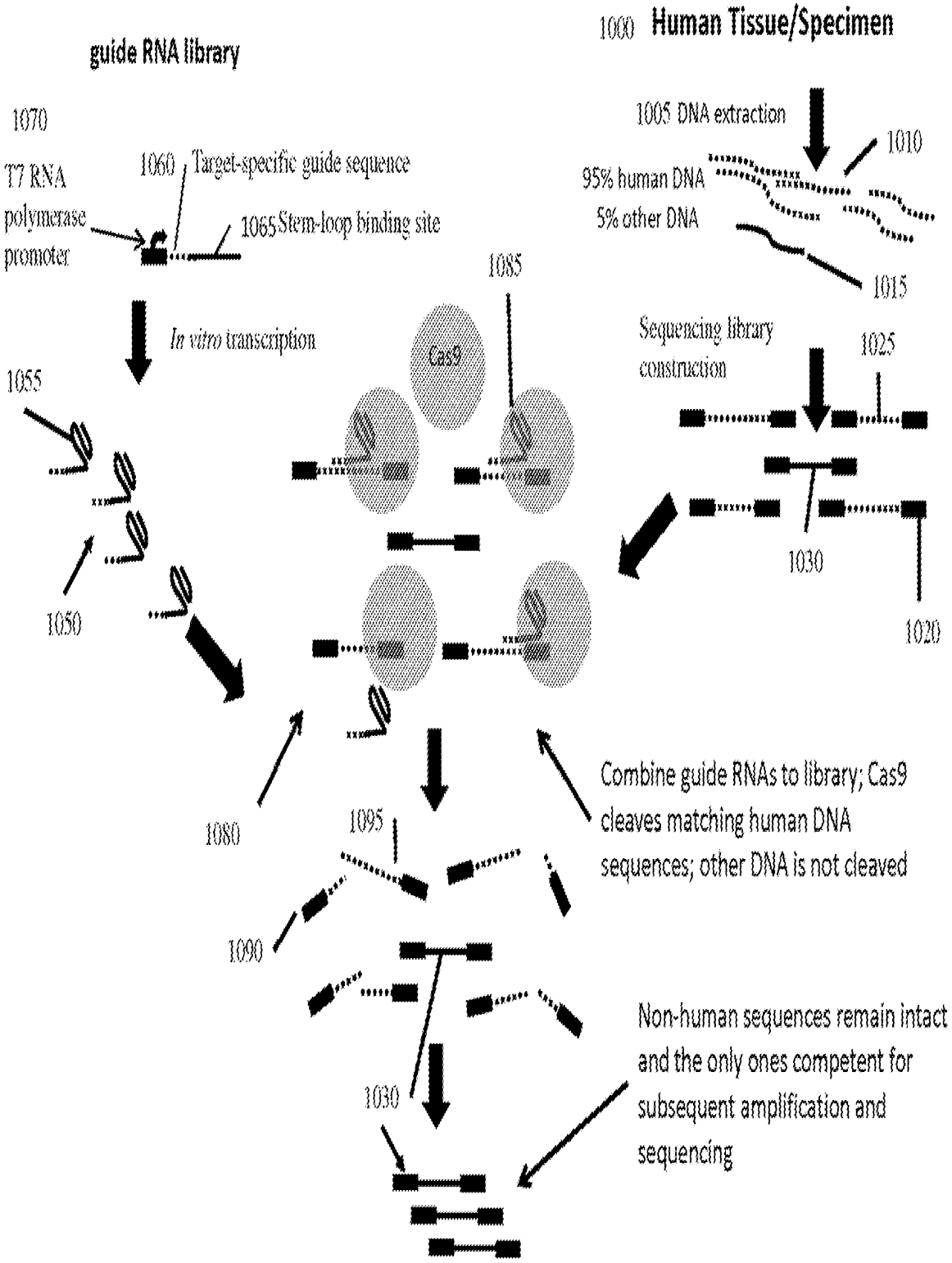
68. The kit of any one of claims 61 to 67, wherein the CRISPR/Cas system protein is Cas9.

69. The kit of any one of claims 61 to 67, comprising at least 10^2 unique gRNAs.

70. The kit of any one of claims 61 to 67, further comprising a collection of Y-shaped adapters or poly-G adapters.

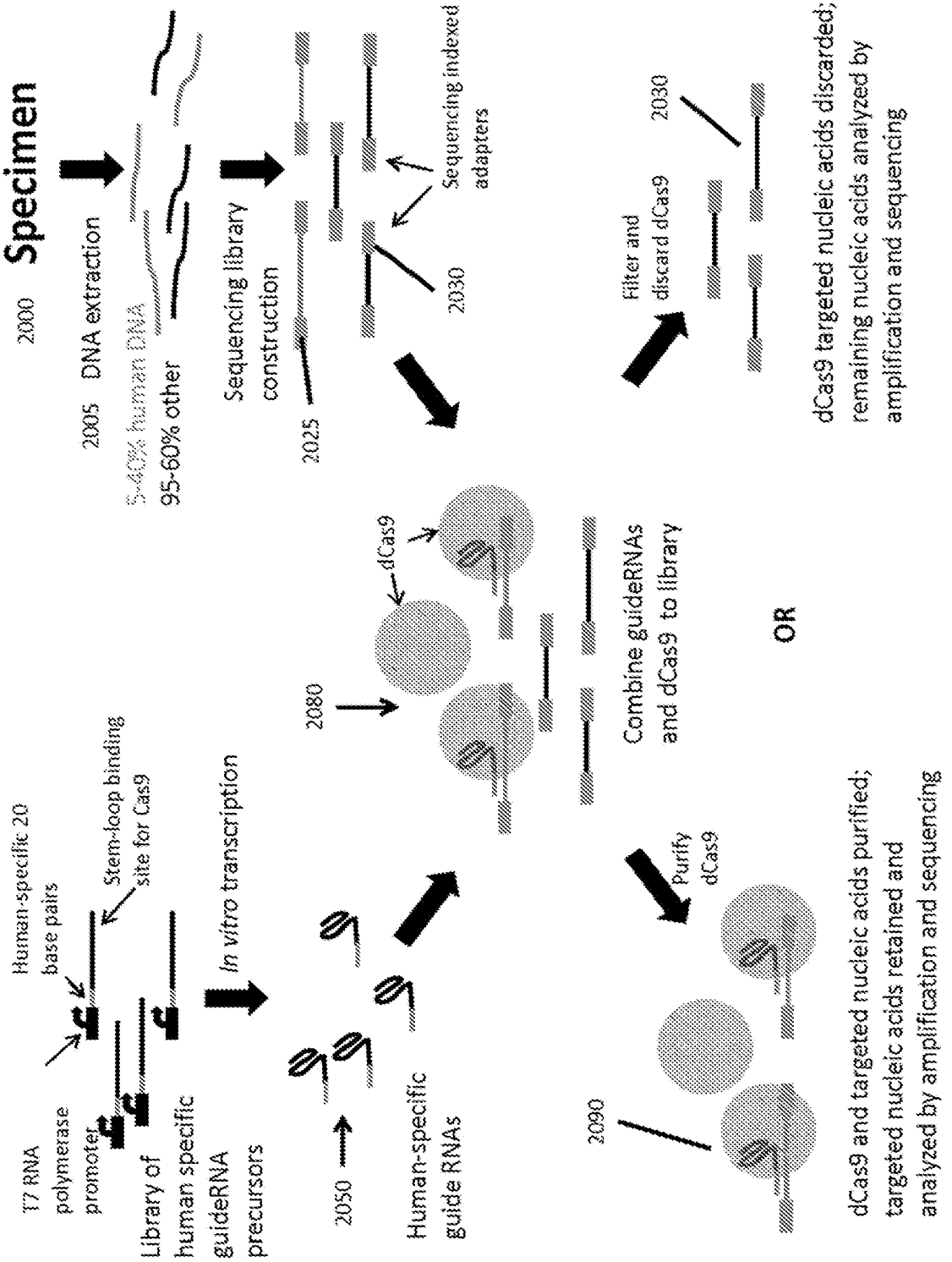
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Fig. 1



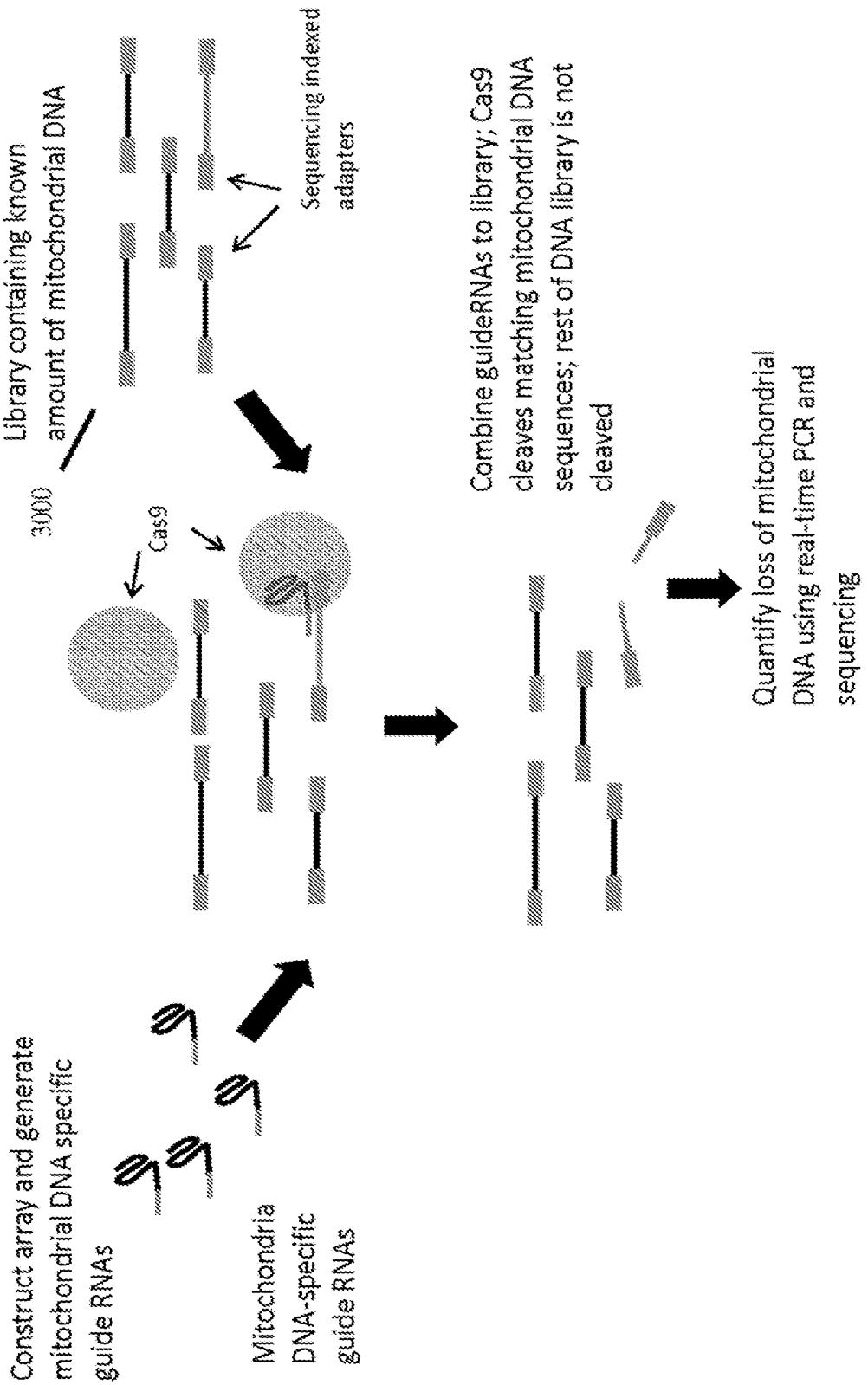
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Fig. 2



IDT Exhibit 1017.0681

Fig. 3



IDT Exhibit 1017.0682

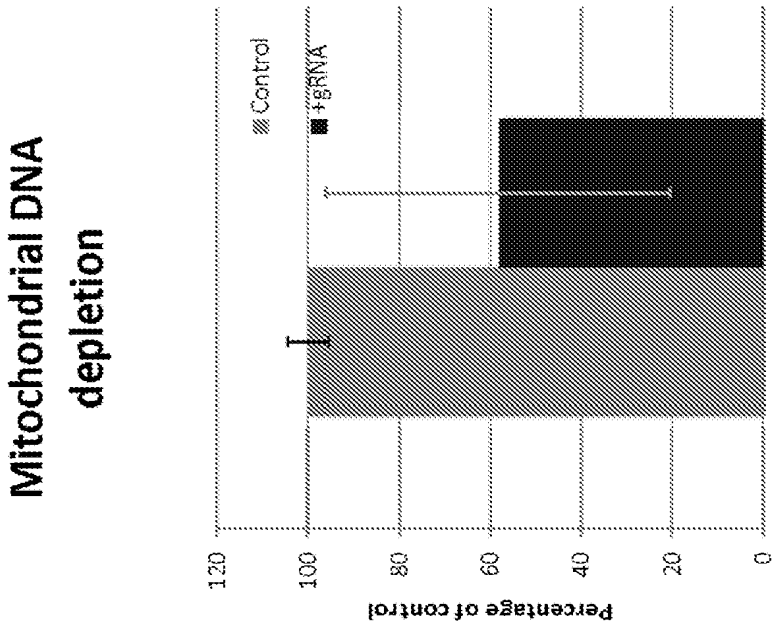


Fig. 4

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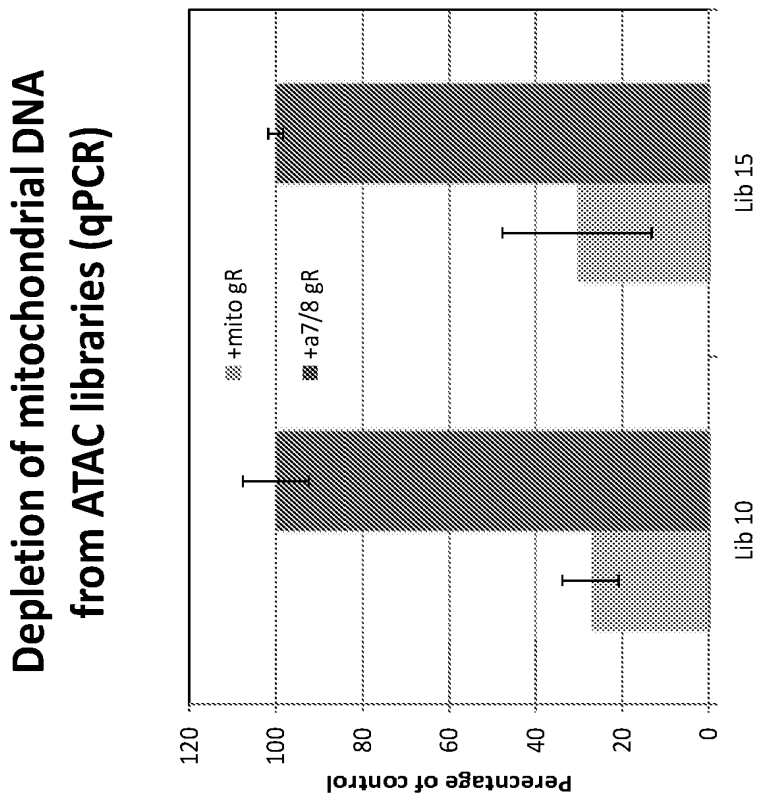


Fig. 5

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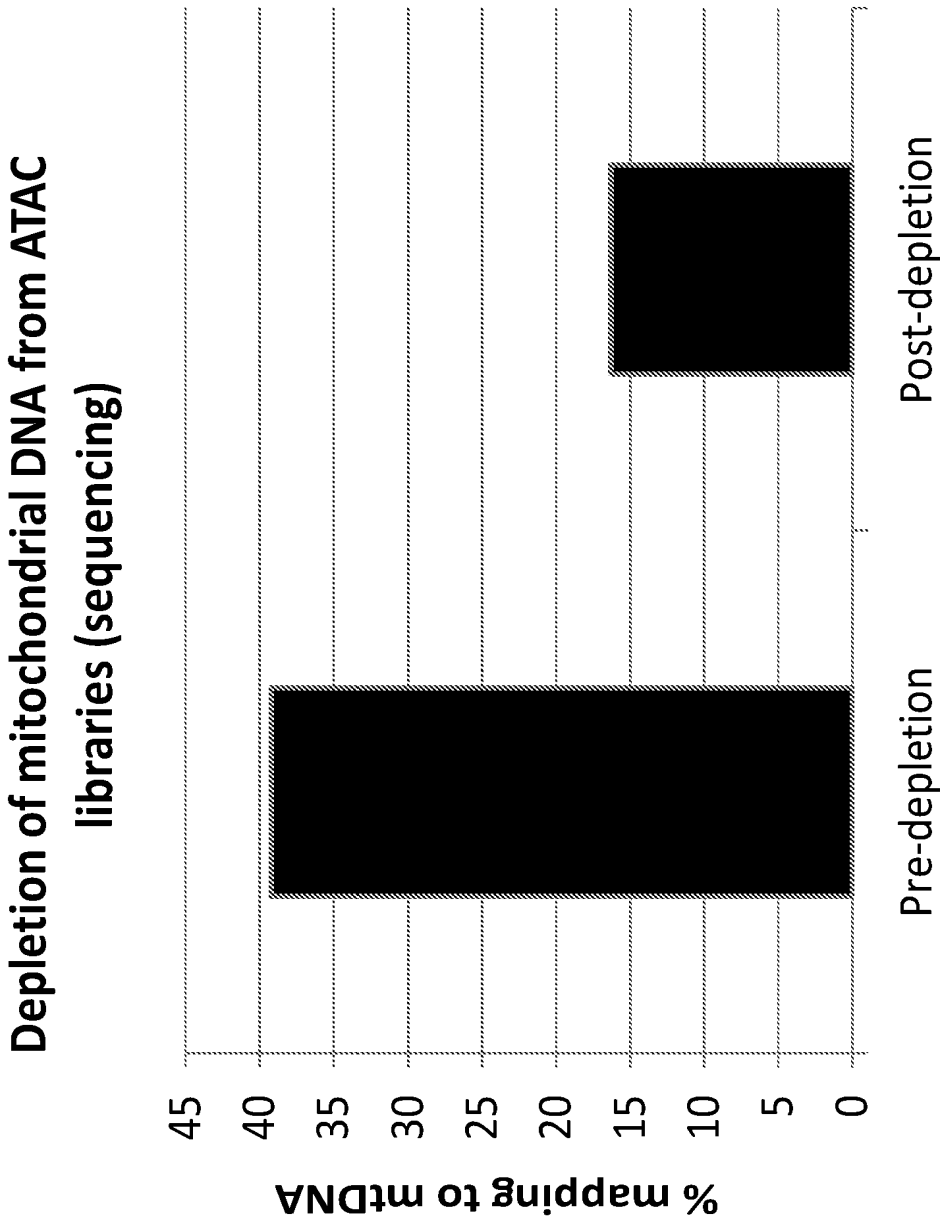


Fig. 6A

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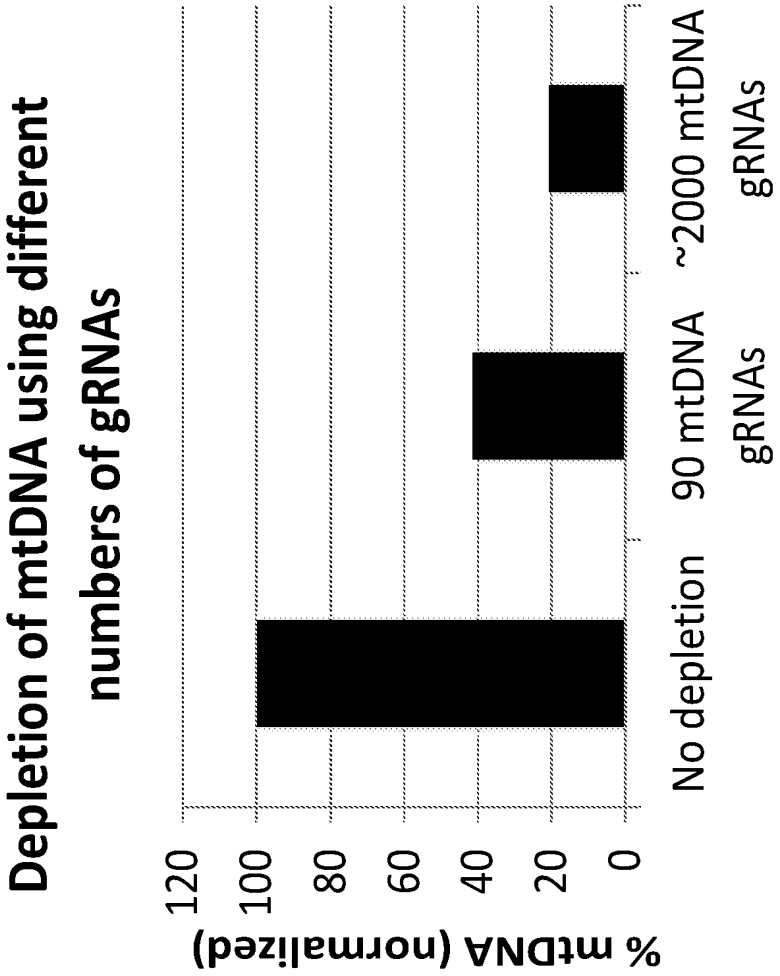


Fig. 6B

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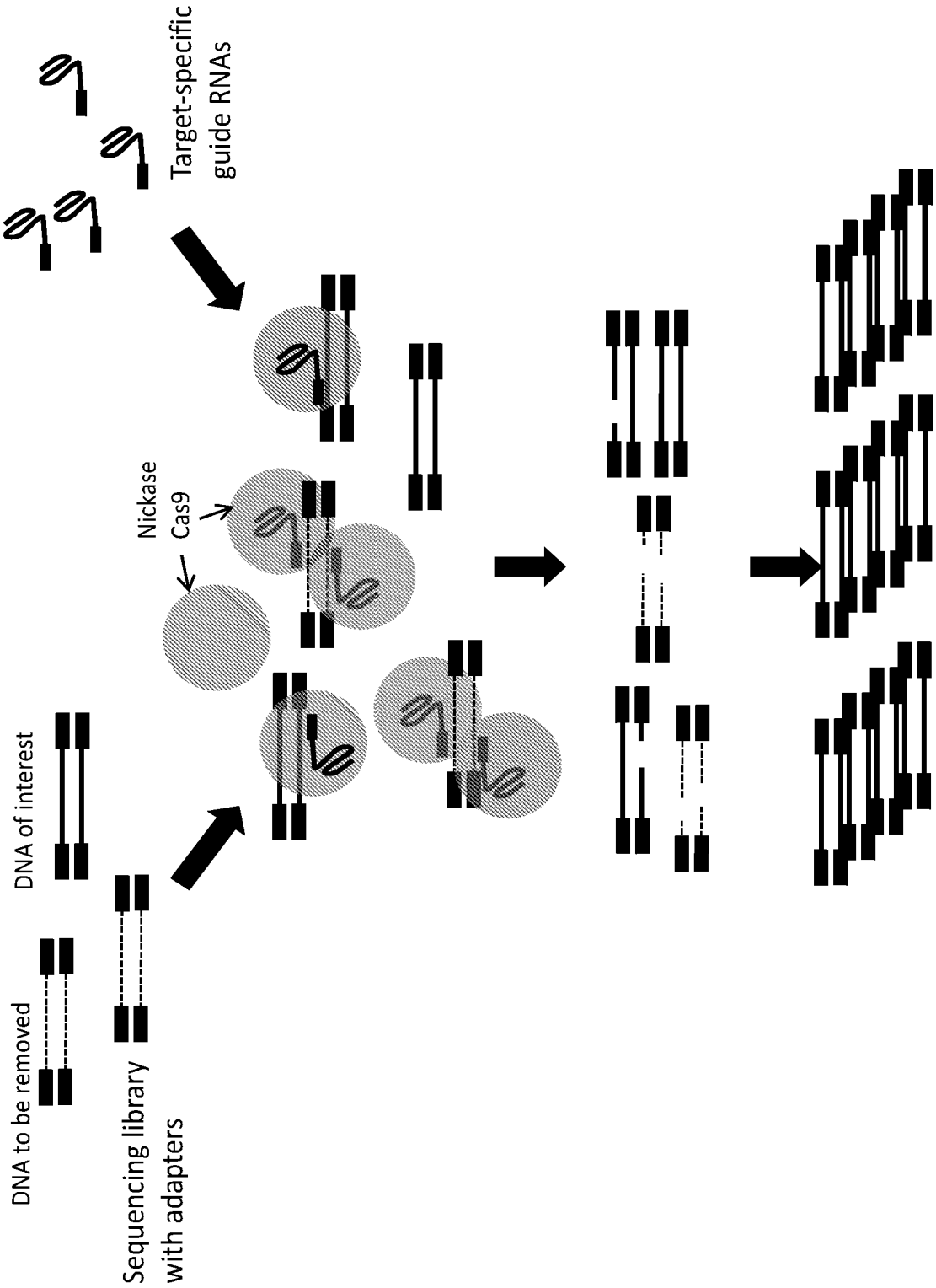
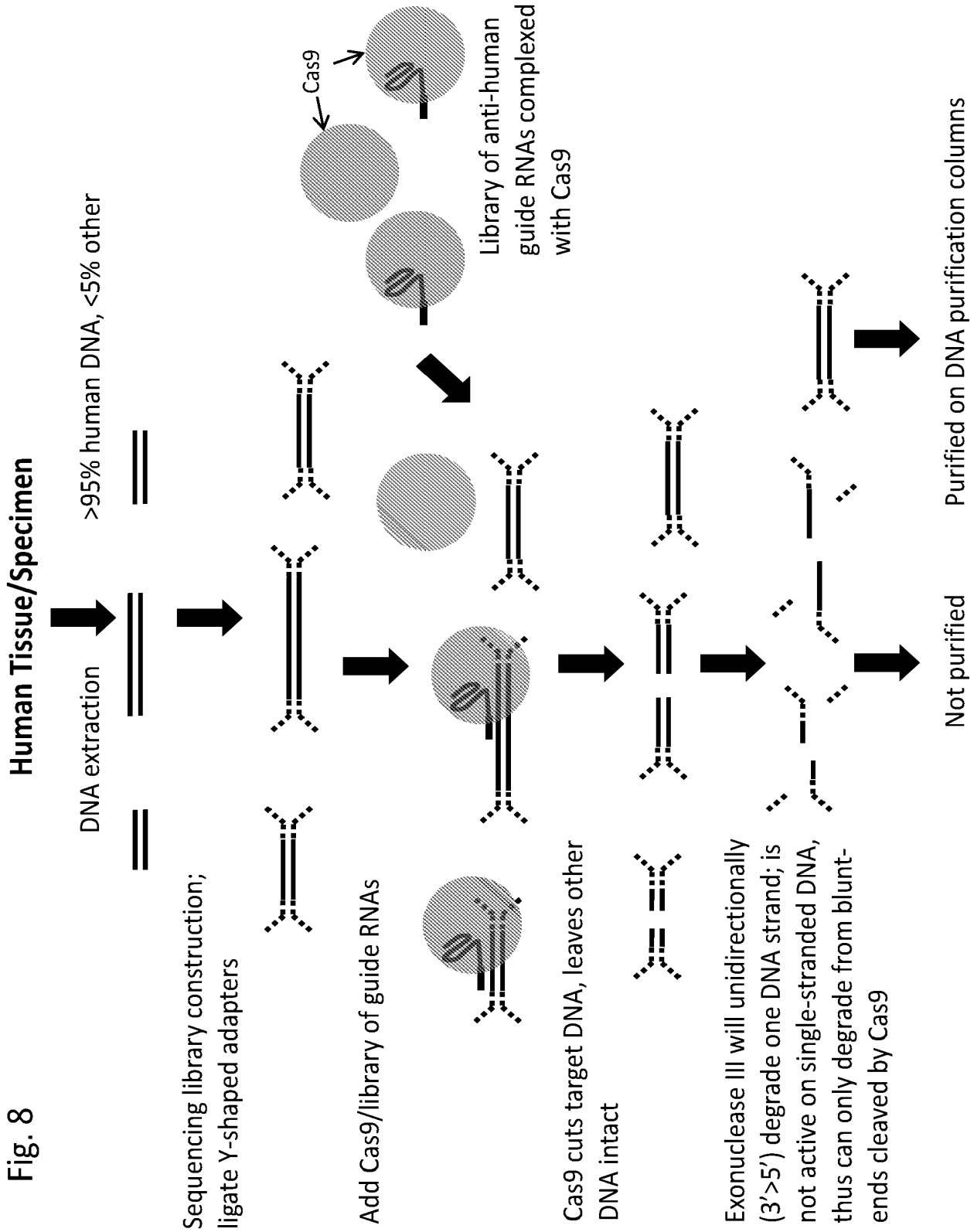


Fig. 7

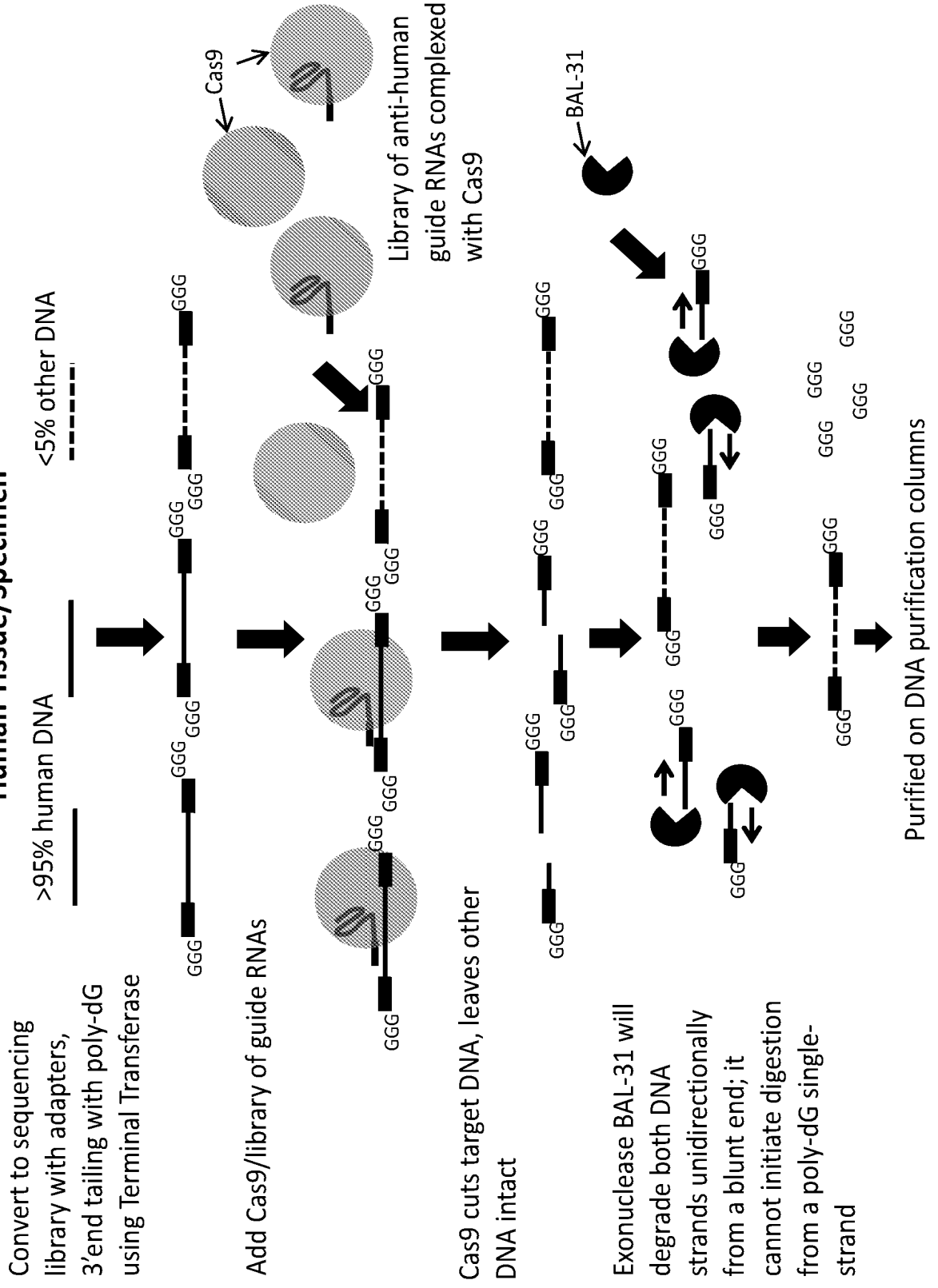
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Fig. 8



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Fig. 9



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Fig. 10

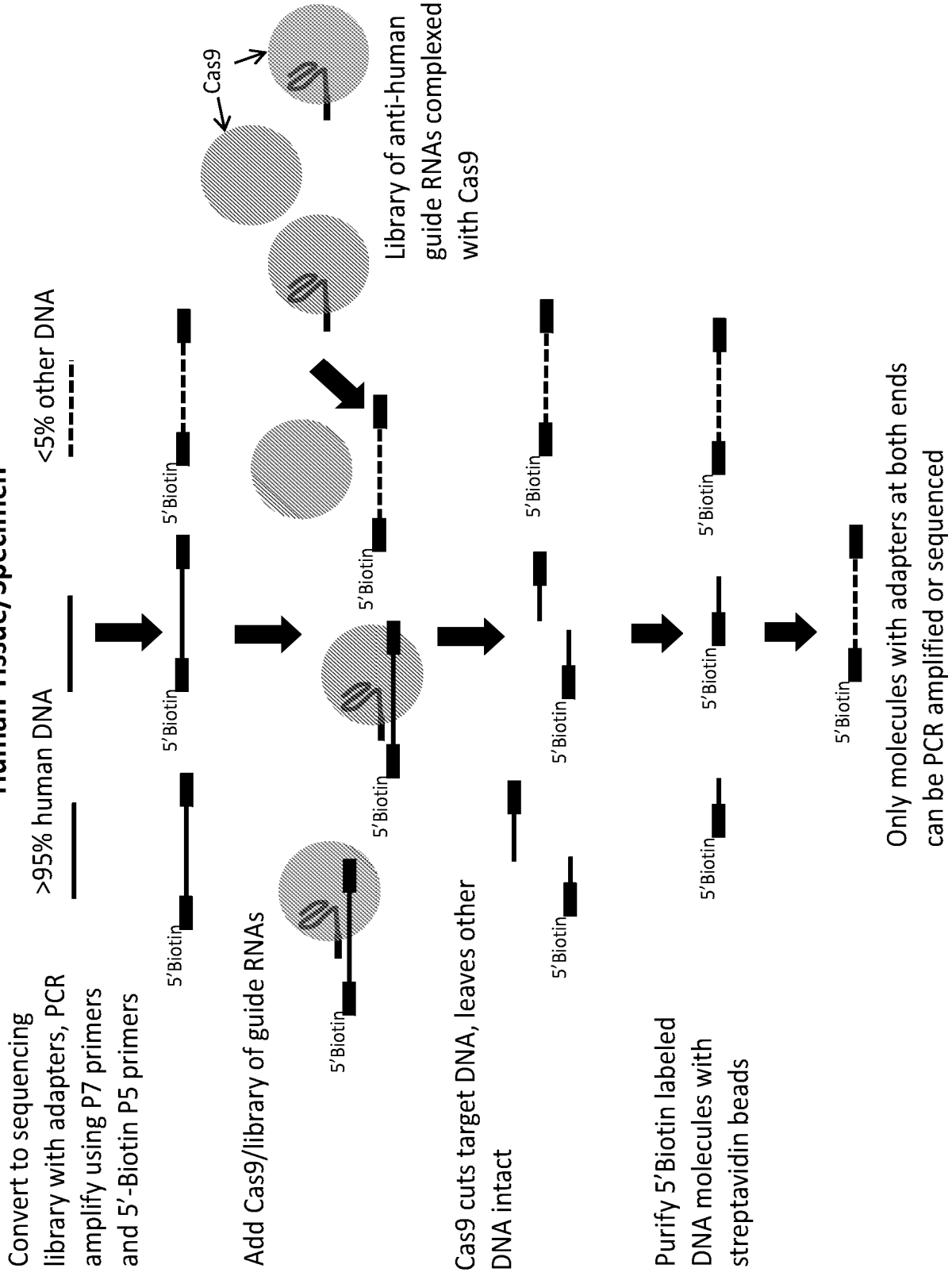
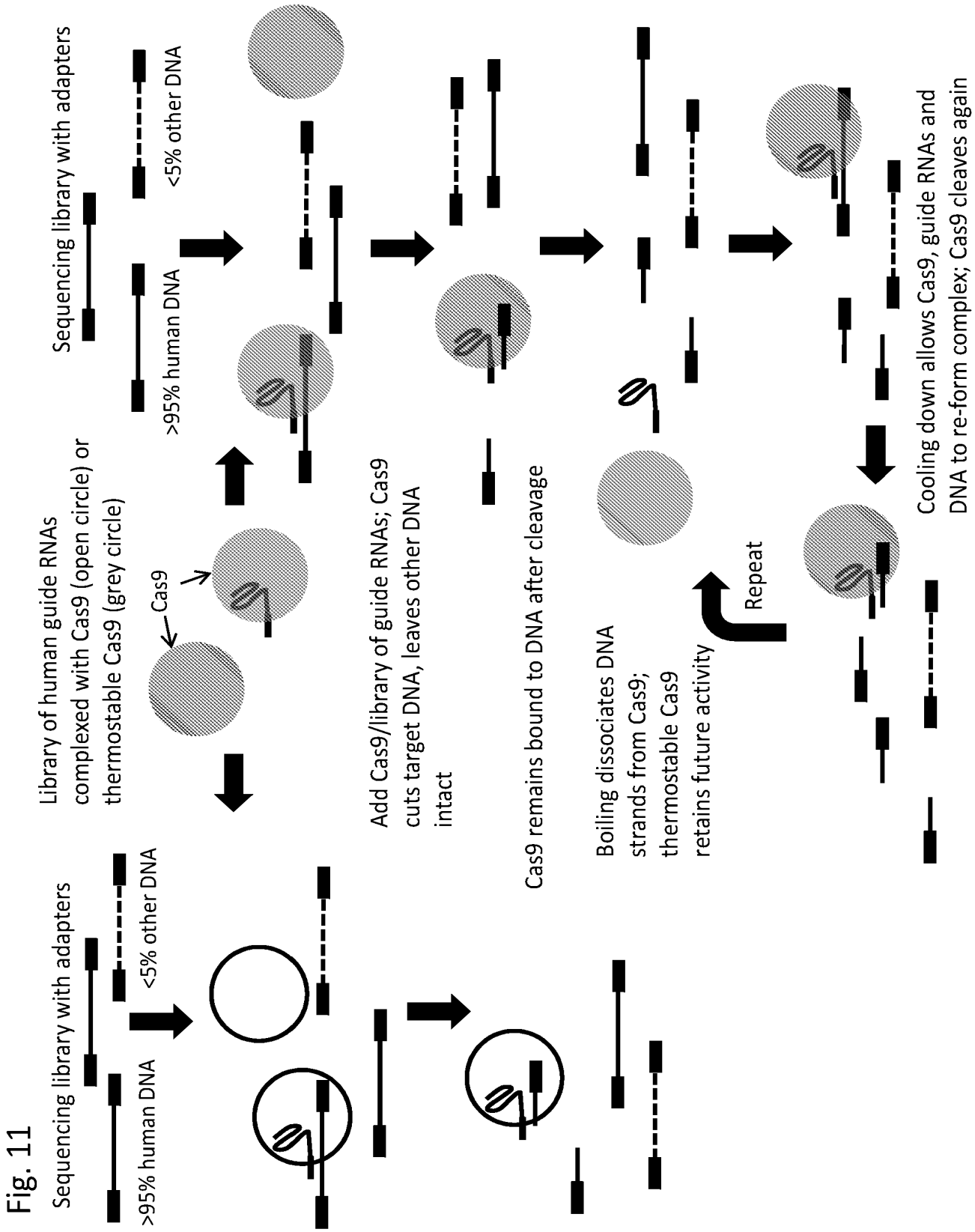

Human Tissue/Specimen
>95% human DNA
<5% other DNA
Convert to sequencing library with adapters, PCR amplify using P7 primers and 5'-Biotin P5 primers
5'Biotin
Add Cas9/library of guide RNAs
Cas9
Library of anti-human guide RNAs complexed with Cas9
Cas9 cuts target DNA, leaves other DNA intact
Purify 5'Biotin labeled DNA molecules with streptavidin beads
Only molecules with adapters at both ends can be PCR amplified or sequenced

Fig. 11



IDT Exhibit 1017.0691

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(54) **Title**: METHOD FOR ARCHIVING AND CLONAL EXPANSION

(57) **Abstract**: The present method provides methods, libraries, and kits related to the archiving and clonal expansion of sequences related to target polynucleotide sequences. The method allow for the attachment of polynucleotides with defined 3' and or 5' sequences to solid surfaces. The polynucleotides attached to the solid substrates can be stored or archived as libraries and can subsequently be retrieved for analysis, for example by clonal expansion. In some embodiments, nucleotides attached to solid surfaces can be used for sequencing of nucleotide sequences related to target RNA or target RNA. The methods are applicable to total RNA and/or total DNA analysis.

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METHOD FOR ARCHIVING AND CLONAL EXPANSION

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application Nos. 61/028,146, filed February 12, 2008, 61/074,991, filed June 23, 2008, and 61/085,811, filed August 1, 2008, which applications are incorporated herein by reference in their entirety. This application is also related to the co-pending patent application [Attorney Docket No 25115-731.201] filed February 12, 2009, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The quality and quantity of nucleic acid sample is important for many studies. High-throughput genomic analysis requires large amounts of template for testing, yet typically the yield of nucleic acids from individual patient samples is limited. Forensic and paleoarcheology work also can be severely limited by nucleic acid sample size. The limitation of starting material impacts the ability to carry out large scale analysis of multiple parameters, as is required for, for example, the genotyping of multiple loci in the study of complex diseases. Moreover, it is well accepted that molecular analysis determination of genomic instability in various pathological condition such as cancer, is most precisely carried out in well defined cell populations, such as that obtained by laser capture micro-dissection or cell sorting. Nucleic acid amplification technologies that provide global amplification of very small polynucleotide samples, for example, from one or a very few cells, may provide a solution to the limited starting materials generally available for analysis.

[0003] Likewise, the ability to amplify ribonucleic acid (RNA) is an important aspect of efforts to elucidate biological processes. 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. Non-coding RNAs have been shown to be of great importance in regulation of various cellular functions and in certain disease pathologies. Such RNAs are often present in very low levels. Thus, amplification methods capable of amplifying low abundance RNAs, are of great importance.

[0004] In addition to the need for amplifying RNA and DNA, there is a need for being able to archive samples, and later retrieve the samples for analysis.

[0005] Various methods for global amplification of DNA target molecules (e.g., whole genome amplification) have been described, including methods based on the polymerase chain reaction (PCR). See, e.g., U.S. Pat. Nos. 5,731,171; 6,365,375; Daigo et al., (2001) Am. J. Pathol. 158 (5):1623-1631; Wang et al, (2001); Cancer Res. 61:4169-4174; Zheng et al, (2001) Cancer Epidemiol. 10:697-700; Dietmaier et al (1999) Am. J. Pathol. 154 (1) 83-95; Stoecklein et al (2002) Am. J. Pathol. 161 (1):43-51; U.S. Pat. Nos. 6,124,120; 6,280,949; Dean et al (2002) PNAS 99 (8):5261-5266. However, PCR-based global amplification methods, such as whole genome amplification (WGA), may generate non-specific amplification artifacts, give incomplete coverage of loci, or generate DNA of insufficient length that cannot be used in many applications. PCR-based methods also suffer from the propensity of the PCR reaction to generate products that are

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preferentially amplified, and thus resulting in biased representation of genomic sequences in the products of the amplification reaction. Methods of global amplification of DNA using composite primers have been described. See e.g. U. S. Patent application 10/824,829.

[0006] Additionally, a number of methods for the analysis of gene expression have been developed in recent years. See, for example, U.S. Pat. Nos. 6,251,639, 6,692,918, 6,686,156, 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; and U.S. published patent applications nos. 2002/0115088, 2003/0186234, 2003/0087251, and 2004/0023271. 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. RNA 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. However, the total amount of sample RNA 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 RNA species is not equal; some species are more abundant than others are, and these are more likely and easier, to analyze. The ability to amplify RNA sequences enables the analysis of less abundant, rare RNA species. The ability to analyze small samples, by means of nucleic acid amplification, is also advantageous for design parameters of large scale screening 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. Methods of amplification from RNA templates have been described, for example in U. S. Patent 6,946,251.

[0007] Sequencing of nucleic acids continues to be one of the most important and useful ways to analyze DNA and RNA samples. Recent developments have made possible highly parallel high throughput sequencing. Many of these approaches use an in vitro cloning step to generate many copies of each individual molecule. Emulsion PCR is one method, isolating individual DNA molecules along with primer-coated beads in aqueous bubbles within an oil phase. A polymerase chain reaction (PCR) then coats each bead with clonal copies of the isolated library molecule and these beads are subsequently immobilized for later sequencing. See, e.g. WO04069849A2, WO05010145A2. In other cases, surface methods of clonal amplification have been developed, for example, by the use of polonies (PCR colonies), or by bridge PCR where fragments are amplified upon primers attached to a solid surface. These methods produce many physically isolated locations which each contain many copies of a single fragment. While these methods have provided improvements in sequencing throughput, there is a continuing need to improve the methods of obtaining samples appropriate for sequencing, and of handling, storing, and amplifying such samples.

[0008] Therefore, there is a need for improved methods of obtaining, storing, amplifying, and analyzing DNA and RNA samples, including methods which can globally amplify DNA or RNA polynucleotide targets. The invention described herein fulfills this need.

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SUMMARY OF THE INVENTION

[0009] One aspect of the invention comprises amplifying a target nucleic acid sequence (DNA or RNA) or the complement of a target nucleic acid sequence on a solid support such as a bead, a magnetic bead, a substantially planar array, an isolated surface, or a well in a plate to form a plurality (e.g. 2; 3; 4; 5; 10; 25; 50; 100; 150; 500; 1,000; 5,000; 25,000 or more) of amplified products. In some cases, the plurality of amplified products comprise clonally amplified products such that a given bead, isolated surface, or well contains a plurality of amplified products of substantially identical sequence. The amplification may be performed by linear amplification such as for example single primer isothermal amplification (SPIA), amplification using a single primer such as for example single primer PCR, rolling circle amplification, or SPIA, amplification from a double stranded nucleic acid having a single stranded 3' overhang, amplification using a DNA-RNA chimeric amplification primer, or any combination thereof. In some embodiments of the method, the amplification step results in at least 1,000; 5,000; 10,000; 100,000; 1,000,000; or more copies of the target nucleic acid or a portion thereof. In some embodiments of the method, the amplification step is performed on a linear template. In some embodiments of the method, the target nucleic acid sequence and/or the amplified product is greater than 100; 200; 400; 500; 1,000; 2,000; 5,000 or more base pairs in length.

[0010] One aspect of the invention comprises a method for clonally amplifying a target nucleic acid sequence or its complement by delivering a target nucleic acid sequence or a set of target nucleic acid sequences into the aqueous phase of a microdroplet of an emulsion and performing linear amplification of the target nucleic acid or a portion thereof in the aqueous phase of the emulsion such that on average each microdroplet amplifies one or less than one of the target nucleic acid sequence or sequences, or such that on average the step of amplification provides microdroplets that comprise a plurality of amplified products that are substantially identical in sequence. In some cases, the amplification within an emulsion is performed in the presence of a solid substrate such as a bead or isolated surface. In some cases, the solid substrate may comprise capture moieties useful for capturing target nucleic acid and/or amplified product. In some cases, the amplification is performed such that on average, a given bead or isolated surface captures one or less than one target nucleic acid, or such that a given bead or isolated surface captures a plurality of amplified products that are substantially identical in sequence.

[0011] One aspect of the invention comprises a kit for performing the methods of the present invention. The kit may be useful for amplifying DNA or RNA for subsequent analysis such as expression analysis including quantitative PCR or microarray analysis; sequencing including thermocycle sequencing, dye terminator sequencing, sequencing using the methods of Illumina/Solexa, SOLiD (ABI), Roche/454 LifeSciences, Helicos, or Sequenom; alternatively the kit may be useful for archiving DNA or RNA sequencing. In another aspect, the kit may be useful for generating clonally expanded target sequences. In yet another aspect, the kit may be useful for generating one or a plurality of amplified products having a defined 3' and 5' end. The kit comprises an RNA-DNA chimeric first primer for creating a first primer extension product, and an RNA-DNA chimeric oligonucleotide for annealing to an end of a double stranded nucleic acid product having a single stranded 3' overhang at one end. In some embodiments, the kit may further

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comprise a polymerase having substantial strand-displacement activity. In some embodiments, the kit may further comprise a second primer. In some embodiments, the kit may further comprise RNase H.

[0012] One aspect of the invention comprises a method for creating a double stranded nucleic acid comprising an RNA-DNA heteroduplex, wherein the double stranded nucleic acid is suitable for creating amplified products by single primer isothermal linear amplification that have a defined 5' and 3' end. The method comprises (a) annealing to a template nucleic acid and extending a first primer or a set of first primers with a DNA polymerase comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target nucleic acid sequence of interest, and a portion of the 5' RNA segment, sequence (A), is not complementary to the target nucleic acid. The annealing and the extension produces a first primer extension product or set of products that is hybridized to the target nucleic acid. The extension step may be performed with an RNA-dependant DNA polymerase from an RNA template or a DNA-dependent DNA polymerase from a DNA template. In some cases, step (a) may be performed in the presence of RNA and DNA and an inhibitor such as for example actinomycin to selectively inhibit the formation of an extension product complementary to the DNA.

[0013] The method further comprises (b) separating or removing the first primer extension product from the target nucleic acid. The separating step may be performed by heat, enzyme, chemical treatment, or a combination thereof.

[0014] The method further comprises step (c) annealing and extending a second primer with a DNA-dependent DNA polymerase. The second primer may comprise a 3' DNA segment and a 5' DNA segment, wherein a portion of the 3' DNA segment is complementary to the first primer extension product or set of first primer extension products and a 5' portion, sequence (B), is not complementary to the first primer extension product or the set of first primer extension products, to produce a double-stranded DNA product or a set of double-stranded DNA products, each product comprising the first primer extension product hybridized to a second primer extension product. The second primer extension product may comprise a DNA sequence (A') that is complementary to and hybridized to the RNA sequence (A) of the first primer extension product at its 3' end, thereby forming a double stranded nucleic acid product or products with an RNA-DNA heteroduplex at one end.

[0015] The method further comprises step (d) cleaving the RNA from the RNA-DNA heteroduplex. The cleaving step may be performed using an enzyme that is specific for the RNA portion of an RNA-DNA heteroduplex such as for example RNase H. The cleaving step may provide a double stranded nucleic acid product or products with a single stranded 3' overhang at one end.

[0016] The method further comprises step (e) annealing a chimeric oligonucleotide comprising a 5' end, wherein the 5' end comprises sequence (C), and the 5' end further comprises RNA, and a 3' end wherein the 3' end comprises the DNA sequence (A), to the single stranded 3' overhang of the double stranded nucleic acid product or products formed in step (d).

[0017] The method further comprises step (f), extending the double stranded nucleic acid product or products with a DNA polymerase to produce a double stranded nucleic acid product with a (C)-

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(C') RNA-DNA heteroduplex at one end and a (B)-(B') double stranded DNA sequence at the other end.

[0018] In some embodiments of the method, the second primer further comprises a ligand at a 5' end of the 5' DNA segment.

[0019] In some embodiments of the method, the method further comprises binding the ligand to a solid surface, whereby the first and second primer extension product or products are bound to the solid surface.

[0020] In some embodiments of the method, the method further comprises step (g), cleaving the RNA portion of the chimeric oligonucleotide in the DNA-RNA heteroduplex to generate a double stranded nucleic acid or a set of double stranded nucleic acids with a single stranded 3' overhang at one end of sequence (C'). The method may further comprise step (h), annealing a DNA-RNA chimeric amplification primer to the single stranded portion of the second primer extension product. The chimeric amplification primer may comprise a sequence (C) that is complementary to the 3' overhang formed in step (g). The chimeric amplification primer may comprise a DNA portion and a 5' RNA portion. The method may further comprises step (i), extending the amplification primer with a DNA polymerase having strand displacement activity to produce an amplified product hybridized to the second primer extension product. The method may further comprise step (j), cleaving the RNA from the amplified product hybridized to the second primer extension product in the RNA-DNA heteroduplex; and step (k), repeating steps (h) to (j) to produce multiple copies of amplified product comprising a 5' end and a 3' end, wherein the 5' end comprises sequence (A) and the end comprises sequence (B').

[0021] In some embodiments of the method, the method comprises use of a first primer that comprises a random annealing sequence such as for example random hexamers or random decamers. Alternatively, the method comprises use of a set of first primers, wherein each member of the set of first primers comprises a distinct 3' DNA annealing sequence, each specific for a target or a region of template nucleic acid. Alternatively, the method comprises use of a first primer that comprises a degenerate annealing sequence for binding to multiple related target sequences. Alternatively, the method comprises use of a first primer that comprises a poly-T sequence, or any sequence that substantially hybridizes to the poly-sequence of messenger RNA. Alternatively, the first primer or set of first primers may comprise a combination thereof.

[0022] In some embodiments of the method, the method comprises use of a second primer that comprises a random annealing sequence such as for example random hexamers or random decamers. Alternatively, the method comprises use of a set of second primers, wherein each member of the set of first primers comprises a distinct 3' DNA annealing sequence, each specific for a target or a region of template nucleic acid. Alternatively, the method comprises use of a second primer that comprises a degenerate annealing sequence for binding to multiple related target sequences. Alternatively, the second primer or set of second primers may comprise a combination thereof.

[0023] In some embodiments of the method, the amplified products are attached, bound, or covalently linked to a solid surface such as a bead. In some cases, the attached, bound, or covalently linked amplified products comprise a clonally amplified sequence on the surface such as a bead or an isolated area on a surface. In some cases, the bead or isolated area on a surface is the only bead

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or isolated area on a surface within an isolated liquid volume such as an aqueous droplet in a water/oil emulsion or a liquid volume in the well of a plate, such that the amplified product is contained with such a liquid volume. In some cases, the bead or isolated area comprises covalently attached oligonucleotides comprising sequence (B), at their 3' ends, whereby upon the amplification of step (k) multiple copies of amplified product comprising sequence (B') at their 5' end are hybridized to the bead or isolated area.

[0024] In some embodiments of the method, the sequence (B) covalently attached to the bead or isolated area is extended to produce multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product, wherein the multiple polynucleotides comprise sequence (A') near their 5' ends and sequence (B) near their 3' ends.

[0025] In some embodiments of the method, the method further comprises removing the amplified product from the bead or isolated area to render the covalently attached polynucleotides single stranded, and extending a sequencing primer, a portion of which is complementary to a portion of sequence (C') to produce a detectable signal or detectable oligonucleotide fragments characteristic of the sequence of the polynucleotide bound to the bead or isolated area, and thereby perform sequencing. In some cases, the sequencing method comprises the use of cleavable labeled terminators, dye terminators, pyrophosphate detection, an isothermal sequencing method, cycle sequencing, or sequencing by ligation.

[0026] One aspect of the invention comprises a method for attaching a target nucleic acid (DNA or RNA) to a solid surface such as a bead or an isolated area. The target nucleic acid attached thereto may be useful for example for clonally amplifying the sequence or a portion thereof. The method comprises step (a), providing a first nucleic acid or a set of first nucleic acids comprising DNA corresponding to a region of a target nucleic acid and further comprising a 5' end and a 3' end, wherein said 5' end of the first nucleic acid or the set of first nucleic acids comprises a sequence (A) and a sequence (C), wherein said sequence (A) is 3' of sequence (C), and wherein sequence (C) comprises RNA, and wherein said 3' end of the first nucleic acid comprises sequence (B'). The first nucleic acid may be from a variety of sources including but not limited to the methods provided herein, single primer polymerase chain reaction products, products, and endonuclease treated rolling circle amplification products. The method further comprises step (b), hybridizing the first nucleic acid or set of first nucleic acids to a solid support comprising an oligonucleotide comprising sequence (B) complementary to sequence (B') of the first nucleic acid. The solid support may be a bead, a substantially planar array, or a magnetic bead. In some cases, the bead or an isolated area of the solid support may comprise only one copy of the first nucleic acid. The method further comprises step (c), extending the oligonucleotide to produce a double stranded product comprising a second nucleic acid hybridized to the first nucleic acid, wherein the second nucleic acid comprises a 3' segment complementary to a portion of the first nucleic acid and a 5' segment complementary to a portion of the first nucleic acid, whereby a portion of the 3' segment comprises a sequence (A') and a portion of the 3' segment comprises a sequence (C'), and wherein the sequence (A') is 5' of the sequence (C'), and whereby a portion of the 5' segment comprises sequence (B). In some cases, the bead or an isolated area of the solid support may thus comprise only one copy of the first and second nucleic acid.

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[0027] In some embodiments, a plurality of first and second nucleic acids are provided corresponding to different sequences in the target nucleic acid. In some cases, the plurality of first and second nucleic acids are bound to one or a plurality of beads or isolated areas on a surface. In some cases, the plurality of first and second nucleic acids are bound to a plurality of beads or isolated areas on a surface such that generally, or on average, one or fewer than one first and second nucleic acids are bound to one bead or isolated area.

[0028] In some embodiments, the method further comprises amplifying the target nucleic acid by treating the first and second nucleic acids bound to the beads or isolated areas with reagents to produce multiple copies of amplification product in an clonal fashion (i.e. multiple copies of substantially one amplification product on each bead or isolated area) that are complementary to all or a portion of the second nucleic acid product. In some cases, the reagents comprise SPIA amplification reagents including but not limited to a DNA-RNA chimeric amplification primer, a DNA-polymerase with substantial strand-displacement activity, and RNase H. In some cases, the bead or isolated area are stored prior to the step of amplification.

[0029] One aspect of the invention comprises a method for attaching a polynucleotide sequence that is representative of a sequence within a nucleic acid target molecule to a solid surface comprising: (a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer, sequence (P), is complementary to a target nucleic acid and a 5' portion of the of the primer, sequence (A), is not complementary to the target nucleic acid, to form a first primer extension product hybridized to the target nucleic acid; (b) separating or removing the first primer extension product from the target nucleic acid; (c) extending a second primer to produce a double stranded product comprising a second primer extension product hybridized to the first primer extension product, wherein the second primer comprises a 3' segment complementary to a portion of the first primer extension product and a ligand, whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer; and (d) binding the ligand to a receptor bound to a solid surface whereby the second primer extension product is attached to the solid surface.

[0030] One aspect of the invention comprises a method comprising: (a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA; to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid; (b) cleaving the target RNA from the RNA/DNA hybrid; and (c) extending a second primer, comprising a ligand and a 3' segment complementary to a portion of the first primer extension product, to produce a double stranded product with a DNA/RNA heteroduplex at one end; wherein the double stranded product comprises a second primer extension product hybridized to the first primer extension product, and whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer.

[0031] In some embodiments of the method, the 3' portion of the primer that is complementary to the target RNA comprises a random nucleotide sequence. In some embodiments of the method the 3' portion of the primer that is complementary to the target RNA comprises a sequence that is

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complementary to polyadenosine (poly-A). In some embodiments of the method the 3' portion of the primer that is complementary to the target RNA comprises a specific sequence that is complementary to a multiplicity of targets. In some embodiments of the method, in step (b), the target RNA is cleaved by heat, enzyme treatment, or chemical treatment.

[0032] In some embodiments of the method the RNA target is in a sample that also comprises DNA, and wherein actinomycin is added prior to step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a).

[0033] In some embodiments of the method the 3' segment of the second primer complementary to a portion of the first primer extension product comprises a random nucleotide sequence, a specific sequence complementary to a specific sequence of the first primer extension product, or a sequence common to multiple first primer extension products. In some embodiments of the method the second primer further comprises a nucleotide sequence (B) that is not complementary to the first primer extension product sequence. In some embodiments of the method the method further comprises step (d) of binding the ligand to a solid surface, whereby the second primer extension product becomes bound to the solid surface. In some embodiments of the method the binding of the ligand results in the double stranded product being bound to the solid surface. In some embodiments of the method the binding of the ligand results in the single stranded second primer extension product being bound to the solid surface.

[0034] In some embodiments of the method the method further comprises treating the solid surface with reagents to produce multiple copies of an amplification product that are substantially complementary the second primer extension product. In some embodiments of the method the solid surface comprises a substantially planar array. In some embodiments of the method the solid surface comprises a bead. In some embodiments of the method the bead comprises a magnetic bead. In some embodiments of the method the bead comprises only one copy of second primer extension product. In some embodiments of the method the second primer extension product is single-stranded. In some embodiments of the method the second primer extension product is double-stranded.

[0035] In some embodiments of the method a plurality of second primer extension products are produced corresponding to different sequences in the target RNA. In some embodiments of the method the plurality of second primer extension products is bound to one or a plurality of beads or a plurality of isolated areas on a surface. In some embodiments of the method the plurality of second primer extension products is bound to a plurality of beads under conditions such that generally, one or fewer second primer extension products is bound to one bead or one isolated area on a surface.

[0036] In some embodiments of the method the method further comprises treating the beads or isolated areas with reagents to produce multiple copies of an amplification product complementary to all or a portion of the second primer extension products. In some embodiments of the method the plurality of beads or isolated areas are contained within a plurality of isolated volumes such that generally one or fewer beads or isolated area is associated with each isolated volume, and whereby the production of multiple copies of amplification product results in multiple copies of substantially one amplification product in each volume. In some embodiments of the method the amplification is

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carried out with a reaction mixture comprising RNase H, an amplification primer comprising a DNA portion and a 5' RNA portion, and a DNA polymerase with strand displacement activity.

[0037] In some embodiments of the method the method further comprises the step of storing the beads or isolated areas comprising generally one or fewer primer extension products per bead or isolated area.

[0038] In some embodiments of the method the method further comprises clonally amplifying the primer extension product bound to the beads or isolated areas after storing them.

[0039] One aspect of the invention comprises a method for amplifying a nucleic acid representative of a target RNA comprising carrying out steps (a) through (d) above and further comprises the steps of: (e) cleaving the RNA in the heteroduplex from the first primer extension product such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded; (f) annealing an amplification primer to the single stranded portion of the second primer extension product complementary to sequence (A), wherein the amplification primer has a DNA portion and a 5' RNA portion; (g) extending the amplification primer with a DNA polymerase having strand displacement activity to produce an amplified product hybridized to the second primer extension product; (h) cleaving the RNA from the amplified product hybridized to the second primer extension product; and repeating steps (f) to (h) to produce multiple copies of amplified product.

[0040] In some embodiments of the method the second primer further comprises a sequence (B that is not complementary to the first primer extension product sequence, whereby the amplification product comprises a sequence at its 3' end which is complementary to (B). In some embodiments of the method the target RNA comprises messenger RNA.

[0041] One aspect of the invention comprises a method comprising:(a) denaturing a double-stranded target DNA; (b) annealing to the target DNA and extending with a DNA polymerase comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the of the primer comprises sequence (A), which is not complementary to the target DNA; to form a plurality of first primer extension products, each with sequence (A) at its 5' end; and (c) extending a second primer comprising a ligand and a 3' DNA region that comprises a random sequence, wherein the primer is optionally a tailed primer comprising a nucleic acid sequence (B) that is 5' of the random sequence, to form a plurality of double-stranded products each comprising a first primer extension product and a second primer extension product whereby the second primer extension product comprises a ligand;

[0042] In some embodiments of the method step (b) comprises a first incubation at a temperature below about 30 °C, and a second incubation at a temperature above about 40 °C. In some embodiments of the method a DNA polymerase which is active at temperatures above about 45 °C is used to extend the first primer.

[0043] In some embodiments of the method the method further comprises step (d) of binding the ligand to a solid surface, whereby the plurality of second primer extension products become bound to the solid surface.

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[0044] In some embodiments of the method the binding of the ligand results in the double stranded product being bound to the solid surface. In some embodiments of the method the binding of the ligand results in the single stranded second primer extension product being bound to the solid surface.

[0045] In some embodiments of the method the method further comprises treating the solid surface with reagents to produce multiple copies of amplification products that are substantially complementary to the plurality of second primer extension products. In some embodiments of the method the solid surface comprises a substantially planar array. In some embodiments of the method the solid surface comprises a plurality of beads or a plurality of isolated areas on a surface. In some embodiments of the method the plurality of second primer extension products is bound to a plurality of beads or isolated areas under conditions such that generally, one or fewer copies of a single second primer extension product is bound to one bead or one isolated area.

[0046] In some embodiments of the method the method further comprises treating the beads or isolated areas with reagents to produce multiple copies of an amplification product substantially complementary to the second primer extension products. In some embodiments of the method the plurality of beads or isolated areas are contained within a plurality of isolated volumes such that generally one or fewer beads or isolated area is associated with each isolated volume, and whereby the production of multiple copies of amplification product results in multiple copies of substantially one amplification product in each volume. In some embodiments of the method the amplification is carried out with a reaction mixture comprising RNase H, an amplification primer with a DNA portion and a 5' RNA portion, and a DNA polymerase with strand displacement activity.

[0047] In some embodiments of the method the method further comprises the step of storing the beads or isolated areas comprising generally one or fewer primer extension products.

[0048] In some embodiments of the method the method further comprises clonally amplifying the primer extension product bound to the beads or isolated areas after storing them.

[0049] One aspect of the invention comprises a method for amplifying a nucleic acid representative of a target DNA comprising carrying out steps (a) through (d) and further comprising the steps of: (e) cleaving the RNA from the first primer extension products such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded; (f) annealing an amplification primer to the single stranded portion of the second primer extension products complementary to sequence (A), wherein the amplification primer has a DNA portion and a 5' RNA portion; (g) extending the amplification primer with a DNA polymerase having strand displacement activity to produce an amplified product hybridized to the second primer extension product; (h) cleaving the RNA from the amplified product hybridized to the second primer extension product; and repeating steps (f) to (h) to produce multiple copies of amplified product

[0050] In some embodiments of the method the second primer comprises a tailed primer comprising a nucleic acid sequence (B) that is 5' of the random sequence, whereby the amplified product comprises a portion complementary to sequence (B) at or near its 3' end. In some embodiments of the method the target DNA is genomic DNA. In some embodiments of the method the target DNA comprises multiple genomes.

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[0051] One aspect of the invention comprises a method for archiving and/or clonal expansion of a nucleotide sequence comprising the steps of: (a) obtaining a plurality of partially double-stranded DNA products comprising a first polynucleotide and a second polynucleotide, wherein the second polynucleotide comprises a sequence (A') at its 3' end and a ligand, wherein the sequence (A') portion of the second polynucleotide is single-stranded, wherein optionally the second polynucleotide comprises a sequence (B) at or near its 5' end; (b) attaching the partially double stranded DNA products to a plurality of beads or a plurality of isolated areas on a surface by binding the ligands to the bead or isolated area; (c) annealing an amplification primer to the single stranded portion of the second polynucleotide complementary to sequence (A'), wherein the amplification primer has a DNA portion and a 5' RNA portion; (d) extending the amplification primer with an enzyme having strand displacement activity to produce a plurality of amplified products hybridized to the second polynucleotide products; (e) cleaving the RNA from the amplified product hybridized to the second polynucleotide products using RNase H; and repeating steps (c) to (e) to produce multiple copies of amplified products.

[0052] In some embodiments of the method the ligands of the DNA products are attached to beads or isolated areas, and on average, one DNA product is attached to one or fewer beads or isolated areas.

[0053] In some embodiments of the method the method further comprises storing the plurality of beads or isolated areas for later analysis.

[0054] In some embodiments of the method the beads or isolated areas are stored after step (b), then later amplified with steps (c) through (f). In some embodiments of the method the amplification is a clonal amplification carried out in multiple isolated volumes wherein on average, one isolated volume has one or fewer beads or isolated areas. In some embodiments of the method the multiple isolated volumes are droplets in a non-aqueous phase.

[0055] One aspect of the invention comprises a method comprising: (a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA; to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid; (b) cleaving the target RNA from the RNA/DNA hybrid; (c) extending a second primer, comprising a ligand and a 3' segment complementary to a portion of the first primer extension product, to produce a double stranded product with a DNA/RNA heteroduplex at one end; wherein the double stranded product comprises a second primer extension product hybridized to the first primer extension product, and wherein a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer; (d) cleaving the RNA in the heteroduplex from the first primer extension product such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded; (e) annealing to the second primer extension product an oligonucleotide comprising a 3'- DNA segment that is complementary to sequence (A') and a 5' RNA segment comprising sequence (C); (f) optionally extending the oligonucleotide to form an oligonucleotide extension product hybridized to the second primer extension product; (g) extending the second primer extension product to create a heteroduplex such that the second primer comprises

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a DNA sequence (C') that is complementary to sequence (C); and (h) cleaving the RNA from the heteroduplex created in step (g) to produce a single-stranded portion of the second primer extension product corresponding to sequence (C').

[0056] In some embodiments of the method the 3' portion of the first primer that is complementary to the target RNA comprises a random nucleotide sequence. In some embodiments of the method the 3' portion of the first primer that is complementary to the target RNA comprises a sequence that is complementary to polyadenosine (poly-A). In some embodiments of the method the 3' portion of the primer that is complementary to the target RNA comprises a specific sequence that is complementary to a multiplicity of targets.

[0057] In some embodiments of the method the RNA target is contained within a sample that also comprises DNA, and actinomycin is added prior to step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a).

[0058] In some embodiments of the method, in step (b), the target RNA is cleaved by chemical heat, or enzyme treatment.

[0059] In some embodiments of the method the 3' segment of the second primer complementary to a portion of the first primer extension product comprises a random nucleotide sequence, a specific sequence complementary to a specific sequence of the first primer extension product, or a sequence common to multiple first primer extension products.

[0060] In some embodiments of the method the method further comprises: (i) binding the ligand on the second primer extension product to a solid surface. In some embodiments of the method, step (i) of binding the ligand to the solid surface is performed before step (h). In some embodiments of the method, step (i) of binding the ligand to the solid surface is performed after step (h).

[0061] One aspect of the invention comprises a method of amplifying a sequence representative of an sequence within an RNA target molecule comprising carrying out steps (a) through (i), and further comprising the steps of: (j) annealing an amplification primer, wherein the amplification primer has a DNA portion and a 5' RNA portion, to the single stranded portion of the second primer extension product complementary to sequence (C'); (k) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product; (l) cleaving the RNA from the amplified product; and (m) repeating steps (j) to (l) to produce multiple copies of amplified product wherein the 5' portion of the amplified product has a sequence complementary to sequence (A').

[0062] In some embodiments of the method, the second primer further comprises a segment (B) that is not complementary to the first primer extension product sequence, whereby the amplified product comprises a sequence (B') at or near its 3' end that is substantially complementary to sequence (B), and a sequence (A) near its 5' end that is complementary to sequence (A'). In some embodiments of the method, the amplification is a clonal amplification. In some embodiments of the method, the solid surface is a bead or an isolated area on a surface. In some embodiments of the method, the bead or isolated area is the only bead or isolated area associated with a isolated liquid volume such that the amplified product is contained within such liquid volume. In some embodiments of the method, the liquid volume is an aqueous droplet within a non-aqueous fluid. In some embodiments of the method, the solid surface is a bead and the droplet is part of a

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microemulsion. In some embodiments of the method, the liquid volume is a well in a plate. In some embodiments of the method, the solid surface is a substantially planar substrate.

[0063] In some embodiments of the method, the bead or isolated area comprises covalently attached thereto multiple oligonucleotides comprising the sequence (B) at their 3' ends, whereby upon the amplification of step (m) multiple copies of amplified product comprising sequence (B') at their 5' end are hybridized to the bead or isolated area.

[0064] One aspect of the invention comprises a method of producing a bead or isolated area with multiple copies of a nucleotide sequence covalently attached thereto by attaching the amplified product as described above, and further comprising extending the (B) sequences to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product and that comprise sequence (A') near their 5' ends.

[0065] One aspect of the invention comprises a sequencing method comprising extending the (B) sequences as described above, further comprising the steps of removing the amplified product to render the covalently attached polynucleotides single-stranded, and extending a primer to sequence (A') to produce detectable oligonucleotide fragments characteristic of the sequence of the polynucleotide bound to the bead or isolated area. In some embodiments of the method, the sequencing method comprises cleavable labeled terminators. In some embodiments of the method, the sequencing method comprises pyrophosphate detection. In some embodiments of the method, the sequencing method is an isothermal sequencing method. In some embodiments of the method the sequencing method comprises cycle sequencing.

[0066] One aspect of the invention comprises a method of performing bridge PCR comprising creating amplified product with defined 3' and 5' ends as described herein, and further comprising the steps of exposing the amplified product to a solid substrate comprising oligonucleotide sequences attached thereto complementary to the A and B' sequences on the amplified product in the presence of components necessary for polymerase chain reaction, and thermal cycling the system to perform bridge PCR amplification.

[0067] One aspect of the invention comprises a method of performing rolling circle amplification comprising creating amplified product with defined 3' and 5' ends as described herein, and further comprising the steps of: (n) hybridizing the amplified product to a nucleic acid sequence comprising regions complementary to A and B' sequences in close proximity; (o) optionally extending the gap with a DNA polymerase enzyme; (p) ligating to form a circular nucleic acid comprising the amplified product, and performing rolling circle amplification by extending a primer that is complementary to a sequence in the circular nucleic acid. In some embodiments of the method, the primer is complementary to sequence (A), sequence (B'), or a sequence that was between sequences (A) and (B') in the amplified product. In some embodiments of the method, the primer is an oligonucleotide attached to a solid surface.

[0068] One aspect of the invention comprises a method of PCR amplification comprising creating amplified product with defined 3' and 5' ends as described herein further comprising the steps of amplifying the amplified product using primers complementary to sequences (A) and (B), or using primers complementary to sequences (A') and (B').

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[0069] One aspect of the invention comprises a method of strand displacement amplification (SDA) creating amplified product with defined 3' and 5' ends as described herein wherein sequences (A) and (B') in the amplified product are designed to be cleaved by a restriction enzyme, and performing strand displacement amplification on the amplified product.

[0070] One aspect of the invention comprises a method comprising: (a) denaturing a double-stranded target DNA; (b) annealing to the target DNA and extending with and enzyme comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the of the primer comprises sequence (A), which is not complementary to the target DNA; to form a plurality of first primer extension products, each with sequence (A) at its 5' end; (c) extending a second primer comprising a ligand and a 3' DNA region that comprises a random sequence, wherein the primer is optionally a tailed primer comprising a nucleic acid sequence (B) that is 5' of the random sequence, to form a plurality of double-stranded products each comprising a first primer extension product and a second primer extension product whereby the second primer extension product comprises a ligand; (d) cleaving the RNA from the first primer extension products such that a portion of the second primer extension products that are complementary to sequence (A) are single stranded; (e) annealing to the second primer extension product an oligonucleotide comprising a 3'-DNA segment that is complementary to sequence (A') and a 5'-RNA segment comprising sequence (C); (f) optionally extending the oligonucleotide to form a plurality of oligonucleotide extension products hybridized to the second primer extension products; (g) extending the second primer extensions product to create a heteroduplex such that the second primer extension products comprise a DNA sequence (C') that is complementary to sequence (C); and (h) cleaving the RNA from the heteroduplex created in step (g).

[0071] In some embodiments of the method the method further comprises: (i) binding the ligand on the second primer extension products to a solid surface. In some embodiments of the method, step (i) of binding the ligand to the solid surface is performed before step (h). In some embodiments of the method, step (i) of binding the ligand to the solid surface is performed after step (h).

[0072] One aspect of the invention comprises a method of amplifying a sequence representative of an sequence within n DNA target molecule carrying out steps (a) through (h) above, and further comprising the steps of: (j) annealing an amplification primer, wherein the amplification primer has a DNA portion and a 5' RNA portion, to the single stranded portion of the second primer extension products complementary to sequence (C'); (k) extending the amplification primer with an enzyme having strand displacement activity to produce a amplified products; (l) cleaving the RNA from the amplified products; and (m) repeating steps (j) to (l) to produce multiple copies of amplified products wherein the 5' portion of the amplified product has a sequence complementary to sequence (A').

[0073] In some embodiments of the method, the second primer comprises the segment (B) that is not complementary to the first primer extension product sequence, whereby the amplified products comprise a sequence (B') at or near their 3' ends that is substantially complementary to sequence (B), and a sequence (A) near their 5' ends that is complementary to sequence (A'). In some embodiments of the method, the amplification is a clonal amplification. In some embodiments of

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the method, the solid surface is a bead or isolated area on a surface. In some embodiments of the method, the bead or isolated area is the only bead or isolated area within isolated liquid volume such that the amplified product is contained within such liquid volume. In some embodiments of the method, the liquid volume is an aqueous droplet within a non-aqueous fluid. In some embodiments of the method, the solid surface is a bead and the droplet is part of a microemulsion. In some embodiments of the method, the liquid volume is a well in a plate. In some embodiments of the method, the solid surface is a substantially planar substrate.

[0074] In some embodiments of the method, the bead or isolated area comprises covalently attached multiple oligonucleotides comprising the sequence (B) at their 3' ends, whereby upon the amplification of step (m) multiple copies of amplified products comprising sequence (B') at their 5' end are hybridized to the bead or isolated area.

[0075] One aspect of the invention comprises a method of producing a bead or isolated area with multiple copies of a nucleotide sequence covalently attached thereto by comprising hybridizing amplified product as described herein further comprising extending the (B) sequences to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product and that comprise sequence (A') near their 5' ends.

[0076] One aspect of the invention comprises a sequencing method comprising hybridizing amplified product to a solid surface as described herein, further comprising the steps of removing the amplified product to render the covalently attached polynucleotides single-stranded, and extending a primer to sequence (A') to produce detectable oligonucleotide fragments characteristic of the sequence of the polynucleotide bound to the bead or isolated area. In some embodiments of the method, the sequencing method comprises cleavable labeled terminators. In some embodiments of the method the sequencing method comprises pyrophosphate detection. In some embodiments of the method, the sequencing method is an isothermal sequencing method. In some embodiments of the method, the sequencing method comprises cycle sequencing.

[0077] One aspect of the invention comprises a method of performing bridge PCR comprising creating amplified product with defined 3' and 5' ends as described herein further comprising the steps of exposing the amplified products to a solid substrate comprising oligonucleotide sequences attached thereto complementary to the A and B' sequences on the amplified products in the presence of components necessary for polymerase chain reaction, and thermal cycling the system to perform bridge PCR amplification.

[0078] One aspect of the invention comprises a method of performing rolling circle amplification comprising creating amplified product with defined 3' and 5' ends as described above further comprising the steps of: (n) hybridizing the amplified products to a target nucleic acid comprising regions complementary to A and B' sequences in close proximity; (o) optionally extending the gap with a polymerase enzyme; (p) ligating to form a circular nucleic acid comprising the amplified product, and performing rolling circle amplification by extending a primer that is complementary to a sequence in the circular nucleic acid. In some embodiments of the method, the primer is complementary to sequence (A), sequence (B'), or a sequence that was between sequences (A) and (B') in the amplified product. In some embodiments of the method, the primer is an oligonucleotide attached to a solid surface.

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[0079] One aspect of the invention comprises a method of PCR amplification comprising creating amplified product with defined 3' and 5' ends as described above further comprising the steps of amplifying the amplified product using primers complementary to sequences (A) and (B), or using primers complementary to sequences (A') and (B').

[0080] One aspect of the invention comprises a method of strand displacement amplification (SDA) comprising creating amplified product with defined 3' and 5' ends as described above wherein sequences (A) and (B') in the amplified product are designed to be cleaved by a restriction enzyme, and performing strand displacement amplification on the amplified product.

[0081] One aspect of the invention provides an alternative method to produce DNA with defined 3' and 5' sequences from RNA.

[0082] One aspect of the invention comprises a method comprising: (a) extending a first primer comprising a 3' portion complementary to a target RNA and a 5' portion, sequence (D), not complementary to the target RNA, to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid; (b) cleaving the target RNA from the RNA/DNA hybrid; (c) extending a second primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to the first primer extension product and a 5' portion, sequence (E), of the of the second primer is not complementary to the first primer extension product, to produce a double-stranded DNA product comprising the first primer extension product hybridized to a second primer extension product, whereby the second primer extension product has a sequence (D') that is complementary to sequence (D) at its 3' end; (d) denaturing the double-stranded DNA product; (e) annealing to the second primer extension product and extending a third primer comprising, from its 5' end, a ligand, optionally a sequence (F), and a sequence (D), wherein sequence (D) is complementary to sequence (D') on the second primer extension product to produce a double-stranded DNA product comprising the second primer extension product hybridized to a third primer extension product, whereby the third primer extension product comprises a sequence (E') at its 3' end complementary to sequence (E).

[0083] In some embodiments of the method the method further comprises binding the ligand to a solid surface, whereby the third primer extension product is bound to the solid surface.

[0084] One aspect of the invention comprises an amplification method comprising carrying out steps (a) through (e) further comprising the steps of: (f) cleaving the RNA portion of the second primer extension product in the DNA-RNA heteroduplex, whereby sequence (E') of the third primer extension product is single stranded. (g) annealing an oligonucleotide comprising a 3' DNA segment (E) that is complementary to sequence (E') and a 5' RNA segment comprising sequence (G); (h) extending the third primer extension product to produce a sequence (G') at its 3' end complementary to sequence (G); cleaving the RNA from the heteroduplex created in step (h) to produce a single-stranded portion of the third primer extension product corresponding to sequence (G'). In some embodiments of the method the method further comprises binding the ligand to a solid surface, whereby the third primer extension product comprising sequence (G') is bound to the solid surface. In some embodiments of the method, the 3' portion of the first primer that is complementary to the target RNA comprises a random nucleotide sequence. In some embodiments of the method, the 3' portion of the first primer that is complementary to the target RNA comprises a sequence that is

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complementary to polyadenosine (poly-A). In some embodiments of the method, the RNA target is contained within a sample that also comprises DNA, and actinomycin is added prior to step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a).

[0085] In some embodiments of the method, in step (b), the target RNA is cleaved by heat, enzyme treatment, or chemical treatment or enzymes.

[0086] In some embodiments of the method, the 3' segment of the second primer complementary to a portion of the first primer extension product comprises a random nucleotide sequence, a specific sequence complementary to a specific sequence of the first primer extension product, or a sequence common to multiple first primer extension products.

[0087] One aspect of the invention comprises a method of amplifying a sequence representative of an sequence within an RNA target molecule carrying out steps (a) through (i) and further comprising the steps of: (j) annealing an amplification primer, wherein the amplification primer has a DNA portion and a 5' RNA portion, to the single stranded portion of the third primer extension product complementary to sequence (G'); (k) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product; (l) cleaving the RNA from the amplified product; and (m) repeating steps (j) to (l) to produce multiple copies of amplified product wherein the 5' portion of the amplified product has a sequence (E) complementary to sequence (E') and the 3' end of the amplified product has sequence (D') complementary to sequence (D) and optionally sequence (F') complementary to sequence (F).

[0088] In some embodiments of the method, the amplification is a clonal amplification. In some embodiments of the method, the solid surface is a bead or an isolated area on a surface. In some embodiments of the method, the bead or isolated area is the only bead or isolated area within isolated liquid volume such that the amplified product is contained within such liquid volume. In some embodiments of the method, the liquid volume is an aqueous droplet within a non-aqueous fluid. In some embodiments of the method, the droplet is part of a microemulsion. In some embodiments of the method, the liquid volume is a well in a plate. In some embodiments of the method, the solid surface is a substantially planar substrate.

[0089] In some embodiments of the method, the bead or isolated area comprises covalently attached thereto multiple oligonucleotides comprising the sequence (D), and/or sequence (F) at their 3' ends, whereby upon the amplification of step (m) multiple copies of amplified product comprising sequence (D') (and/or F') at their 5' end are hybridized to the bead or isolated area.

[0090] One aspect of the invention comprises a method of producing a bead or isolated area with multiple copies of a nucleotide sequence covalently attached thereto by hybridizing amplified product as described above, further comprising extending the oligonucleotide at the (D), and/or (F) sequences to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product comprising sequence (E') near their 5' ends.

[0091] One aspect of the invention comprises a sequencing method comprising producing a bead with multiple copies of a nucleotide sequence covalently attached thereto, further comprising the steps of removing the amplified product to render the covalently attached polynucleotides single-stranded, and extending a primer to sequence (E') to produce detectable oligonucleotide fragments

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characteristic of the sequence of the polynucleotide bound to the bead or isolated area. In some embodiments of the method, the sequencing method comprises cleavable labeled terminators. In some embodiments of the method, the sequencing method comprises pyrophosphate detection. In some embodiments of the method, the sequencing method is an isothermal sequencing method. In some embodiments of the method, the sequencing method comprises cycle sequencing.

[0092] One aspect of the invention comprises alternative methods to produce DNA with defined 3' and 5' sequences from a DNA target

[0093] One aspect of the invention comprises a method comprising: (a) denaturing a double-stranded target DNA; (b) annealing to the target DNA and extending a first primer comprising a 3' portion comprising a random sequence and a 5' portion, sequence (D), which is not complementary to the target DNA, to form a plurality of first primer extension products, each comprising sequence (D) at its 3' end; (c) extending a second primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion comprises a random sequence, and a 5' portion, sequence (E), of the of the second primer is not complementary to the first primer extension products, to produce a plurality of double-stranded DNA products comprising a first primer extension product hybridized to a second primer extension product, whereby the second primer extension products have a sequence (D') that is complementary to sequence (D) at their 3' ends; (d) denaturing the double-stranded DNA products; e) annealing to the second primer extension products and extending a third primer comprising, from its 5' end, a ligand, optionally a sequence (F), and a sequence (D), wherein sequence (D) is complementary to sequence (D') on the second primer extension products to produce double-stranded DNA products comprising second primer extension products hybridized to third primer extension products, whereby the third primer extension products comprise a sequence (E') at its 3' end complementary to sequence (E) in a DNA-RNA heteroduplex.

[0094] In some embodiments of the method the method further comprises binding the ligand to a solid surface, whereby the third primer extension products are bound to the solid surface.

[0095] In some embodiments of the method the method further comprises the steps of: (f) cleaving the RNA portion of the second primer extension products in the DNA-RNA heteroduplex, whereby sequence (E') of the third primer extension products is single stranded. (g) annealing an oligonucleotide comprising a 3' DNA segment (E) that is complementary to sequence (E') and a 5' RNA segment comprising sequence (G); (h) extending the third primer extension products to produce a sequence (G') at their 3' ends complementary to sequence (G); (i) cleaving the RNA from the heteroduplex created in step (h) to produce single-stranded portions of the third primer extension products corresponding to sequence (G').

[0096] In some embodiments of the method the method further comprises binding the ligand to a solid surface, whereby the third primer extension products comprising sequence (G') are bound to the solid surface.

[0097] One aspect of the invention comprises a method of amplifying a sequence representative of a sequence within a DNA target molecule comprising following the steps (a) through (i), further comprising the steps of: (j) annealing an amplification primer to the single stranded portion of the third primer extension products complementary to sequence (G'); wherein the amplification primer has a DNA portion and a 5' RNA portion, (k) extending the amplification primer with an enzyme

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having strand displacement activity to produce a amplified products; (l) cleaving the RNA from the amplified products; and (m) repeating steps (j) to (l) to produce multiple copies of amplified products wherein the 5' portion of the amplified products have a sequence (E) complementary to sequence (E') and the 3' end of the amplified products have sequence (D') complementary to sequence (D) and optionally sequence (F') complementary to sequence (F).

[0098] In some embodiments of the method, the amplification is a clonal amplification. In some embodiments of the method, the solid surface is a plurality of beads or a plurality of isolated areas on a surface.

[0099] In some embodiments of the method, each bead or isolated area in the plurality of beads or isolated areas is the only bead or isolated area within isolated liquid volume such that the amplified product corresponding to the sequence on that bead or isolated area is associated with such liquid volume. In some embodiments of the method, the liquid volume is an aqueous droplet within a non-aqueous fluid. In some embodiments of the method, the droplet is part of a microemulsion. In some embodiments of the method, the liquid volume is a well in a plate. In some embodiments of the method, the solid surface is a substantially planar substrate.

[00100] In some embodiments of the method, the bead or isolated area comprises covalently attached thereto multiple oligonucleotides comprising the sequence (D), and/or sequence (F) at their 3' ends, whereby upon the amplification of step (m) multiple copies of amplified product comprising sequence (D') (and/or sequence (F') at their 5' end are hybridized to the bead or isolated area.

[00101] One aspect of the invention comprises a method of producing a bead or isolated area with multiple copies of a nucleotide sequence covalently attached thereto further comprising extending the (D), and/or (F) sequences to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product comprising sequence (E') near their 5' ends.

[00102] One aspect of the invention comprises a sequencing method comprising creating amplified product with defined 3' and 5' ends as described above, further comprising the steps of removing the amplified product to render the covalently attached polynucleotides single-stranded, and extending a primer to sequence (E') to produce detectable oligonucleotide fragments characteristic of the sequence of the polynucleotide bound to the bead or isolated area. In some embodiments of the method, the sequencing method comprises cleavable labeled terminators. In some embodiments of the method, the sequencing method comprises pyrophosphate detection. In some embodiments of the method, the sequencing method is an isothermal sequencing method. In some embodiments of the method, the sequencing method comprises cycle sequencing.

[00103] In one aspect the invention comprises a library of nucleic acid sequences.

[00104] One aspect of the invention comprises a library comprising a plurality of double-stranded oligonucleotides each of the oligonucleotides comprising: (a) a first strand comprising DNA which has, proceeding from its 5' end (i) a ligand, (ii) a specific sequence (B, D, or DF), (iii) a sequence corresponding to or complementary to a sequence within a nucleic acid target, (iv) a specific sequence (A' or E'); and a specific sequence (C' or G'); and (b) a second strand having from its 5' end, (i) a specific RNA sequence (C or G) complementary to specific sequence C' or G', (ii) a specific sequence (A or E) complementary to sequence (A' or E'), (iii) a sequence complementary to

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or corresponding to a sequence within a target nucleic acid. In some embodiments, the ligands are bound to a solid surface. In some embodiments, the solid surface comprises a plurality of beads. In some embodiments, each of the plurality of beads comprises a single molecule of double-stranded oligonucleotide. In some embodiments, the solid surface comprises a plurality of isolated areas on a surface.

[00105] One aspect of the invention relates to kits comprising reagents that can be used, for example, for carrying out the methods of the invention.

[00106] One aspect of the invention comprises kit comprising: (a) a first primer comprising a 3'-DNA portion and a 5'-RNA portion, wherein the 5' RNA portion further comprises sequence (A); (b) a second primer comprising a 5'-ligand; (c) an RNA dependent DNA polymerase; (d) a DNA dependent DNA polymerase with strand displacement activity; (e) RNase H; and (f) an amplification chimeric primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the sequence of the amplification primer is the substantial the same sequence as the (A) sequence. In some embodiments, the 3'-DNA portion of the first primer comprises a random sequence. In some embodiments, the second primer is a DNA primer that comprises a random sequence at its 3' end. In some embodiments, the 3'-DNA portion of the first primer comprises a random sequence, and the second primer is a DNA primer that comprises a random sequence at its 3' end.

[00107] One aspect of the invention comprises a kit comprising: (a) a first primer comprising a 3'-DNA portion and a 5'-RNA portion, wherein the 5' RNA portion further comprises sequence (A); (b) a second primer comprising a 5'-ligand; (c) an RNA dependent DNA polymerase; (d) a DNA dependent DNA polymerase with strand displacement activity; (e) RNase H; (f) a chimeric oligonucleotide comprising a 3'-DNA portion substantially comprising sequence (A) and a 5'-RNA sequence (C); and (g) a chimeric amplification primer comprising a 3'-DNA portion and a 5'-RNA portion, wherein the chimeric amplification primer comprises a sequence which is substantially the same as sequence (C). In some embodiments, the 3'-DNA portion of the first primer comprises a random sequence. In some embodiments, the second primer is a DNA primer that comprises a random sequence at its 3' end. In some embodiments, the 3'-DNA portion of the first primer comprises a random sequence, and the second primer is a DNA primer that comprises a random sequence at its 3' end. In some embodiments, the second primer further comprises a sequence (B) at or near the 5'-end. In some embodiments, the kit further comprises solid support with immobilized receptor to the ligand on it surface. In some embodiments, the kit further comprises solid surface with an oligonucleotide attached to the surface by the 5'-end and comprising a sequence substantially the same as sequence (B).

[00108] In some embodiments, the kit further comprises solid surface with an oligonucleotide attached to the surface by the 5'-end hybridizable to sequence (A).

[00109] In some embodiments, the kit further comprises an inhibitor of the DNA dependent DNA polymerase. In some embodiments, the inhibitor of the DNA dependent DNA polymerase is Actinomycin.

[00110] One aspect of the invention comprises a kit comprising: (a) a first primer that is a tailed DNA primer comprising a 5'-tail sequence (D); (b) a second primer that is a chimeric primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the 5'-end comprises a tail sequence

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(E); (c) a third primer which is a tailed primer comprising a 3'-sequence that comprises a sequence substantially the same as sequence (D), optionally a 5'-tail sequence (F), and 5'-ligand; (d) an RNA dependent DNA polymerase; e) a DNA dependent DNA polymerase with strand displacement activity; (f) RNase H; and (g) a chimeric amplification primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the chimeric amplification primer comprises a sequence which is substantially the same a sequence (E).

[00111] In some embodiments, the 3'-DNA portion of the first primer comprises a random sequence. In some embodiments, the second primer is a DNA primer that comprises a random sequence at its 3' end. In some embodiments, the 3'-DNA portion of the first primer comprises a random sequence, and the second primer is a DNA primer that comprises a random sequence at its 3' end.

[00112] One aspect of the invention comprises a kit comprising: (a) a first primer that is a tailed DNA primer comprising a 5'-tail sequence (D); (b) a second primer that is a chimeric primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the 5'-end comprises a tail sequence (E); (c) a third primer which is a tailed primer comprising a 3'-sequence that comprises a sequence substantially the same as sequence (D), optionally a 5'-tail sequence (F), and 5'-ligand; (d) an RNA dependent DNA polymerase; e) a DNA dependent DNA polymerase with strand displacement activity; (f) RNase H; (g) a chimeric oligonucleotide comprising a 3'-DNA sequence (E) and a 5'-RNA sequence G; and (h) a chimeric amplification primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the chimeric amplification primer comprises a sequence which is substantially the same as sequence (G). In some embodiments, the 3'-DNA portion of the first primer comprises a random sequence. In some embodiments, the second primer is a DNA primer that comprises a random sequence at its 3' end. In some embodiments, the 3'-DNA portion of the first primer comprises a random sequence, and the second primer is a DNA primer that comprises a random sequence at its 3' end. In some embodiments, the kit further comprises an inhibitor of the DNA dependent DNA polymerase, In some embodiments, the inhibitor of the DNA dependent DNA polymerase is Actinomycin.

[00113] One aspect of the invention comprises a method for attaching a polynucleotide sequence that is representative of a sequence within a nucleic acid target molecule to a solid surface comprising: (a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer, sequence (P), is complementary to a target nucleic acid and a 5' portion of the of the primer, sequence (A), is not complementary to the target nucleic acid, to form a first primer extension product hybridized to the target nucleic acid; (b) separating or removing the first primer extension product from the target nucleic acid; (c) extending a second primer to produce a double-stranded product comprising a second primer extension product hybridized to the first primer extension product, wherein the second primer comprises a 3' segment complementary to a portion of the first primer extension product and 5' segment non-complementary sequence (B) to the first primer extension product, whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer and a portion of the 5' end of the second primer extension product comprises non-complementary sequence (B); (d) adding an exonuclease to the double-stranded DNA/RNA hybrid, whereby single

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stranded 3' nucleotides are removed from the first primer extension product; (e) extending the first primer extension product to produce a sequence (B'), complementary to sequence (B) on the second primer extension product; (f) denaturing the first and second primer extension products; (g) binding the sequence (B') of the first primer extension product to a third primer comprising sequence (B) bound to a solid surface, whereby the first primer extension product is attached to the solid surface; and (h) extending the sequence (B) of the third primer to produce a double-stranded product comprising a third primer extension product hybridized to the first primer extension product, wherein the 5'end of the third primer comprises a sequence (B) complementary to the sequence (B') of the first primer extension product, whereby a portion of the 3' end of the third primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer.

[00114] One aspect of the invention comprises a method comprising: (a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA; to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid; (b) removing the target RNA from the RNA/DNA hybrid; (c) extending a second primer, comprising a 3' segment complementary to a portion of the first primer extension product and a 5' segment non-complementary to the first primer extension product comprising sequence (B), to produce a double-stranded DNA product with a DNA/RNA heteroduplex at one end, wherein the double-stranded product comprises a second primer extension product hybridized to the first primer extension product and wherein a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer; (d) adding an exonuclease to the double-stranded DNA product, whereby single stranded 3' nucleotides are removed from the 3' region of the first primer extension product that is not hybridized to the second primer extension product; (e) extending the first primer extension product to produce a sequence (B'), complementary to sequence (B) on the second primer extension product; (f) denaturing the double-stranded DNA product; (g) attaching the single-stranded first primer extension product to a solid support by annealing sequence (B') to the solid support comprising an oligonucleotide attached thereto, comprising a sequence (B); and (h) extending sequence (B) on the solid support to produce a third primer extension product, hybridized to the first extension product, wherein the third primer extension product comprises a 3' sequence (A'), whereby a DNA/RNA heteroduplex at one end is generated.

[00115] One aspect of the invention comprises a method for amplifying a nucleic acid representative of a target RNA comprising carrying out steps (a) through (h) above and further comprising the steps of: (i) cleaving the RNA region from the first polynucleotide product hybridized to the third primer extension product using RNase H; (j) annealing an amplification primer to sequence (A') on the single-stranded portion of the third primer extension product, wherein the amplification primer has a DNA portion and a 5' RNA portion; (k) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product hybridized to the third primer extension product on the solid support; (l) repeating steps (i) to (k) to produce multiple copies of an amplified product wherein the amplified product comprises

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sequence (B') at its 3' end; and (m) capturing the amplified product on the solid support wherein the solid support comprises sequence (B).

[00116] In some embodiments, the 3' portion of the primer that is complementary to the target RNA comprises a random nucleotide sequence. In some embodiments, the 3' portion of the primer that is complementary to the target RNA comprises a sequence that is complementary to polyadenosine (poly-A). In some embodiments, the 3' portion of the primer that is complementary to the target RNA comprises a specific sequence that is complementary to a multiplicity of targets. In some embodiments, the target RNA is cleaved by heat, enzyme treatment, or chemical treatment in step (b).

[00117] In some embodiments, the RNA target is in a sample that also comprises DNA, and wherein actinomycin is added prior to step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a). . In some embodiments, the 3' portion of the second primer complementary to a portion of the first primer extension product comprises a random nucleotide sequence, a specific sequence complementary to a specific sequence of the first primer extension product, or a sequence common to multiple first primer extension products.

[00118] In some embodiments, the solid support comprises a bead. In other embodiments, the solid support comprises an isolated area. In some embodiments, the solid support comprises a plurality of beads or a plurality of isolated areas on a surface. In some embodiments, the solid support comprises a substantially planar array. In some embodiments, the bead comprises a magnetic bead. In some embodiments, the bead comprises only one copy of the first primer extension product.

[00119] In some embodiments, the first primer extension product is single-stranded. In some embodiments, a plurality of first primer extension products are produced corresponding to different sequences in the target RNA. In some embodiments, the plurality of first primer extension products is bound to a solid support comprising either one or a plurality of beads or a plurality of isolated areas on a surface. In some embodiments, the plurality of first primer extension products is bound to a plurality of beads under conditions such that generally, one or fewer first primer extension products is bound to one bead or one isolated area on a surface. In some embodiments, the plurality of beads or isolated areas are contained within a plurality of isolated volumes such that generally one or fewer beads or isolated area is associated with each isolated volume, and whereby the production of multiple copies of amplification product results in multiple copies of substantially one amplification product in each volume.

[00120] In some embodiments, the method further comprises the step of storing the beads or isolated areas comprising generally one or fewer primer extension products per bead or isolated area. In some embodiments, the method further comprises clonally amplifying the primer extension product bound to the beads or isolated areas after storing them. In other embodiments, the target RNA comprises messenger RNA.

[00121] One aspect of the invention comprises a method comprising: (a) denaturing a double-stranded target DNA; (b) annealing to the target DNA and extending with a DNA polymerase comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the primer comprises sequence (A), which is not complementary to the target DNA; to form a plurality

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of first primer extension product hybridized to the target DNA and comprising sequence (A) at its 5' end; (c) separating the first primer extension product from the target DNA; (d) annealing to the first primer extension product and extending a second primer comprising a 3' complementary DNA region that comprises a random sequence, wherein the second primer is a tailed primer comprising a 5' sequence (B), to form a double-stranded product comprising a first primer extension product and a second primer extension product, whereby a double-stranded product with a DNA/RNA heteroduplex at one end is generated; (e) adding an exonuclease to the double-stranded DNA product, whereby single stranded 3' nucleotides are removed from the 3' region of the first primer extension product that is not hybridized to the second primer extension product; (f) extending the first primer extension product to produce a sequence (B'), complementary to sequence (B) on the second primer extension product; (g) denaturing the double-stranded DNA product; (h) attaching the single-stranded first primer extension product to a solid support by annealing sequence (B') to the solid support comprising an oligonucleotide attached thereto, comprising a sequence (B), whereby a plurality of first primer extension products become bound to the solid surface; and (i) extending sequence (B) on the solid support to produce a third primer extension product, hybridized to the first primer extension product, comprising a 3' sequence (A'), whereby a DNA/RNA heteroduplex at one end is generated.

[00122] One aspect of the invention comprises a method for amplifying a nucleic acid representative of a target RNA comprising carrying out steps (a) through (i) above and further comprising the steps of: (j) cleaving the RNA from the first polynucleotide product hybridized to the amplified product using RNase H; (k) annealing an amplification primer to the single-stranded portion of the amplified product complementary to sequence (A'), wherein the amplification primer has a DNA portion and a 5' RNA portion; (l) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product hybridized to the third primer extension product on the bead or isolated area; (m) repeating steps (j) to (l) to produce multiple copies of an amplified product wherein the amplified product comprises sequence (B') at its 3' end; and (n) capturing the amplified product on the solid support comprising sequence (B).

[00123] In some embodiments, step (b) above comprises a first incubation at a temperature below about 30 °C, and a second incubation at a temperature above about 40 °C. In some embodiments, a DNA polymerase which is active at temperatures above about 45 °C is used to extend the first primer.

[00124] In some embodiments, the solid support comprises a bead. In some embodiments, the solid support comprises an isolated area. In some embodiments, the solid support comprises a plurality of beads or a plurality of isolated areas on a surface. In some embodiments, the solid support comprises a substantially planar array. In some embodiments, the bead comprises a magnetic bead.

[00125] In some embodiments, the plurality of first primer extension products is bound to a plurality of beads or isolated areas under conditions such that generally, one or fewer copies of a single first primer extension product is bound to one bead or one isolated area. In some embodiments, the plurality of beads or isolated areas are contained within a plurality of isolated volumes such that generally one or fewer beads or isolated area is associated with each isolated volume, and whereby

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the production of multiple copies of amplification product results in multiple copies of substantially one amplification product in each volume.

[00126] In some embodiments, the method further comprises the step of storing the beads or isolated areas comprising generally one or fewer primer extension products. In some embodiments, the method further comprises clonally amplifying the primer extension product bound to the beads or isolated areas after storing them. In some embodiments, the target DNA is genomic DNA. In some embodiments, the target DNA comprises multiple genomes.

[00127] One aspect of the invention for preparing DNA with defined 3' and 5' sequences comprises a method comprising: (a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA; to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid; (b) removing the target RNA from the RNA/DNA hybrid; (c) extending a second primer, comprising a 3' segment complementary to a portion of the first primer extension product and a 5' segment non-complementary to the first primer extension product comprising sequence (B), to produce a double-stranded product with a DNA/RNA heteroduplex at one end; wherein the double-stranded product comprises a second primer extension product hybridized to the first primer extension product, and whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer; (d) cleaving the RNA in the heteroduplex from the first primer extension product such that a portion of the second primer extension product that is complementary to sequence (A) is single-stranded; (e) annealing to the second primer extension product an oligonucleotide comprising a 3'- DNA sequence (A) that is complementary to sequence (A') and a 5'-RNA segment comprising sequence (C) that is non-complementary to the second primer extension product; (f) extending the oligonucleotide at the 3' segment to form an oligonucleotide extension product hybridized to the second primer extension product; (g) denaturing the double-stranded DNA product; (h) attaching the single-stranded first primer extension product to a solid support by annealing sequence (B') to the solid support comprising a sequence (B); and (i) extending sequence (B) on the solid support to produce a third primer extension product, comprising a 3' sequence (A') and (C'), whereby a DNA/RNA heteroduplex at one end is generated.

[00128] One aspect of the invention comprises a method for amplifying a nucleic acid representative of a target RNA comprising carrying out steps (a) through (i) above and further comprising the steps of: (j) cleaving the RNA from the heteroduplex polynucleotide product hybridized to the amplified product using RNase H to produce a single-stranded portion of the third primer extension product corresponding to sequence (C'); (k) annealing an amplification primer to the single-stranded portion of the third primer extension product complementary to sequence (C'), wherein the amplification primer has a DNA portion and a 5' RNA portion; (l) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product hybridized to the third primer extension product on the solid support; (m) repeating steps (j) to (l) to produce multiple copies of the amplified product comprising sequences (A) and (B'); and

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(n) capturing the amplified product on the solid support wherein the solid support comprises sequence (B).

[00129] In some embodiments, the method further comprises extending sequence (B), whereby multiple copies are bound to solid support through sequence (B) having sequence (A') at 3' end. In some embodiments, the method further comprises sequencing by synthesis using a sequence (A) complementary to (A') as the priming sequence. In some embodiments, the 3' portion of the first primer that is complementary to the target RNA comprises a random nucleotide sequence. In some embodiments, the 3' portion of the first primer that is complementary to the target RNA comprises a sequence that is complementary to polyadenosine (poly-A). In some embodiments, the 3' portion of the primer that is complementary to the target RNA comprises a specific sequence that is complementary to a multiplicity of targets. In some embodiments, the RNA target is contained within a sample that also comprises DNA, and actinomycin is added prior to step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a). In some embodiments, the target RNA is cleaved by chemical heat, or enzyme treatment in step (b)

[00130] In some embodiments, the 3' segment of the second primer complementary to a portion of the first primer extension product comprises a random nucleotide sequence, a specific sequence complementary to a specific sequence of the first primer extension product, or a sequence common to multiple first primer extension products. In some embodiments, the amplification is a clonal amplification.

[00131] In some embodiments, the solid support is a bead. In some embodiments, the solid support is an isolated area on a surface. In some embodiments, the bead or isolated area is the only bead or isolated area associated with an isolated liquid volume such that the amplified product is contained within such liquid volume. In some embodiments, the liquid volume is an aqueous droplet within a non-aqueous fluid. In some embodiments, the solid surface is a bead and the droplet is part of a microemulsion. In some embodiments, the liquid volume is a well in a plate. In some embodiments, the solid support is a substantially planar substrate. In some embodiments, a method of producing a solid support with multiple copies of a nucleotide sequence covalently attached thereto by performing the method described above, and further comprising extending the (B) sequences to produce multiple polynucleotides covalently attached to the solid support that are substantially complementary to the amplified product and that comprise sequence (A') near their 3' ends.

[00132] In some embodiments, a sequencing method comprises performing the method described above and further comprises the steps of removing the third primer extension product to render the covalently attached polynucleotides single-stranded, and extending a primer to sequence (A) to produce detectable oligonucleotide fragments characteristic of the sequence of the polynucleotide bound to the bead or isolated area. In some embodiments the sequencing method comprises cleavable labeled terminators. In some embodiments the sequencing method comprises pyrophosphate detection. In some embodiments the sequencing method is an isothermal sequencing method. In some embodiments the sequencing method comprises cycle sequencing.

[00133] In some embodiments a method of performing bridge PCR comprising performing the method described above and further comprising the steps of exposing the amplified product to a solid substrate comprising oligonucleotide sequences attached thereto complementary to the A and

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B' sequences on the amplified product in the presence of components necessary for polymerase chain reaction, and thermal cycling the system to perform bridge PCR amplification.

[00134] In some embodiments a method of performing rolling circle amplification comprising performing the method described previously and further comprising the steps of: (o) hybridizing the amplified product to a nucleic acid sequence comprising regions complementary to A and B' sequences in close proximity; (p) optionally extending the gap with a DNA polymerase enzyme; (q) ligating to form a circular nucleic acid comprising the amplified product, and performing rolling circle amplification by extending a primer that is complementary to a sequence in the circular nucleic acid.

[00135] In some embodiments the primer is complementary to sequence (A), sequence (B'), or a sequence that was between sequences (A) and (B') in the amplified product. In some embodiments the primer is an oligonucleotide attached to a solid surface.

[00136] In some embodiments, a method of PCR amplification comprising performing the method previously described and further comprising the steps of amplifying the amplified product using primers complementary to sequences (A) and (B), or using primers complementary to sequences (A') and (B'). In some embodiments, a method of strand displacement amplification (SDA) comprising performing the method previously described wherein sequences (A) and (B') in the amplified product are designed to be cleaved by a restriction enzyme, and performing strand displacement amplification on the amplified product.

[00137] One aspect of the invention for preparing DNA with defined 3' and 5' sequences comprises a method comprising: (a) denaturing a double-stranded target DNA; (b) annealing to the target DNA and extending with a DNA polymerase comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the of the primer comprises sequence (A), which is not complementary to the target DNA; to form a first primer extension product hybridized to the target DNA and comprising sequence (A) at its 5' end; (c) separating the first primer extension product from the target DNA; (d) annealing to the first primer extension product and extending a second primer comprising a 3' complementary DNA region that comprises a random sequence, wherein the second primer is a tailed primer comprising a 5' sequence (B), to form a double-stranded product comprising a first primer extension product and a second primer extension product, whereby a double-stranded product with a DNA/RNA heteroduplex at one end is generated; (e) cleaving the RNA in the heteroduplex from the first primer extension product such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded; (f) annealing to the second primer extension product an oligonucleotide comprising a 3'- DNA segment that is complementary to sequence (A') and a 5' RNA segment comprising sequence (C); (g) extending the oligonucleotide along the second primer extension product to form an oligonucleotide extension product comprising a sequence (B'), complementary to sequence (B) on the second primer extension product; (h) denaturing the double-stranded DNA product; (i) attaching the single-stranded first primer extension product to solid support by annealing sequence (B') to the bead or isolated area comprising a sequence (B); and (j) extending sequence (B) on the solid support to produce a third

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primer extension product, hybridized to the oligonucleotide extension product, comprising a 3' sequence (A') and (C'), whereby a DNA/RNA heteroduplex at one end is generated.

[00138] One aspect of the invention comprises a method for amplifying a nucleic acid representative of a target DNA comprising carrying out steps (a) through (j) above and further comprising the steps of: (k) cleaving the RNA from the heteroduplex polynucleotide product hybridized to the amplified product using RNase H to produce a single-stranded portion of the second primer extension product corresponding to sequence (C'); (l) annealing an amplification primer to the single-stranded portion of the amplified product complementary to sequence (C'), wherein the amplification primer has a DNA portion and a 5' RNA portion; (m) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product hybridized to the amplified product on the bead or isolated area; and (n) repeating steps (k) to (m) to produce multiple copies of the second polynucleotide product comprising sequences (A) and (B').

[00139] In some embodiments, the amplification is a clonal amplification. In some embodiments, the solid support is a bead or isolated area on a surface. In some embodiments, the bead or isolated area is the only bead or isolated area within isolated liquid volume such that the amplified product is contained within such liquid volume. In some embodiments, the liquid volume is an aqueous droplet within a non-aqueous fluid. In some embodiments, the solid surface is a bead and the droplet is part of a microemulsion. In some embodiments, the liquid volume is a well in a plate. In some embodiments, the solid support is a substantially planar substrate. In some embodiments, the bead or isolated area comprises covalently attached multiple oligonucleotides comprising the sequence (B) at their 3' ends, whereby upon the amplification of step (m), multiple copies of amplified products comprising sequence (B') at their 5' end are hybridized to the bead or isolated area.

[00140] In some embodiments, a method of producing a bead or isolated area with multiple copies of a nucleotide sequence covalently attached thereto by performing the method previously described and further comprising extending the (B) sequences to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product and that comprise sequence (A') near their 5' ends.

[00141] In some embodiments, a sequencing method comprising performing the method previously described, further comprising the steps of removing the amplified product to render the covalently attached polynucleotides single-stranded, and extending a primer to sequence (A') to produce detectable oligonucleotide fragments characteristic of the sequence of the polynucleotide bound to the bead or isolated area. In some embodiments, the sequencing method comprises cleavable labeled terminators. In some embodiments, the sequencing method comprises pyrophosphate detection. In some embodiments, the sequencing method is an isothermal sequencing method. In some embodiments, the sequencing method comprises cycle sequencing.

[00142] In some embodiments, a method of performing bridge PCR comprising performing the method previously described and further comprising the steps of exposing the amplified products to a solid substrate comprising oligonucleotide sequences attached thereto complementary to the A and B' sequences on the amplified products in the presence of components necessary for polymerase chain reaction, and thermal cycling the system to perform bridge PCR amplification.

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[00143] In some embodiments, a method of performing rolling circle amplification comprising performing the method previously described and further comprising the steps of: (o) hybridizing the amplified products to a target nucleic acid comprising regions complementary to A and B' sequences in close proximity; (p) optionally extending the gap with a polymerase enzyme; (q) ligating to form a circular nucleic acid comprising the amplified product, and performing rolling circle amplification by extending a primer that is complementary to a sequence in the circular nucleic acid.

[00144] In some embodiments, the primer is complementary to sequence (A), sequence (B'), or a sequence that was between sequences (A) and (B') in the amplified product. In some embodiments, the primer is an oligonucleotide attached to a solid surface.

[00145] In some embodiments, a method of PCR amplification comprising performing the method previously described and further comprising the steps of amplifying the amplified product using primers complementary to sequences (A) and (B), or using primers complementary to sequences (A') and (B').

[00146] In some embodiments, a method of strand displacement amplification (SDA) comprising performing the method previously described wherein sequences (A) and (B') in the amplified product are designed to be cleaved by a restriction enzyme, and performing strand displacement amplification on the amplified product.

INCORPORATION BY REFERENCE

[00147] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[00148] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[00149] Figure 1 illustrates a method of producing a polynucleotide bound to a solid support wherein the polynucleotide comprises sequences related to a target nucleic acid and comprises a defined sequence (A') at its 3' end.

[00150] Figure 2 illustrates a method of producing a polynucleotide comprising a ligand, sequences related to a target RNA, and a defined sequence (A') at its 3' end. The Figure also illustrates binding such polynucleotide to a solid surface.

[00151] Figure 3 illustrates a method of producing a polynucleotide comprising a ligand, sequences related to a target DNA and a defined sequence (A') at its 3' end. The Figure also illustrates binding such polynucleotide to a solid surface.

[00152] Figure 4 shows a method of producing amplified product from a nucleotide, bound to the bead through a ligand, wherein the amplified product comprises sequences related to the target nucleic acid and a defined sequence (B') at its 3' end.

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[00153] Figure 5A shows a method of producing a second primer extension product comprising, from its 5' end, a ligand, a defined sequence (B), a sequence representative of a target polynucleotide, a sequence (A') and a sequence (C'). The Figure also illustrates binding the second primer extension product to a solid surface.

[00154] Figure 5B illustrates an isothermal amplification using a composite primer utilizing the second primer extension product produced as illustrated in Figure 5A bound to the bead.

[00155] Figure 6 illustrates the capture by the solid surface comprising oligonucleotides of sequence (B) of amplification product having defined 3' and 5' sequences by hybridization to (B'), the 3' defined sequence of the amplified product. The Figure also shows the extension of the sequence (B) bound to the solid surface to produce a polynucleotide bound to the solid surface. The Figure also illustrates the use of sequencing primers (A) to sequence the bound polynucleotide.

[00156] Figure 7 shows the capture by the solid surface comprising oligonucleotides of sequence (A') of amplification product having defined 3' and 5' sequences by hybridization to (A), the 5' defined sequence of the amplified product.

[00157] Figure 8 shows how the of amplification product having defined 3' and 5' sequences can be used for analysis of sequence D by hybridization, gap ligation, and rolling circle amplification.

[00158] Figure 9 illustrates an alternative method of producing a polynucleotide comprising a ligand that can be bound to a solid support that comprises sequences related to a target nucleic acid and comprises a defined sequence (E') at its 3' end.

[00159] Figure 10 shows how the polynucleotide produced as shown in Figure 9 can be used to produce amplified product.

[00160] Figure 11 shows how the amplification illustrated in Figure 10 can be carried out while the polynucleotide is bound to a solid surface.

[00161] Figure 12 shows how a chimeric RNA/DNA oligonucleotide (G/E) can be used with the product of the method illustrated in Figure 9 to produce amplified product with defined sequences at its 3' and 5' ends.

[00162] Figure 13 illustrates an alternative method of producing a polynucleotide bound to a solid support wherein the polynucleotide comprises sequences related to a target nucleic acid and comprises a defined sequence (A') at its 3' end.

[00163] Figure 14 illustrates an alternative method of producing a polynucleotide bound to a solid support wherein the polynucleotide comprises sequence B, sequences related to a target RNA, and a defined sequence (A') at its 3' end.

[00164] Figure 15 shows a method of producing amplified product from a polynucleotide, bound to the bead through sequence B, wherein the amplified product comprises sequences complementary to the target nucleic acid and a defined sequence (B') at its 3' end.

[00165] Figure 16 shows an alternative method of producing a polynucleotide bound to a solid support wherein the polynucleotide comprises sequence B, sequences related to a target DNA and a defined sequence (A') at its 3' end. The figure also illustrates production of amplified product from the bound polynulcleotide, wherein the amplified product comprises defined sequence B' at its 3' end.

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[00166] Figure 17 shows an alternative method of producing a polynucleotide having defined 3' and 5' sequences from a RNA target and a chimeric oligonucleotide primer. A chimeric oligonucleotide extension product is bound to a solid support through sequence B' and is used to generate a bound polynucleotide which comprises from its 5' end, a defined sequence (B), a sequence representative of a target polynucleotide, a sequence (A') and a sequence (C').

[00167] Figure 18 illustrates an isothermal amplification using a composite primer and the bound polynucleotide product produced in Figure 17.

[00168] Figure 19 shows an alternative method of producing a polynucleotide having defined 3' and 5' sequences from a DNA target and a chimeric oligonucleotide primer. A chimeric oligonucleotide extension product is bound to a solid support through sequence B' and is used to generate a bound polynucleotide which comprises from its 5' end, a defined sequence (B), a sequence representative of a target polynucleotide, a sequence (A') and a sequence (C').

DETAILED DESCRIPTION OF THE INVENTION

[00169] The invention provides methods, compositions, and kits useful in the analysis of nucleic acids. Some aspects of the invention relate to the preparation of polynucleotides comprising ligands and binding such ligands to solid surfaces for analysis and for archiving. The polynucleotides bound to the surface are generally produced in a manner that allows for them to be readily manipulated, for example, by incorporating defined 3' and/or 5' regions into the polynucleotides. The bound polynucleotides can then treated by the methods described herein to produce amplified product. The methods of the invention provide for producing amplified product with defined 3' and/or 5' ends. The amplified products having defined 3' and 5' ends can be used for further manipulation and analysis. They can be used, for example in molecular inversion probe (MIP) analysis.

[00170] The polynucleotides and amplified product of the present invention generally comprise sequences that are related to (i.e. either equivalent to or complementary to) the target RNA or target DNA from which they are derived. In some embodiments, the methods of the invention can be used for the global isolation and amplification of target RNA or DNA, e.g. messenger RNA (mRNA) or genomic DNA. Thus, the methods can be used to produce a plurality of polynucleotides and/or amplification products having sequence related to the target RNA or DNA, and also having defined, e.g. universal, sequences at their 3' and/or 5' ends. This plurality of polynucleotides and or amplification products can be representative of the target nucleic acid or subset of the target nucleic acid such as to comprise a library of representative sequences.

[00171] One aspect of the invention relates to the clonal amplification of nucleic acid sequences of interest. The polynucleotides of the present invention attached to solid substrates can be attached in a manner in which polynucleotides of different specific sequences representative of the target nucleic acid are isolated from one another, for example, each attached to a different bead, or each attached to an isolated area on a surface. In one embodiment, a plurality of polynucleotides, each comprising a specific sequence is bound to a plurality of beads or isolated areas such that only one copy of each polynucleotide is bound to each bead or each isolated surface area. These isolated polynucleotides can then be clonally amplified such that, for example each bead or surface area is retained within an isolated volume. This can be accomplished with beads, for example, by using a

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microemulsion in which, on average, each droplet comprises one or fewer beads per droplet. The methods of the present invention allow for the archiving of the polynucleotides bound to the beads and the subsequent analysis of the polynucleotides, for example, using clonal amplification.

[00172] In one aspect of the invention, the polynucleotide bound to the surface is produced in a manner that is amenable to the sequencing of the polynucleotide, thus revealing information about the target nucleic acid from which it is derived.

Method for generating polynucleotide bound to a solid surface

[00173] One aspect of the invention is a method for attaching a polynucleotide sequence that is representative of a sequence within a nucleic acid target molecule to a solid surface. The polynucleotide sequence that is produced is representative of the sequence within a nucleic acid target molecule if it is either the same as, or complementary to the sequence within the target nucleic acid. Where the target nucleic acid is double stranded, the method can produce sequences that are representative of both of the strands. The polynucleotide can be, for example either DNA or RNA.

[00174] The first step of the method comprises: (a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer, sequence (P), is complementary to a target nucleic acid, and a 5' portion of the of the primer, sequence (A), is not complementary to the target nucleic acid, to form a first primer extension product hybridized to the target nucleic acid. The first primer may be extended by a DNA polymerase such as an RNA-dependent DNA polymerase for extending the first primer along an RNA target nucleic acid, or a DNA-dependent DNA polymerase for extending the first primer along a DNA target nucleic acid. In some embodiments, the 3' portion of the primer that is complementary to the target nucleic acid is a specific sequence. For example, where a specific region of interest of a target nucleic acid that is known or suspected to be upstream of a specific sequence on the target nucleic acid, sequence (P) of the composite primer can be designed to hybridize to this specific sequence on the target nucleic acid such that extension of the primer results in producing a first primer extension product that is complementary to such upstream region. The specific sequence may be common to a family of target RNA. A combination of primers with various specific sequences at the 3' end can also be useful. The specific sequence may be common to a family of target RNA. A combination of primers with various specific sequences at the 3' end can also be useful. In some embodiments, such as where the target nucleic acid comprises mRNA, and the mRNA comprises a plurality of sequences, each having a 3' poly-A segment; the specific sequence (P) can comprise a sequence that can hybridize to the poly-A region of the mRNA, thus allowing the extension of the first primer to produce a plurality of first primer extension products, each of which is complementary to the region of an mRNA molecule adjacent to the poly-A region. In some embodiments, the sequence (P) comprises a random sequence, such that the extension of the first primer results in a plurality of first primer extension products complementary to the sequences adjacent to the sequence where each random species hybridizes. The use of a random sequence such as sequence (P) at the 3' end of the primer can be useful for performing a global amplification of a nucleic acid target, generating a plurality of sequences which together can represent, for example substantially the whole sequence of the target nucleic acid. In some embodiments, the relative amounts of the various sequences can be

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used to quantitate the relative amount of a given sequence in a sample, for example to determine the level of expression in an mRNA sample, or to determine gene copy number in a DNA sample.

[00175] The first primer extension product comprises a 5' portion comprising sequence (A). Sequence A comprises RNA. In some embodiments, sequence (A) is RNA, and sequence (P) is DNA. In other embodiments, sequence (A) may comprise some (DNA). In some embodiments, sequence (P) may comprise some RNA. In some embodiments, sequence (A) and sequence (P) are adjacent.

[00176] The method further comprises step (b) separating or removing the first primer extension product from the target nucleic acid. The first primer extension product can be separated from the target nucleic acid by a variety of methods. In some cases the separation can be affected by denaturing the complex comprising the first primer extension product and the nucleic acid. Denaturation can be performed, for example by heating the sample, or by adding a denaturing agent, or using a combination of heating the sample and adding denaturing agents. Other methods of separating the first primer extension product from the target nucleic acid involve selectively cleaving or degrading the target nucleic acid. Where the target nucleic acid is RNA, the cleaving or degrading can be accomplished with an enzyme that cleaves RNA from an RNA/DNA hybrid such as RNase H, or chemically, for example with the addition of alkali. In some embodiments, the target nucleic acid is completely cleaved or degraded. In other embodiments, the target nucleic acid is only partly cleaved or degraded. The amount of cleavage or degradation required is that amount which allows the extension of the second primer. In some embodiments, the cleavage or degradation is carried out partially, and the fragments of the target nucleic acid that remain can constitute the second primer for step (c).

[00177] The method further comprises step (c) extending a second primer to produce a double stranded product comprising a second primer extension product hybridized to the first primer extension product, wherein the second primer comprises a 3' segment complementary to a portion of the first primer extension product and a ligand, whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer. The extension of the second primer is carried out with a DNA polymerase as described herein. The second primer can comprise RNA, DNA, or can be a composite primer comprising both RNA and DNA. The second primer can be a tailed primer having a 3' portion which is complementary to the first primer extension product, and a 5' portion, sequence (B), which is not complementary to the first primer extension product. In some embodiments, the second primer can comprise a specific primer sequence that is designed to hybridize to a specific sequence in the first primer extension product. In some embodiments the second primer comprises a random primer sequence that randomly binds to the first primer extension product. Extension of the second primer comprising a random sequence produces a plurality of second primer extension products. The use of a random sequence at the 3' end of the primer is useful, for example, in performing global amplification of a target RNA or target DNA, whereby a plurality of second primer extension products are produced which is representative of the sequence of the target nucleic acid. In some embodiments, for example where the first primer is designed to hybridize to a specific sequence on a target RNA, or a sequence common to a family of RNA targets, random priming by the second

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primer may ensure amplification of the entire selected target or family of selected targets. In some cases, random primer by the second primer in combination with extension by a polymerase having substantial strand-displacement activity may ensure amplification of the entire selected target, an entire transcriptome, an entire genome, an entire family of selected targets, or a substantial portion thereof. In this embodiment, the second primer extension products comprise sequences which are the same or substantially the same as the sequences in the target nucleic acid (sense copies), for example, the second primer extension products or the amplicons therefrom may comprise sequences that are 95%, 99%, 99.9%, 99.999%, 99.9999%, 99.999999% or more identical to the sequences in the target nucleic acid or their complement. The second primer comprises a ligand that is a member of a ligand-receptor pair. In some embodiments, the ligand is attached to the primer at the 5' end of the primer. In some embodiments, the ligand is a small molecule, such as biotin or digoxigenin. In some embodiments, the receptor is an antibody, and the ligand is a molecule or portion of a molecule recognized by the antibody.

[00178] The second primer extension product is extended such that the 3' portion of the second primer extension product comprises a sequence (A') which is complementary to sequence (A) of the first primer. Since sequence (A) on the first primer extension product comprises RNA, both DNA dependent DNA polymerase activity and RNA dependent DNA polymerase activity are used in step (c). The primer extension results in a product that is at least partially double stranded.

[00179] The method further comprises step (d) binding the ligand to a receptor bound to a solid surface whereby the second primer extension product is attached to the solid surface. The solid surface may comprise a bead or a set of beads, a magnetic bead or a set of beads, a substantially planar array, a well in a plate, a series of wells in a plate, an isolated surface, or a set of isolated surfaces. The ligand-receptor pair may comprise any pair of binding agents that are capable of specifically associating. For example, the ligand receptor pair may be avidin and biotin or biotin and avidin respectively. Similarly, the ligand-receptor pair may be any pair of proteins, peptides or small molecules that bind to each other. Additionally, the ligand-receptor pair may comprise nucleic acid sequences such a RNA, DNA, peptide nucleic acids or their analogs that bind or hybridize specifically. Other examples of receptors include aptamers, antibodies, affibodies, enzymes, or any protein, peptide, macromolecule, or small molecule specifically capable of binding to a ligand. Other examples of ligands include any protein, peptide, macromolecule, or small molecule specifically capable of binding to a receptor.

[00180] The receptor is a member of the ligand-receptor pair such that binding of the ligand results in attaching the second primer extension product to the solid surface. In some embodiments, the second primer extension product is still hybridized to the first primer extension product when it is attached to the solid surface. In some embodiments, the first primer extension product is removed from the second primer extension product such that a single stranded polynucleotide is attached to the solid surface. The method produces a nucleic acid that is bound to a solid surface that has a specific sequence (A') at its 3' end. The specific, or universal, sequence (A') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. As described above, in some embodiments, the nucleic acid bound to the bead also comprises sequence (B) at or near its 5' end.

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[00181] One aspect of the invention comprises amplification of the nucleic acid bound to the bead. In some embodiments, the amplification is carried out using isothermal amplification using a composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (A') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification. In some embodiments, for example where random sequences at the 3' end of the first and/or second primer are used, a plurality of different nucleic acids bound to a solid surface is created in which each of the nucleic acids has a specific sequence (A') at its 3' end (and in some embodiments also a specific sequence (B) at its 5' end), and where the different nucleic acids have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target nucleic acid. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target nucleic acid.

[00182] Step (d) of binding the polynucleotides to the solid surface through the ligand can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

[00183] A schematic exemplary of an embodiment of the invention relating to method for generating polynucleotide bound to a solid surface is shown in Figure 1. Step I shows the extension of a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer, sequence (P), is complementary to a target nucleic acid and a 5' portion of the of the primer, sequence (A), is not complementary to the target nucleic acid, to form a first primer extension product hybridized to the target nucleic acid. The target nucleic acid can be, for example, DNA or RNA. The target nucleic acid as shown is single stranded. The target nucleic acid may be single stranded in the sample, or can be double stranded, and rendered single stranded, for example, but denaturation with heat. The sequence (P) can represent a specific sequence, a sequence that will hybridize to Poly-A, a sequence common to a plurality of regions (consensus sequence), or a random sequence. Step II illustrates the separating or removing of the first primer extension product from the target nucleic acid. The separation or removing can be accomplished for instance by denaturation of the complex, or by selective degradation of the target nucleic acid. For example, where the target nucleic acid is RNA, the separation/removing/degradation can be accomplished by alkali, or by enzymatic cleavage, for example by RNase H. Step III illustrates extending a second primer to produce a double stranded product comprising a second primer extension product hybridized to the first primer extension product, wherein the second primer comprises a 3' segment complementary to a portion of the first primer extension product and a ligand, whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer. In the embodiment shown the second primer comprises a sequence (B) that is 5' to the segment that is complementary to the first primer extension product, and the ligand is attached 5' to the sequence (B). This step requires both DNA dependent DNA polymerase activity and RNA dependent DNA polymerase activity to product the

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second primer extension product. Step V illustrates binding of the ligand to a receptor bound to a solid surface whereby the second primer extension product is attached to the solid surface. In the embodiment shown, the second primer extension product is bound to the solid substrate in double stranded form while still hybridized to the first primer extension product. In some embodiments, the second primer extension product is rendered single stranded before binding of the ligand to the solid surface. The bound second primer extension product comprises defined sequence (B) at its 5' end and defined sequence (A') at its 3' end. The bound second primer extension product can be stored or archived, and later used for analysis and amplification, for example clonal amplification as described herein.

Method for generating a polynucleotide comprising a ligand for binding to a solid surface from an RNA target

[00184] The invention provides methods, compositions and kits for copying, storing, and amplifying polynucleotides having sequences related to target ribonucleic acid (RNA) sequences. The methods provide for amplification of a single RNA species or pool of RNA species. The methods are suitable for, for example, generation of libraries, including cDNA libraries. The methods can generate single stranded RNA or DNA products, which are readily suitable for multiplex analysis by microarray technologies, as well as electrophoresis-based technologies such as differential display, and for sequencing.

[00185] The methods of the invention can copy, store, and amplify 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 whole transcriptome or total RNA) sequences in a biological sample. Thus, one of the major advantages of the methods of the invention is the ability to copy, store, and 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 sample of interest. The methods of the invention have the potential of amplifying a multiplicity, a large multiplicity, and in some embodiments all RNA (such as whole transcriptome or total RNA in a sample) sequences in a sample.

[00186] 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. The methods are also useful for whole transcriptome amplification. The methods of the invention can be used for the total RNA in samples such as viral RNA.

[00187] The method for generating a polynucleotide comprising a ligand for binding to a solid surface from an RNA target comprise the step of (a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA; to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid. In some embodiments, the 3' portion of the primer that is complementary to the target RNA is a

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specific sequence. For example, where a specific region of interest of a target RNA that is known or suspected to be upstream of a specific sequence on the target RNA, the sequence that is complementary to the target RNA of the first primer can be designed to hybridize to this specific sequence on the target RNA such that extension of the primer results in producing a first primer extension product that is complementary to such upstream region. The specific sequence may be common to a family of target RNA. A combination of primers with various specific sequences at the 3' end can also be useful. The specific sequence may be common to a family of target RNA. A combination of primers with various specific sequences at the 3' end can also be useful. In some embodiments, such as where the target RNA comprises mRNA, and the mRNA comprises a plurality of sequences, each having a 3' poly-A segment; the specific sequence that is complementary to the target RNA can comprise a sequence that will hybridize to the poly-A region of the mRNA, thus allowing the extension of the first primer to produce a plurality of first primer extension products, each of which is complementary to the region of an mRNA molecule adjacent to the poly-A region. In some embodiments, the sequence that is complementary to the target RNA comprises a random sequence, such that the extension of the first primer results in a plurality of first primer extension products complementary to the sequences adjacent to the sequence where each random species hybridizes. The use of a random sequence at the 3' end of the primer can be useful for performing a global amplification of a target RNA, generating a plurality of sequences which together can represent, for example substantially the whole sequence of the target RNA. In some embodiments, the relative amounts of the various sequences can be used to quantitate the relative amount of a given sequence in a sample, for example to determine the level of expression in an mRNA sample. In some embodiments more than one type of sequence that is complementary to the target RNA can be used, for instance both a primer with a random sequence and a primer, or combination of primers with a specific sequence complementary to RNA can be used. In some embodiments, multiple primers comprising different specific sequences can be used.

[00188] The method further comprises the step of: (b) cleaving the target RNA from the RNA/DNA hybrid. In some embodiments, the cleaving of the target RNA from the RNA/DNA hybrid involves selectively cleaving or degrading the target RNA. In some cases the cleaving can be affected by denaturing the complex comprising the first primer extension product and the nucleic acid. Denaturation can be performed, for example by heating the sample, or by adding a denaturing agent, or using a combination of heating the sample and adding denaturing agents. The cleaving can be accomplished with an enzyme that cleaves RNA from an RNA/DNA hybrid such as RNase H, or a combination of RNase enzymes, or chemically. In some embodiments, the target RNA is completely cleaved. In other embodiments, the target RNA is only partly cleaved or degraded. The amount of cleaving required is that amount which will allow the extension of the second primer. In some embodiments, the cleaving is carried out partially, and the fragments of the target RNA that remain can constitute the second primer for step (c).

[00189] The method further comprises: step (c) extending a second primer, comprising a ligand and a 3' segment complementary to a portion of the first primer extension product, to produce a double stranded product with a DNA/RNA heteroduplex at one end; wherein the double stranded product comprises a second primer extension product hybridized to the first primer extension product, and

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whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer. The extension of the second primer is carried out with a DNA polymerase as described herein. The second primer can comprise RNA, DNA, or can be a composite primer comprising both RNA and DNA. The second primer can be a tailed primer having a 3' portion which is complementary to the first primer extension product, and a 5' portion, sequence (B), which is not complementary to the first primer extension product. In some embodiments, the second primer can comprise a specific primer sequence that is designed to hybridize to a specific sequence in the first primer extension product. In some embodiments the second primer comprises a random primer sequence that randomly binds to the first primer extension product. Extension of the second primer comprising a random sequence produces a plurality of second primer extension products. The use of a random sequence at the 3' end of the primer is useful, for example, in performing global amplification of a target RNA, whereby a plurality of second primer extension products are produced which is representative of the sequence of the target RNA. In some embodiments, for example where the first primer is designed to hybridize to a specific sequence on a target RNA, or a sequence common to a family of RNA targets, random priming by the second primer ensures amplification of the entire selected target or family of selected targets. In this embodiment, the second primer extension products comprise sequences which are the same or substantially the same as the sequences in the target RNA(sense copies). The second primer comprises a ligand that is a member of a ligand-receptor pair. In some embodiments, the ligand is attached to the primer at the 5' end of the primer. In some embodiments, the ligand is a small molecule, such as biotin or digoxigenin. In some embodiments, the receptor is an antibody, and the ligand is a molecule or portion of a molecule recognized by the antibody.

[00190] The second primer extension product is extended such that the 3' portion of the second primer extension product comprises a sequence (A') which is complementary to sequence (A) of the first primer. Since sequence (A) on the first primer extension product comprises RNA, both DNA dependent DNA polymerase activity and RNA dependent DNA polymerase activity are used in step (c). The primer extension results in a product that is at least partially double stranded. The method produces a nucleic acid that comprises a ligand allowing it to be bound to a solid surface and that has a specific sequence (A') at its 3' end. The specific, or universal, sequence (A') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. As described above, in some embodiments, the nucleic acid attached to the ligand also comprises sequence (B) at or near its 5' end.

[00191] In some embodiments, the sample comprising the target RNA is in a sample that also comprises DNA. In such cases, it can be advantageous to add a selective DNA dependent DNA polymerase inhibitor such as actinomycin such that it is present during step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a). The presence of a DNA dependent DNA polymerase inhibitor such as actinomycin is particularly advantageous when a first primer comprising a random sequence is used, as the inhibitor allows for the selective creation of first primer extension products to RNA without the need of separating the RNA from the DNA. This is also advantageous when the priming is carried out at specific target sequences since the sequence may be the same on the DNA when the DNA and RNA in the sample represent total

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nucleic acid from the same biological entity, for example, human tissue, animal tissue, and the like. The use of DNA dependent DNA polymerase inhibitors such as actinomycin is described in copending applications.

[00192] In some embodiments, the method further comprises step (d) binding the ligand to a receptor bound to a solid surface whereby the second primer extension product is attached to the solid surface. The receptor bound to the solid surface is a member of the ligand-receptor pair such that binding of the ligand results in attaching the second primer extension product to the solid surface. In some embodiments, the second primer extension product is still hybridized to the first primer extension product when it is attached to the solid surface. In some embodiments, the first primer extension product is removed from the second primer extension product such that a single stranded polynucleotide is attached to the solid surface. The method produces a nucleic acid that is bound to a solid surface that has a specific sequence (A') at its 3' end. The specific, or universal, sequence (A') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. As described above, in some embodiments, the nucleic acid bound to the bead also comprises sequence (B) at or near its 5' end. One aspect of the invention comprises amplification of the nucleic acid bound to the bead. In some embodiments, the amplification is carried out using isothermal amplification using a composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (A') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification.

[00193] In some embodiments, the amplification of the second primer extension product comprises the steps of: (e) cleaving the RNA in the heteroduplex from the first primer extension product such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded; (f) annealing an amplification primer to the single stranded portion of the second primer extension product complementary to sequence (A), wherein the amplification primer has a DNA portion and a 5' RNA portion; (g) extending the amplification primer with a DNA polymerase having strand displacement activity to produce an amplified product hybridized to the second primer extension product; (h) cleaving the RNA from the amplified product hybridized to the second primer extension product; and (i) repeating steps (f) to (h) to produce multiple copies of amplified product. Where this method of amplification is used, and a sequence (B) is incorporated into the second primer extension product as described above, amplification product is generated which comprises sequence (B') complementary to sequence B at or near its 3' end.

[00194] In some embodiments, for example where random sequences at the 3' end of the first and/or second primer are used, a plurality of different nucleic acids bound to a solid surface is created in which each of the nucleic acids has a specific sequence (A') at its 3' end (and in some embodiments also a specific sequence (B) at its 5' end), and where the different nucleic acids have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target RNA. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target RNA.

[00195] The solid surface can be any of a variety of surfaces, some described in more detail below. The solid surface can be, for example a planar surface, for example, a planar array. In some

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embodiments the solid surface comprises a plurality of beads. In some embodiments the beads are magnetic.

[00196] The step of binding the polynucleotides to the solid surface through the ligand, step (d), can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

[00197] In some embodiments, the method further comprises treating the solid surface with reagents to produce multiple copies of an amplification product that are substantially complementary the second primer extension product. This step comprises carrying out an amplification reaction wherein the bound nucleic acid acts as a template for the amplification. Generally, the amplification is carried out using the sequence (A') on the second primer extension product for the hybridization of primer. In some embodiments, the amplification produces single stranded amplified product, In some embodiments, the amplification provides double stranded product. In some embodiments, the second primer comprises a specific sequence (B), which becomes incorporated into the second primer extension product. In some embodiments the amplification is an isothermal amplification reaction comprising a composite RNA/DNA primer, RNase H, and a DNA polymerase with strand displacement activity. In some embodiments, the amplification is carried out using polymerase chain reaction, (PCR). For example where the second primer extension product comprises both as sequence (B) at or near its 5' end and a sequence (A') at or near its 3' end, a set of primers, one designed to hybridize to all or a portion of the sequence (A') and the other designed to hybridize to sequence (B'), the complement of sequence (B), can be used to carry out a PCR reaction to exponentially produce double stranded amplified product.

[00198] In some embodiments the amplification is carried out such that the amplified product is not attached to the substrate, but is freely dissolved in the solution. In other embodiments, the amplification is carried out such that the amplified product remains bound to the substrate, for example by performing solid phase PCR such as bridge PCR. In yet other embodiments, an amplified product is generated that may float freely in solution, but which comprises a sequence, for example sequence (A) or sequence (B'), that allows it to be captured to another solid surface or other portion of the solid surface by hybridization to a complementary sequence bound to such surface (e.g. sequence (A') or sequence (B). In some embodiments, the amplified product is a single-stranded product and, because it is generated at the solid surface, the amplified product readily captured by complementary sequences, e.g. sequence (B), bound to the surface.

[00199] In one aspect of the invention, a plurality of beads is used, and the methods described above are carried out such that on average, one or fewer second primer extension product molecules are bound per bead. The beads are dispersed into an aqueous solution, and a plurality of microreactors, e.g. droplets, are produced such that on average one or fewer beads is contained within each of the plurality of microreactors. The amplification of the second primer extension products bound to the beads is then carried out such that the clonal amplification of each of the plurality of second primer extension products in the separate microreactors is achieved. This clonal amplification in

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microreactors can be performed on a sample of target RNA, such as whole transcriptome or total RNA, wherein the plurality of second primer extension products comprise sequences that correspond to most, to substantially all, or to all of the sequences in the target RNA. In some embodiments, the amplified products are captured by bead having attached thereto a plurality of oligonucleotides comprising complementary sequences bound to such surface (e.g. sequence (A') or sequence (B)), which are complementary to sequence (A) or sequence (B') on the amplified product.

[00200] In some embodiments, the plurality of beads, produced as described above, with each bead comprising a single second primer extension product can comprise a library. These libraries can be stored, then later clonally amplified. In some embodiments, a library of beads can comprise a plurality of beads wherein each bead had multiple copies of a single amplification product generated from a second primer extension product. These libraries can be analyzed, for example by sequencing. The libraries can be stored, and later analyzed. In some embodiments the libraries can be stored, then analyzed multiple times.

[00201] A schematic exemplary of an embodiment of the invention relating to method for generating a polynucleotide comprising a ligand for binding to a solid surface from an RNA target is shown in Figure 2. Figure 2 shows a target RNA and a chimeric RNA/DNA first primer. The primer is first annealed to the target RNA. Step I illustrates extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA; to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid. The sequence complementary to a target RNA can be a specific sequence, a sequence that will hybridize to Poly-A, a sequence common to a plurality of regions (consensus sequence), or a random sequence. Step II represents cleaving the target RNA from the RNA/DNA hybrid. Cleaving can be accomplished thermally, chemically, or enzymatically, e.g. with RNase H. The second primer comprising a ligand is then annealed to the first primer extension product. Step III(a) and step III(b) illustrate extending a second primer, comprising a ligand and a 3' segment complementary to a portion of the first primer extension product, to produce a double stranded product with a DNA/RNA heteroduplex at one end; wherein the double stranded product comprises a second primer extension product hybridized to the first primer extension product, and whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer. Step III(a) shows step III for a second primer that does not comprise sequence (B). Step III(b) shows step III for a second primer that comprises a sequence (B) that is 5' of the segment complementary to a portion of the first primer extension product. In each case the second primer is shown with a ligand attached to the 5' end. The second primer extension product comprising a ligand and a defined sequence (A) at its 3' end is useful for storage, archiving and analysis as it has a ligand capable of binding to a solid surface. Such second primer extension product also comprises a sequence that is representative of (identical to or substantially identical to) a sequence in the target RNA, so analysis of this product provides information about the target RNA. Step IV shows the binding the ligand to a solid surface, whereby the second primer extension product becomes bound to the solid surface.

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Method for generating a polynucleotide comprising a ligand for binding to a solid surface from a DNA target

[00202] The methods of the present invention can be used to analyze the DNA (e.g. genomic DNA) samples that are important for many studies. The methods can be used for high-throughput genomic analysis, and can be used for forensic and paleoarcheology work which can be severely limited by nucleic acid sample size. The methods can be used, for example, for the genotyping of multiple loci in the study of complex diseases. The methods can also be used for the determination of genomic instability in various pathological conditions such as cancer, which is most precisely carried out in well defined cell populations, such as that obtained by laser capture micro-dissection or cell sorting. The DNA amplification technologies described herein provide global amplification of very small polynucleotide samples, for example, from one or a very few cells.

[00203] One aspect of the invention is a method for generating from a DNA target a polynucleotide comprising a ligand for binding to a solid surface.

[00204] The method comprises the steps of: (a) denaturing a double-stranded target DNA. Double stranded DNA can be denatured, for example by heating, or by the addition of denaturing agents.

[00205] The method further comprises step (b) annealing to the target DNA and extending with a DNA polymerase comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the of the primer comprises sequence (A), which is not complementary to the target DNA; to form a plurality of first primer extension products, each with sequence (A) at its 5' end. The enzyme that carries out step (b) is generally a DNA polymerase. In some cases a mixture of DNA polymerases can be used. The sequence that is complementary to the target DNA comprises a random sequence, such that the extension of the first primer results in a plurality of first primer extension products complementary to the sequences adjacent to the sequence where each random species hybridizes. The use of a random sequence at the 3' end of the primer as sequence can be useful for performing a global amplification of a DNA target, generating a plurality of sequences which together can represent, for example substantially the whole sequence of the target DNA. In some embodiments, the relative amounts of the various sequences can be used to quantitate the relative amount of a given sequence in a sample, for example to determine the number of gene copies in a DNA sample, or obtaining sequence information. In some embodiments, the extension of one first primer, will result in the release of a downstream first primer extension product. This can occur throughout the target DNA resulting in the release of multiple first primer extension products from the target DNA. This process can occur simultaneously on both of the strands of the double-stranded DNA target, thus creating first primer extension products complementary to sequences in both strands.

[00206] In some embodiments, the first primer extension step is carried out with a DNA polymerase capable of extension at elevated temperature that is not compatible with subsequent hybridization of the random sequence to the displaced primer-extension product. For example, Bst DNA polymerase can be used which is active at elevated temperature. The reaction can be carried out stepwise, first with an incubation at a lower temperature such as about 25 °C, followed by an incubation at higher temperature such as about 50 °C. In some embodiments, the first incubation is carried out below about 30 °C, and the second incubation is carried out above about 40 °C. In some embodiments, a

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DNA polymerase which is active at temperatures above about 45 °C is used to extend the first primer. Mixtures of DNA polymerases can also be useful.

[00207] The method further comprises step (c) extending a second primer comprising a ligand and a 3' DNA region that comprises a random sequence, wherein the primer is optionally a tailed primer comprising a nucleic acid sequence (B) that is 5' of the random sequence, to form a plurality of double-stranded products each comprising a first primer extension product and a second primer extension product whereby the second primer extension product comprises a ligand. This step may be carried out with or without prior denaturation. If carried out without denaturation, generally, only the single stranded displaced first primer extension product will hybridize to the second primer. Generally the second primer does not comprise RNA. The extension of the second primer is carried out with a DNA polymerase as described herein. The second primer can be a tailed primer having a 3' portion which is complementary to the first primer extension product, and a 5' portion, sequence (B), which is not complementary to the first primer extension product. The second primer comprises a random primer sequence that randomly binds to the first primer extension product. Extension of the second primer comprising a random sequence produces a plurality of second primer extension products. The use of a random sequence at the 3' end of the primer is useful, for example, in performing global amplification of a target DNA, whereby a plurality of second primer extension products are produced which is representative of the sequence of the target DNA. In some embodiments, for example where the first primer is designed to hybridize to a specific sequence on a target RNA, or a sequence common to a family of RNA targets, random priming by the second primer ensures amplification of the entire selected target or family of selected targets. In this embodiment, the second primer extension products comprise sequences which are the same or substantially the same as the sequences in the target DNA. The second primer comprises a ligand that is a member of a ligand-receptor pair. In some embodiments, the ligand is attached to the primer at the 5' end of the primer. In some embodiments, the ligand is a small molecule, such as biotin or digoxigenin. In some embodiments, the receptor is an antibody, and the ligand is a molecule or portion of a molecule recognized by the antibody.

[00208] The second primer extension product is extended such that the 3' portion of the second primer extension product comprises a sequence (A') which is complementary to sequence (A) of the first composite primer. Since sequence (A) on the first primer extension product comprises RNA, both DNA dependent DNA polymerase activity and RNA dependent DNA polymerase activity are used in step (c). The primer extension results in a product that is at least partially double stranded. The method produces a nucleic acid that comprises a ligand allowing it to be bound to a solid surface and that has a specific sequence (A') at its 3' end. The specific, or universal, sequence (A') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. As described above, in some embodiments, the nucleic acid attached to the ligand also comprises sequence (B) at or near its 5' end.

[00209] In some embodiments, The method further comprises step (d) binding the ligand to a receptor bound to a solid surface whereby the second primer extension product is attached to the solid surface. The receptor bound to the solid surface is a member of the ligand-receptor pair such that binding of the ligand results in attaching the second primer extension product to the solid

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surface. In some embodiments, the second primer extension product is still hybridized to the first primer extension product when it is attached to the solid surface. In some embodiments, the first primer extension product is removed from the second primer extension product such that a single stranded polynucleotide is attached to the solid surface. The method produces a nucleic acid that is bound to a solid surface that has a specific sequence (A') at its 3' end. The specific, or universal, sequence (A') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. As described above, in some embodiments, the nucleic acid bound to the bead also comprises sequence (B) at or near its 5' end. One aspect of the invention comprises amplification of the nucleic acid bound to the bead. In some embodiments, the amplification is carried out using isothermal amplification using a composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (A') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification.

[00210] In some embodiments, the amplification of the second primer extension product comprises the steps of: (c) cleaving the RNA in the heteroduplex from the first primer extension product such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded; (f) annealing an amplification primer to the single stranded portion of the second primer extension product complementary to sequence (A), wherein the amplification primer has a DNA portion and a 5' RNA portion; (g) extending the amplification primer with a DNA polymerase having strand displacement activity to produce an amplified product hybridized to the second primer extension product; (h) cleaving the RNA from the amplified product hybridized to the second primer extension product; and (i) repeating steps (f) to (h) to produce multiple copies of amplified product. Where this method of amplification is used, and a sequence (B) is incorporated into the second primer extension product as described above, amplification product is generated which comprises sequence (B') complementary to sequence B at or near its 3' end.

[00211] In some embodiments, a plurality of different nucleic acids bound to a solid surface is created in which each of the nucleic acids has a specific sequence (A') at its 3' end (and in some embodiments also a specific sequence (B) at its 5' end), and where the different nucleic acids have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target DNA. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target DNA.

[00212] The solid surface can be any of a variety of surfaces, some described in more detail below. The solid surface can be, for example a planar surface, for example, a planar array. In some embodiments the solid surface comprises a plurality of beads. In some embodiments the beads are magnetic.

[00213] The step of binding the polynucleotides to the solid surface through the ligand, step (d), can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

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[00214] In some embodiments, the method further comprises treating the solid surface with reagents to produce multiple copies of an amplification product that are substantially complementary the second primer extension product. This step comprises carrying out an amplification reaction wherein the bound nucleic acid acts as a template for the amplification. Generally, the amplification is carried out using the sequence (A') on the second primer extension product for the hybridization of primer. In some embodiments, the amplification produces single stranded amplified product, In some embodiments, the amplification provides double stranded product. In some embodiments, the second primer comprises a specific sequence (B), which becomes incorporated into the second primer extension product. In some embodiments the amplification is an isothermal amplification reaction comprising a composite RNA/DNA primer, RNase H, and a DNA polymerase with strand displacement activity. In some embodiments, the amplification is carried out using polymerase chain reaction, (PCR). For example where the second primer extension product comprises both as sequence (B) at or near its 5' end and a sequence (A') at or near its 3' end, a set of primers, one designed to hybridize to all or a portion of the sequence (A') and the other designed to hybridize to sequence (B'), the complement of sequence (B), can be used to carry out a PCR reaction to exponentially produce double stranded amplified product.

[00215] In some embodiments the amplification is carried out such that the amplified product is not attached to the substrate, but is freely dissolved in the solution. In other embodiments, the amplification is carried out such that the amplified product remains bound to the substrate, for example by performing solid phase PCR such as bridge PCR. In yet other embodiments, an amplified product is generated that may float freely in solution, but which comprises a sequence, for example sequence (A) or sequence (B'), that allows it to be captured to another solid surface or other portion of the solid surface by hybridization to a complementary sequence bound to such surface (e.g. sequence (A') or sequence (B)). In some embodiments, the amplified product is a single-stranded product and, because it is generated at the solid surface, the amplified product readily captured by complementary sequences, e.g. sequence (B), bound to the surface.

[00216] In one aspect of the invention, a plurality of beads is used, and the methods described above are carried out such that on average, one or fewer second primer extension product molecules are bound per bead. The beads are dispersed into an aqueous solution, and a plurality of microreactors, e.g. droplets, are produced such that on average one or fewer beads is contained within each of the plurality of microreactors. The amplification of the second primer extension products bound to the beads is then carried out such that the clonal amplification of each of the plurality of second primer extension products in the separate microreactors is achieved. This clonal amplification in microreactors can be performed on a sample of target DNA, such as genomic DNA, wherein the plurality of second primer extension products comprise sequences that correspond to most, to substantially all, or to all of the sequences in the target DNA. In some embodiments, the amplified products are captured by bead having attached thereto a plurality of oligonucleotides comprising complementary sequences bound to such surface (e.g. sequence (A') or sequence (B)), which are complementary to sequence (A) or sequence (B') on the amplified product.

[00217] In some embodiments, the plurality of beads, produced as described above, with each bead comprising a single second primer extension product can comprise a library. These libraries can be

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stored, then later clonally amplified. In some embodiments, a library of beads can comprise a plurality of beads wherein each bead had multiple copies of a single amplification product generated from a second primer extension product. These libraries can be analyzed, for example by sequencing. The libraries can be stored, and later analyzed. In some embodiments the libraries can be stored, then analyzed multiple times.

[00218] A schematic exemplary of an embodiment of the invention relating to method for generating a polynucleotide comprising a ligand for binding to a solid surface from a DNA target is shown in Figure 3. Step I represents denaturing a double-stranded target DNA, for example by raising the temperature. Steps II and III illustrate annealing to the target DNA and extending with a DNA polymerase comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the of the primer comprises sequence (A), which is not complementary to the target DNA; to form a plurality of first primer extension products, each with sequence (A) at its 5' end. The enzyme that carries out step (b) is generally a DNA polymerase. In some cases a mixture of DNA polymerases can be used. In some embodiments, a DNA polymerase with strand displacement activity is used such that a growing first primer extension product can displace a downstream first primer extension product, producing a plurality of first primer extension products, representing different regions of the sequence of the target DNA are produced. Step IV illustrates extending a second primer comprising a ligand and a 3' DNA region that comprises a random sequence, wherein the primer is optionally a tailed primer comprising a nucleic acid sequence (B) that is 5' of the random sequence, to form a plurality of double-stranded products each comprising a first primer extension product and a second primer extension product whereby the second primer extension product comprises a ligand. Step IV(b) shows step IV in which the second primer comprises a segment (B) that is 5' of the 3' DNA region that comprises a random sequence. Step IV(a) shows step IV where the second primer does not comprise a (B) sequence. The second primer extension products comprising a ligand and a defined sequence (A) at its 3' end are useful for storage, archiving and analysis as they have a ligand capable of binding to a solid surface. Such second primer extension products also comprises sequences that are representative of (identical to or substantially identical to) a sequence in the target DNA, so analysis of these products provides information about the target DNA. Step V shows the binding the ligand to a solid surface, whereby the plurality of second primer extension products become bound to the solid surface.

Method for archiving and clonal expansion

[00219] An aspect of the invention is a method for archiving and clonal expansion of a nucleotide sequence.

[00220] The method comprises the steps of: (a) obtaining a plurality of partially double-stranded DNA products comprising a first polynucleotide and a second polynucleotide, wherein the second polynucleotide comprises a sequence (A') at its 3' end and a ligand, and the sequence (A') portion of the second polynucleotide is single-stranded, wherein optionally the second polynucleotide comprises a sequence (B) at or near its 5' end. The plurality of partially double-stranded DNA products comprising a first polynucleotide and a second polynucleotide, wherein the second

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polynucleotide comprises a sequence (A') at its 3' end and a ligand, and the sequence (A') portion of the second polynucleotide is single-stranded, wherein optionally the second polynucleotide comprises a sequence (B) at or near its 5' end may be obtained, for example, by the methods described above.

[00221] The method further comprises step (b) attaching the partially double stranded DNA products to a plurality of beads or a plurality of isolated areas on a surface by binding the ligands to the bead or isolated area. The receptor bound to the solid surface is a member of the ligand-receptor pair such that binding of the ligand results in attaching the second polynucleotide to the solid surface. In some embodiments, the second polynucleotide is still hybridized to the first polynucleotide when it is attached to the solid surface. In some embodiments, the first polynucleotide is removed from the second polynucleotide such that a single stranded polynucleotide is attached to the solid surface. The method produces a polynucleotide that is bound to a solid surface that has a specific sequence (A') at its 3' end. The specific, or universal, sequence (A') can be a site for primer hybridization and further analysis or amplification of the polynucleotide bound to the bead. As described above, in some embodiments, the polynucleotide bound to the bead also comprises sequence (B) at or near its 5' end. One aspect of the invention comprises amplification of the polynucleotide bound to the bead. In some embodiments, a plurality of different polynucleotides bound to a solid surface is created in which each of the polynucleotides has a specific sequence (A') at its 3' end (and in some embodiments also a specific sequence (B) at its 5' end), and where the different polynucleotides have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target nucleic acid. The set of bound polynucleotides thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target nucleic acid.

[00222] The solid surface can be any of a variety of surfaces, some described in more detail below. The solid surface can be, for example a planar surface, for example, a planar array. In some embodiments the solid surface comprises a plurality of beads. In some embodiments the beads are magnetic.

[00223] The step of binding the polynucleotides to the solid surface through the ligand, step (b), can be carried out such that only one polynucleotide is bound to an isolated area of a surface or only one polynucleotide is bound to a single bead. This isolated binding of polynucleotides can be used for clonal amplification of the specific bound polynucleotide in that area or on that bead. Such bound, isolated polynucleotides can also be stored and archived for later analysis, for example by sequencing. The bound, isolated polynucleotides can be amplified, stored, and analyzed multiple times.

[00224] The method further comprises the steps of: (c) annealing an amplification primer to the single stranded portion of the second polynucleotide complementary to sequence (A'), wherein the amplification primer has a DNA portion and a 5' RNA portion; (d) extending the amplification primer with an enzyme having strand displacement activity to produce a plurality of amplified products hybridized to the second polynucleotide products; (e) cleaving the RNA from the amplified product hybridized to the second polynucleotides using RNase H; and (f) repeating steps (c) to (e) to produce multiple copies of amplified products.

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[00225] In some embodiments, the DNA products are attached to beads or isolated areas of the solid surface, and on average, one DNA product is attached to one or fewer beads or isolated areas. The plurality of beads or isolated areas can be stored for later analysis, and in some cases can later be amplified with steps (c) through (f). In some embodiments, the amplification is a clonal amplification carried out in multiple isolated volumes wherein, on average, one isolated volume has one or fewer beads or isolated areas.

[00226] A schematic exemplary of an embodiment of the invention relating to amplification of polynucleotides bound to a solid surface is shown in Figure 4. The polynucleotides bound to the solid surface can be generated, for example from RNA or from DNA by the methods described above. A polynucleotides bound to the solid surface can be a second primer extension product comprising a sequence (A') at its 3' end. In some embodiments, as in those shown in Figure 4, the polynucleotides bound to the surface are hybridized to a first primer extension product comprising and RNA sequence (A). For clarity, the polynucleotides are referred to as second primer extension products. It will be understood, however, by those of ordinary skill in the art that polynucleotides of the same structure as shown here can be used in the present amplification methods. Where the polynucleotides are single stranded, it would be understood by those skilled in the art that the amplification can be carried out without the first RNase H step (Step I). In Figure 4, the steps labeled (b), e.g. Step I(b), represents the method for the case in which the second primer comprises a sequence (B) 5' to the 3' sequence that is complementary to the first primer extension product, while steps labeled (a), e.g. Step I(a) represent the method in which the second primer, and therefore the second primer extension product, does not include sequence (B).

[00227] In Figure 4, Step I illustrates cleaving the RNA from the first primer extension products such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded. As shown, the cleavage is performed using RNase H. Chemical and thermal means can alternatively be employed. Step II shows annealing an amplification primer to the single stranded portion of the second primer extension products complementary to sequence (A), wherein the amplification primer has a DNA portion and a 5' RNA portion. Step III illustrates extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product hybridized to the second primer extension product. Step IV illustrates the step of cleaving the RNA from the amplified product hybridized to the second primer extension product. The product of step IV can be the starting point for another round of amplification in that Step II, the hybridization of and amplification primer, can occur, followed by the subsequent steps. Thus, steps II – IV can be repeated to produce multiple copies of amplified product. Note that for case (b) the amplified product that is produced comprises the sequence (B'), complementary to sequence (B) at its 3' end.

[00228] In some embodiments, the amplification illustrated in Figure 4 can be carried out on beads. In some embodiments, the beads have only one bound second primer extension product. In some embodiments such beads are individually in isolated volumes or microreactors allowing for clonal amplification.

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Method for generating a polynucleotide having a defined 3' and 5' sequences from an RNA target

[00229] One aspect of the invention is a method for generating a polynucleotide having a defined 3' and 5' sequences from an RNA target. The method utilizes a composite RNA/DNA oligonucleotide in order to extend the second primer extension product such that the second primer extension product comprises a sequence (C') at its 3' end than can be used as a site for isothermal amplification in a manner such that the sequence (A) is present at or near the 5' end of the amplified product produced in this amplification.

[00230] The method comprises the steps: (a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA; to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid. . In some embodiments, the 3' portion of the primer that is complementary to the target RNA is a specific sequence. For example, where a specific region of interest of a target RNA that is known or suspected to be upstream of a specific sequence on the target RNA, the sequence that is complementary to the target RNA of the first primer can be designed to hybridize to this specific sequence on the target RNA such that extension of the primer results in producing a first primer extension product that is complementary to such upstream region. The specific sequence may be common to a family of target RNA. A combination of primers with various specific sequences at the 3' end can also be useful. In some embodiments, such as where the target RNA comprises mRNA, and the mRNA comprises a plurality of sequences, each having a 3' poly-A segment; the specific sequence that is complementary to the target RNA can comprise a sequence that will hybridize to the poly-A region of the mRNA, thus allowing the extension of the first primer to produce a plurality of first primer extension products, each of which is complementary to the region of an mRNA molecule adjacent to the poly-A region. In some embodiments, the sequence that is complementary to the target RNA comprises a random sequence, such that the extension of the first primer results in a plurality of first primer extension products complementary to the sequences adjacent to the sequence where each random species hybridizes. The use of a random sequence at the 3' end of the primer can be useful for performing a global amplification of a RNA target, generating a plurality of sequences which together can represent, for example substantially the whole sequence of the target RNA. In some embodiments, the relative amounts of the various sequences can be used to quantitate the relative amount of a given sequence in a sample, for example to determine the level of expression in an mRNA sample. In some embodiments more than one type of sequence that is complementary to the target RNA can be used, for instance both a primer with a random sequence and a primer, or combination of primers with a specific sequence complementary to RNA can be used. In some embodiments, multiple primers comprising different specific sequences can be used.

[00231] The method further comprises step (b) cleaving the target RNA from the RNA/DNA hybrid. In some embodiments, the cleaving of the target RNA from the RNA/DNA hybrid involves selectively cleaving or degrading the target RNA. In some cases the cleaving can be affected by denaturing the complex comprising the first primer extension product and the nucleic acid. Denaturation can be performed, for example by heating the sample, or by adding a denaturing agent,

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or using a combination of heating the sample and adding denaturing agents. The cleaving can be accomplished with an enzyme that cleaves RNA from an RNA/DNA hybrid such as RNase H, or a combination of RNase enzymes, or chemically. In some embodiments, the target RNA is completely cleaved. In other embodiments, the target RNA is only partly cleaved or degraded. The amount of cleaving required is that amount which will allow the extension of the second primer. In some embodiments, the cleaving is carried out partially, and the fragments of the target RNA that remain can constitute the second primer for step (c).

[00232] The method further comprises step (c) extending a second primer, comprising a ligand and a 3' segment complementary to a portion of the first primer extension product, to produce a double stranded product with a DNA/RNA heteroduplex at one end; wherein the double stranded product comprises a second primer extension product hybridized to the first primer extension product, and wherein a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer. The extension of the second primer is carried out with a DNA polymerase as described herein. The second primer can comprise RNA, DNA, or can be a composite primer comprising both RNA and DNA. The second primer can be a tailed primer having a 3' portion which is complementary to the first primer extension product, and a 5' portion, sequence (B), which is not complementary to the first primer extension product. In some embodiments, the second primer can comprise a specific primer sequence that is designed to hybridize to a specific sequence in the first primer extension product. In some embodiments the second primer comprises a random primer sequence that randomly binds to the first primer extension product. Extension of the second primer comprising a random sequence produces a plurality of second primer extension products. The use of a random sequence at the 3' end of the primer is useful, for example, in performing global amplification of a target RNA, whereby a plurality of second primer extension products are produced which is representative of the sequence of the target RNA. In some embodiments, for example where the first primer is designed to hybridize to a specific sequence on a target RNA, or a sequence common to a family of RNA targets, random priming by the second primer ensures amplification of the entire selected target or family of selected targets. In this embodiment, the second primer extension products comprise sequences which are the same or substantially the same as the sequences in the target RNA (sense copies). The second primer comprises a ligand that is a member of a ligand-receptor pair. In some embodiments, the ligand is attached to the primer at the 5' end of the primer. In some embodiments, the ligand is a small molecule, such as biotin or digoxigenin. In some embodiments, the receptor is an antibody, and the ligand is a molecule or portion of a molecule recognized by the antibody.

[00233] The second primer extension product is extended such that the 3' portion of the second primer extension product comprises a sequence (A') which is complementary to sequence (A) of the first primer. Since sequence (A) on the first primer extension product comprises RNA, both DNA dependent DNA polymerase activity and RNA dependent DNA polymerase activity are used in step (c). The primer extension results in a product that is at least partially double stranded. The product further comprises a DNA-RNA heteroduplex region. The method produces a nucleic acid that comprises a ligand allowing it to be bound to a solid surface and that has a specific sequence (A') at its 3' end. The specific, or universal, sequence (A') can be a site for primer hybridization and

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further analysis or amplification of the nucleic acid bound to the bead. As described above, in some embodiments, the nucleic acid attached to the ligand also comprises sequence (B) at or near its 5' end.

[00234] In some embodiments, the sample comprising the target RNA is in a sample that also comprises DNA. In such cases, it can be advantageous to add a selective DNA dependent DNA polymerase inhibitor such as actinomycin such that it is present during step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a). The presence of a DNA dependent DNA polymerase inhibitor such as actinomycin is particularly advantageous when a first primer comprising a random sequence is used, as the inhibitor allows for the selective creation of first primer extension products to RNA without the need of separating the RNA from the DNA. This is also advantageous when the priming is carried out at specific target sequences since the sequence may be the same on the DNA when the DNA and RNA in the sample represent total nucleic acid from the same biological entity, for example, human tissue, animal tissue, and the like. The use of DNA dependent DNA polymerase inhibitors such as actinomycin is described in copending application.

[00235] The method further comprises step (d) cleaving the RNA in the heteroduplex from the first primer extension product such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded. The cleaving of RNA can be performed, for example by treatment with RNase H, which will selectively cleave the RNA portion of the DNA/RNA partial heteroduplex formed in step (c).

[00236] The method further comprises step (e) annealing to the second primer extension product an oligonucleotide comprising a 3'- DNA segment that is complementary to sequence (A') and a 5' RNA segment comprising sequence (C). The oligonucleotide comprises at least one DNA and at least one RNA portion. In some embodiments the 5' DNA segment is complementary to all of sequence (A'), in other embodiments, the 5' DNA segment is complementary to portion of sequence (A'). In some embodiments, 5' RNA segment comprising sequence (C) is partly complementary to sequence (A').

[00237] The method optionally comprises step: (f) extending the oligonucleotide to form an oligonucleotide extension product hybridized to the second primer extension product. In some embodiments, the oligonucleotide is optionally extended from its 3' end to produce an oligonucleotide extension product hybridized to the second primer extension product and displacing the DNA portion of the first primer extension product. In some embodiments, the second primer comprises a sequence (B), such that the oligonucleotide extension product will comprise a sequence (B') at or near its 3' end that is complementary to sequence (B).

[00238] The method further comprises step (g) extending the second primer extension product to create a heteroduplex such that the second primer comprises a DNA sequence (C') that is complementary to sequence (C). The DNA sequence (C') is created by a DNA polymerase that has RNA dependent DNA polymerase activity. This step creates an RNA/DNA heteroduplex region that can be used for further manipulation of the second primer extension product.

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[00239] The method further comprises step (h) cleaving the RNA from the heteroduplex created in step (g) to produce a single-stranded portion of the second primer extension product corresponding to sequence (C'). The RNA can be cleaved from the heteroduplex, for example, by RNase H.

[00240] In some embodiments, The method further comprises step (i) binding the ligand on the second primer extension product to a solid surface. The binding can be performed at various stages or steps during the procedure depending, for example, on whether it is advantageous to carry out the subsequent steps on a solid surface. The binding step is generally performed after step (c). In some embodiments, the binding the ligand to the solid surface is performed before step (h). In some embodiments, the binding the ligand to the solid surface is performed after step (h). The receptor bound to the solid surface is a member of the ligand-receptor pair such that binding of the ligand results in attaching the second primer extension product to the solid surface. In some embodiments, the second primer extension product is still hybridized to the first primer extension product when it is attached to the solid surface. In some embodiments, the first primer extension product is removed from the second primer extension product such that a single stranded polynucleotide is attached to the solid surface. The method produces a nucleic acid that is bound to a solid surface that has a specific sequence (A') and (C') at its 3' end. The specific sequence (C') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. As described above, in some embodiments, the nucleic acid bound to the bead also comprises sequence (B) at or near its 5' end. One aspect of the invention comprises amplification of the nucleic acid bound to the bead. In some embodiments, the amplification is carried out using isothermal amplification using a composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (C') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification. When the sequence (C') acts as a site to which a composite amplification primer binds, the amplified product that is produced has the sequence (A) (and a portion of sequence (C) at its 5' end. Where the second primer comprises the sequence (B), the amplified product also has the sequence (B'), complementary to (B) at or near its 3' end. Thus the method produced amplified product with defined sequences at or near both its 3' and 5' ends.

[00241] In some embodiments, for example where random sequences at the 3' end of the first and/or second primer are used, a plurality of different nucleic acids bound to a solid surface is created in which each of the nucleic acids has a specific sequence (A') and (C') at its 3' end (and in some embodiments also a specific sequence (B) at its 5' end), and where the different nucleic acids have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target RNA. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target RNA.

[00242] The solid surface can be any of a variety of surfaces, some described in more detail below. The solid surface can be, for example a planar surface, for example, a planar array. In some embodiments the solid surface comprises a plurality of beads. In some embodiments the beads are magnetic.

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[00243] The step of binding the polynucleotides to the solid surface through the ligand, step (i), can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

[00244] In some embodiments, the method further comprises treating the solid surface with reagents to produce multiple copies of an amplification product that are substantially complementary the second primer extension product. This step comprises carrying out an amplification reaction wherein the bound nucleic acid acts as a template for the amplification. Generally, the amplification is carried out using the sequence (C') on the second primer extension product for the hybridization of primer. In some embodiments, the amplification produces single stranded amplified product, In some embodiments, the amplification provides double stranded product. In some embodiments, the second primer comprises a specific sequence (B), which becomes incorporated into the second primer extension product. In some embodiments the amplification is an isothermal amplification reaction comprising a composite RNA/DNA primer, RNase H, and a DNA polymerase with strand displacement activity. In some embodiments, the amplification is carried out using polymerase chain reaction, (PCR). For example where the second primer extension product comprises both as sequence (B) at or near its 5' end and a sequence (C') at or near its 3' end, a set of primers, one designed to hybridize to all or a portion of the sequence (C') and the other designed to hybridize to sequence (B'), the complement of sequence (B), can be used to carry out a PCR reaction to exponentially produce double stranded amplified product.

[00245] In some embodiments, the amplification is performed by a method comprising the following steps: (j) annealing an amplification primer, wherein the amplification primer has a DNA portion and a 5' RNA portion, to the single stranded portion of the second primer extension product complementary to sequence (C'); (k) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product; (l) cleaving the RNA from the amplified product; and (m) repeating steps (j) to (l) to produce multiple copies of amplified product wherein the 5' portion of the amplified product has a sequence complementary to sequence (A'). Where the second primer further comprises a segment (B) that is not complementary to the first primer extension product sequence, this amplification method, utilizing sequence (C'), allows for the production of an amplified product comprises a sequence (B') at or near its 3' end that is substantially complementary to sequence (B), and a sequence (A) near its 5' end that is complementary to sequence (A'), thus producing an amplified polynucleotide product with defined 3' and 5' ends.

[00246] In some embodiments the amplification is carried out such that the amplified product is not attached to the substrate, but is freely dissolved in the solution. In other embodiments, the amplification is carried out such that the amplified product remains bound to the substrate, for example by performing solid phase PCR such as bridge PCR. In yet other embodiments, an amplified product is generated that may float freely in solution, but which comprises a sequence, for example sequence (A) or sequence (B'), that allows it to be captured to another solid surface or other

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portion of the solid surface by hybridization to a complementary sequence bound to such surface (e.g. sequence (A') or sequence (B). In some embodiments, the amplified product is a single-stranded product and, because it is generated at the solid surface, the amplified product readily captured by complementary sequences, e.g. sequence (B), bound to the surface.

[00247] In one aspect of the invention, a plurality of beads is used, and the methods described above are carried out such that on average, one or fewer second primer extension product molecules are bound per bead. The beads are dispersed into an aqueous solution, and a plurality of microreactors, e.g. droplets, are produced such that on average one or fewer beads is contained within each of the plurality of microreactors. The amplification of the second primer extension products bound to the beads is then carried out such that the clonal amplification of each of the plurality of second primer extension products in the separate microreactors is achieved. This clonal amplification in microreactors can be performed on a sample of target RNA, such as whole transcriptome or total RNA, wherein the plurality of second primer extension products comprise sequences that correspond to most, to substantially all, or to all of the sequences in the target RNA. In some embodiments, the amplified products are captured by bead having attached thereto a plurality of oligonucleotides comprising complementary sequences bound to such surface (e.g. sequence (A') or sequence (B)), which are complementary to sequence (A) or sequence (B') on the amplified product.

[00248] In some embodiments, the plurality of beads, produced as described above, with each bead comprising a single second primer extension product can comprise a library. These libraries can be stored, then later clonally amplified. In some embodiments, a library of beads can comprise a plurality of beads wherein each bead had multiple copies of a single amplification product generated from a second primer extension product. These libraries can be analyzed, for example by sequencing. The libraries can be stored, and later analyzed. In some embodiments the libraries can be stored, then analyzed multiple times.

[00249] In some embodiments, a bead or isolated area of the solid surface comprises covalently attached thereto multiple oligonucleotides comprising the sequence (B) at their 3' ends, whereby upon the amplification of step (m) multiple copies of amplified product comprising sequence (B') at their 5' end are hybridized to the bead or isolated area. For example, where beads are used, a plurality of beads in a plurality of microreactors wherein, the plurality of beads has, on average one or fewer second primer extension products bound to it and there are, on average, one or fewer beads in each microreactor, a clonal amplification of the plurality of second primer extension products can be carried out, and the amplified products in each of the microreactors will bind to the bead through the sequence (B') on the amplified product to the sequence (B) on the beads. This approach produces a plurality of beads, each with multiple copies of a different sequence bound to it. Where these sequences are representative of the target RNA, the plurality of beads can constitute a library representative of such RNA.

[00250] After the amplified products are bound to the beads by hybridization, the (B) sequences on the beads can be extended along the amplified product by a DNA polymerase or mixture of polymerases to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product and also comprise sequence (A') near their 5' ends. Where the (B) sequences are covalently attached to the beads, this method provides

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for the production of beads with polynucleotides complementary to amplified product covalently attached to the beads. Covalently attached polynucleotides such as those produce here are more robust than nucleotides that are attached only by hybridization to the beads. Thus, the covalently attached polynucleotides can be more stable and can be used with analysis methods and sequencing methods that have harsher conditions which would result in the displacement of polynucleotides bound only by hybridization.

[00251] In some embodiments, the amplified product is removed from the covalently bound polynucleotide to render the polynucleotide single stranded. Such single stranded covalently bound polynucleotides comprise a specific sequence at their 3' ends comprising sequence (A') and a portion of sequence (C'). This specific sequence at the 3' end of the covalently bound polynucleotide can act as a hybridization site for a primer complementary to sequence (A') that can act as a primer to carry out sequencing by any of a variety of sequencing methods, for example, those described herein.

[00252] The sequencing methods can comprise the use of cleavable labeled terminators. The sequencing method can comprise pyrophosphate detection. The sequencing method can comprise an isothermal sequencing method, for example using chimeric primers, RNase H, and a polymerase with strand displacement activity. The sequencing method can also comprise cycle sequencing.

[00253] In some embodiments, the sequencing methods comprise sequencing by ligation. Sequencing by ligation involves using a DNA ligase. Although commonly represented as joining two pairs of ends at once, as in the ligation of restriction enzyme fragments, ligase can also join the ends on only one of the two strands (for example, when the other strand either already continuous or lacks a terminal phosphate necessary for ligation). DNA ligase is sensitive to the structure of DNA and has very low efficiency when there are mismatches between the bases of the two strands. Sequencing by ligation relies upon the sensitivity of DNA ligase for base-pairing mismatches.

[00254] The target molecule to be sequenced is a single strand of unknown DNA sequence, flanked on at least one end by a known sequence. A short "anchor" strand is brought in to bind the known sequence. A mixed pool of probe oligonucleotides is then brought in (eight or nine bases long), labeled (typically with fluorescent dyes or other detection means) according to the position that will be sequenced. These molecules hybridize to the target DNA sequence, next to the anchor sequence, and DNA ligase preferentially joins the molecule to the anchor when its bases match the unknown DNA sequence. Based on the fluorescence or other signal produced by the molecule, one can infer the identity of the nucleotide at this position in the unknown sequence. The oligonucleotide probes may also be constructed with cleavable linkages which can be cleaved after identifying the label. This will both remove the label and regenerate a 5' phosphate on the end of the ligated probe, preparing the system for another round of ligation.

[00255] This cycle can be repeated several times to read longer sequences. This sequences every Nth base, where N is the length of the probe left behind after cleavage. To sequence the skipped positions, the anchor and ligated oligonucleotides may be stripped off the target DNA sequence, and another round of sequencing by ligation started with an anchor one or more bases shorter. A simpler, albeit more limited, technique is to do repeated rounds of a single ligation where the label corresponds to different position in the probe, followed by stripping the anchor and ligated probe.

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Sequencing by ligation can proceed in either direction (either 5'-3' or 3'-5') depending on which end of the probe oligonucleotides are blocked by the label. The 3'-5' direction is more efficient for doing multiple cycles of ligation. Note that this is the opposite direction to polymerase based sequencing methods.

[00256] One feature unique to sequencing by ligation is the possibility of labeling the probe oligonucleotides according to various combinations of bases at more than one position. This has error detection capabilities not available to polymerase-based sequencing methods. The Applied Biosystems SOLiD sequencing system uses 2-base encoding to improve its error rates.

[00257] In some embodiments the methods of the invention provide for performing bridge PCR comprising making amplified product as described above with defined 3' and 5' ends, and further comprising the steps of exposing the amplified product to a solid substrate comprising oligonucleotide sequences attached thereto complementary to the defined 3' and 5' sequences, for example, A and B' sequences, on the amplified product in the presence of components necessary for polymerase chain reaction, and thermal cycling the system to perform bridge PCR amplification.

[00258] In some embodiments the methods of the invention provide for making amplified product as described above with defined 3' and 5' ends and further performing rolling circle amplification comprising performing the steps of: (n) hybridizing the amplified product to an oligonucleotide comprising regions complementary to A and B' sequences in close proximity; (o) optionally extending the gap with a polymerase enzyme; (p) ligating to form a circular nucleic acid comprising the amplified product, and performing rolling circle amplification by extending a primer that is complementary to a sequence in the circular nucleic acid. In some embodiments, the rolling circle amplification uses primers complementary to sequence (A), sequence (B'), or a sequence that was between sequences (A) and (B') in the amplified product. In some cases, such a primer can be an oligonucleotide attached to a solid surface, thus resulting in amplified product bound to the surface

[00259] In some embodiments the methods of the invention provide for performing PCR comprising making amplified product as described above with defined 3' and 5' ends, further comprising the steps of amplifying the amplified product using primers complementary to sequences (A) and (B), or using primers complementary to sequences (A') and (B').

[00260] In some embodiments the methods of the invention provide for performing strand displacement amplification (SDA) comprising making amplified product as described above with defined 3' and 5' ends, wherein the defined 3' and 5' ends, for example, sequences (A) and (B'), in the amplified product are designed to be cleaved by a restriction enzyme, and performing strand displacement amplification on the amplified product.

Method for generating a polynucleotide having a defined 3' and 5' sequences from a DNA target

[00261] One aspect of the invention is a method for generating a polynucleotide having a defined 3' and 5' sequences from a DNA target. The method utilizes a composite RNA/DNA oligonucleotide in order to extend the second primer such that the second primer extension products comprise a sequence (C') at its 3' end than can be used as a site for isothermal amplification in a manner such that the sequence (A) is present at or near the 5' end of the amplified product produced in this

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amplification, and where a second primer comprising sequence (B) is used, amplified products with defined sequences at both the 3' and 5' ends can be produced.

[00262] The method comprises the step: (a) denaturing a double-stranded target DNA. Double stranded DNA can be denatured, for example by heating, or by the addition of denaturing agents.

[00263] The method further comprises step (b) annealing to the target DNA and extending with a DNA polymerase comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the of the primer comprises sequence (A), which is not complementary to the target DNA; to form a plurality of first primer extension products, each with sequence (A) at its 5' end. The sequence that is complementary to the target DNA comprises a random sequence, such that the extension of the first primer results in a plurality of first primer extension products complementary to the sequences adjacent to the sequence where each random species hybridizes. The use of a random sequence at the 3' end of the primer can be useful for performing a global amplification of a target DNA, generating a plurality of sequences which together can represent, for example substantially the whole sequence of the target DNA. In some embodiments, the relative amounts of the various sequences can be used to quantitate the relative amount of a given sequence in a sample, for example to determine the number of gene copies in a DNA sample, or obtaining sequence information. In some embodiments, the extension of one first primer, will result in the release of a downstream first primer extension product. This can occur throughout the target DNA resulting in the release of multiple first primer extension products from the target DNA. This process can occur simultaneously on both of the strands of the double-stranded DNA target, thus creating first primer extension products complementary to sequences in both strands.

[00264] In some embodiments, the first primer extension step is carried out with a DNA polymerase capable of extension at elevated temperature that is not compatible with subsequent hybridization of the random sequence to the displaced primer-extension product. For example, Bst DNA polymerase can be used which is active at elevated temperature. The reaction can be carried out stepwise, first with an incubation at a lower temperature such as about 25 °C, followed by an incubation at higher temperature such as about 50 °C. In some embodiments, the first incubation is carried out below about 30 °C, and the second incubation is carried out above about 40 °C. In some embodiments, a DNA polymerase which is active at temperatures above about 45 °C is used to extend the first primer. Mixtures of DNA polymerases can also be useful.

[00265] The method further comprises step (c), extending a second primer, comprising a ligand and a 3' segment complementary to a portion of the first primer extension product, to produce a double stranded product with a DNA/RNA heteroduplex at one end; wherein the double stranded product comprises a second primer extension product hybridized to the first primer extension product, and wherein a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer. In this step, a second primer comprising a ligand and a 3' DNA region that comprises a random sequence is used. The primer is optionally a tailed primer comprising a nucleic acid sequence (B) that is 5' of the random sequence, to form a plurality of double-stranded products each comprising a first primer extension product and a second primer extension product whereby the second primer extension products comprise a ligand.

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This step may be carried out with or without prior denaturation. If carried out without denaturation, generally, only the single stranded displaced first primer extension products will hybridize to the second primer. Generally the second primer does not comprise RNA. The extension of the second primer is carried out with a DNA polymerase as described herein. The second primer can be a tailed primer having a 3' portion which is complementary to the first primer extension products, and a 5' portion, sequence (B), which is not complementary to the first primer extension products. The second primer comprises a random primer sequence that randomly binds to the first primer extension products. Extension of the second primer comprising a random sequence produces a plurality of second primer extension products. The use of a random sequence at the 3' end of the primer is useful, for example, in performing global amplification of a target DNA, whereby a plurality of second primer extension products are produced which is representative of the sequence of the target DNA. In some embodiments, for example where the first primer is designed to hybridize to a specific sequence on a target RNA, or a sequence common to a family of RNA targets, random priming by the second primer ensures amplification of the entire selected target or family of selected targets. In this embodiment, the second primer extension products comprise sequences which are the same or substantially the same as the sequences in the target DNA. The second primer comprises a ligand that is a member of a ligand-receptor pair. In some embodiments, the ligand is attached to the primer at the 5' end of the primer. In some embodiments, the ligand is a small molecule, such as biotin or digoxigenin. In some embodiments, the receptor is an antibody, and the ligand is a molecule or portion of a molecule recognized by the antibody.

[00266] The second primer extension products are extended such that the 3' portion of the second primer extension product comprises a sequence (A') which is complementary to sequence (A) of the first composite primer. Since sequence (A) on the first primer extension products comprise RNA, both DNA dependent DNA polymerase activity and RNA dependent DNA polymerase activity are used in step (c). The primer extension results in a products that are at least partially double stranded. The method produces a nucleic acid that comprises a ligand allowing it to be bound to a solid surface and that has a specific sequence (A') at its 3' end. The specific, or universal, sequence (A') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. As described above, in some embodiments, the nucleic acid attached to the ligand also comprises sequence (B) at or near its 5' end.

[00267] The method further comprises step (d) cleaving the RNA from the first primer extension products such that a portion of the second primer extension products that are complementary to sequence (A) is single stranded. The cleaving of RNA can be performed, for example by treatment with RNase H, which will selectively cleave the RNA portion of the DNA/RNA partial heteroduplex formed in step (c).

[00268] The method further comprises step (e) annealing to the second primer extension products an oligonucleotide comprising a 3'- DNA segment that is complementary to sequence (A') and a 5' RNA segment comprising sequence (C). The oligonucleotide comprises at least one DNA and at least one RNA portion. In some embodiments the 5' DNA segment is complementary to all of sequence (A'), in other embodiments, the 5' DNA segment is complementary to portion of sequence

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(A'). In some embodiments, 5' RNA segment comprising sequence (C) is partly complementary to sequence (A').

[00269] The method optionally comprises step: (f) extending the oligonucleotide to form a plurality of oligonucleotide extension products hybridized to the second primer extension products. In some embodiments, the oligonucleotide is optionally extended from its 3' end to produce a plurality of oligonucleotide extension products hybridized to the second primer extension product and displacing the DNA portion of the first primer extension product. In some embodiments, the second primer comprises a sequence (B), such that the oligonucleotide extension products will comprise a sequence (B') at or near their 3' ends that are complementary to sequence (B).

[00270] The method further comprises step (g) extending the second primer extension products to create a heteroduplex such that the second primer comprises a DNA sequence (C') that is complementary to sequence (C). The DNA sequence (C') is created by a DNA polymerase that has RNA dependent DNA polymerase activity. This step creates an RNA/DNA heteroduplex region that can be used for further manipulation of the second primer extension products.

[00271] The method further comprises step (h) cleaving the RNA from the heteroduplex created in step (g) to produce a single-stranded portion of the second primer extension products corresponding to sequence (C'). The RNA can be cleaved from the heteroduplex, for example, by RNase H.

[00272] In some embodiments, The method further comprises step (i) binding the ligand on the second primer extension products to a solid surface. The binding step can be performed at various stages or steps during the procedure depending, for example, on whether it is advantageous to carry out the subsequent steps on a solid surface. The binding step is generally performed after step (c). In some embodiments, the binding the ligand to the solid surface is performed before step (h). In some embodiments, the binding the ligand to the solid surface is performed after step (h). The receptor bound to the solid surface is a member of the ligand-receptor pair such that binding of the ligand results in attaching the second primer extension products to the solid surface. In some embodiments, the second primer extension products are still hybridized to the first primer extension products when they are attached to the solid surface. In some embodiments, the first primer extension products are removed from the second primer extension products such that a single stranded polynucleotide is attached to the solid surface. The method produces polynucleotides that are bound to a solid surface that have a specific sequence (A') and (C') at their 3' ends. The specific sequence (C') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. As described above, in some embodiments, the nucleic acid bound to the bead also comprises sequence (B) at or near its 5' end. One aspect of the invention comprises amplification of the nucleic acid bound to the bead. In some embodiments, the amplification is carried out using isothermal amplification using a composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (C') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification. When the sequence (C') acts as a site to which a composite amplification primer binds, the amplified products that are produced have the sequence (A) (and a portion of sequence (C) at their 5' ends)). Where the second primer comprises the sequence (B), the amplified products also have the sequence

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(B'), complementary to (B) at or near their 3' ends. Thus the method produced amplified products with defined sequences at or near both its 3' and 5' ends.

[00273] Due to the use of random priming, a plurality of different nucleic acids bound to a solid surface is created in which each of the nucleic acids has a specific sequence (A') and (C') at its 3' end (and in some embodiments also a specific sequence (B) at its 5' end), and where the different nucleic acids have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target DNA. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target DNA.

[00274] The solid surface can be any of a variety of surfaces, some described in more detail below. The solid surface can be, for example a planar surface, for example, a planar array. In some embodiments the solid surface comprises a plurality of beads. In some embodiments the beads are magnetic.

[00275] The step of binding the polynucleotides to the solid surface through the ligand, step (i), can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times. This allows, for example, for the analysis of an individuals genes, or a portion of the individual genes at one time, then allows for archiving the genetic material at a later date by the same or by different tests.

[00276] In some embodiments, the method further comprises treating the solid surface with reagents to produce multiple copies of amplification products that are substantially complementary to the second primer extension products. This step comprises carrying out an amplification reaction wherein the bound nucleic acid acts as a template for the amplification. Generally, the amplification is carried out using the sequence (C') on the second primer extension products as for primer hybridization. In some embodiments, the amplification produces single stranded amplified products. In some embodiments, the amplification provides double stranded products. In some embodiments, the second primer comprises a specific sequence (B), which becomes incorporated into the second primer extension products. In some embodiments the amplification is an isothermal amplification reaction comprising a composite RNA/DNA primer, RNase H, and a DNA polymerase with strand displacement activity. In some embodiments, the amplification is carried out using polymerase chain reaction, (PCR). For example where the second primer extension products comprises both as sequence (B) at or near its 5' end and a sequence (C') at or near its 3' end, a set of primers, one designed to hybridize to all or a portion of the sequence (C') and the other designed to hybridize to sequence (B'), the complement of sequence (B), can be used to carry out a PCR reaction to exponentially produce double stranded amplified products.

[00277] In some embodiments, the amplification is performed by a method comprising the following steps: (j) annealing an amplification primer, wherein the amplification primer has a DNA portion and a 5' RNA portion, to the single stranded portion of the second primer extension products complementary to sequence (C'); (k) extending the amplification primer with an enzyme having

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strand displacement activity to produce an amplified products; (l) cleaving the RNA from the amplified products; and (m) repeating steps (j) to (l) to produce multiple copies of amplified products wherein the 5' portion of the amplified products have a sequence complementary to sequence (A'). Where the second primer further comprises a segment (B) that is not complementary to the first primer extension products sequence, this amplification method, utilizing sequence (C'), allows for the production of amplified products comprises a sequence (B') at or near their 3' ends that is substantially complementary to sequence (B), and a sequence (A) near their 5' end that are complementary to sequence (A'), thus producing an amplified polynucleotide products with defined 3' and 5' ends.

[00278] In some embodiments the amplification is carried out such that the amplified products are not attached to the substrate, but is freely dissolved in the solution. In other embodiments, the amplification is carried out such that the amplified products remains bound to the substrate, for example by performing solid phase PCR such as bridge PCR. In yet other embodiments, amplified products are generated that may float freely in solution, but which comprise a sequence, for example sequence (A) or sequence (B'), that allows them to be captured to another solid surface or other portion of the solid surface by hybridization to a complementary sequence bound to such surface (e.g. sequence (A') or sequence (B). In some embodiments, the amplified product is a single-stranded product and, because it is generated at the solid surface, the amplified product readily captured by complementary sequences, e.g. sequence (B), bound to the surface.

[00279] In one aspect of the invention, a plurality of beads is used, and the methods described above are carried out such that on average, one or fewer second primer extension product molecules are bound per bead. The beads are dispersed into an aqueous solution, and a plurality of microreactors, e.g. droplets, are produced such that on average one or fewer beads is contained within each of the plurality of microreactors. The amplification of the second primer extension products bound to the beads is then carried out such that the clonal amplification of each of the plurality of second primer extension products in the separate microreactors is achieved. This clonal amplification in microreactors can be performed on a sample of target DNA, such as genomic DNA, wherein the plurality of second primer extension products comprise sequences that correspond to most, to substantially all, or to all of the sequences in the target DNA. In some embodiments, the amplified products are captured by bead having attached thereto a plurality of oligonucleotides comprising complementary sequences bound to such surface (e.g. sequence (A') or sequence (B)), which are complementary to sequence (A) or sequence (B') on the amplified products.

[00280] In some embodiments, the plurality of beads, produced as described above, with each bead comprising a single second primer extension product can comprise a library. The library can represent, for example, the genomic DNA of an individual, or the genomic DNA from a group of cells or from a single cell. These libraries can be stored, then later clonally amplified. In some embodiments, a library of beads can comprise a plurality of beads wherein each bead had multiple copies of a single amplification product generated from a second primer extension product. These libraries can be analyzed, for example by sequencing. The libraries can be stored, and later analyzed. In some embodiments the libraries can be stored, then analyzed multiple times.

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[00281] In some embodiments, a bead or isolated area of the solid surface comprises covalently attached thereto multiple oligonucleotides comprising the sequence (B) at their 3' ends, whereby upon the amplification of step (m) multiple copies of amplified product comprising sequence (B') at their 5' end are hybridized to the bead or isolated area. For example, where beads are used, a plurality of beads in a plurality of microreactors wherein, the plurality of beads has, on average one or fewer second primer extension products bound to it and there are, on average, one or fewer beads in each microreactor, a clonal amplification of the plurality of second primer extension products can be carried out, and the amplified products in each of the microreactors will bind to the bead through the sequence (B') on the amplified product to the sequence (B) on the beads. This approach produces a plurality of beads, each with multiple copies of a different sequence bound to it. Where these sequences are representative of the target DNA, the plurality of beads can constitute a library representative of such DNA.

[00282] After the amplified products are bound to the beads by hybridization, the (B) sequences on the beads can be extended to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product and also comprise sequence (A') near their 5' ends. Where the (B) sequences are covalently attached to the beads, this method provides for the production of beads with polynucleotides complementary to amplified product covalently attached to the beads. Covalently attached polynucleotides such as those produce here are more robust than nucleotides that are attached only by hybridization to the beads. Thus, the covalently attached polynucleotides can be more stable and can be used with analysis methods and sequencing methods that have harsher conditions which would result in the displacement of polynucleotides bound only by hybridization.

[00283] In some embodiments, the amplified product is removed from the covalently bound polynucleotide to render the polynucleotide single stranded. Such single stranded covalently bound polynucleotides comprise a specific sequence at their 3' ends comprising sequence (A') and a portion of sequence (C'). This specific sequence at the 3' end of the covalently bound polynucleotide can act as a hybridization site for a primer complementary to sequence (A) that can act as a primer to carry out sequencing by any of a variety of sequencing methods, for example, those described herein. The single stranded covalently bound polynucleotides derived from DNA can be sequenced as described above for the single stranded covalently bound polynucleotides derived from DNA, such as pyrosequencing, cycle sequencing, isothermal sequencing and other methods such as those described below.

[00284] The sequencing methods can comprise the use of cleavable labeled terminators. The sequencing method can comprise pyrophosphate detection. The sequencing method can comprise an isothermal sequencing method, for example using chimeric primers, RNase H, and a polymerase with strand displacement activity. The sequencing method can also comprise cycle sequencing.

[00285] In some embodiments the methods of the invention provide for performing bridge PCR comprising making amplified product as described above with defined 3' and 5' ends, and further comprising the steps of exposing the amplified product to a solid substrate comprising oligonucleotide sequences attached thereto complementary to the defined 3' and 5' sequences, for example, A and B' sequences, on the amplified product in the presence of components necessary for

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polymerase chain reaction, and thermal cycling the system to perform bridge PCR amplification. The bridge PCR can be carried out such that isolated lawns of amplified product are obtained wherein each lawn comprises polynucleotides having sequences representative of a portion of the sequence of the target DNA.

[00286] In some embodiments the methods of the invention provide for making amplified product as described above with defined 3' and 5' ends and further performing rolling circle amplification comprising performing the steps of: (n) hybridizing the amplified product to a target DNA comprising regions complementary to A and B' sequences in close proximity; (o) optionally extending the gap with a polymerase enzyme; (p) ligating to form a circular nucleic acid comprising the amplified product, and performing rolling circle amplification by extending a primer that is complementary to a sequence in the circular nucleic acid. In some embodiments, the rolling circle amplification uses primers complementary to sequence (A), sequence (B'), or a sequence that was between sequences (A) and (B') in the amplified product. In some cases, such a primer can be an oligonucleotide attached to a solid surface, thus resulting in amplified product bound to the surface

[00287] In some embodiments the methods of the invention provide for performing PCR comprising making amplified product as described above with defined 3' and 5' ends, further comprising the steps of amplifying the amplified product using primers complementary to sequences (A) and (B), or using primers complementary to sequences (A') and (B').

[00288] In some embodiments the methods of the invention provide for performing strand displacement amplification (SDA) comprising making amplified product as described above with defined 3' and 5' ends, wherein the defined 3' and 5' ends, for example, sequences (A) and (B'), in the amplified product are designed to be cleaved by a restriction enzyme, and performing strand displacement amplification on the amplified product.

[00289] A schematic exemplary of an embodiment of the invention relating to generating a polynucleotide having a defined 3' and 5' sequences is shown in Figures 5A and 5B. The starting material in 5A can be generated as described in the methods herein. The starting material can be generated, for example, from the methods above from a target RNA or a target DNA. While the starting material is described as, for example first primer extension product, it will be understood that a complex of the same structure as shown and described can be used in the method described here.

[00290] In Figure 5A, step I shows the step of: cleaving the RNA from the first primer extension product in the DNA-RNA heteroduplex such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded. As shown, the cleavage is performed using RNase H. Chemical and thermal means can alternatively be employed. Step II illustrates annealing to the second primer extension product an oligonucleotide comprising a 3'- DNA segment that is complementary to sequence (A') and a 5' RNA segment comprising sequence (C). Step III illustrates extending the second primer extension product to create a DNA-RNA heteroduplex such that the second primer comprises a DNA sequence (C') that is complementary to sequence (C). Step IV illustrates the optional step of extending the oligonucleotide to form an oligonucleotide extension product hybridized to the second primer extension product. Step V illustrates binding the ligand to a solid surface resulting in binding of the second primer extension product to the solid surface. While

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the step of binding the ligand to the solid surface is shown at step V is to be understood that the binding step can be performed at many steps in the process. For example, in this case, the binding step could alternately have been carried out before step I , or could be carried out after amplification.

[00291] In Figure 5B, step VI illustrates cleaving the RNA from the DNA-RNA heteroduplex created in step III to produce a single-stranded portion of the second primer extension product corresponding to sequence (C'). Step VII illustrates annealing an amplification primer, wherein the amplification primer has a DNA portion and a 5' RNA portion, to the single stranded portion of the second primer extension product complementary to sequence (C'). Step VIII illustrates extending the amplification primer with a DNA polymerase having strand displacement activity to produce an amplified product. Step IX illustrates the step of cleaving the RNA from the extended hybridized amplification primer in the DNA-RNA heteroduplex. The product of step IX is now capable of hybridizing another amplification primer, allowing for steps VII to IX to occur again, resulting in the generation of another amplified product. These steps can thus be repeated to produce multiple copies of amplified product wherein the 5' portion of the amplified product has a sequence complementary to sequence (A'). In the embodiment illustrated in Figures 5A and 5B the second primer comprised a sequence (B), and as such, the amplified product shown in Figure 5B comprises a defined sequences on both the 5' and 3' ends.

[00292] A schematic exemplary of an embodiment of the invention relating to the capture of amplification product on a solid surface is shown in Figure 6. In some embodiments, the amplified product can be captured onto a surface by hybridization to the defined sequences on the 5' and/or 3' ends of the amplified product. This approach can be useful for example, where a clonal amplification of a given sequence or plurality of sequences that are representative of a target RNA or target DNA produces multiple copies of a specific sequence having defined 5' and 3' ends. The clonal amplification can be carried out in an isolated volume, or on in an isolated area on a surface. In some embodiments, the clonal amplification is carried out with beads in droplets of an inverse emulsion, wherein each droplet has on average one or fewer beads. In some embodiments, it is useful to capture the amplified product on a solid surface, for example a bead. Figure 6 illustrates how a solid surface, for example the surface of a bead can have multiple oligonucleotides attached thereto. In the embodiment illustrated, the amplified product has a defined sequence (B') at its 3' end and a defined sequence (A) near its 5' end. The bead has multiple oligonucleotides attached to its surface comprising the sequence (B), which is complementary to the sequence (B') at the 3' end of an amplified product. The bead can capture the amplified product as shown by hybridization under the right conditions. The capture of amplified product is illustrated in step I. This type of bead can be used as a library, and can be used for sequencing, for example, by extending from sequence (B). Figure 6 also illustrates in step II that the (B) sequences on the bead can be extended along the amplified product to produce a polynucleotide attached to the bead which has sequence (A') at or near its 3' end and sequence (B) at its 5' end. Such a polynucleotide can be made single stranded, for example by heat or chemical denaturation, and washing as illustrated in step III. The bead with single stranded polynucleotide which has sequence (A') at or near its 3' end and sequence (B) at its 5' can be more stable than a bead to which a polynucleotide is held by hybridization alone. The polynucleotide attached to the bead can be covalently bound. The beads made in this fashion

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can be used to create libraries with long storage life. The single stranded product of step III can be used for archiving, storage, and a variety of manipulations made possible by the defined sequences at the 5' and 3' ends. For example, as shown in Figure 6, a sequencing primer (A) can be hybridized to sequence (A') to allow the sequencing of the polynucleotide attached to the bead by methods such as those described herein.

[00293] Figure 7 illustrates that the amplified product with a defined sequence (B') at its 3' end and a defined sequence (A) near its 5' end can also be captured onto surfaces such as beads by hybridization to an oligonucleotide attached to the solid surface that comprises the sequence (A'). In some cases, the capture is by specific nucleic acid hybridization of sequence (A) of the amplified product to the complementary sequence (A'). In other cases, the oligonucleotide attached to the surface of the solid surface may not be a perfect complement of at least a portion of the 5' end of the amplified product. For example, the oligonucleotide may differ by about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, or more nucleotides from the perfect complement of sequence (A). The oligonucleotide may be attached to the solid surface by any method known in the art. For example, the oligonucleotide may be attached via non-covalent interaction such as by ionic, hydrogen, or hydrophobic interaction or a combination thereof. In some cases, the oligonucleotide may be attached by one or more covalent bonds between the oligonucleotide and the solid surface. In some cases, the oligonucleotide may be attached by interaction between a receptor ligand pair such as for example an avidin molecule on the surface and a biotin molecule on the oligonucleotide, or any receptor ligand pair known in the art.

[00294] Figure 8 illustrates that the amplified products with defined 3' and 5' sequences can be used for gap ligation to create a circular polynucleotide which can then be amplified and characterized using rolling circle amplification. In Figure 8, the amplified product with a defined sequence (B') at its 3' end and a defined sequence (A) near its 5' end is hybridized to a sequence D with sequences complementary to sequence (A) and sequence (B') (with B' hybridizes upstream from (A), wherein the hybridization of sequences (A) and (B') leave a gap. The gap is closed with a ligase and alternatively with a polymerase for larger gaps. The circular polynucleotide, e.g. DNA, can then be amplified and characterized using rolling circle amplification. Primers for rolling circle amplification can hybridize (3) to sequence (A), (2) to sequence (B'), or (1) to the sequence between (A) and (B') on the amplified product. These methods can be used for the accurate determination of mutations such as single nucleotide polymorphisms (SNPs).

Alternative method for generating a polynucleotide having a defined 3' and 5' sequences from an RNA target

[00295] One aspect of the present invention is an alternative method for generating a polynucleotide having a defined 3' and 5' sequences from an RNA target. Unlike the methods described in detail above, this method generally does not use a composite RNA/DNA primer as the first primer. Here, a tailed primer, usually not comprising RNA, composed, for example, of DNA is used to create the first primer extension product. In this method, the second primer comprises a composite RNA/DNA primer, and the third primer comprises a ligand for binding the third primer extension product to a solid surface.

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[00296] The method comprises step: (a) extending a first primer comprising a 3' portion complementary to a target RNA and a 5' portion, sequence (D), not complementary to the target RNA, to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid. The first primer generally does not comprise RNA, and may be all DNA. The first primer is a tailed primer comprising a 5' portion, sequence (D) which is generally not complementary to the target RNA, and does not hybridize to the target RNA. In some embodiments, the 3' portion of the primer that is complementary to the target RNA is a specific sequence. For example, where a specific region of interest of a target RNA that is known or suspected to be upstream of a specific sequence on the target RNA, the sequence that is complementary to the target RNA of the first primer can be designed to hybridize to this specific sequence on the target RNA such that extension of the primer results in producing a first primer extension product that is complementary to such upstream region. The specific sequence may be common to a family of target RNA. A combination of primers with various specific sequences at the 3' end can also be useful. In some embodiments, such as where the target RNA comprises mRNA, and the mRNA comprises a plurality of sequences, each having a 3' poly-A segment; the specific sequence that is complementary to the target RNA can comprise a sequence that will hybridize to the poly-A region of the mRNA, thus allowing the extension of the first primer to produce a plurality of first primer extension products, each of which is complementary to the region of an mRNA molecule adjacent to the poly-A region. In some embodiments, the sequence that is complementary to the target RNA comprises a random sequence, such that the extension of the first primer results in a plurality of first primer extension products complementary to the sequences adjacent to the sequence where each random species hybridizes. The use of a random sequence at the 3' end of the primer can be useful for performing a global amplification of a RNA target, generating a plurality of sequences which together can represent, for example substantially the whole sequence of the target RNA. In some embodiments, the relative amounts of the various sequences can be used to quantitate the relative amount of a given sequence in a sample, for example to determine the level of expression in an mRNA sample. In some embodiments more than one type of sequence that is complementary to the target RNA can be used, for instance both a primer with a random sequence and a primer, or combination of primers with a specific sequence complementary to RNA can be used. In some embodiments, multiple primers comprising different specific sequences can be used.

[00297] The method further comprises step (b) cleaving the target RNA from the RNA/DNA hybrid. In some cases the cleaving can be accomplished by denaturing the complex comprising the first primer extension product and the nucleic acid. Denaturation can be performed, for example by heating the sample, or by adding a denaturing agent, or using a combination of heating the sample and adding denaturing agents. The cleaving can be accomplished with an enzyme that cleaves RNA from an RNA/DNA hybrid such as RNase H, or a combination of RNase enzymes, or chemically. In some embodiments, the target RNA is completely cleaved. In other embodiments, the target RNA is only partly cleaved or degraded. The amount of cleaving required is that amount which will allow the extension of the second primer.

[00298] The method further comprises step (c) extending a second primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to the first

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primer extension product and a 5' portion, sequence (E), of the of the second primer is not complementary to the first primer extension product, to produce a double-stranded DNA product comprising the first primer extension product hybridized to a second primer extension product, whereby the second primer extension product has a sequence (D') that is complementary to sequence (D) at its 3' end. The extension of the second primer is carried out with a DNA polymerase as described herein. The second primer is a composite RNA/DNA primer having a 3' portion which is complementary to the first primer extension product, and a 5' portion, sequence (E), which is not complementary to the first primer extension product. In some embodiments, the second primer can comprise a specific primer sequence that is designed to hybridize to a specific sequence in the first primer extension product. In some embodiments the second primer comprises a random primer sequence that randomly binds to the first primer extension product. Extension of the second primer comprising a random sequence produces a plurality of second primer extension products. The use of a random sequence at the 3' end of the primer is useful, for example, in performing global amplification of a target RNA, whereby a plurality of second primer extension products are produced which is representative of the sequence of the target RNA. In some embodiments, for example where the first primer is designed to hybridize to a specific sequence on a target RNA, or a sequence common to a family of RNA targets, random priming by the second primer ensures amplification of the entire selected target or family of selected targets. In this embodiment, the second primer extension products comprise sequences which are the same or substantially the same as the sequences in the target RNA (sense copies).

[00299] The second primer extension product is extended such that the 3' portion of the second primer extension product comprises a sequence (D') which is complementary to sequence (D) of the first primer. The primer extension results in a product that is at least partially double stranded.

[00300] In some embodiments, the sample comprising the target RNA is in a sample that also comprises DNA. In such cases, it can be advantageous to add a selective DNA dependent DNA polymerase inhibitor such as actinomycin such that it is present during step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a). The presence of a DNA dependent DNA polymerase inhibitor such as actinomycin is particularly advantageous when a first primer comprising a random sequence is used, as the inhibitor allows for the selective creation of first primer extension products to RNA without the need of separating the RNA from the DNA. This is also advantageous when the priming is carried out at specific target sequences since the sequence may be the same on the DNA when the DNA and RNA in the sample represent total nucleic acid from the same biological entity, for example, human tissue, animal tissue, and the like. The use of DNA dependent DNA polymerase inhibitors such as actinomycin is described in copending application.

[00301] The method further comprises step (d) denaturing the double-stranded DNA product. Double stranded DNA can be denatured, for example by heating, or by the addition of denaturing agents.

[00302] The method further comprises step (e) annealing to the second primer extension product and extending a third primer comprising, from its 5' end, a ligand, optionally a sequence (F), and a sequence (D), wherein sequence (D) is complementary to sequence (D') on the second primer

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extension product to produce a double-stranded DNA product comprising the second primer extension product hybridized to a third primer extension product, whereby the third primer extension product comprises a sequence (E') at its 3' end complementary to sequence (E) in a DNA-RNA heteroduplex. The third primer comprises a ligand that is a member of a ligand-receptor pair. In some embodiments, the ligand is attached to the primer at the 5' end of the primer. In some embodiments, the ligand is a small molecule, such as biotin or digoxigenin. In some embodiments, the receptor is an antibody, and the ligand is a molecule or portion of a molecule recognized by the antibody. The method produces a nucleic acid that comprises a ligand allowing it to be bound to a solid surface and that has a specific sequence (E') at its 3' end. The specific sequence (E') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. As described above, the nucleic acid attached to the ligand also comprises sequence (D') at or near its 5' end, and in some embodiments comprises the sequence (F').

[00303] In some embodiments, the method further comprises the step of binding the ligand to a solid surface, whereby the third primer extension product is bound to the solid surface.

[00304] In some embodiments, the method further comprises the steps of: (f) cleaving the RNA portion of the second primer extension product in the DNA-RNA heteroduplex, whereby sequence (E') of the third primer extension product is single stranded, (g) annealing an oligonucleotide comprising a 3' DNA segment (E) that is complementary to sequence (E') and a 5' RNA segment comprising sequence (G), (h) extending the third primer extension product to produce a sequence (G') at its 3' end complementary to sequence (G), and (i) cleaving the RNA from the heteroduplex created in step (h) to produce a single-stranded portion of the third primer extension product corresponding to sequence (G'). The cleaving of RNA in steps (f) and (i) can be performed, for example by treatment with RNase H, which will selectively cleave the RNA portion of the DNA/RNA partial heteroduplex formed in step (e). The oligonucleotide comprises at least one DNA and at least one RNA portion. In some embodiments the 3' DNA segment is complementary to all of sequence (E'), in other embodiments, the 3' DNA segment is complementary to portion of sequence (E'). In some embodiments, 5' RNA segment comprising sequence (G) is partly complementary to sequence (E'). In some embodiments, the oligonucleotide is optionally extended from its 3' end to produce an oligonucleotide extension product hybridized to the third primer extension product and displacing the DNA portion of the second primer extension product. In some embodiments, the third primer comprises a sequence (F), such that the oligonucleotide extension product will comprise a sequence (F') at or near its 3' end that is complementary to sequence (F). The DNA sequence (G') is created by a DNA polymerase that has RNA dependent DNA polymerase activity. This step creates an RNA/DNA heteroduplex region that can be used for further manipulation of the second primer extension product.

[00305] In some embodiments the method further comprises binding the ligand to a solid surface, whereby the third primer extension product comprising sequence (G') is bound to the solid surface. The solid surface can be any of a variety of surfaces, some described in more detail below. The solid surface can be, for example a planar surface, for example, a planar array. In some embodiments the solid surface comprises a plurality of beads. In some embodiments the beads are magnetic. The receptor bound to the solid surface is a member of the ligand-receptor pair such that

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binding of the ligand results in attaching the second primer extension product to the solid surface. In some embodiments, the second primer extension product is still hybridized to the first primer extension product when it is attached to the solid surface. In some embodiments, the second primer extension product is removed from the third primer extension product such that a single stranded polynucleotide is attached to the solid surface. The method produces a nucleic acid that is bound to a solid surface that has a specific sequence (E') and (G') at its 3' end. The specific sequence (G') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. As described above, in some embodiments, the nucleic acid bound to the bead also comprises sequence (F) at or near its 5' end. One aspect of the invention comprises amplification of the nucleic acid bound to the bead. In some embodiments, the amplification is carried out using isothermal amplification using a composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (G') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification. When the sequence (G') acts as a site to which a composite amplification primer binds, the amplified product that is produced has the sequence (E) (and a portion of sequence (G) at its 5' end. The amplified product has a sequence (D') at or near its 3' end. Where the third primer comprises the sequence (F), the amplified product also has the sequence (F'), complementary to (F) at or near its 3' end. Thus the method produced amplified product with defined sequences at or near both its 3' and 5' ends.

[00306] In some embodiments, for example where random sequences at the 3' end of the first and/or second primer are used, a plurality of different nucleic acids bound to a solid surface is created in which each of the nucleic acids has a specific sequence (E') and (G') at its 3' end, the sequence (D) at or near its 5' end (and in some embodiments also a specific sequence (F) at its 5' end), and where the different nucleic acids have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target RNA. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target RNA.

[00307] In some embodiments, the method further comprises treating the solid surface with reagents to produce multiple copies of an amplification product that are substantially complementary the second primer extension product. This step comprises carrying out an amplification reaction wherein the bound nucleic acid acts as a template for the amplification. Generally, the amplification is carried out using the sequence (G') on the third primer extension product for the hybridization of primer. In some embodiments, the amplification produces single stranded amplified product, In some embodiments, the amplification provides double stranded product. The third primer extension product comprises the specific sequence (D). In some embodiments, the third primer comprises a specific sequence (F), which thus becomes incorporated into the third primer extension product. In some embodiments the amplification is an isothermal amplification reaction comprising a composite RNA/DNA primer, RNase H, and a DNA polymerase with strand displacement activity. In some embodiments, the amplification is carried out using polymerase chain reaction, (PCR). For example where the third primer extension product comprises both as sequence (F) at or near its 5' end and a sequence (G') at or near its 3' end, a set of primers, one designed to hybridize to all or a portion of

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the sequence (G') and the other designed to hybridize to sequence (F'), complementary to sequence (F), and/or to sequence (D') to exponentially produce double stranded amplified product.

[00308] One aspect of the invention is a method of amplifying a sequence representative of a sequence within a target RNA comprising the above steps and further comprising: (j) annealing an amplification primer, wherein the amplification primer has a DNA portion and a 5' RNA portion, to the single stranded portion of the third primer extension product complementary to sequence (G'); (k) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product; (l) cleaving the RNA from the amplified product; and (m) repeating steps (j) to (l) to produce multiple copies of amplified product wherein the 5' portion of the amplified product has a sequence (E) complementary to sequence (E') and the 3' end of the amplified product has sequence (D') complementary to sequence (D) and optionally sequence (F') complementary to sequence (F).

[00309] The step of binding the polynucleotides to the solid surface through the ligand can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

[00310] In some embodiments the amplification is carried out such that the amplified product is not attached to the substrate, but is freely dissolved in the solution. In other embodiments, the amplification is carried out such that the amplified product remains bound to the substrate, for example by performing solid phase PCR such as bridge PCR. In yet other embodiments, an amplified product is generated that may float freely in solution, but which comprises a sequence, for example sequence (E) or sequence (D'), that allows it to be captured to another solid surface or other portion of the solid surface by hybridization to a complementary sequence bound to such surface (e.g. sequence (E') or sequence (D). In some embodiments, the amplified product is a single-stranded product and, because it is generated at the solid surface, the amplified product readily captured by complementary sequences, e.g. sequence (B), bound to the surface.

[00311] In one aspect of the invention, a plurality of beads is used, and the methods described above are carried out such that on average, one or fewer third primer extension product molecules are bound per bead. The beads are dispersed into an aqueous solution, and a plurality of microreactors, e.g. droplets, are produced such that on average one or fewer beads is contained within each of the plurality of microreactors. The amplification of the third primer extension products bound to the beads is then carried out such that the clonal amplification of a plurality of third primer extension products is achieved. This clonal amplification in microreactors can be performed on a sample of target RNA, such as whole transcriptome or total RNA, wherein the plurality of third primer extension products comprise sequences that correspond to most, to substantially all, or to all of the sequences in the target RNA. In some embodiments, the amplified products are captured by bead having attached thereto a plurality of oligonucleotides comprising complementary sequences bound to such surface (e.g. sequence (E') or sequence (D)), which are complementary to sequence (E) or sequence (D') on the amplified product.

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[00312] In some embodiments, the plurality of beads, produced as described above, with each bead comprising a single third primer extension product can comprise a library. These libraries can be stored, then later clonally amplified. In some embodiments, a library of beads can comprise a plurality of beads wherein each bead had multiple copies of a single amplification product generated from a third primer extension product. These libraries can be analyzed, for example by sequencing. The libraries can be stored, and later analyzed. In some embodiments the libraries can be stored, then analyzed multiple times.

[00313] In some embodiments, a bead or isolated area of the solid surface comprises covalently attached thereto multiple oligonucleotides comprising the sequence (D) (or F) at their 3' ends, whereby upon the amplification of step (m) multiple copies of amplified product comprising sequence (D') (or F') at their 5' end are hybridized to the bead or isolated area. For example, where beads are used, a plurality of beads in a plurality of microreactors wherein, the plurality of beads has, on average one or fewer third primer extension products bound to it and there are, on average, one or fewer beads in each microreactor, a clonal amplification of the plurality of third primer extension products can be carried out, and the amplified products in each of the microreactors will bind to the bead through the sequence (D') (and/or F') on the amplified product to the sequence (D) (and/or F) on the beads. This approach produces a plurality of beads, each with multiple copies of a different sequence bound to it. Where these sequences are representative of the target RNA, the plurality of beads can constitute a library representative of such RNA.

[00314] After the amplified products are bound to the beads by hybridization, the (D) sequences on the beads can be extended to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product and also comprise sequence (E') near their 5' ends. Where the (D) (and or F) sequences are covalently attached to the beads, this method provides for the production of beads with polynucleotides complementary to amplified product covalently attached to the beads. Covalently attached polynucleotides such as those produce here are more robust than nucleotides that are attached only by hybridization to the beads. Thus, the covalently attached polynucleotides can be more stable and can be used with analysis methods and sequencing methods that have harsher conditions which would result in the displacement of polynucleotides bound only by hybridization.

[00315] In some embodiments, the amplified product is removed from the covalently bound polynucleotide to render the polynucleotide single stranded. Such single stranded covalently bound polynucleotides comprise a specific sequence at their 3' ends comprising sequence (E') and a portion of sequence (G'). This specific sequence at the 3' end of the covalently bound polynucleotide can act as a hybridization site for a primer complementary to sequence (E) that can act as a primer to carry out sequencing by any of a variety of sequencing methods, for example, those described herein.

[00316] The sequencing methods can comprise the use of cleavable labeled terminators. The sequencing method can comprise pyrophosphate detection. The sequencing method can comprise an isothermal sequencing method, for example using chimeric primers, RNase H, and a polymerase with strand displacement activity. The sequencing method can also comprise cycle sequencing.

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[00317] The amplified products with defined 3' and 5' ends can be used in the methods described herein for the amplified products produced by the other methods. They can be used, for example, for bridge PCR, rolling circle amplification, and strand displacement amplification.

[00318] A schematic exemplary of an embodiment of the invention relating to an alternative method for generating a polynucleotide having a defined 3' and 5' sequences from an RNA target is shown in Figure 9. Step I illustrates the step of extending a DNA first primer comprising a 3' portion complementary to a target RNA and a 5' portion, sequence (D), not complementary to the target RNA, to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid. Step II illustrates cleaving the target RNA from the RNA/DNA hybrid, and hybridizing the second primer. Step III illustrates extending a second primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to the first primer extension product and a 5' portion, sequence (E), of the of the second primer is not complementary to the first primer extension product, to produce a double-stranded DNA product comprising the first primer extension product hybridized to a second primer extension product, whereby the second primer extension product has a sequence (D') that is complementary to sequence (D) at its 3' end. Step IV illustrates denaturing the double-stranded DNA product. Step V illustrates annealing to the second primer extension product and extending a third primer comprising, from its 5' end, a ligand, optionally a sequence (F), and a sequence (D), wherein sequence (D) is complementary to sequence (D') on the second primer extension product to produce a double-stranded DNA product comprising the second primer extension product hybridized to a third primer extension product, whereby the third primer extension product comprises a sequence (E') at its 3' end complementary to sequence (E). The products of steps V and VI may be useful for SPIA amplification of a target RNA sequence or its complement.

[00319] Step VI illustrates the step of cleaving the RNA portion of the second primer extension product in the DNA-RNA heteroduplex, whereby sequence (E') of the third primer extension product is single stranded.

[00320] Figure 10 illustrates the isothermal amplification starting from the partially double-stranded polynucleotide produced either from RNA described above, or from DNA as described below. Step VII comprises annealing an amplification primer comprising 5' RNA and 3' DNA segments. Step VIII shows the extension of the amplification primer by a DNA polymerase with strand displacement activity. Step IX shows the cleavage of the RNA portion of the amplification primer. Step X illustrates the continued extension of the amplification primer by a DNA polymerase with strand displacement activity to produce an amplified product comprising sequence (D') and sequence (F') at its 3' end. The product of step X can hybridize to another amplification primer, and steps VII through X can be repeated to produce multiple copies of amplified product. While the ligand is unbound in this embodiment, it is to be understood that the ligand can be bound to the surface at various steps in the process. For example, it can be advantageous to have the species bound during the amplification step. Figure 11 illustrates the process shown in Figure 10 where the ligand and third primer extension product are bound to the support during amplification.

Alternative method for generating a polynucleotide having a defined 3' and 5' sequences from a DNA target

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[00321] One aspect of the invention is an alternative method for generating a polynucleotide having a defined 3' and 5' sequences from a DNA target. This method generally does not use a composite RNA/DNA primer as the first primer. Here, a tailed primer, usually not comprising RNA, composed, for example, of DNA is used to create a plurality of first primer extension products. In this method, the second primer comprises a composite RNA/DNA primer, and the third primer comprises a ligand for binding the third primer extension products to a solid surface.

[00322] The method comprises step: (a) denaturing a double-stranded target DNA. Double stranded DNA can be denatured, for example by heating, or by the addition of denaturing agents.

[00323] The method further comprise step: (b) annealing to the target DNA and extending a first primer comprising a 3' portion comprising a random sequence and a 5' portion, sequence (D), which is not complementary to the target DNA, to form a plurality of first primer extension products, each comprising sequence (D) at its 3' end. The first primer generally does not comprise RNA, and may be all DNA. The first primer is a tailed primer comprising a 5' portion, sequence (D) which is generally not complementary to the target DNA, and does not hybridize to the target DNA. The sequence that is complementary to the target DNA comprises a random sequence, such that the extension of the first primer results in a plurality of first primer extension products complementary to the sequences adjacent to the sequence where each random species hybridizes. The use of a random sequence at the 3' end of the primer can be useful for performing a global amplification of a DNA target, generating a plurality of sequences which together can represent, for example substantially the whole sequence of the target DNA. In some embodiments, the relative amounts of the various sequences can be used to quantitate the relative amount of a given sequence in a sample, for example to determine the number of gene copies in a target DNA sample. In some embodiments more than one type of sequence that is complementary to the target DNA can be used, for instance both a primer with a random sequence and a primer with a sequence complementary to DNA can be used. In some embodiments, multiple primers comprising different specific sequences can be used.

[00324] The method further comprises step (c): extending a second primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion comprises a random sequence, and a 5' portion, sequence (E), of the of the second primer is not complementary to the first primer extension products, to produce a plurality of double-stranded DNA products comprising a first primer extension product hybridized to a second primer extension product, whereby the second primer extension products have a sequence (D') that is complementary to sequence (D) at their 3' ends. The extension of the second primer is carried out with a DNA polymerase as described herein. The second primer is a composite RNA/DNA primer having a 3' portion which is complementary to the first primer extension product, and a 5' portion, sequence (E), which is not complementary to the first primer extension product. The second primer comprises a random primer sequence that randomly binds to the first primer extension product. Extension of the second primer comprising a random sequence produces a plurality of second primer extension products. The use of a random sequence at the 3' end of the primer is useful, for example, in performing global amplification of a target DNA, whereby a plurality of second primer extension products are produced which is representative of the sequence of the target DNA. In some embodiments, for example where the first primer is designed to hybridize to a specific sequence on a target RNA, or a sequence common

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to a family of RNA targets, random priming by the second primer ensures amplification of the entire selected target or family of selected targets. In this embodiment, the second primer extension products comprise sequences which are the same or substantially the same as the sequences in the target DNA (sense copies).

[00325] The second primer extension products are extended such that the 3' portion of the second primer extension products comprises a sequence (D') which is complementary to sequence (D) of the first primer. The primer extension results in products that are at least partially double stranded.

[00326] The method further comprises step (d) denaturing the double-stranded DNA product. Double stranded DNA can be denatured, for example by heating, or by the addition of denaturing agents.

[00327] The method further comprises step (e) annealing to the second primer extension product and extending a third primer comprising, from its 5' end, a ligand, optionally a sequence (F), and a sequence (D), wherein sequence (D) is complementary to sequence (D') on the second primer extension products to produce double-stranded DNA products comprising second primer extension products hybridized to a third primer extension products, whereby the third primer extension products comprise a sequence (E') at its 3' end complementary to sequence (E). The third primer comprises a ligand that is a member of a ligand-receptor pair. In some embodiments, the ligand is attached to the primer at the 5' end of the primer. In some embodiments, the ligand is a small molecule, such as biotin or digoxigenin. In some embodiments, the receptor is an antibody, and the ligand is a molecule or portion of a molecule recognized by the antibody. The method produces nucleic acids that comprise a ligand allowing them to be bound to a solid surface and that has a specific sequence (E') at its 3' end. The specific sequence (E') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. As described above, the nucleic acid attached to the ligand also comprises sequence (D') at or near its 5' end, and in some embodiments comprises the sequence (F').

[00328] In some embodiments, the method further comprises the step of binding the ligand to a solid surface, whereby the third primer extension product is bound to the solid surface.

[00329] In some embodiments, the method further comprises the steps of: (f) cleaving the RNA portion of the second primer extension products in the DNA-RNA heteroduplex, whereby sequence (E') of the third primer extension products is single stranded, (g) annealing an oligonucleotide comprising a 3' DNA segment (E) that is complementary to sequence (E') and a 5' RNA segment comprising sequence (G), (h) extending the third primer extension products to produce a sequence (G') at their 3' end complementary to sequence (G), and (i) cleaving the RNA from the heteroduplex created in step (h) to produce a single-stranded portion of the third primer extension products corresponding to sequence (G'). The cleaving of RNA in steps (f) and (i) can be performed, for example by treatment with RNase H, which will selectively cleave the RNA portion of the DNA/RNA partial heteroduplex formed in step (e). The oligonucleotide comprises at least one DNA and at least one RNA portion. In some embodiments the 3' DNA segment is complementary to all of sequence (E'), in other embodiments, the 3' DNA segment is complementary to portion of sequence (E'). In some embodiments, 5' RNA segment comprising sequence (G) is partly complementary to sequence (E'). In some embodiments, the oligonucleotide is optionally extended

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from its 3' end to produce a plurality of oligonucleotide extension products hybridized to the third primer extension products and displacing the DNA portion of the second primer extension products. In some embodiments, the third primer comprises a sequence (F), such that the oligonucleotide extension products will comprise a sequence (F') at or near their 3' end that is complementary to sequence (F). The DNA sequence (G') is created by a DNA polymerase that has RNA dependent DNA polymerase activity. This step creates an RNA/DNA heteroduplex region that can be used for further manipulation of the second primer extension products.

[00330] In some embodiments the method further comprises binding the ligand to a solid surface, whereby the third primer extension products comprising sequence (G') are bound to the solid surface. The solid surface can be any of a variety of surfaces, some described in more detail below. The solid surface can be, for example a planar surface, for example, a planar array. In some embodiments the solid surface comprises a plurality of beads. In some embodiments the beads are magnetic. The receptor bound to the solid surface is a member of the ligand-receptor pair such that binding of the ligand results in attaching the second primer extension products to the solid surface. In some embodiments, the second primer extension products are still hybridized to the first primer extension product when they are attached to the solid surface. In some embodiments, the second primer extension products are removed from the third primer extension products such that single stranded polynucleotides are attached to the solid surface. The method produces nucleic acids that are bound to a solid surface having a defined sequence (E') and (G') at their 3' ends. The specific sequence (G') can be a site for primer hybridization and further analysis or amplification of the nucleic acids bound to the beads. As described above, in some embodiments, the nucleic acid bound to the bead also comprises sequence (F) at or near its 5' end. One aspect of the invention comprises amplification of the nucleic acids bound to the bead. In some embodiments, the amplification is carried out using isothermal amplification using a composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (G') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification. When the sequence (G') acts as a site to which a composite amplification primer binds, the amplified products that are produced have the sequence (E) (and a portion of sequence (G) at their 5' ends. The amplified products have a sequence (D') at or near their 3' ends. Where the third primer comprises the sequence (F), the amplified products also have the sequence (F'), complementary to (F) at or near their 3' ends. Thus the method produces amplified product with defined sequences at or near both its 3' and 5' ends.

[00331] In some embodiments, for example where random sequences at the 3' end of the first and/or second primer are used, a plurality of different nucleic acids bound to a solid surface is created in which each of the nucleic acids has a specific sequence (E') and (G') at its 3' end, the sequence (D) at or near its 5' end (and in some embodiments also a specific sequence (F) at its 5' end), and where the different nucleic acids have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target DNA. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target DNA.

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[00332] In some embodiments, the method further comprises treating the solid surface with reagents to produce multiple copies of amplification products that are substantially complementary the second primer extension products. This step comprises carrying out an amplification reaction wherein the bound nucleic acid acts as a template for the amplification. Generally, the amplification is carried out using the sequence (G') on the third primer extension product for the hybridization of primer. In some embodiments, the amplification produces single stranded amplified products, In some embodiments, the amplification provides double stranded products. The third primer extension products comprise the specific sequence (D'). In some embodiments, the third primer comprises a specific sequence (F), which thus becomes incorporated into the third primer extension products. In some embodiments the amplification is an isothermal amplification reaction comprising a composite RNA/DNA primer, RNase H, and a DNA polymerase with strand displacement activity. In some embodiments, the amplification is carried out using polymerase chain reaction, (PCR). For example where the third primer extension products comprise both as sequence (F) at or near its 5' end and a sequence (G') at or near its 3' end, a set of primers, one designed to hybridize to all or a portion of the sequence (G') and the other designed to hybridize to sequence (F'), the complement of sequence (B), or sequence (D), can be used to carry out a PCR reaction to exponentially produce double stranded amplified products.

[00333] One aspect of the invention is a method of amplifying a sequence representative of a sequence within a target DNA comprising the above steps and further comprising: (j) annealing an amplification primer, wherein the amplification primer has a DNA portion and a 5' RNA portion, to the single stranded portion of the third primer extension products complementary to sequence (G'); (k) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified products; (l) cleaving the RNA from the amplified products; and (m) repeating steps (j) to (l) to produce multiple copies of amplified products wherein the 5' portion of the amplified products have a sequence (E) complementary to sequence (E') and the 3' end of the amplified products have sequence (D') complementary to sequence (D) and optionally sequence (F') complementary to sequence (F).

[00334] The step of binding the polynucleotides to the solid surface through the ligand can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

[00335] In some embodiments the amplification is carried out such that the amplified products are not attached to the substrate, but is freely dissolved in the solution. In other embodiments, the amplification is carried out such that the amplified products remains bound to the substrate, for example by performing solid phase PCR such as bridge PCR. In yet other embodiments, amplified products are generated that may float freely in solution, but which comprise a sequence, for example sequence (E) or sequence (D'), that allows them to be captured to another solid surface or other portion of the solid surface by hybridization to a complementary sequence bound to such surface (e.g. sequence (E') or sequence (D). In some embodiments, the amplified product is a single-

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stranded product and, because it is generated at the solid surface, the amplified product readily captured by complementary sequences, e.g. sequence (B), bound to the surface.

[00336] In one aspect of the invention, a plurality of beads is used, and the methods described above are carried out such that on average, one or fewer third primer extension product molecules are bound per bead. The beads are dispersed into an aqueous solution, and a plurality of microreactors, e.g. droplets, are produced such that on average one or fewer beads is contained within each of the plurality of microreactors. The amplification of the third primer extension products bound to the beads is then carried out such that the clonal amplification of a plurality of third primer extension products is achieved. This clonal amplification in microreactors can be performed on a sample of target DNA such as genomic, wherein the plurality of third primer extension products comprise sequences that correspond to most, to substantially all, or to all of the sequences in the target DNA. In some embodiments, the amplified products are captured by bead having attached thereto a plurality of oligonucleotides comprising complementary sequences bound to such surface (e.g. sequence (E') or sequence (D)), which are complementary to sequence (E) or sequence (D') on the amplified product.

[00337] In some embodiments, the plurality of beads, produced as described above, with each bead comprising a single third primer extension product can comprise a library. These libraries can be stored, then later clonally amplified. In some embodiments, a library of beads can comprise a plurality of beads wherein each bead had multiple copies of a single amplification product generated from a third primer extension product. These libraries can be analyzed, for example by sequencing. The libraries can be stored, and later analyzed. In some embodiments the libraries can be stored, then analyzed multiple times.

[00338] In some embodiments, a bead or isolated area of the solid surface comprises covalently attached thereto multiple oligonucleotides comprising the sequence (D) (or F) at their 3' ends, whereby upon the amplification of step (m) multiple copies of amplified product comprising sequence (D') (or F') at their 5' end are hybridized to the bead or isolated area. For example, where beads are used, a plurality of beads in a plurality of microreactors wherein, the plurality of beads has, on average one or fewer third primer extension products bound to it and there are, on average, one or fewer beads in each microreactor, a clonal amplification of the plurality of third primer extension products can be carried out, and the amplified products in each of the microreactors will bind to the bead through the sequence (D') (and/or F') on the amplified product to the sequence (D) (and/or F) on the beads. This approach produces a plurality of beads, each with multiple copies of a different sequence bound to it. Where these sequences are representative of the target DNA, the plurality of beads can constitute a library representative of such DNA.

[00339] After the amplified products are bound to the beads by hybridization, the (D) sequences on the beads can be extended to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product and also comprise sequence (E') near their 5' ends. Where the (D) (and or F) sequences are covalently attached to the beads, this method provides for the production of beads with polynucleotides complementary to amplified product covalently attached to the beads. Covalently attached polynucleotides such as those produce here are more robust than nucleotides that are attached only by hybridization to the

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beads. Thus, the covalently attached polynucleotides can be more stable and can be used with analysis methods and sequencing methods that have harsher conditions which would result in the displacement of polynucleotides bound only by hybridization.

[00340] In some embodiments, the amplified product is removed from the covalently bound polynucleotide to render the polynucleotide single stranded. Such single stranded covalently bound polynucleotides comprise a specific sequence at their 3' ends comprising sequence (E') and a portion of sequence (G'). This specific sequence at the 3' end of the covalently bound polynucleotide can act as a hybridization site for a primer complementary to sequence (E) that can act as a primer to carry out sequencing by any of a variety of sequencing methods, for example, those described herein.

[00341] The sequencing can be used to reveal information about the target DNA, for example the genomic DNA. The sequencing methods can comprise the use of cleavable labeled terminators. The sequencing method can comprise pyrophosphate detection. The sequencing method can comprise an isothermal sequencing method, for example using chimeric primers, RNase H, and a polymerase with strand displacement activity. The sequencing method can also comprise cycle sequencing.

[00342] The amplified products with defined 3' and 5' ends can be used in the methods described herein for the amplified products produced by the other methods. They can be used, for example, for bridge PCR, rolling circle amplification, and strand displacement amplification.

[00343] A schematic exemplary of an embodiment of the invention relating to an alternative method for generating a polynucleotide having a defined 3' and 5' sequences from an RNA target is shown in Figure 12. The steps shown in Figure 12 are applicable to product that is generated from the alternative method for either RNA or from DNA as described above. Step I comprises the steps of annealing an oligonucleotide comprising a 3' DNA segment (E) that is complementary to sequence (E') and a 5' RNA segment comprising sequence (G). Step II comprises extending the third primer extension product to produce a sequence (G') at its 3' end complementary to sequence (G). Step III comprises cleaving the RNA from the heteroduplex created in step II to produce a single-stranded portion of the third primer extension product corresponding to sequence (G').

[00344] Step IV comprises annealing an amplification primer, wherein the amplification primer has a DNA portion and a 5' RNA portion, to the single stranded portion of the third primer extension product complementary to sequence (G'). Step V comprises extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product. Step VI comprises cleaving the RNA from the amplified product. The product of step VI can hybridize to another amplification primer, thus allowing steps IV to VI to be repeated to produce multiple copies of amplified product wherein the 5' portion of the amplified product has a sequence (E) complementary to sequence (E') and the 3' end of the amplified product has sequence (D') complementary to sequence (D) and optionally sequence (F') complementary to sequence (F).

Alternative method for generating a polynucleotide bound to a solid surface

[00345] One aspect of the invention is a method for attaching a polynucleotide sequence that is representative of a sequence within a nucleic acid target molecule to a solid surface. The terms solid

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surface and solid support are used interchangeably herein. The polynucleotide sequence that is produced is representative of the sequence within a nucleic acid target molecule if it is either the same as, or complementary to the sequence within the target nucleic acid. Where the target nucleic acid is double stranded, the method can produce sequences that are representative of both of the strands. The polynucleotide can be, for example either DNA or RNA.

[00346] The first step of the method comprises step: (a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer, sequence (P), is complementary to a target nucleic acid and a 5' portion of the of the primer, sequence (A), is not complementary to the target nucleic acid, to form a first primer extension product hybridized to the target nucleic acid. In some embodiments, the 3' portion of the primer that is complementary to the target nucleic acid is a specific sequence. For example, where a specific region of interest of a target nucleic acid that is known or suspected to be upstream of a specific sequence on the target nucleic acid, sequence (P) of the composite primer can be designed to hybridize to this specific sequence on the target nucleic acid such that extension of the primer results in producing a first primer extension product that is complementary to such upstream region. The specific sequence may be common to a family of target RNA. A combination of primers with various specific sequences at the 3' end can also be useful. In some embodiments, such as where the target nucleic acid comprises mRNA, and the mRNA comprises a plurality of sequences, each having a 3' poly-A segment; the specific sequence (P) can comprise a sequence that will hybridize to the poly-A region of the mRNA, thus allowing the extension of the first primer to produce a plurality of first primer extension products, each of which is complementary to the region of an mRNA molecule adjacent to the poly-A region. In some embodiments, the sequence (P) comprises a random sequence, such that the extension of the first primer results in a plurality of first primer extension products complementary to the sequences adjacent to the sequence where each random species hybridizes. The use of a random sequence such as sequence (P) at the 3' end of the primer can be useful for performing a global amplification of a nucleic acid target, generating a plurality of sequences which together can represent, for example substantially the whole sequence of the target nucleic acid. In some embodiments, the relative amounts of the various sequences can be used to quantitate the relative amount of a given sequence in a sample, for example to determine the level of expression in an mRNA sample, or to determine gene copy number in a DNA sample.

[00347] The first primer extension product comprises a 5' portion comprising sequence (A). Sequence A comprises RNA. In some embodiments, sequence (A) is RNA, and sequence (P) is DNA. In other embodiments, sequence (A) will comprise some DNA. In some embodiments, sequence (P) will comprise some RNA. In some embodiments, sequence (A) and sequence (P) are adjacent.

[00348] The method further comprises step: (b) separating or removing the first primer extension product from the target nucleic acid. The first primer extension product can be separated from the target nucleic acid by a variety of methods. In some cases the separation can be affected by denaturing the complex comprising the first primer extension product and the nucleic acid. Denaturation can be performed, for example by heating the sample, or by adding a denaturing agent, or using a combination of heating the sample and adding denaturing agents. Other methods of

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separating the first primer extension product from the target nucleic acid involve selectively cleaving or degrading the target nucleic acid. Where the target nucleic acid is RNA, the cleaving or degrading can be accomplished by denaturing or heating the sample to degrade RNA or with an enzyme that cleaves RNA from an RNA/DNA hybrid such as RNase H, or chemically. In some embodiments, the target nucleic acid is completely cleaved or degraded. In other embodiments, the target nucleic acid is only partly cleaved or degraded. The amount of cleavage or degradation required is that amount which will allow the extension of the second primer. In some embodiments, the cleavage or degradation is carried out partially, and the fragments of the target nucleic acid that remain can constitute the second primer for step (c).

[00349] The method further comprises step: (c) extending a second primer to produce a double-stranded product comprising a second primer extension product hybridized to the first primer extension product, wherein the second primer comprises a 3' segment complementary to a portion of the first primer extension product and 5' segment non-complementary sequence (B) to the first primer extension product, whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer and a portion of the 5' end of the second primer extension product comprises non-complementary sequence (B).

[00350] The extension of the second primer is carried out with a DNA polymerase as described herein. The second primer can comprise RNA, DNA, or can be a composite primer comprising both RNA and DNA. The second primer is generally a tailed primer having a 3' portion which is complementary to the first primer extension product, and a 5' portion, sequence (B), which is not complementary to the first primer extension product. In some embodiments, the second primer can comprise a specific primer sequence that is designed to hybridize to a specific sequence in the first primer extension product. In some embodiments the second primer comprises a random primer sequence that randomly binds to the first primer extension product. Extension of the second primer comprising a random sequence produces a plurality of second primer extension products. The use of a random sequence at the 3' end of the primer is useful, for example, in performing global amplification of a target RNA or target DNA, whereby a plurality of second primer extension products are produced which is representative of the sequence of the target nucleic acid. In some embodiments, for example where the first primer is designed to hybridize to a specific sequence on a target RNA, or a sequence common to a family of RNA targets, random priming by the second primer ensures amplification of the entire selected target or family of selected targets. In this embodiment, the second primer extension products comprise sequences which are the same or substantially the same as the sequences in the target nucleic acid (sense copies). The second primer comprises a sequence (B) that is homologous to a sequence (B) on a solid support.

[00351] The second primer extension product is extended such that the 3' portion of the second primer extension product comprises a sequence (A') which is complementary to sequence (A) of the first primer. Since sequence (A) on the first primer extension product comprises RNA, both DNA dependent DNA polymerase activity and RNA dependent DNA polymerase activity are used in step (c). The primer extension results in a product that is at least partially double stranded since sequence (B) is not hybridized to the first primer extension product.

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[00352] The method further comprises step: (d) adding an exonuclease to the double-stranded DNA/RNA hybrid, whereby single stranded 3' nucleotides are removed from the first primer extension product. Non-limiting examples of an exonuclease include single-strand specific 3'-exonucleases such as exonuclease 1. The exonuclease should remove all of the single-stranded 3' nucleotides which are not hybridized to sequence (B). In some embodiments, the exonuclease may remove additional 3' nucleotides which are hybridized to the second primer extension product. In other embodiments, a polymerase comprising exonuclease activity may be used. Non-limiting examples include a T4 polymerase comprising 3' exonuclease activity.

[00353] The method further comprises step: (e) extending the first primer extension product to produce a sequence (B'), complementary to sequence (B) on the second primer extension product. The extension of the first primer extension product is carried out with a DNA polymerase as described herein and is generally carried out with a DNA-dependent DNA polymerase if the second primer extension product contains only DNA or with RNA-dependent DNA polymerase if the second primer extension product contains a RNA sequence . The primer extension results in a product that is double stranded and comprises sequences (A) and (B') on the first primer extension product and sequences (B) and (A') on the second primer extension product.

[00354] The method further comprises step: (f) denaturing the first and second primer extension products. The first primer extension product can be separated from the second primer extension product by denaturation. Denaturation can be performed, for example by heating the sample, or by adding a denaturing agent, or using a combination of heating the sample and adding denaturing agents.

[00355] The method further comprises step: (g) binding the sequence (B') of the first primer extension product to a third primer comprising sequence (B) bound to a solid surface, whereby the first primer extension product is attached to the solid surface. The third primer comprises an oligonucleotide with sequence (B) that is complementary to the sequence (B') of the first primer extension product and results in attaching the single stranded first primer extension product to the solid surface. The method produces a nucleic acid that is bound to a solid surface that has a specific sequence (A) at its 5' end. As described above, in some embodiments, the nucleic acid bound to the bead also comprises sequence (B') at or near its 3' end.

[00356] Step (g) of binding the polynucleotides to the solid surface sequence (B) can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

[00357] The method further comprises step: (h) extending the sequence (B) of the third primer to produce a double-stranded product comprising a third primer extension product hybridized to the first primer extension product, wherein the 5'end of the third primer comprises a sequence (B) complementary to the sequence (B') of the first primer extension product, whereby a portion of the 3' end of the third primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer.

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[00358] The extension of the third primer is carried out with a DNA polymerase as described herein. The primer extension results in a product that is double stranded and comprises sequences (A) and (B') on the first primer extension product and sequences (B) and (A') on the third primer extension product.

[00359] The specific, or universal, sequence (A') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. One aspect of the invention comprises amplification of the nucleic acid bound to the bead. In some embodiments, the amplification is carried out using isothermal amplification using a composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (A') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification. In some embodiments, for example where random sequences at the 3' end of the first and/or second primer are used, a plurality of different nucleic acids bound to a solid surface is created in which each of the nucleic acids has a specific sequence (A') at its 3' end (and in some embodiments also a specific sequence (B) at its 5' end), and where the different nucleic acids have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target nucleic acid. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target nucleic acid.

[00360] A schematic exemplary of an embodiment of the invention relating to an alternative method for generating polynucleotide bound to a solid surface is shown in Figure 13. The figure shows a target nucleic acid (RNA or DNA) and a chimeric RNA/DNA first primer. The primer is first annealed to the target nucleic. Step Ia (RNA target) and Ib (DNA target) illustrates extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target nucleic acid and a 5' portion, sequence (A), of the of the primer is not complementary to the target nucleic acid, to form a first primer extension product hybridized to the target nucleic acid, forming an RNA/DNA hybrid. The sequence complementary to a target nucleic acid can be a specific sequence, a sequence that will hybridize to Poly-A, a sequence common to a plurality of regions (consensus sequence), or a random sequence. Step IIa (RNA target) and IIb (DNA target) represent separating the target nucleic acid from the RNA/DNA hybrid. Separation of the target nucleic acid can be accomplished thermally, chemically, or enzymatically, e.g. with RNase H. The second primer comprising a 5' sequence (B) is then annealed to the first primer extension product. Step IIIa and IIIb illustrate extending a second primer, comprising a 5' sequence (B) and a 3' segment complementary to a portion of the first primer extension product, to produce a double stranded product with a DNA/RNA heteroduplex at one end; wherein the double stranded product comprises a second primer extension product hybridized to the first primer extension product, and whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer. This embodiment provides for attachment of the first primer extension product to the solid surface by creating a sequence (B'), allowing attachment to a solid surface comprising sequences (B) attached thereto. Step IV shows the removal of 3' nucleotides from the 3' region of the first primer extension product that is not hybridized to the second primer extension product. This step may be done using exonucleases or

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using DNA polymerase comprising exonuclease activity. Step V shows extension of the first primer extension product by DNA polymerase to generate a sequence (B'), complementary to the sequence (B) of the second primer extension product. Step VI shows the denaturation of the first and second primer extension product by methods described previously. The first primer extension product comprising a sequence (B') and a defined sequence (A) at its 3' end is useful for storage, archiving and analysis as it has a sequence (B') capable of binding to a solid surface. Such first primer extension product also comprises a sequence that is representative of (identical to or substantially identical to) a sequence in the target RNA, so analysis of this product provides information about the target RNA. Step VII shows the binding of sequence (B') of the first primer extension product to a sequence (B) on a solid surface, whereby the first primer extension product becomes bound to the solid surface. Step VIII shows extension of the immobilized sequence (B) oligonucleotide on the solid support using DNA and RNA-dependent DNA polymerase, resulting in another DNA/RNA heteroduplex.

Alternative method for generating a polynucleotide for binding to a solid surface from an RNA target

[00361] The invention provides methods, compositions and kits for copying, storing, and amplifying polynucleotides having sequences related to target ribonucleic acid (RNA) sequences. The methods provide for amplification of a single RNA species or pool of RNA species. The methods are suitable for, for example, generation of libraries, including cDNA libraries. The methods can generate single stranded RNA or DNA products, which are readily suitable for multiplex analysis by microarray technologies, as well as electrophoresis-based technologies such as differential display, and for sequencing.

[00362] The methods of the invention can copy, store, and amplify 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 whole transcriptome or total RNA) sequences in a biological sample. Thus, one of the major advantages of the methods of the invention is the ability to copy, store, and 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 sample of interest. The methods of the invention have the potential of amplifying a multiplicity, a large multiplicity, and in some embodiments all RNA (such as whole transcriptome or total RNA in a sample) sequences in a sample.

[00363] 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. The methods are also useful for whole transcriptome amplification. The methods of the invention can be used for the total RNA in samples such as viral RNA.

[00364] An aspect of the invention is a method for generating a polynucleotide comprising a sequence (B') for binding to a solid surface from a RNA target comprising the step of: (a) extending

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a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA; to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid. This extension is generally performed with an enzyme comprising RNA-dependent DNA polymerase activity.

[00365] In some embodiments, the 3' portion of the primer that is complementary to the target RNA is a specific sequence. For example, where a specific region of interest of a target RNA that is known or suspected to be upstream of a specific sequence on the target RNA, the sequence that is complementary to the target RNA of the first primer can be designed to hybridize to this specific sequence on the target RNA such that extension of the primer results in producing a first primer extension product that is complementary to such upstream region. The specific sequence may be common to a family of target RNA. A combination of primers with various specific sequences at the 3' end can also be useful. In some embodiments, such as where the target RNA comprises mRNA, and the mRNA comprises a plurality of sequences, each having a 3' poly-A segment; the specific sequence that is complementary to the target RNA can comprise a sequence that will hybridize to the poly-A region of the mRNA, thus allowing the extension of the first primer to produce a plurality of first primer extension products, each of which is complementary to the region of an mRNA molecule adjacent to the poly-A region. In some embodiments, the sequence that is complementary to the target RNA comprises a random sequence, such that the extension of the first primer results in a plurality of first primer extension products complementary to the sequences adjacent to the sequence where each random species hybridizes. The use of a random sequence at the 3' end of the primer can be useful for performing a global amplification of a target RNA, generating a plurality of sequences which together can represent, for example substantially the whole sequence of the target RNA. In some embodiments, the relative amounts of the various sequences can be used to quantitate the relative amount of a given sequence in a sample, for example to determine the level of expression in an mRNA sample. In some embodiments more than one type of sequence that is complementary to the target RNA can be used, for instance both a primer with a random sequence and a primer, or combination of primers with a specific sequence complementary to RNA can be used. In some embodiments, multiple primers comprising different specific sequences can be used.

[00366] The method further comprises the step of: (b) removing the target RNA from the RNA/DNA hybrid. In some embodiments, the removal of the target RNA from the RNA/DNA hybrid involves selectively cleaving or degrading the target RNA. In some cases, the complex comprising the first primer extension product and the nucleic acid can be heated in reaction conditions comprising Mg, which leads to cleaving the RNA including the RNA in the RNA/DNA hybrid. The RNA can also be removed from the RNA/DNA hybrid by denaturation, performed, for example by heating the sample (thermal methods), or by adding denaturing agents or using a combination of heating the sample and adding denaturing agents. The cleaving can be accomplished with an enzyme that cleaves RNA from an RNA/DNA hybrid such as RNase H, or a combination of RNase enzymes, or chemically. In some embodiments, the target RNA is completely cleaved. In

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other embodiments, the target RNA is only partly cleaved or degraded. The amount of cleaving required is that amount which will allow the extension of the second primer.

[00367] The method further comprises the step of: (c) extending a second primer, comprising a 3' segment complementary to a portion of the first primer extension product and a 5' segment non-complementary to the first primer extension product comprising sequence (B), to produce a double-stranded DNA product with a DNA/RNA heteroduplex at one end, wherein the double-stranded product comprises a second primer extension product hybridized to the first primer extension product and wherein a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer.

[00368] The extension of the second primer is carried out with a DNA polymerase as described herein. In some embodiments, a DNA polymerase comprising both DNA and RNA dependent DNA polymerase activities is used here. In other embodiments, both a RNA dependent DNA polymerase and a DNA dependent DNA polymerase are used. The second primer can comprise RNA, DNA, or can be a composite primer comprising both RNA and DNA. In some embodiments, the second primer can comprise a specific primer sequence that is designed to hybridize to a specific sequence in the first primer extension product. In some embodiments the second primer comprises a random primer sequence that randomly binds to the first primer extension product. Extension of the second primer comprising a random sequence produces a plurality of second primer extension products. The use of a random sequence at the 3' end of the primer is useful, for example, in performing global amplification of a target RNA, whereby a plurality of second primer extension products are produced which is representative of the sequence of the target RNA. In some embodiments, for example where the first primer is designed to hybridize to a specific sequence on a target RNA, or a sequence common to a family of RNA targets, random priming by the second primer ensures amplification of the entire selected target or family of selected targets. In this embodiment, the second primer extension products comprise sequences which are the same or substantially the same as the sequences in the target RNA (sense copies). The second primer comprises a sequence (B) that is homologous to a sequence (B) on a solid support.

[00369] The second primer extension product is extended such that the 3' portion of the second primer extension product comprises a sequence (A') which is complementary to sequence (A) of the first primer. Since sequence (A) on the first primer extension product comprises RNA, both DNA dependent DNA polymerase activity and RNA dependent DNA polymerase activity are used in step (c). The primer extension results in a product that is at least partially double stranded since sequence (B) on the second primer does not hybridize to the first primer extension product.

[00370] The method further comprises the step of: (d) adding an exonuclease to the double-stranded DNA product, whereby single stranded 3' nucleotides are removed from the 3' region of the first primer extension product that is not hybridized to the second primer extension product. Non-limiting examples of an exonuclease include single-strand specific 3'-exonucleases such as exonuclease 1. The exonuclease should remove all of the single-stranded 3' nucleotides which are not hybridized to sequence (B). In some embodiments, the exonuclease may remove additional 3' nucleotides which are hybridized to the second primer extension product. In other embodiments, a

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polymerase comprising exonuclease activity may be used. Non-limiting examples include a T4 polymerase comprising 3' exonuclease activity.

[00371] The method further comprises the step of: (e) extending the first primer extension product to produce a sequence (B'), complementary to sequence (B) on the second primer extension product. The extension of the first primer extension product is carried out with a DNA polymerase as described herein and is generally carried out with a DNA-dependent DNA polymerase if the second primer extension product contains only DNA or with RNA-dependent DNA polymerase if the second primer extension product contains a RNA sequence. The primer extension results in a product that is double stranded and comprises sequences (A) and (B') on the first primer extension product and sequences (B) and (A') on the second primer extension product.

[00372] The method further comprises the step of: (f) denaturing the double-stranded DNA product. The first primer extension product can be separated from the second primer extension product by denaturation. Denaturation can be performed, for example by heating the sample, or by adding a denaturing agent, or using a combination of heating the sample and adding denaturing agents.

[00373] The method further comprises the step of: (g) attaching the single-stranded first primer extension product to a solid support by annealing sequence (B') to the solid support comprising an oligonucleotide attached thereto, comprising a sequence (B). The oligonucleotide or third primer comprises an oligonucleotide sequence (B) that is complementary to the sequence (B') of the first primer extension product and results in attaching the single stranded first primer extension product to the solid surface. The method produces a nucleic acid that is bound to a solid surface that has a specific sequence (A) at its 5' end. As described above, in some embodiments, the nucleic acid bound to the bead also comprises sequence (B') at or near its 3' end.

[00374] Step (g) of binding the polynucleotides to the solid surface sequence (B) can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

[00375] The method further comprises the step of: (h) extending sequence (B) on the solid support to produce a third primer extension product, hybridized to the second extension product, wherein the third primer extension product comprises a 3' sequence (A'), whereby a DNA/RNA heteroduplex at one end is generated. The extension of the third primer is carried out with a DNA polymerase as described herein. In some embodiments, a DNA polymerase comprising both DNA and RNA dependent DNA polymerase activities is used here. In other embodiments, both a RNA dependent DNA polymerase and a DNA dependent DNA polymerase are used. The primer extension results in a product that is double stranded and comprises sequences (A) and (B') on the first primer extension product and sequences (B) and (A') on the third primer extension product.

[00376] The method produces a nucleic acid that is bound to a solid surface that has a specific sequence (A') at its 3' end. The specific, or universal, sequence (A') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. One aspect of the invention comprises amplification of the nucleic acid bound to the bead. In some

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embodiments, the amplification is carried out using isothermal amplification using a composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (A') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification. In some embodiments, for example where random sequences at the 3' end of the first and/or second primer are used, a plurality of different nucleic acids bound to a solid surface is created in which each of the nucleic acids has a specific sequence (A') at its 3' end (and in some embodiments also a specific sequence (B) at its 5' end), and where the different nucleic acids have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target nucleic acid. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target nucleic acid.

[00377] In some embodiments, the sample comprising the target RNA is in a sample that also comprises DNA. In such cases, it can be advantageous to add a selective DNA dependent DNA polymerase inhibitor such as actinomycin such that it is present during step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a). The presence of a DNA dependent DNA polymerase inhibitor such as actinomycin is particularly advantageous when a first primer comprising a random sequence is used, as the inhibitor allows for the selective creation of first primer extension products to RNA without the need of separating the RNA from the DNA. This is also advantageous when the priming is carried out at specific target sequences since the sequence may be the same on the DNA when the DNA and RNA in the sample represent total nucleic acid from the same biological entity, for example, human tissue, animal tissue, and the like. The use of DNA dependent DNA polymerase inhibitors such as actinomycin is described in co-pending application.

[00378] One aspect of the invention comprises amplification of the nucleic acid bound to the bead. In some embodiments, the amplification is carried out using isothermal amplification using a composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (A') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification.

[00379] In some embodiments, the amplification comprises the steps of : (i) cleaving the RNA region from the first polynucleotide product hybridized to the third primer extension product using RNase H; (j) annealing an amplification primer to sequence (A') on the single-stranded portion of the third primer extension product, wherein the amplification primer has a DNA portion and a 5' RNA portion; (k) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product hybridized to the third primer extension product on the solid support; (l) repeating steps (i) to (k) to produce multiple copies of an amplified product wherein the amplified product comprises sequence (B') at its 3' end; and (m) capturing the amplified product on the solid support wherein the solid support comprises sequence (B).

[00380] In some embodiments, for example where random sequences at the 3' end of the first and/or second primer are used, a plurality of different nucleic acids bound to a solid surface is created in which each of the bound nucleic acids has a specific sequence (A') at its 3' end and also a specific sequence (B) at its 5' end, and where the different nucleic acids have different intervening

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sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target RNA. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target RNA.

[00381] The solid surface can be any of a variety of surfaces, some described in more detail below. The solid surface can be, for example a planar surface, for example, a planar array. In some embodiments the solid surface comprises a plurality of beads. In some embodiments the beads are magnetic.

[00382] The step of attaching or binding the polynucleotides to the solid surface through the sequence (B), step (g), can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

[00383] In some embodiments, the method further comprises treating the solid surface with reagents to produce multiple copies of an amplification product that are substantially complementary to the third primer extension product. This step comprises carrying out an amplification reaction wherein the bound nucleic acid acts as a template for the amplification. Generally, the amplification is carried out using the sequence (A') on the third primer extension product for the hybridization of primer. In some embodiments the amplification is an isothermal amplification reaction comprising a composite RNA/DNA primer, RNase H, and a DNA polymerase with strand displacement activity. In some embodiments, the amplification is carried out using polymerase chain reaction (PCR). For example where the third primer extension product comprises both as sequence (B) at or near its 5' end and a sequence (A') at or near its 3' end, a set of primers, one designed to hybridize to all or a portion of the sequence (A') and the other designed to hybridize to sequence (B'), the complement of sequence (B), can be used to carry out a PCR reaction to exponentially produce double stranded amplified product.

[00384] In some embodiments the amplification is carried out such that the amplified product is not attached to the substrate, but is freely dissolved in the solution. In other embodiments, the amplification is carried out such that the amplified product remains bound to the substrate, for example by performing solid phase PCR such as bridge PCR. In yet other embodiments, an amplified product is generated that may float freely in solution, but which comprises a sequence, for example sequence (A) or sequence (B'), that allows it to be captured to another solid surface or other portion of the solid surface by hybridization to a complementary sequence bound to such surface, e.g. sequence (A') or sequence (B). In some embodiments, the amplified product is a single-stranded product and, because it is generated at the solid surface, the amplified product readily captured by complementary sequences, e.g. sequence (B), bound to the surface.

[00385] In one aspect of the invention, a plurality of beads is used, and the methods described above are carried out such that on average, one or fewer first primer extension product molecules are bound per bead. The beads are dispersed into an aqueous solution, and a plurality of microreactors, e.g. droplets, are produced such that on average one or fewer beads is contained within each of the plurality of microreactors. The amplification of the first primer extension products bound to the

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beads is then carried out such that the clonal amplification of each of the plurality of second primer extension products in the separate microreactors is achieved. This clonal amplification in microreactors can be performed on a sample of target RNA, such as whole transcriptome or total RNA, wherein the plurality of first primer extension products comprise sequences that correspond to most, to substantially all, or to all of the sequences in the target RNA. In some embodiments, the amplified products are captured by bead having attached thereto a plurality of oligonucleotides comprising complementary sequences bound to such surface, e.g. sequence (A') or sequence (B), which are complementary to sequence (A) or sequence (B') on the amplified product.

[00386] In some embodiments, the plurality of beads, produced as described above, with each bead comprising a single first primer extension product can comprise a library. These libraries can be stored, then later clonally amplified. In some embodiments, a library of beads can comprise a plurality of beads wherein each bead had multiple copies of a single amplification product generated from a second primer extension product. These libraries can be analyzed, for example by sequencing. The libraries can be stored, and later analyzed. In some embodiments the libraries can be stored, then analyzed multiple times.

[00387] A schematic exemplary of an embodiment of the invention relating to this method for generating a polynucleotide for binding to a solid surface from an RNA target is shown in Figure 14. The figure shows a target RNA and a chimeric RNA/DNA first primer. The primer is first annealed to the target RNA. Step I illustrates extension of the first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA, to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid. The sequence complementary to a target RNA can be a specific sequence, a sequence that will hybridize to Poly-A, a sequence common to a plurality of regions (consensus sequence), or a random sequence. Step II represents separation of the target RNA from the RNA/DNA hybrid. The separation can be accomplished thermally, chemically, or enzymatically, e.g. with RNase H. The second primer comprising a 5' sequence (B) is then annealed to the first primer extension product. Step III illustrate extending a second primer, comprising a 5' sequence (B) and a 3' segment complementary to a portion of the first primer extension product, to produce a double stranded product with a DNA/RNA heteroduplex at one end; wherein the double stranded product comprises a second primer extension product hybridized to the first primer extension product, and whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer. This embodiment provides for attachment of the first primer extension product to the solid surface by creating a sequence (B'), allowing attachment to a solid surface comprising sequences (B) attached thereto. Step IV shows the removal of 3' nucleotides from the 3' region of the first primer extension product that is not hybridized to the second primer extension product. This step may be done using exonucleases or using DNA polymerase comprising exonuclease activity. Step V shows extension of the first primer extension product by DNA polymerase to generate a sequence (B'), complementary to the sequence (B) of the second primer extension product. Step VI shows the denaturation of the first and second primer extension product by methods described previously. The first primer extension product

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comprising a sequence (B') and a defined sequence (A) at its 3' end is useful for storage, archiving and analysis as it has a sequence (B') capable of binding to a solid surface. Such first primer extension product also comprises a sequence that is representative of (identical to or substantially identical to) a sequence in the target RNA, so analysis of this product provides information about the target RNA. Step VII shows the binding of sequence (B') of the first primer extension product to a sequence (B) on a solid surface, whereby the first primer extension product becomes bound to the solid surface. Step VIII shows extension of the immobilized sequence (B) oligonucleotide on the solid support using DNA and RNA-dependent DNA polymerase, resulting in another DNA/RNA heteroduplex.

[00388] In Figure 15, Step I illustrates amplification using single primer isothermal linear amplification (SPIA) wherein RNaseH cleaves the RNA from the DNA/RNA heteroduplex, a chimeric RNA/DNA primer binds to sequence (A'), and DNA polymerase with strand displacement activity is used to extend the chimeric primer to produce amplified product of the first primer extension product. Step II illustrates amplified products that are produced from repeated rounds of SPIA from Step I of the figure. Since the amplification products are generated in close proximity to access sequence (B) on the solid support, the amplified products are captured on the solid support through hybridization of the 3' sequence (B') to sequence (B) for further manipulations as described herein such as clonal amplification.

Alternative method for generating a polynucleotide for binding to a solid surface from a DNA target

[00389] The methods of the present invention can be used to analyze the DNA (e.g. genomic DNA) samples that are important for many studies. The methods can be used for high-throughput genomic analysis, and can be used for forensic and paleoarcheology work which can be severely limited by nucleic acid sample size. The methods can be used, for example, for the genotyping of multiple loci in the study of complex diseases. The methods can also be used for the determination of genomic instability in various pathological conditions such as cancer, which is most precisely carried out in well defined cell populations, such as that obtained by laser capture micro-dissection or cell sorting. The DNA amplification technologies described herein provide global amplification of very small polynucleotide samples, for example, from one or a very few cells.

[00390] One aspect of the invention is a method for generating from a DNA target a polynucleotide comprising a sequence for binding to a solid surface comprising the steps of: (a) denaturing a double-stranded target DNA. Double stranded DNA can be denatured, for example by heating, or by the addition of denaturing agents, or using a combination of heating the sample and adding denaturing agents.

[00391] The method further comprises step: (b) annealing to the target DNA and extending with a DNA polymerase comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the primer comprises sequence (A), which is not complementary to the target DNA; to form a plurality of first primer extension product hybridized to the target DNA and comprising sequence (A) at its 5' end. The enzyme that carries out step (b) is generally a DNA polymerase. In some cases a mixture of DNA polymerases can be used. This extension is generally performed with

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an enzyme comprising DNA-dependent DNA polymerase activity. The sequence that is complementary to the target DNA comprises a random sequence, such that the extension of the first primer results in a plurality of first primer extension products complementary to the sequences adjacent to the sequence where each random species hybridizes. The use of a random sequence at the 3' end of the primer can be useful for performing a global amplification of a DNA target, generating a plurality of sequences which together can represent, for example substantially the whole sequence of the target DNA. In some embodiments, the relative amounts of the various sequences can be used to quantitate the relative amount of a given sequence in a sample, for example to determine the number of gene copies in a DNA sample, or obtaining sequence information. In some embodiments, the extension of one first primer, will result in the release of a downstream first primer extension product. This can occur throughout the target DNA resulting in the release of multiple first primer extension products from the target DNA. This process can occur simultaneously on both of the strands of the double-stranded DNA target, thus creating first primer extension products complementary to sequences in both strands.

[00392] In some embodiments, the first primer extension step is carried out with a DNA polymerase capable of extension at elevated temperature that is not compatible with subsequent hybridization of the random sequence to the displaced primer-extension product. For example, Bst DNA polymerase can be used which is active at elevated temperature. The reaction can be carried out stepwise, first with incubation at a lower temperature such as about 25 °C, followed by incubation at higher temperature such as about 50 °C. In some embodiments, the first incubation is carried out below about 30 °C, and the second incubation is carried out above about 40 °C. In some embodiments, a DNA polymerase which is active at temperatures above about 45 °C is used to extend the first primer. Mixtures of DNA polymerases can also be useful.

[00393] The method further comprises step: (c) separating the first primer extension product from the target DNA. In some embodiments, the separation can be affected by denaturing the complex comprising the first primer extension product and the nucleic acid. Denaturation can be performed, for example by heating the sample, or by adding a denaturing agent, or using a combination of heating the sample and adding denaturing agents. The amount of cleaving required is that amount which will allow the extension of the second primer.

[00394] The method further comprises step: (d) annealing to the first primer extension product and extending a second primer comprising a 3' complementary DNA region that comprises a random sequence, wherein the second primer is a tailed primer comprising a 5' sequence (B), to form a double stranded product comprising a first primer extension product and a second primer extension product, whereby a double-stranded product with a DNA/RNA heteroduplex at one end is generated. In some embodiments, a DNA polymerase comprising both DNA and RNA dependent DNA polymerase activities is used here. In other embodiments, both a RNA dependent DNA polymerase and a DNA dependent DNA polymerase are used. This step may be carried out with or without prior denaturation. If carried out without denaturation, generally, only the single stranded displaced first primer extension product will hybridize to the second primer. Generally the second primer does not comprise RNA. The extension of the second primer is carried out with a DNA polymerase as described herein. The second primer comprises a random primer sequence that randomly binds to

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the first primer extension product. Extension of the second primer comprising a random sequence produces a plurality of second primer extension products. The use of a random sequence at the 3' end of the primer is useful, for example, in performing global amplification of a target DNA, whereby a plurality of second primer extension products are produced which is representative of the sequence of the target DNA. In some embodiments, for example where the first primer is designed to hybridize to a specific sequence on a target DNA, or a sequence common to a family of DNA targets, random priming by the second primer ensures amplification of the entire selected target or family of selected targets. In this embodiment, the second primer extension products comprise sequences which are the same or substantially the same as the sequences in the target DNA.

[00395] The second primer extension product is extended such that the 3' portion of the second primer extension product comprises a sequence (A') which is complementary to sequence (A) of the first composite primer. Since sequence (A) on the first primer extension product comprises RNA, both DNA dependent DNA polymerase activity and RNA dependent DNA polymerase activity are used in step (d). The primer extension results in a product that is at least partially double stranded since sequence (B) not does hybridize to the first primer extension product. The method produces a nucleic acid that comprises a sequence (B), allowing it to be bound to a solid surface by hybridization to its complement, which is immobilized on the solid surface and that has a specific sequence (A') at its 3' end. The specific, or universal, sequence (A') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead.

[00396] The method further comprises step: (e) adding an exonuclease to the double-stranded DNA product, whereby single stranded 3' nucleotides are removed from the 3' region of the first primer extension product that is not hybridized to the second primer extension product. Non-limiting examples of an exonuclease include single-strand specific 3'-exonucleases such as exonuclease 1. The exonuclease should remove all of the single-stranded 3' nucleotides which are not hybridized to sequence (B). In some embodiments, the exonuclease may remove additional 3' nucleotides which are hybridized to the second primer extension product. In other embodiments, a polymerase comprising exonuclease activity may be used. Non-limiting examples include a T4 polymerase comprising 3' exonuclease activity.

[00397] The method further comprises step: (f) extending the first primer extension product to produce a sequence (B'), complementary to sequence (B) on the second primer extension product. The extension of the first primer extension product is carried out with a DNA polymerase as described herein and is generally carried out with a DNA-dependent DNA polymerase if the second primer extension product contains only DNA or with RNA-dependent DNA polymerase if the second primer extension product contains a RNA sequence. The primer extension results in a product that is double stranded and comprises sequences (A) and (B') on the first primer extension product and sequences (B) and (A') on the second primer extension product.

[00398] The method further comprises step: (g) denaturing the double-stranded DNA product. The first primer extension product can be separated from the second primer extension product by denaturation. Denaturation can be performed, for example by heating the sample, or by adding a denaturing agent, or using a combination of heating the sample and adding denaturing agents.

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[00399] The method further comprises step: (h) attaching the single-stranded first primer extension product to a solid support by annealing sequence (B') to the solid support comprising an oligonucleotide attached thereto, comprising a sequence (B), whereby a plurality of first primer extension products become bound to the solid surface. The oligonucleotide or third primer comprises a sequence or oligo (B) that is complementary to the sequence (B') of the first primer extension product and results in attaching the single stranded first primer extension product to the solid surface. The method produces a nucleic acid that is hybridize to a sequence on the solid surface and has a specific sequence (A) at its 5' end.

[00400] Step (h) of binding the polynucleotides to the solid surface sequence (B) can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

[00401] The method further comprises step: (i) extending sequence (B) on the solid support to produce a third primer extension product, hybridized to the first primer extension product, wherein the third primer extension product comprises a 3' sequence (A'), whereby a DNA/RNA heteroduplex at one end is generated.

[00402] The extension of the third primer is carried out with a DNA polymerase as described herein. In some embodiments, a DNA polymerase comprising both DNA and RNA dependent DNA polymerase activities is used here. In other embodiments, both a RNA dependent DNA polymerase and a DNA dependent DNA polymerase are used. The primer extension results in a product that is double stranded and comprises sequences (A) and (B') on the first primer extension product and sequences (B) and (A') on the third primer extension product.

[00403] The method produces a nucleic acid that is bound to a solid surface that has a specific sequence (A') at its 3' end. The specific, or universal, sequence (A') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. One aspect of the invention comprises amplification of the nucleic acid bound to the bead. In some embodiments, the amplification is carried out using isothermal amplification using a composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (A') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification. In some embodiments, for example where random sequences at the 3' end of the first and/or second primer are used, a plurality of different nucleic acids bound to a solid surface is created in which each of the nucleic acids has a specific sequence (A') at its 3' end (and in some embodiments also a specific sequence (B) at its 5' end), and where the different nucleic acids have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target nucleic acid. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target nucleic acid.

[00404] One aspect of the invention comprises amplification of the nucleic acid bound to the bead. In some embodiments, the amplification is carried out using isothermal amplification using a

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composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (A') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification.

[00405] In some embodiments, the amplification comprises the steps of: (j) cleaving the RNA from the first polynucleotide product hybridized to the amplified product using RNase H; (k) annealing an amplification primer to the single-stranded portion of the amplified product complementary to sequence (A'), wherein the amplification primer has a DNA portion and a 5' RNA portion; (l) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product hybridized to the third primer extension product on the bead or isolated area; (m) repeating steps (j) to (l) to produce multiple copies of an amplified product wherein the amplified product comprises sequence (B') at its 3' end; and (n) capturing the amplified product on the solid support comprising sequence (B).

[00406] In some embodiments, a plurality of different nucleic acids bound to a solid surface is created in which each of the nucleic acids has a specific sequence (A') at its 3' end and also a specific sequence (B) at its 5' end, and where the different nucleic acids have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target DNA. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target DNA.

[00407] The solid surface can be any of a variety of surfaces, some described in more detail below. The solid surface can be, for example a planar surface, for example, a planar array. In some embodiments the solid surface comprises a plurality of beads. In some embodiments the beads are magnetic.

[00408] The step of binding the polynucleotides to the solid surface through the sequence (B), step (h), can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

[00409] In some embodiments, the method further comprises treating the solid surface with reagents to produce multiple copies of an amplification product that are substantially complementary to the third primer extension product. This step comprises carrying out an amplification reaction wherein the bound nucleic acid acts as a template for the amplification. Generally, the amplification is carried out using the sequence (A') on the third primer extension product for the hybridization of primer. In some embodiments the amplification is an isothermal amplification reaction comprising a composite RNA/DNA primer, RNase H, and a DNA polymerase with strand displacement activity. In some embodiments, the amplification is carried out using polymerase chain reaction (PCR). For example where the third primer extension product comprises both as sequence (B) at or near its 5' end and a sequence (A') at or near its 3' end, a set of primers, one designed to hybridize to all or a portion of the sequence (A') and the other designed to hybridize to sequence (B'), the complement of sequence (B), can be used to carry out a PCR reaction to exponentially produce double stranded amplified product.

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[00410] In some embodiments the amplification is carried out such that the amplified product is not attached to the substrate, but is freely dissolved in the solution. In other embodiments, the amplification is carried out such that the amplified product remains bound to the substrate, for example by performing solid phase PCR such as bridge PCR. In yet other embodiments, an amplified product is generated that may float freely in solution, but which comprises a sequence, for example sequence (A) or sequence (B'), that allows it to be captured to another solid surface or other portion of the solid surface by hybridization to a complementary sequence bound to such surface, e.g. sequence (A') or sequence (B). In some embodiments, the amplified product is a single-stranded product and, because it is generated at the solid surface, the amplified product readily captured by complementary sequences, e.g. sequence (B), bound to the surface.

[00411] In one aspect of the invention, a plurality of beads is used, and the methods described above are carried out such that on average, one or fewer first primer extension product molecules are bound per bead. The beads are dispersed into an aqueous solution, and a plurality of microreactors, e.g. droplets, are produced such that on average one or fewer beads is contained within each of the plurality of microreactors. The amplification of the second primer extension products bound to the beads is then carried out such that the clonal amplification of each of the plurality of second primer extension products in the separate microreactors is achieved. This clonal amplification in microreactors can be performed on a sample of target DNA, such as genomic DNA, wherein the plurality of second primer extension products comprise sequences that correspond to most, to substantially all, or to all of the sequences in the target DNA. In some embodiments, the amplified products are captured by bead having attached thereto a plurality of oligonucleotides comprising complementary sequences bound to such surface (e.g. sequence (A') or sequence (B)), which are complementary to sequence (A) or sequence (B') on the amplified product.

[00412] In some embodiments, the plurality of beads, produced as described above, with each bead comprising a single first primer extension product can comprise a library. These libraries can be stored, then later clonally amplified. In some embodiments, a library of beads can comprise a plurality of beads wherein each bead had multiple copies of a single amplification product generated from a second primer extension product. These libraries can be analyzed, for example by sequencing. The libraries can be stored, and later analyzed. In some embodiments the libraries can be stored, then analyzed multiple times.

[00413] A schematic exemplary of an embodiment of the invention relating to method for generating a polynucleotide comprising a sequence (B) for binding to a solid surface from a DNA target is shown in Figure 16. Step I represents denaturing a double-stranded target DNA, for example by raising the temperature. Steps II illustrate annealing to the target DNA and extending with a DNA polymerase comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the of the primer comprises sequence (A), which is not complementary to the target DNA; to form a plurality of first primer extension products, each with sequence (A) at its 5' end. The enzyme that carries out Step II is generally a DNA polymerase. In some cases a mixture of DNA polymerases can be used. In some embodiments, a DNA polymerase with strand displacement activity is used such that a growing first primer extension product can displace a

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downstream first primer extension product, producing a plurality of first primer extension products, representing different regions of the sequence of the target DNA are produced. Step III illustrates annealing a second primer to the first primer extension product that has been denatured from the target DNA. Step IV illustrates extending a second primer comprising a 3' DNA region that comprises a random sequence, wherein the primer is a tailed primer comprising a nucleic acid sequence (B) that is 5' of the random sequence, to form a plurality of double-stranded products each comprising a first primer extension product and a second primer extension product. Step V shows the removal of single stranded 3' nucleotides from the 3' region of the first primer extension product that is not hybridized to the second primer extension product. This step may be done using exonucleases. Step VI shows extension of the first primer extension product by DNA polymerase to generate a sequence (B'), complementary to sequence (B) of the second primer extension product. Step VII shows the denaturation of the first and second primer extension product by methods described previously. Step VIII shows the binding of sequence (B') of the first primer extension product to a sequence (B) on a solid surface, whereby the first primer extension product becomes hybridized to the solid surface. The immobilized sequence (B) oligonucleotide on the solid support is extended using DNA and RNA-dependent DNA polymerase, resulting in another DNA/RNA heteroduplex, as illustrated in step IX. Amplification of the first primer extension product takes place using repeated cycles of single primer isothermal linear amplification (SPIA), which comprises the following steps not shown in the figure: RNaseH cleaves the RNA from the DNA/RNA heteroduplex, a chimeric RNA/DNA primer binds to sequence (A'), and DNA polymerase with strand displacement activity is used to extend the chimeric primer to produce amplified product of the first primer extension product. As shown in step X, since the amplification products are generated in close proximity to access sequence (B) on the solid support, the amplified products are captured on the solid support through hybridization of the 3' sequence (B') to sequence (B) for further manipulations as described herein such as clonal amplification.

Alternative method for generating a polynucleotide having a defined 3' and 5' sequences from an RNA target

[00414] One aspect of the invention is a method for generating a polynucleotide having defined 3' and 5' sequences from a RNA target. The method utilizes a composite RNA/DNA oligonucleotide to generate an oligonucleotide extension product comprising sequences (A) and (C), which will allow extension of the third primer on the solid surface such that the third primer extension product comprises a sequence (C') at its 3' end than can be used as a site for isothermal amplification in a manner such that the sequence (A) is present at or near the 5' end of the amplified product produced in this amplification.

[00415] The method comprises the steps: (a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA; to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid. This extension is generally performed with an enzyme comprising RNA-dependent DNA polymerase activity. In some embodiments, the 3' portion of the primer that is complementary to the target RNA is a specific sequence. For example, where a specific region of interest of a target RNA that is

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known or suspected to be upstream of a specific sequence on the target RNA, the sequence that is complementary to the target RNA of the first primer can be designed to hybridize to this specific sequence on the target RNA such that extension of the primer results in producing a first primer extension product that is complementary to such upstream region. The specific sequence may be common to a family of target RNA. A combination of primers with various specific sequences at the 3' end can also be useful. In some embodiments, such as where the target RNA comprises mRNA, and the mRNA comprises a plurality of sequences, each having a 3' poly-A segment; the specific sequence that is complementary to the target RNA can comprise a sequence that will hybridize to the poly-A region of the mRNA, thus allowing the extension of the first primer to produce a plurality of first primer extension products, each of which is complementary to the region of an mRNA molecule adjacent to the poly-A region. In some embodiments, the sequence that is complementary to the target RNA comprises a random sequence, such that the extension of the first primer results in a plurality of first primer extension products complementary to the sequences adjacent to the sequence where each random species hybridizes. The use of a random sequence at the 3' end of the primer can be useful for performing a global amplification of a RNA target, generating a plurality of sequences which together can represent, for example substantially the whole sequence of the target RNA. In some embodiments, the relative amounts of the various sequences can be used to quantitate the relative amount of a given sequence in a sample, for example to determine the level of expression in an mRNA sample. In some embodiments more than one type of sequence that is complementary to the target RNA can be used, for instance both a primer with a random sequence and a primer, or combination of primers with a specific sequence complementary to RNA can be used. In some embodiments, multiple primers comprising different specific sequences can be used.

[00416] The method further comprises step: (b) removing the target RNA from the RNA/DNA hybrid. In some embodiments, the cleaving of the target RNA from the RNA/DNA hybrid involves selectively cleaving or degrading the target RNA. In some cases the complex comprising the first primer extension product and the nucleic acid can be heated in reaction conditions comprising Mg, which leads to cleaving the RNA including the RNA in the RNA/DNA hybrid. The RNA can also be removed from the RNA/DNA hybrid by denaturation, performed, for example by heating the sample (thermal methods), or by adding a denaturing agent, or using a combination of heating the sample and adding denaturing agents. The cleaving can be accomplished with an enzyme that cleaves RNA from an RNA/DNA hybrid such as RNase H, or a combination of RNase enzymes, or cleaving can be accomplished chemically or by heating. In some embodiments, the target RNA is completely cleaved. In other embodiments, the target RNA is only partly cleaved or degraded. The amount of cleaving required is that amount which will allow the extension of the second primer.

[00417] The method further comprises step: (c) extending a second primer, comprising a 3' segment complementary to a portion of the first primer extension product and a 5' segment non-complementary to the first primer extension product comprising sequence (B), to produce a double-stranded product with a DNA/RNA heteroduplex at one end; wherein the double-stranded product comprises a second primer extension product hybridized to the first primer extension product, and

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whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer.

[00418] The extension of the second primer is carried out with a DNA polymerase as described herein. In some embodiments, a DNA polymerase comprising both DNA and RNA dependent DNA polymerase activities is used here. In other embodiments, both a RNA dependent DNA polymerase and a DNA dependent DNA polymerase are used.

[00419] The second primer can comprise RNA, DNA, or can be a composite primer comprising both RNA and DNA. In some embodiments, the second primer can comprise a specific primer sequence that is designed to hybridize to a specific sequence in the first primer extension product. In some embodiments the second primer comprises a random primer sequence that randomly binds to the first primer extension product. Extension of the second primer comprising a random sequence produces a plurality of second primer extension products. The use of a random sequence at the 3' end of the primer is useful, for example, in performing global amplification of a target RNA, whereby a plurality of second primer extension products are produced which is representative of the sequence of the target RNA. In some embodiments, for example where the first primer is designed to hybridize to a specific sequence on a target RNA, or a sequence common to a family of RNA targets, random priming by the second primer ensures amplification of the entire selected target or family of selected targets. In this embodiment, the second primer extension products comprise sequences which are the same or substantially the same as the sequences in the target RNA (sense copies).

[00420] The second primer extension product is extended such that the 3' portion of the second primer extension product comprises a sequence (A') which is complementary to sequence (A) of the first primer. Since sequence (A) on the first primer extension product comprises RNA, both DNA dependent DNA polymerase activity and RNA dependent DNA polymerase activity are used in step (c). The primer extension results in a product that is at least partially double stranded since sequence (B) does not hybridize to the first primer extension product. The product further comprises a DNA-RNA heteroduplex region. The specific, or universal, sequence (A') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead.

[00421] In some embodiments, the sample comprising the target RNA is in a sample that also comprises DNA. In such cases, it can be advantageous to add a selective DNA dependent DNA polymerase inhibitor such as actinomycin such that it is present during step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a). The presence of a DNA dependent DNA polymerase inhibitor such as actinomycin is particularly advantageous when a first primer comprising a random sequence is used, as the inhibitor allows for the selective creation of first primer extension products to RNA without the need of separating the RNA from the DNA. This is also advantageous when the priming is carried out at specific target sequences since the sequence may be the same on the DNA when the DNA and RNA in the sample represent total nucleic acid from the same biological entity, for example, human tissue, animal tissue, and the like. The use of DNA dependent DNA polymerase inhibitors such as actinomycin is described in copending application.

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[00422] The method further comprises step: (d) cleaving the RNA in the heteroduplex from the first primer extension product such that a portion of the second primer extension product that is complementary to sequence (A) is single-stranded. The cleaving of RNA can be performed, for example by treatment with RNase H, which will selectively cleave the RNA portion of the DNA/RNA partial heteroduplex formed in step (c).

[00423] The method further comprises step: (e) annealing to the second primer extension product an oligonucleotide comprising a 3'- DNA sequence (A) that is complementary to sequence (A') and a 5'-RNA segment comprising sequence (C) that is non-complementary to the second primer extension product. The oligonucleotide comprises at least one DNA and at least one RNA portion. In some embodiments the 5' DNA segment is complementary to all of sequence (A'), in other embodiments, the 5' DNA segment is complementary to portion of sequence (A'). In some embodiments, 5' RNA segment comprising sequence (C) is partly complementary to sequence (A').

[00424] The method further comprises step: (f) extending the oligonucleotide at the 3' segment to form an oligonucleotide extension product hybridized to the second primer extension product. In some embodiments, the oligonucleotide is extended from its 3' end to produce an oligonucleotide extension product hybridized to the second primer extension product and displaces the first primer extension product. The second primer comprises a sequence (B), such that the oligonucleotide extension product will comprise a sequence (B') at or near its 3' end that is complementary to sequence (B). An optional step that may be added using a DNA polymerase that has RNA dependent DNA polymerase activity is extension of the second primer extension product to create a heteroduplex such that the second primer comprises a DNA sequence (C') that is complementary to sequence (C). This step creates an RNA/DNA heteroduplex region.

[00425] The method further comprises step: (g) denaturing the double-stranded DNA product. The first primer extension product can be separated from the second primer extension product by denaturation. Denaturation can be performed, for example by heating the sample, or by adding a denaturing agent, or using a combination of heating the sample and adding denaturing agents.

[00426] The method further comprises step: (h) attaching the single-stranded first primer extension product to a solid support by annealing sequence (B') to the solid support comprising a sequence (B). The oligonucleotide bound to the solid surface comprises sequence (B) such that binding of sequence (B') of the oligonucleotide primer extension product results in attaching the oligonucleotide primer extension product to the solid surface. The method produces a nucleic acid that is hybridized to sequence (B) on a solid surface that has a specific sequence (A) and (C) at its 5' end.

[00427] The method further comprises step: (i) extending sequence (B) on the solid support to produce a third primer extension product, comprising a 3' sequence (A') and (C'), whereby a DNA/RNA heteroduplex at one end is generated.

[00428] The extension of the third primer is carried out with a DNA polymerase as described herein. In some embodiments, a DNA polymerase comprising both DNA and RNA dependent DNA polymerase activities is used here. In other embodiments, both a RNA dependent DNA polymerase and a DNA dependent DNA polymerase are used.

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[00429] The primer extension results in a product that is double stranded and comprises sequences (B'), (A), (C) on the oligonucleotide primer extension product and sequences (B), (A') and (C') on the third primer extension product.

[00430] In some embodiments, the sample comprising the target RNA is in a sample that also comprises DNA. In such cases, it can be advantageous to add a selective DNA dependent DNA polymerase inhibitor such as actinomycin such that it is present during step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a). The presence of a DNA dependent DNA polymerase inhibitor such as actinomycin is particularly advantageous when a first primer comprising a random sequence is used, as the inhibitor allows for the selective creation of first primer extension products to RNA without the need of separating the RNA from the DNA. This is also advantageous when the priming is carried out at specific target sequences since the sequence may be the same on the DNA when the DNA and RNA in the sample represent total nucleic acid from the same biological entity, for example, human tissue, animal tissue, and the like. The use of DNA dependent DNA polymerase inhibitors such as actinomycin is described in co-pending application.

[00431] The method produces a nucleic acid that is bound to a solid surface that has a specific sequence (A') and (C') at its 3' end and a sequence (B) at or near its 5' end. The specific, or universal, sequence (A') or (C') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. The specific sequence (C') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. One aspect of the invention comprises amplification of the nucleic acid bound to the bead. In some embodiments, the amplification is carried out using isothermal amplification using a composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (C') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification. When the sequence (C') acts as a site to which a composite amplification primer binds, the amplified product that is produced has the sequence (A) and a portion of sequence (C) at its 5' end. The third primer comprises the sequence (B) and the amplified product also has the sequence (B'), complementary to (B) at or near its 3' end. Thus the method produced amplified product with defined sequences at or near both its 3' and 5' ends.

[00432] In some embodiments, for example where random sequences at the 3' end of the first and/or second primer are used, a plurality of different nucleic acids hybridized to a solid surface is created in which each of the nucleic acids has a specific sequence (A) and (C) at its 5' end and also a specific sequence (B') at its 3' end, and where the different nucleic acids have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target RNA. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target RNA.

[00433] The solid surface can be any of a variety of surfaces, some described in more detail below. The solid surface can be, for example a planar surface, for example, a planar array. In some embodiments the solid surface comprises a plurality of beads. In some embodiments the beads are magnetic.

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[00434] The step of binding the polynucleotides to the solid surface through sequence (B'), step (i), can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

[00435] In some embodiments, the method further comprises treating the solid surface with reagents to produce multiple copies of an amplification product that are substantially complementary to the third primer extension product. This step comprises carrying out an amplification reaction wherein the bound nucleic acid acts as a template for the amplification.

[00436] Generally, the amplification is carried out using the sequence (C') on the third primer extension product for the hybridization of a primer such as a composite RNA/DNA amplification primer hybridizes, allowing for amplification. In some embodiments the amplification is an isothermal amplification reaction comprising a composite RNA/DNA primer, RNase H, and a DNA polymerase with strand displacement activity. In some embodiments, the amplification is carried out using polymerase chain reaction, (PCR). For example where the third primer extension product comprises both a sequence (B) at or near its 5' end and a sequence (C') at or near its 3' end, a set of primers, one designed to hybridize to all or a portion of the sequence (C') and the other designed to hybridize to sequence (B), can be used to carry out a PCR reaction to exponentially produce double stranded amplified product.

[00437] In some embodiments, the amplification is performed by a method comprising the following steps: (j) cleaving the RNA from the heteroduplex polynucleotide product hybridized to the amplified product using RNase H to produce a single-stranded portion of the third primer extension product corresponding to sequence (C'); (k) annealing an amplification primer to the single-stranded portion of the third primer extension product complementary to sequence (C'), wherein the amplification primer has a DNA portion and a 5' RNA portion; (l) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product hybridized to the third primer extension product on the solid support; (m) repeating steps (j) to (l) to produce multiple copies of the amplified product comprising sequences (A) and (B'); and (n) capturing the amplified product on the solid support wherein the solid support comprises sequence (B).

[00438] This amplification method, utilizing a sequence (B) and (C'), allows for the production of an amplified product comprising a sequence (B') at or near its 3' end that is substantially complementary to sequence (B), and a sequence (A) near its 5' end that is complementary to sequence (A'), thus producing an amplified polynucleotide product with defined 3' and 5' ends.

[00439] In some embodiments the amplification is carried out such that the amplified product is not attached to the substrate, but is freely dissolved in the solution. In other embodiments, the amplification is carried out such that the amplified product remains bound to the substrate, for example by performing solid phase PCR such as bridge PCR. In yet other embodiments, an amplified product is generated that may float freely in solution, but which comprises a sequence, for example sequence (A) or sequence (B'), that allows it to be captured to another solid surface or other

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portion of the solid surface by hybridization to a complementary sequence bound to such surface, e.g. sequence (A') or sequence (B). In some embodiments, the amplified product is a single-stranded product and, because it is generated at the solid surface, the amplified product readily captured by complementary sequences, e.g. sequence (B), bound to the surface.

[00440] In one aspect of the invention, a plurality of beads is used, and the methods described above are carried out such that on average, one or fewer oligonucleotide primer extension product molecules are bound per bead. The beads are dispersed into an aqueous solution, and a plurality of microreactors, e.g. droplets, are produced such that on average one or fewer beads is contained within each of the plurality of microreactors. The amplification of the third primer extension products bound to the beads is then carried out such that the clonal amplification of each of the plurality of second primer extension products in the separate microreactors is achieved. This clonal amplification in microreactors can be performed on a sample of target RNA, such as whole transcriptome or total RNA, wherein the plurality of third primer extension products comprise sequences that correspond to most, to substantially all, or to all of the sequences in the target RNA. In some embodiments, the amplified products are captured by bead having attached thereto a plurality of oligonucleotides comprising complementary sequences bound to such surface (e.g. sequence (A') or sequence (B)), which are complementary to sequence (A) or sequence (B') on the amplified product.

[00441] In some embodiments, the plurality of beads, produced as described above, with each bead comprising a single third primer extension product can comprise a library. These libraries can be stored, then later clonally amplified. In some embodiments, a library of beads can comprise a plurality of beads wherein each bead had multiple copies of a single amplification product generated from a second primer extension product. These libraries can be analyzed, for example by sequencing. The libraries can be stored, and later analyzed. In some embodiments the libraries can be stored, then analyzed multiple times.

[00442] In some embodiments, a bead or isolated area of the solid surface comprises covalently attached thereto multiple oligonucleotides comprising the sequence (B) at their 5' ends, whereby upon the amplification of step (n) multiple copies of amplified product comprising sequence (B') at their 3' end are hybridized to the bead or isolated area. For example, where beads are used, a plurality of beads in a plurality of microreactors wherein, the plurality of beads has, on average one or fewer third primer extension products bound to it and there are, on average, one or fewer beads in each microreactor, a clonal amplification of the plurality of third primer extension products can be carried out, and the amplified products in each of the microreactors will bind to the bead through the sequence (B') on the amplified product to the sequence (B) on the beads. This approach produces a plurality of beads, each with multiple copies of a different sequence bound to it. Where these sequences are representative of the target RNA, the plurality of beads can constitute a library representative of such RNA.

[00443] Since the amplification products are generated in close proximity to access sequence (B) on the solid support, the amplified products are captured on the solid support through hybridization of the 3' sequence (B') to sequence (B) for further manipulations as described herein. The (B) sequences on the beads can be extended along the amplified product by a DNA polymerase or

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mixture of polymerases to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product and also comprise sequence (A') near their 3' ends. This method provides for the production of beads with polynucleotides complementary to amplified product covalently attached to the beads. Covalently attached polynucleotides such as those produce here are more robust than nucleotides that are attached only by hybridization to the beads. Thus, the covalently attached polynucleotides can be more stable and can be used with analysis methods and sequencing methods that have harsher conditions which would result in the displacement of polynucleotides bound only by hybridization.

[00444] In some embodiments, the amplified product is removed from the covalently bound polynucleotide to render the polynucleotide single stranded. Such single stranded covalently bound polynucleotides comprise a specific sequence at their 3' ends comprising sequence (A') and a portion of sequence (C'). Here, the portion of sequence (C') is the DNA portion of the chimeric amplification primer (C) that does is generally not cleaved by RNase H and therefore becomes incorporated into the amplified product. This specific sequence at the 3' end of the covalently bound polynucleotide can act as a hybridization site for a primer complementary to sequence (A') that can act as a primer to carry out sequencing by any of a variety of sequencing methods, for example, those described herein.

[00445] The sequencing methods can comprise the use of cleavable labeled terminators. The sequencing method can comprise pyrophosphate detection. The sequencing method can comprise an isothermal sequencing method, for example using chimeric primers, RNase H, and a polymerase with strand displacement activity. The sequencing method can also comprise cycle sequencing.

[00446] In some embodiments the methods of the invention provide for performing bridge PCR comprising making amplified product as described above with defined 3' and 5' ends, and further comprising the steps of exposing the amplified product to a solid substrate comprising oligonucleotide sequences attached thereto complementary to the defined 3' and 5' sequences, for example, A and B' sequences, on the amplified product in the presence of components necessary for polymerase chain reaction, and thermal cycling the system to perform bridge PCR amplification.

[00447] In some embodiments the methods of the invention provide for making amplified product as described above with defined 3' and 5' ends and further performing rolling circle amplification comprising performing the steps of: (o) hybridizing the amplified product to a nucleic acid sequence comprising regions complementary to A and B' sequences in close proximity; (p) optionally extending the gap with a DNA polymerase enzyme; (q) ligating to form a circular nucleic acid comprising the amplified product, and performing rolling circle amplification by extending a primer that is complementary to a sequence in the circular nucleic acid.

[00448] In some embodiments, the rolling circle amplification uses primers complementary to sequence (A), sequence (B'), or a sequence that was between sequences (A) and (B') in the amplified product. In some cases, such a primer can be an oligonucleotide attached to a solid surface, thus resulting in amplified product bound to the surface

[00449] In some embodiments the methods of the invention provide for performing PCR comprising making amplified product as described above with defined 3' and 5' ends, further comprising the

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steps of amplifying the amplified product using primers complementary to sequences (A) and (B), or using primers complementary to sequences (A') and (B').

[00450] In some embodiments the methods of the invention provide for performing strand displacement amplification (SDA) comprising making amplified product as described above with defined 3' and 5' ends, wherein the defined 3' and 5' ends, for example, sequences (A) and (B'), in the amplified product are designed to be cleaved by a restriction enzyme, and performing strand displacement amplification on the amplified product.

[00451] A schematic exemplary of an embodiment of the invention relating to generating a polynucleotide having a defined 3' and 5' sequences is shown in Figure 17. The figure shows a target RNA and a chimeric RNA/DNA first primer. The primer is first annealed to the target RNA. Step I illustrates extension of the first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA, to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid. The sequence complementary to a target RNA can be a specific sequence, a sequence that will hybridize to Poly-A, a sequence common to a plurality of regions (consensus sequence), or a random sequence. Step II represents separation of the target RNA from the RNA/DNA hybrid. The separation can be accomplished thermally, chemically, or enzymatically, e.g. with RNase H. The second primer comprising a 5' sequence (B) is then annealed to the first primer extension product. Step III illustrate extending a second primer, comprising a 5' sequence (B) and a 3' segment complementary to a portion of the first primer extension product, to produce a double stranded product with a DNA/RNA heteroduplex at one end; wherein the double stranded product comprises a second primer extension product hybridized to the first primer extension product, and whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer. In step IV, cleavage of the RNA from the first primer extension product in the DNA-RNA heteroduplex occurs such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded. As shown, the cleavage is performed using RNase H. Chemical and thermal means can alternatively be employed. Step V illustrates annealing to the second primer extension product a chimeric oligonucleotide comprising a 3'- DNA segment that is complementary to sequence (A') and a 5' RNA segment comprising sequence (C). Step VI is an optional step and may occur when there is DNA polymerase comprising RNA-dependent DNA polymerase activity. In this step the second primer extension product is extended along sequence C. Step VII illustrates extension of the oligonucleotide at the 3' end, generating an oligonucleotide extension product which is hybridized to the second primer extension product and comprises a sequence (B'), complementary to sequence (B) on the second primer extension product. The first primer extension product is displaced during 3'-extension of the oligonucleotide. Step VIII illustrates the denaturation of the chimeric oligonucleotide extension product from the second primer extension product. Step IX illustrates binding of the chimeric oligonucleotide extension product to a third primer comprising sequence (B) on the solid surface. Step X illustrates extension of the third primer to create a strand complementary to the chimeric oligonucleotide extension product comprising sequence (C') and (A').

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[00452] In Figure 18, Step I illustrates the steps of: cleaving the RNA from the DNA-RNA heteroduplex created in step X above to produce a single-stranded portion of the third primer extension product corresponding to sequence (C'); annealing an amplification primer to sequence (C'), wherein the amplification primer has a DNA portion and a 5' RNA portion, to the single stranded portion of the second primer extension product complementary to sequence (C'); extending the amplification primer with a DNA polymerase having strand displacement activity to produce an amplified product. These steps can be repeated to produce multiple copies of amplified product wherein the 5' portion of the amplified product has a sequence complementary to sequence (A'). In the embodiment illustrated, the amplified product shown comprises a defined sequences on both the 5' and 3' ends. Step II illustrates that the amplification products are generated in close proximity to the sequence (B) immobilized on the bead and will thus allow the amplification product to be captured on the bead via hybridization of its sequence (B'). DNA polymerase extends immobilized sequence (B) along the hybridized amplification product to generate bound nucleic acid comprising specific sequences (B) and (A'). Also shown on the amplified product is a portion of sequence (C') that is the DNA portion of the chimeric amplification primer (C). This portion does not generally become cleaved by RNase H and therefore becomes incorporated into the amplified product.

Alternative method for generating a polynucleotide having a defined 3' and 5' sequences from a DNA target

[00453] The method utilizes a composite RNA/DNA oligonucleotide to generate an oligonucleotide extension product comprising sequences (A) and (C), which will allow extension of the third primer on the solid surface such that the third primer extension product comprises a sequence (C') at its 3' end than can be used as a site for isothermal amplification in a manner such that the sequence (A) is present at or near the 5' end of the amplified product produced in this amplification, and where a second primer comprising sequence (B) is used, amplified products with defined sequences at both the 3' and 5' ends can be produced.

[00454] The method comprises the step: (a) denaturing a double-stranded target DNA. Double stranded DNA can be denatured, for example by heating, or by the addition of denaturing agents.

[00455] The method further comprises step: (b) annealing to the target DNA and extending with a DNA polymerase comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the of the primer comprises sequence (A), which is not complementary to the target DNA; to form a first primer extension product hybridized to the target DNA and comprising sequence (A) at its 5' end. This extension is generally performed with an enzyme comprising DNA-dependent DNA polymerase activity. The sequence that is complementary to the target DNA comprises a random sequence, such that the extension of the first primer results in a plurality of first primer extension products complementary to the sequences adjacent to the sequence where each random species hybridizes. The use of a random sequence at the 3' end of the primer can be useful for performing a global amplification of a target DNA, generating a plurality of sequences which together can represent, for example substantially the whole sequence of the target DNA. In some embodiments, the relative amounts of the various sequences can be used to quantitate the relative amount of a given sequence in a sample, for example to determine the number of gene copies in a

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DNA sample, or obtaining sequence information. In some embodiments, the extension of one first primer, will result in the release of a downstream first primer extension product. This can occur throughout the target DNA resulting in the release of multiple first primer extension products from the target DNA. This process can occur simultaneously on both of the strands of the double-stranded DNA target, thus creating first primer extension products complementary to sequences in both strands.

[00456] In some embodiments, the first primer extension step is carried out with a DNA polymerase capable of extension at elevated temperature that is not compatible with subsequent hybridization of the random sequence to the displaced primer-extension product. For example, Bst DNA polymerase can be used which is active at elevated temperature. The reaction can be carried out stepwise, first with an incubation at a lower temperature such as about 25 °C, followed by an incubation at higher temperature such as about 50 °C. In some embodiments, the first incubation is carried out below about 30 °C, and the second incubation is carried out above about 40 °C. In some embodiments, a DNA polymerase which is active at temperatures above about 45 °C is used to extend the first primer. Mixtures of DNA polymerases can also be useful.

[00457] The method further comprises step: (c) separating the first primer extension product from the target DNA. In some embodiments, the separation can be affected by denaturing the complex comprising the first primer extension product and the nucleic acid. Denaturation can be performed, for example by heating the sample, or by adding a denaturing agent, or using a combination of heating the sample and adding denaturing agents. The amount of cleaving required is that amount which will allow the extension of the second primer.

[00458] The method further comprises step: (d) annealing to the first primer extension product and extending a second primer comprising a 3' complementary DNA region that comprises a random sequence, wherein the second primer is a tailed primer comprising a 5' sequence (B), to form a double-stranded product comprising a first primer extension product and a second primer extension product, whereby a double-stranded product with a DNA/RNA heteroduplex at one end is generated. In some embodiments, a DNA polymerase comprising both DNA and RNA dependent DNA polymerase activities is used here. In other embodiments, both a RNA dependent DNA polymerase and a DNA dependent DNA polymerase are used.

[00459] This step may be carried out with or without prior denaturation. If carried out without denaturation, generally, only the single stranded displaced first primer extension product will hybridize to the second primer. Generally the second primer does not comprise RNA. The extension of the second primer is carried out with a DNA polymerase as described herein. The second primer comprises a random primer sequence that randomly binds to the first primer extension product. Extension of the second primer comprising a random sequence produces a plurality of second primer extension products. The use of a random sequence at the 3' end of the primer is useful, for example, in performing global amplification of a target DNA, whereby a plurality of second primer extension products are produced which is representative of the sequence of the target DNA. In some embodiments, for example where the first primer is designed to hybridize to a specific sequence on a target DNA, or a sequence common to a family of DNA targets, random priming by the second primer ensures amplification of the entire selected target or family of selected

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targets. In this embodiment, the second primer extension products comprise sequences which are the same or substantially the same as the sequences in the target DNA.

[00460] The second primer extension product is extended such that the 3' portion of the second primer extension product comprises a sequence (A') which is complementary to sequence (A) of the first composite primer. Since sequence (A) on the first primer extension product comprises RNA, both DNA dependent DNA polymerase activity and RNA dependent DNA polymerase activity are used in step (d). The primer extension results in a product that is at least partially double stranded since sequence (B) does not hybridize to the first primer extension product. The method produces a nucleic acid that comprises a sequence (B), allowing it to be bound to a solid surface by hybridization to its complement, which is immobilized on the solid surface and that has a specific sequence (A') at its 3' end. The specific, or universal, sequence (A') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead.

[00461] The method further comprises step: (e) cleaving the RNA in the heteroduplex from the first primer extension product such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded. The cleaving of RNA can be performed, for example by treatment with RNase H, which will selectively cleave the RNA portion of the DNA/RNA partial heteroduplex formed in step (d).

[00462] The method further comprises step: (f) annealing to the second primer extension product an oligonucleotide comprising a 3'- DNA segment that is complementary to sequence (A') and a 5' RNA segment comprising sequence (C).

[00463] The oligonucleotide comprises at least one DNA and at least one RNA portion. In some embodiments the 5' DNA segment is complementary to all of sequence (A'), in other embodiments, the 5' DNA segment is complementary to portion of sequence (A'). In some embodiments, 5' RNA segment comprising sequence (C) is partly complementary to sequence (A').

[00464] The method further comprises step: (g) extending the oligonucleotide along the second primer extension product to form an oligonucleotide extension product comprising a sequence (B'), complementary to sequence (B) on the second primer extension product. In some embodiments, the oligonucleotide is extended from its 3' end to produce an oligonucleotide extension product hybridized to the second primer extension product and displaces the first primer extension product. The second primer comprises a sequence (B), such that the oligonucleotide extension product will comprise a sequence (B') at or near its 3' end that is complementary to sequence (B). An optional step that may be added using a DNA polymerase that has RNA dependent DNA polymerase activity is extension of the second primer extension product to create a heteroduplex such that the second primer comprises a DNA sequence (C') that is complementary to sequence (C). This step creates an RNA/DNA heteroduplex region.

[00465] The method further comprises step: (h) denaturing the double-stranded DNA product. The oligonucleotide primer extension product can be separated from the second primer extension product by denaturation. Denaturation can be performed, for example by heating the sample, or by adding a denaturing agent, or using a combination of heating the sample and adding denaturing agents.

[00466] The method further comprises step: (i) attaching the single-stranded oligonucleotide extension product to solid support by annealing sequence (B') to the bead or isolated area

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comprising a sequence (B). The third primer comprises an oligonucleotide sequence (B) that is complementary to the sequence (B') of the oligonucleotide primer extension product and results in attaching the single stranded oligonucleotide primer extension product to the solid surface. The method produces a nucleic acid that is hybridized to a solid surface that has a specific sequence (A) at its 5' end.

[00467] Step (i) of binding the polynucleotides to the solid surface sequence (B) can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

[00468] The method further comprises the step of: (j) extending sequence (B) on the solid support to produce a third primer extension product, hybridized to the oligonucleotide extension product, comprising a 3' sequence (A') and (C'), whereby a DNA/RNA heteroduplex at one end is generated. The extension of the third primer is carried out with a DNA polymerase as described herein. In some embodiments, a DNA polymerase comprising both DNA and RNA dependent DNA polymerase activities is used here. In other embodiments, both a RNA dependent DNA polymerase and a DNA dependent DNA polymerase are used. The primer extension results in a product that is double stranded and comprises sequences (B'), (A), (C) on the first primer extension product and sequences (B), (A') and (C') on the third primer extension product.

[00469] The method produces a nucleic acid that is bound to a solid surface that has a specific sequence (A') and (C') at its 3' end and a sequence (B) at or near its 5' end. The specific, or universal, sequence (A') or (C') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. The specific, or universal, sequence (C') can be a site for primer hybridization and further analysis or amplification of the nucleic acid bound to the bead. One aspect of the invention comprises amplification of the nucleic acid bound to the bead. In some embodiments, the amplification is carried out using isothermal amplification using a composite RNA/DNA primer, RNase H, and a polymerase with strand displacement activity. For this embodiment, the sequence (C') acts as the site to which the composite RNA/DNA amplification primer hybridizes, allowing for amplification. In some embodiments, for example where random sequences at the 3' end of the first and/or second primer are used, a plurality of different nucleic acids bound to a solid surface is created in which each of the nucleic acids has a specific sequence (C') at its 3' end and also a specific sequence (B) at its 5' end, and where the different nucleic acids have different intervening sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target nucleic acid. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target nucleic acid.

[00470] In some embodiments, for example where random sequences at the 3' end of the first and/or second primer are used, a plurality of different nucleic acids hybridized to a solid surface is created in which each of the nucleic acids has a specific sequence (A) and (C) at its 5' end and also a specific sequence (B') at its 3' end, and where the different nucleic acids have different intervening

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sequences, wherein the intervening sequences are identical to or substantially identical to the sequences in the target DNA. The set of bound nucleic acids thus generated can be analyzed, for example, by sequencing in order to provide information about the sequence of the target DNA.

[00471] The solid surface can be any of a variety of surfaces, some described in more detail below. The solid surface can be, for example a planar surface, for example, a planar array. In some embodiments the solid surface comprises a plurality of beads. In some embodiments the beads are magnetic.

[00472] The step of binding the polynucleotides to the solid surface through sequence (B'), step (i), can be carried out such that only one nucleic acid is bound to an isolated area of a surface or only one nucleic acid is bound to a single bead. This isolated binding of nucleic acids can be used for clonal amplification of the specific bound nucleic acid in that area or on that bead. Such bound, isolated nucleic acids can also be stored and archived for later analysis, for example by sequencing. The bound, isolated nucleic acids can be amplified, stored, and analyzed multiple times.

[00473] In some embodiments, the method further comprises treating the solid surface with reagents to produce multiple copies of an amplification product that are substantially complementary to the third primer extension product. This step comprises carrying out an amplification reaction wherein the bound nucleic acid acts as a template for the amplification.

[00474] Generally, the amplification is carried out using the sequence (C') on the third primer extension product for the hybridization of a primer such as a composite RNA/DNA amplification primer hybridizes, allowing for amplification. In some embodiments the amplification is an isothermal amplification reaction comprising a composite RNA/DNA primer, RNase H, and a DNA polymerase with strand displacement activity. In some embodiments, the amplification is carried out using polymerase chain reaction, (PCR). For example where the second primer extension product comprises both as sequence (B) at or near its 5' end and a sequence (C') at or near its 3' end, a set of primers, one designed to hybridize to all or a portion of the sequence (C') and the other designed to hybridize to sequence (B), can be used to carry out a PCR reaction to exponentially produce double stranded amplified product.

[00475] In some embodiments, the amplification is performed by a method comprising the following steps: (k) cleaving the RNA from the heteroduplex polynucleotide product hybridized to the amplified product using RNase H to produce a single-stranded portion of the second primer extension product corresponding to sequence (C'); (l) annealing an amplification primer to the single-stranded portion of the amplified product complementary to sequence (C'), wherein the amplification primer has a DNA portion and a 5' RNA portion; (m) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product hybridized to the amplified product on the bead or isolated area; and (n) repeating steps (k) to (m) to produce multiple copies of the second polynucleotide product comprising sequences (A) and (B'). This amplification method, utilizing a sequence (B) and (C'), allows for the production of an amplified product comprises a sequence (B') at or near its 3' end that is substantially complementary to sequence (B), and a sequence (A) near its 5' end that is complementary to sequence (A'), thus producing an amplified polynucleotide product with defined 3' and 5' ends.

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[00476] In some embodiments the amplification is carried out such that the amplified product is not attached to the substrate, but is freely dissolved in the solution. In other embodiments, the amplification is carried out such that the amplified product remains bound to the substrate, for example by performing solid phase PCR such as bridge PCR. In yet other embodiments, an amplified product is generated that may float freely in solution, but which comprises a sequence, for example sequence (A) or sequence (B'), that allows it to be captured to another solid surface or other portion of the solid surface by hybridization to a complementary sequence bound to such surface, e.g. sequence (A') or sequence (B). In some embodiments, the amplified product is a single-stranded product and, because it is generated at the solid surface, the amplified product readily captured by complementary sequences, e.g. sequence (B), bound to the surface.

[00477] In one aspect of the invention, a plurality of beads is used, and the methods described above are carried out such that on average, one or fewer oligonucleotide primer extension product molecules are bound per bead. The beads are dispersed into an aqueous solution, and a plurality of microreactors, e.g. droplets, are produced such that on average one or fewer beads is contained within each of the plurality of microreactors. The amplification of the third primer extension products bound to the beads is then carried out such that the clonal amplification of each of the plurality of second primer extension products in the separate microreactors is achieved. This clonal amplification in microreactors can be performed on a sample of target DNA, such as whole transcriptome or total DNA, wherein the plurality of third primer extension products comprise sequences that correspond to most, to substantially all, or to all of the sequences in the target RNA. In some embodiments, the amplified products are captured by bead having attached thereto a plurality of oligonucleotides comprising complementary sequences bound to such surface (e.g. sequence (A') or sequence (B)), which are complementary to sequence (A) or sequence (B') on the amplified product.

[00478] In some embodiments, the plurality of beads, produced as described above, with each bead comprising a single oligonucleotide primer extension product can comprise a library. These libraries can be stored, then later clonally amplified. In some embodiments, a library of beads can comprise a plurality of beads wherein each bead had multiple copies of a single amplification product generated from a third primer extension product. These libraries can be analyzed, for example by sequencing. The libraries can be stored, and later analyzed. In some embodiments the libraries can be stored, then analyzed multiple times.

[00479] In some embodiments, a bead or isolated area of the solid surface comprises covalently attached thereto multiple oligonucleotides comprising the sequence (B) at their 5' ends, whereby upon the amplification of step (m) multiple copies of amplified product comprising sequence (B') at their 3' end are hybridized to the bead or isolated area. For example, where beads are used, a plurality of beads in a plurality of microreactors wherein, the plurality of beads has, on average one or fewer oligonucleotide primer extension products bound to it and there are, on average, one or fewer beads in each microreactor, a clonal amplification of the plurality of third primer extension products can be carried out, and the amplified products in each of the microreactors will bind to the bead through the sequence (B') on the amplified product to the sequence (B) on the beads. This approach produces a plurality of beads, each with multiple copies of a different sequence bound to it.

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Where these sequences are representative of the target DNA, the plurality of beads can constitute a library representative of such DNA.

[00480] After the amplified products are bound to the beads by hybridization, the (B) sequences on the beads can be extended along the amplified product by a DNA polymerase or mixture of polymerases to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product and also comprise sequence (A') near their 3' ends. This method provides for the production of beads with polynucleotides complementary to amplified product covalently attached to the beads. Covalently attached polynucleotides such as those produce here are more robust than nucleotides that are attached only by hybridization to the beads. Thus, the covalently attached polynucleotides can be more stable and can be used with analysis methods and sequencing methods that have harsher conditions which would result in the displacement of polynucleotides bound only by hybridization.

[00481] In some embodiments, the amplified product is removed from the covalently bound polynucleotide to render the polynucleotide single stranded. Such single stranded covalently bound polynucleotides comprise a specific sequence at their 3' ends comprising sequence (A') and a portion of sequence (C'). Here, the portion of sequence (C') is the DNA portion of the chimeric amplification primer (C) that does is generally not cleaved by RNase H and therefore becomes incorporated into the amplified product. This specific sequence at the 3' end of the covalently bound polynucleotide can act as a hybridization site for a primer complementary to sequence (A') that can act as a primer to carry out sequencing by any of a variety of sequencing methods, for example, those described herein.

[00482] The sequencing methods can comprise the use of cleavable labeled terminators. The sequencing method can comprise pyrophosphate detection. The sequencing method can comprise an isothermal sequencing method, for example using chimeric primers, RNase H, and a polymerase with strand displacement activity. The sequencing method can also comprise cycle sequencing.

[00483] In some embodiments the methods of the invention provide for performing bridge PCR comprising making amplified product as described above with defined 3' and 5' ends, and further comprising the steps of exposing the amplified product to a solid substrate comprising oligonucleotide sequences attached thereto complementary to the defined 3' and 5' sequences, for example, A and B' sequences, on the amplified product in the presence of components necessary for polymerase chain reaction, and thermal cycling the system to perform bridge PCR amplification.

[00484] In some embodiments the methods of the invention provide for making amplified product as described above with defined 3' and 5' ends and further performing rolling circle amplification comprising performing the steps of: (o) hybridizing the amplified products to a target nucleic acid comprising regions complementary to A and B' sequences in close proximity; (p) optionally extending the gap with a polymerase enzyme; and (q) ligating to form a circular nucleic acid comprising the amplified product, and performing rolling circle amplification by extending a primer that is complementary to a sequence in the circular nucleic acid.

[00485] In some embodiments, the rolling circle amplification uses primers complementary to sequence (A), sequence (B'), or a sequence that was between sequences (A) and (B') in the

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amplified product. In some cases, such a primer can be an oligonucleotide attached to a solid surface, thus resulting in amplified product bound to the surface

[00486] In some embodiments the methods of the invention provide for performing PCR comprising making amplified product as described above with defined 3' and 5' ends, further comprising the steps of amplifying the amplified product using primers complementary to sequences (A) and (B), or using primers complementary to sequences (A') and (B').

[00487] In some embodiments the methods of the invention provide for performing strand displacement amplification (SDA) comprising making amplified product as described above with defined 3' and 5' ends, wherein the defined 3' and 5' ends, for example, sequences (A) and (B'), in the amplified product are designed to be cleaved by a restriction enzyme, and performing strand displacement amplification on the amplified product.

[00488] A schematic exemplary of an embodiment of the invention relating to generating a polynucleotide having a defined 3' and 5' sequences is shown in Figure 19. The figure shows a double stranded target DNA that is denatured in step I. In step II, a chimeric RNA/DNA first primer is first annealed to the target DNA and is extended to form a first primer extension product hybridized to the target DNA, forming a RNA/DNA hybrid. The first primer comprises a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target DNA. The sequence complementary to a target DNA can be a specific sequence, a sequence that will hybridize to Poly-A, a sequence common to a plurality of regions (consensus sequence), or a random sequence. Step III represents separation of the target DNA from the RNA/DNA hybrid. The separation can be accomplished thermally, chemically, or enzymatically, e.g. with RNase H. A second primer comprising a 5' sequence (B) is then annealed to the first primer extension product. Step IV illustrate extension of the second primer, comprising a 5' sequence (B) and a 3' segment complementary to a portion of the first primer extension product, to produce a double stranded product with a DNA/RNA heteroduplex at one end; wherein the double stranded product comprises a second primer extension product hybridized to the first primer extension product, and whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer. In step V, cleavage of the RNA from the first primer extension product in the DNA-RNA heteroduplex occurs such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded. As shown, the cleavage is performed using RNase H. Chemical and thermal means can alternatively be employed. Step VI illustrates annealing to the second primer extension product a chimeric oligonucleotide comprising a 3'- DNA segment that is complementary to sequence (A') and a 5' RNA segment comprising sequence (C). Step VII is an optional step and may occur when there is DNA polymerase comprising RNA-dependent DNA polymerase activity. In this step the second primer extension product is extended along sequence C. Step VIII illustrates extension of the oligonucleotide at the 3' end, generating an oligonucleotide extension product which is hybridized to the second primer extension product and comprises a sequence (B'), complementary to sequence (B) on the second primer extension product. The first primer extension product is displaced during 3'-extension of the oligonucleotide. Step IX illustrates the denaturation of the chimeric

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oligonucleotide extension product from the second primer extension product. Step X illustrates binding of the chimeric oligonucleotide extension product to a third primer comprising sequence (B) on the solid surface. Step XI illustrates extension of the third primer to create a strand complementary to the chimeric oligonucleotide extension product comprising sequence (C') and (A'). Amplification of the oligonucleotide extension product comprising specific sequences (B) and (A') at the 3' and 5' ends, respectively, take place by carrying out the steps illustrated in Figure 18.

[00489] In one aspect of the present invention, methods are provided for amplifying a target nucleic acid or its complement on a solid support to form a plurality of amplified products comprising clonally amplifying said target sequence or its complement by linear amplification. In some embodiments, the method comprises amplification using a single primer. In some embodiments, the method comprises amplification from a double-stranded nucleic acid having a single-stranded 3' overhang at one end. In some embodiments, the method comprises amplification using a DNA-RNA chimeric primer. In some embodiments, the method comprises a combination of linear amplification using a single primer from a double-stranded nucleic acid having a single-stranded 3' overhang at one end using a DNA-RNA chimeric primer. In some embodiments, the target nucleic acid sequence is coupled to a solid support. In some cases, the amplification is isothermal. In some cases, the solid support is a bead. In some cases, the amplification results in at least 10,000; 100,000; one million or more copies of the target sequence, its complement, or a portion thereof. In some cases, the solid surface comprises a plurality of primers of substantially identical sequence. In some cases, the solid surface consists of a plurality of primers of substantially identical sequence. In some cases, the target nucleic acid sequence is a linear template. In some cases, the target nucleic acid sequence is greater than about 150, 200, 250, 300, 400, 500, 1kb, 2kb, 5kb, 10kb or more in length. In some cases, the target nucleic acid is less than about 1 megabase, 100kb, 50kb, 10kb, 5kb, or less in length.

[00490] In one aspect of the present invention, methods are provided for clonally amplifying a target nucleic acid sequence or its complement by delivering the target nucleic acid sequence into an emulsion and performing linear amplification of the target nucleic acid sequence inside the emulsion. In some cases, the method further comprises the step of forming the emulsion first. In some cases, the emulsion is formed around the target nucleic acid and/or the target nucleic acid in a reaction mixture comprising amplification reagents including but not limited to buffers; one or more enzymes such as a DNA polymerase with substantial strand displacement activity, exonuclease, and RNase H; salts; primers including chimeric primers, amplification primers, and all-DNA primers, oligonucleotides including chimeric oligonucleotides; and dNTPs. In some cases, the method may further comprise amplifying the target nucleic acid in the presence of a solid surface such as a bead, a substantially planar array, an isolated surface, or a well in a plate. In some cases the amplification results in a plurality of non-multimerized individual amplification products. These are physically separated or chemically separated amplified products. In some cases, the amplification results in a lower error rate than PCR such as for example fewer errors than 2 in every 100,000 nucleotides incorporated into amplified product.

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General Techniques

[00491] 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).

[00492] The terms "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, carbocyclic sugar analogs, .alpha.-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.sub.2 ("amidate"), P(O)R, P(O)OR', CO or CH.sub.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.

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Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

[00493] "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. The oligonucleotide(s) are generally comprised of a sequence of at least 5 nucleotides, generally from about 10 to about 100 nucleotides, about 20 to about 50 nucleotides, and often about 10 to about 30 nucleotides in length. The oligonucleotides of the invention can be DNA, RNA, DNA-RNA, or other polynucleotide. The terms oligo or sequence may be used interchangeable herein.

[00494] Various techniques can be employed for preparing an oligonucleotide utilized in the present invention. Such oligonucleotide can be obtained by biological synthesis or by chemical synthesis. For short sequences (up to about 100 nucleotides) chemical synthesis will frequently be more economical as compared to the biological synthesis. In addition to economy, chemical synthesis provides a convenient way of incorporating low molecular weight compounds and/or modified bases during the synthesis step. Furthermore, chemical synthesis is very flexible in the choice of length and region of the target polynucleotide binding sequence. The oligonucleotide can be synthesized by standard methods such as those used in commercial automated nucleic acid synthesizers. Chemical synthesis of DNA on a suitably modified glass or resin can result in DNA covalently attached to the surface. This may offer advantages in washing and sample handling. For longer sequences standard replication methods employed in molecular biology can be used such as the use of M13 for single stranded DNA as described by J. Messing (1983) Methods Enzymol, 101, 20-78.

[00495] In the present invention, nucleoside triphosphates are incorporated by a polymerase enzyme in the extension of the primer to produce an extension product. Nucleoside triphosphates are generally nucleosides having a 5'-triphosphate substituent. The nucleosides are pentose sugar derivatives of nitrogenous bases of either purine or pyrimidine derivation, covalently bonded to the 1'-carbon of the pentose sugar, which is usually a deoxyribose or a ribose. The purine bases include adenine(A), guanine (G), inosine (I), and derivatives and analogs thereof. The pyrimidine bases include cytosine (C), thymine (T), uracil (U), and derivatives and analogs thereof. Nucleoside triphosphates include deoxyribonucleoside triphosphates such as the four common triphosphates dATP, dCTP, dGTP and dTTP and ribonucleoside triphosphates such as the four common triphosphates rATP, rCTP, rGTP and rUTP. The term "nucleoside triphosphates" also includes derivatives and analogs thereof, which are exemplified by those derivatives that are recognized in a similar manner to the underivatized nucleoside triphosphates. Examples of such derivatives or analogs, by way of illustration and not limitation, are those which are biotinylated, amine modified, alkylated, and the like and also include phosphorothioate, phosphite, ring atom modified derivatives, and the like.

[00496] As used herein, the term "nucleotide" generally refers to a base-sugar-phosphate combination that is the monomeric unit of nucleic acid polymers, i.e., DNA and RNA. In some aspects of the invention modified nucleotides are used, for example, where a nucleotide is connected to a ligand. A modified nucleotide is generally the unit in a nucleic acid polymer that results from

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the incorporation of a modified nucleoside triphosphate during an amplification reaction and therefore becomes part of the nucleic acid polymer.

[00497] As used herein, a nucleoside is generally a base-sugar combination or a nucleotide lacking a phosphate moiety.

[00498] 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.

[00499] The primers of the invention are usually oligonucleotide primers. A primer is generally an oligonucleotide that is employed in an extension on a polynucleotide template. The oligonucleotide primer is usually a synthetic nucleotide that is single stranded, containing a sequence at its 3'-end that is capable of hybridizing with a sequence of the target polynucleotide. Normally, the 3' region of the primer that hybridizes with the target nucleic acid has at least 80%, preferably 90%, more preferably 95%, most preferably 100%, complementarity to a sequence or primer binding site. The number of nucleotides in the hybridizable sequence of a specific oligonucleotide primer should be such that stringency conditions used to hybridize the oligonucleotide primer will prevent excessive random non-specific hybridization. Usually, the number of nucleotides in the hybridizing portion of the oligonucleotide primer will be at least as great as the defined sequence of the target polynucleotide, namely, at least ten nucleotides, at least about 15 nucleotides and generally from about 10 to about 200, usually about 20 to about 50 nucleotides.

[00500] 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 random primers used herein are generally tailed random primers comprising a 3' segment that acts as a random primer to the target polynucleotide, and a 5' sequence that generally does not hybridize to the target polynucleotide. The sequence of a random primer (or its complement) may or may not be naturally-occurring, or may or may not be present in a pool of sequences in a sample of interest. The amplification of a plurality of polynucleotides, e.g. DNA or 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. In some embodiments one portion of a primer is random, and another portion of the primer comprises a defined sequence. For

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example, in some embodiments, a 3' portion of the primer will comprise a random sequence, while the 5' portion of the primer comprises a defined sequence. In some embodiments a 3' random portion of the primer will comprise DNA, and a 5' portion defined portion of the primer will comprise RNA, in other embodiments, both the 3' and 5' portions will comprise DNA.

[00501] Composite primers are employed in certain embodiments of the invention. Composite primers are primers that are composed of RNA and DNA portions. In some aspects, the composite primer is a tailed composite primer comprising, for example, a 3' DNA portion and a 5' RNA portion. In the tailed composite primer, a 3' portion, all or a portion of which comprises DNA is complementary to a polynucleotide; and a 5' portion, all or a portion of which comprises RNA, is not complementary to the polynucleotide and does not hybridize to the polynucleotide under conditions in which the 3' portion of the tailed composite primer hybridizes to the polynucleotide target. When the tailed composite primer is extended with a DNA polymerase, a primer extension product with a 5' RNA portion comprising a defined sequence can be created. This primer extension product can then have a second primer anneal to it, which can be extended with a DNA polymerase to create a double stranded product with an RNA/DNA heteroduplex comprising a defined sequence at one end. The RNA portion can be selectively cleaved from the partial heteroduplex to create a double stranded DNA with a 3' single stranded overhang which can be useful for a various aspects of the present invention including allowing for isothermal amplification using a composite amplification primer.

[00502] In other aspects, the composite primer is an amplification composite primer. In the amplification composite primer, both the RNA and the DNA portions are generally complementary and hybridize to a sequence in the polynucleotide to be copied or amplified. In some embodiments, a 3' portion of the amplification composite primer is DNA and a 5' portion of the composite amplification primer is RNA. The composite amplification primer is designed such that the primer is extended from the 3' DNA portion to create a primer extension product. The 5 'RNA portion of this primer extension product, in a DNA-RNA heteroduplex is susceptible to cleavage by RNase H, thus freeing a portion of the polynucleotide to the hybridization of an additional composite amplification primer. The extension of the additional composite primer by a DNA polymerase with strand displacement activity releases the primer extension product from the original primer and creates another copy of the sequence of the polynucleotide. Repeated rounds of primer hybridization, primer extension with strand displacement DNA synthesis, and RNA cleavage create multiple copies of the sequence of the polynucleotide. Composite primers are described in more detail below.

[00503] Polymerases are used in the methods of the invention, for example to extend primers to produce extension products. A polymerase, or nucleotide polymerase, is a catalyst, usually an enzyme, for forming an extension of a polynucleotide along a DNA or RNA template where the extension is complementary thereto. The nucleotide polymerase is a template dependent polynucleotide polymerase and utilizes nucleoside triphosphates as building blocks for extending the 3'-end of a polynucleotide to provide a sequence complementary with the polynucleotide template. Usually, the catalysts are enzymes, such as DNA polymerases, for example, prokaryotic DNA polymerase (I, II, or III), T4 DNA polymerase, T7 DNA polymerase, Klenow fragment, Bst DNA

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polymerase, reverse transcriptase, Bca polymerase, Vent DNA polymerase, Pfu DNA polymerase, Taq DNA polymerase, and the like, derived from any source such as cells, bacteria, such as E. coli, plants, animals, virus, thermophilic bacteria, and so forth. RNA polymerases include T7 RNA polymerase, AMV polymerase, Q-beta-replicase, and so forth. In some cases, Bst DNA Polymerase Large Fragment can be used. Bst DNA polymerase Large Fragment is the portion of the *Bacillus stearothermophilus* DNA Polymerase protein that contains the 5′ → 3′ polymerase activity, but lacks the 5′ →3′ exonuclease domain. Where the polymerase forms an extension product on a DNA template, it is referred to herein as a DNA dependent polymerase. Where the polymerase forms an extension product on a RNA template, it is referred to herein as a RNA dependent polymerase.

[00504] 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 ^{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.

[00505] The term "double stranded product" is used herein to refer to products that are produced by the extension of a primer. It is understood that the products are at least partially double stranded, for example, in the region comprising the primer extension product and its complement. The double stranded product need not be completely double-stranded, and may have single stranded regions. It is also understood that the double stranded product can have heteroduplex regions in which one strand comprises RNA and the complementary strand comprised DNA in that region.

Amplification

[00506] Some aspects of the invention comprise the amplification of polynucleotide molecules or sequences within the polynucleotide molecules. Amplification generally refers to a method that results in the formation of one or more copies of a nucleic acid or polynucleotide molecule or in the formation of one or more copies of the complement of a nucleic acid or polynucleotide molecule. Amplifications can be used in the invention, for example, to amplify or analyze a polynucleotide bound to a solid surface. The amplifications can be performed, for example, after archiving the samples in order to analyze the archived polynucleotide.

[00507] In some aspects of the invention, exponential amplification of nucleic acids or polynucleotides is used. These methods often depend on the product catalyzed formation of multiple copies of a nucleic acid or polynucleotide molecule or its complement. The amplification products are sometimes referred to as "amplicons." One such method for the enzymatic amplification of specific double stranded sequences of DNA is known as the polymerase chain reaction (PCR). This

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in vitro amplification procedure is based on repeated cycles of denaturation, oligonucleotide primer annealing, and primer extension by thermophilic template dependent polynucleotide polymerase, resulting in the exponential increase in copies of the desired sequence of the polynucleotide analyte flanked by the primers. The two different PCR primers, which anneal to opposite strands of the DNA, are positioned so that the polymerase catalyzed extension product of one primer can serve as a template strand for the other, leading to the accumulation of a discrete double stranded fragment whose length is defined by the distance between the 5' ends of the oligonucleotide primers.

[00508] Another method for amplification involves amplification of a single stranded polynucleotide using a single oligonucleotide primer. The single stranded polynucleotide that is to be amplified contains two non-contiguous sequences that are complementary to one another and, thus, are capable of hybridizing together to form a stem-loop structure. This single stranded polynucleotide already may be part of a polynucleotide analyte or may be created as the result of the presence of a polynucleotide analyte.

[00509] Another method for achieving the result of an amplification of nucleic acids is known as the ligase chain reaction (LCR). This method uses a ligase enzyme to join pairs of preformed nucleic acid probes. The probes hybridize with each complementary strand of the nucleic acid analyte, if present, and ligase is employed to bind each pair of probes together resulting in two templates that can serve in the next cycle to reiterate the particular nucleic acid sequence.

[00510] Another method for achieving a nucleic acid amplification is the nucleic acid sequence based amplification (NASBA). This method is a promoter-directed, enzymatic process that induces in vitro continuous, homogeneous and isothermal amplification of a specific nucleic acid to provide RNA copies of the nucleic acid. The reagents for conducting NASBA include a first DNA primer with a 5' tail comprising a promoter, a second DNA primer, reverse transcriptase, RNAse-H, T7 RNA polymerase, NTPs and dNTPs.

[00511] Another method for amplifying a specific group of nucleic acids is the Q-beta-replicase method, which relies on the ability of Q-beta-replicase to amplify its RNA substrate exponentially. The reagents for conducting such an amplification include "midi-variant RNA" (amplifiable hybridization probe), NTPs, and Q-beta-replicase.

[00512] Another method for amplifying nucleic acids is known as 3SR and is similar to NASBA except that the RNAse-H activity is present in the reverse transcriptase. Amplification by 3SR is an RNA specific target method whereby RNA is amplified in an isothermal process combining promoter directed RNA polymerase, reverse transcriptase and RNase H with target RNA.

[00513] Another method for amplifying nucleic acids is the Transcription Mediated Amplification (TMA) used by Gen-Probe. The method is similar to NASBA in utilizing two enzymes in a self-sustained sequence replication. See U.S. Pat. No. 6,946,254.

[00514] Another method for amplification of nucleic acids is Strand Displacement Amplification (SDA) (Westin et al 2000, Nature Biotechnology, 18, 199-202; Walker et al 1992, Nucleic Acids Research, 20, 7, 1691-1696), which is an isothermal amplification technique based upon the ability of a restriction endonuclease such as HincII or BsoBI to nick the unmodified strand of a hemiphosphorothioate form of its recognition site, and the ability of an exonuclease deficient DNA polymerase such as Klenow exo minus polymerase, or Bst polymerase, to extend the 3'-end at the

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nick and displace the downstream DNA strand. Exponential amplification results from coupling sense and antisense reactions in which strands displaced from a sense reaction serve as targets for an antisense reaction and vice versa.

[00515] Another method for amplification of nucleic acids is Rolling Circle Amplification (RCA) (Lizardi et al. 1998, Nature Genetics, 19:225-232). RCA can be used to amplify single stranded molecules in the form of circles of nucleic acids. In its simplest form, RCA involves the hybridization of a single primer to a circular nucleic acid. Extension of the primer by a DNA polymerase with strand displacement activity results in the production of multiple copies of the circular nucleic acid concatenated into a single DNA strand.

[00516] In some embodiments of the invention, RCA is coupled with ligation. For example, a single oligonucleotide can be used both for ligation and as the circular template for RCA. This type of polynucleotide can be referred to as a "padlock probe" or a "RCA probe". For a padlock probe, both termini of the oligonucleotide contains sequences complementary to a domain within a nucleic acid sequence of interest. The first end of the padlock probe is substantially complementary to a first domain on the nucleic acid sequence of interest, and the second end of the padlock probe is substantially complementary to a second domain, adjacent to the first domain near the first domain. Hybridization of the oligonucleotide to the target nucleic acid results in the formation of a hybridization complex. Ligation of the ends of the padlock probe results in the formation of a modified hybridization complex containing a circular polynucleotide. In some cases, prior to ligation, a polymerase can fill in the gap by extending one end of the padlock probe. The circular polynucleotide thus formed can serve as a template for RCA that with the addition of a polymerase results in the formation of an amplified product nucleic acid. The methods of the invention described herein, can produce amplified products with defined sequences on both the 5' and 3' ends. Such amplified products can be used as padlock probes.

[00517] Some aspects of the invention utilize the linear amplification of nucleic acids or polynucleotides. Linear amplification generally refers to a method that involve the formation of one or more copies of the complement of only one strand of a nucleic acid or polynucleotide molecule, usually a nucleic acid or polynucleotide analyte. Thus, the primary difference between linear amplification and exponential amplification is that the latter is auto catalyzed, that is, the product serves to catalyze the formation of more product, whereas in the former process the starting sequence catalyzes the formation of product but is not itself replicated. In linear amplification the amount of product formed increases as a linear function of time as opposed to exponential amplification where the amount of product formed is an exponential function of time.

[00518] In some embodiments, amplification methods can be solid-phase amplification, polony amplification, colony amplification, emulsion PCR, bead RCA, surface RCA, surface SDA, etc., as will be recognized by one of skill in the art. In some embodiments, amplification methods that results in amplification of free DNA molecules in solution or tethered to a suitable matrix by only one end of the DNA molecule can be used. Methods that rely on bridge PCR, where both PCR primers are attached to a surface (see, e.g., Adessi et al., Nucleic Acids Research (2000): 28(20): E87) can be used. In some cases the methods of the invention can create a "polymerase colony technology", or "polony", referring to a multiplex amplification that maintains spatial clustering of

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identical amplicons (see Harvard Molecular Technology Group and Lipper Center for Computational Genetics website). These include, for example, in situ polonies (Mitra and Church, Nucleic Acid Research 27, e34, Dec. 15, 1999), in situ rolling circle amplification (RCA) (Lizardi et al., Nature Genetics 19, 225, July 1998), bridge PCR (U.S. Pat. No. 5,641,658), picotiter PCR (Leamon et al., Electrophoresis 24, 3769, November 2003), and emulsion PCR (Dressman et al., PNAS 100, 8817, Jul. 22, 2003). The methods of the invention provide new methods for generating and using polonies.

[00519] In some embodiments, amplification methods such as amplification that maintains spatial clustering of identical amplicons, amplification that produces in situ polonies, picotiter amplification, amplification inside microdroplets, amplification inside the aqueous phase of microdroplets of an oil and water emulsion or the like, may provide clonal amplification. "Clonal amplification" as used herein is the amplification of a target sequence or a set of target sequences in a spatially or physically separated manner such that the amplified products are spatially or physically separated from the amplified products of other target sequences. For example, some of the methods of the present invention provide for amplification of a set of target sequences attached to, bound to, or hybridized to a solid surface such as a set of beads, isolated surfaces, or wells in a plate such that each surface on average contains one or fewer target sequences. In some cases, the amplification is carried out under conditions that the amplified products of the target sequence of a given surface are attached to, bound to, or hybridized to that surface. Further, in some cases, the given surface comprises few, substantially no, or no other amplified products from other target sequences. Similarly, clonal amplification may be carried out by separating the target sequences and their products from other target sequences and their products in other ways such as in a set of microreactors such as microdroplets such as in an emulsion, in a set of wells, or by dilution. Additionally clonal amplification may be performed using a combination of compositions and techniques such as by amplifying a set of target sequences on a solid surface or set of solid surfaces such as a bead or set of beads in a set of microdroplets in an emulsion.

Single Primer Isothermal Amplification Using a Complex Comprising an RNA/DNA Partial Heteroduplex as a Template

[00520] In some aspects of the invention, the amplification method that is used is a single primer isothermal amplification using a complex comprising an RNA/DNA partial heteroduplex as a template. In this method, termed single primer isothermal amplification, a complex comprising an RNA/DNA partial heteroduplex is a substrate for further amplification as follows: an enzyme which cleaves RNA sequence from an RNA/DNA hybrid (such as RNase H) cleaves RNA from the partial heteroduplex, leaving a partially double stranded polynucleotide complex comprising a 3' single stranded DNA sequence. The 3' single stranded sequence (formed by cleavage of RNA in the complex comprising an RNA/DNA partial heteroduplex) is generally the complement of the amplification composite primer, and thus forms a specific binding site for a composite primer. Extension of a bound composite primer by a DNA-dependent DNA polymerase with strand displacement activity produces a primer extension product, which displaces the previously bound

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cleaved primer extension product, whereby polynucleotide (generally, DNA) product accumulates. See, for example, U.S. Pat. Nos. 6,251,639 and 6,692,918.

[00521] Amplification using a complex comprising an RNA/DNA partial heteroduplex as a template for further amplification (also termed single primer isothermal amplification) generally occurs under conditions permitting composite primer hybridization, primer extension by a DNA polymerase with strand displacement activity, cleavage of RNA from an RNA/DNA hybrid and strand displacement. In so far as the composite primer hybridizes to the 3' single stranded portion (of the partially double stranded polynucleotide which is formed by cleaving RNA in the complex comprising an RNA/DNA partial heteroduplex) comprising, generally, the complement of at least a portion of the composite primer sequence, composite primer hybridization may be under conditions permitting specific hybridization.

[00522] In some embodiments, the methods of the invention result in amplification of a multiplicity, a large multiplicity, or a very large multiplicity of template polynucleotide sequences. In some embodiments, essentially all of the template polynucleotide present in the initial sample (e.g., all of the mRNA or all of the genomic DNA) is amplified. In other embodiments, at least 1, at least 5, at least 10, at least 20. at least 50, at least 100, at least 200, at least 300, or more distinct sequences (such as a gene or other subsegment of a polynucleotide, transcripts of a nucleic acid target, a marker (such as a SNP or other polymorphism) are amplified, as assessed, e.g., by analysis of marker sequences known to be present in the template sample under analysis, using methods known in the art. Template polynucleotide sequences that are amplified may be present on the same polynucleotide (e.g., a chromosome or portion of a chromosome for genomic DNA template or on the same RNA for RNA template) or on different template polynucleotides (e.g., different chromosome or portions of chromosomes for DNA template, or different RNAs for RNA template). In some case, amplification of genomic DNA is exemplified herein, it will be understood by those of skill in the art, however, that the global amplification methods of the invention are suitable for amplification of any pool or subset of polynucleotides.

[00523] In some embodiments, the methods of the invention are used to globally amplify double stranded DNA target. It is understood that in these cases, the amplified product generally is a mixture of sense and antisense copies of the template DNA. In some embodiments, the methods of the invention are used to globally amplify a single stranded DNA or RNA target. In these cases, the amplification product will generally be a copy of either the target polynucleotide (sense copy) or of the complement to the target nucleotide (antisense copy). Whether the sense or antisense copy is produced will depend on the method, as will be understood by one of ordinary skill in the art. In some embodiments, the amplification product of different senses can be annealed to form a double stranded (or partially double stranded) complex. In other embodiments, they can be prevented from annealing (or subsequently denatured) to produce a mixture of single stranded amplification products. The amplified products may be of differing lengths.

[00524] As illustrated in these embodiments, all steps are isothermal (in the sense that thermal cycling is not required), although the temperatures for each of the steps may or may not be the same. It is understood that various other embodiments may be practiced, given the general description

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provided above. For example, as described and exemplified herein, certain steps may be performed as temperature is changed (e.g., raised, or lowered).

[00525] For simplicity, the isothermal amplification methods of the invention are described as two distinct steps or phases, above. It is understood that the two phases may occur simultaneously in some embodiments (for example, if the enzyme that cleaves RNA from RNA/DNA hybrid is included in the first reaction mixture).

[00526] Although generally only one composite primer is described above, it is further understood that the amplification methods may be performed in the presence of two or more different composite primers that randomly prime template polynucleotide. In addition, the amplification polynucleotide products of two or more separate amplification reactions conducted using two or more different composite primers that randomly prime template polynucleotide can be combined.

Extension of primers

[00527] The methods of the present invention involve the extension of primers. In general, primers hybridize to, and are extended along (chain extended), a sequence within the target polynucleotide and, thus, the target polynucleotide acts as a template. The extended primers are chain "extension products." The sequence over which the primer is extended may lie between two defined sequences but need not be. In general, the primers hybridize with a sequence within the target polynucleotide. The target sequence usually contains from about 30 to 5,000 or more nucleotides, often 50 to 1,000 nucleotides. The target polynucleotide may be a fraction of a larger molecule or it may be substantially the entire molecule (polynucleotide analyte).

Composite Primers

[00528] 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, 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 when hybridized to a polynucleotide template. 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).

Composite Amplification Primers

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[00529] Composite amplification primers are RNA/DNA composite primers that can be used to create multiple copies of (amplify) a polynucleotide sequence isothermally using RNA cleavage, and DNA polymerase activity with strand displacement. Amplification with such primers is described, for example in U. S. Patents 6,251,639, 6,692,918, and 6,946,251. The composite amplification primer comprises sequences capable of hybridizing to a portion of a DNA template, and most often comprises sequences hybridizable to a defined 3'-portion of the DNA.

[00530] A composite amplification primer comprises at least one RNA portion that is capable of (a) binding (hybridizing) to a sequence on a DNA template independent of hybridization of the DNA portion(s) to a sequence on the same extension product; and being cleaved with a ribonuclease when hybridized to the DNA template. The composite amplification primers bind to the DNA template 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 DNA template remains intact, thus enabling annealing of another composite primer.

[00531] The composite amplification primers also comprise a 3' DNA portion that is capable of hybridization to a sequence on the DNA template such that its hybridization to the DNA is favored over that of the nucleic acid strand that is displaced from the DNA template 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.

Ligands and Receptors

[00532] The present invention utilizes ligands to bind polynucleotides to a solid surface. A ligand is a compound which can bind to a receptor. .The ligand and receptor (antiligand) can be members of a specific binding pair of two different molecules. In some cases, the receptor and or ligand have one or more areas on the surface or in a cavity which gives rise to specific binding. The ligand can be complementary with a particular spatial and polar organization of the receptor. The specific binding pair may be members of an immunological pair such as antigen-antibody, or may be operator-repressor, nuclease-nucleotide, biotin-avidin, hormones-hormone receptors, nucleic acid duplexes, IgG-protein A, DNA-DNA, DNA-RNA, and the like. Examples of ligands and/or receptors include, but are not limited to, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones such as steroids, hormone receptors, peptides, enzymes and other catalytic polypeptides, enzyme substrates, cofactors, drugs including small organic molecule drugs, opiates, opiate receptors, lectins, sugars, saccharides including polysaccharides, proteins, and antibodies including monoclonal antibodies and synthetic antibody fragments, cells, cell membranes and moieties therein including cell membrane receptors, and organelles. Examples of ligand-receptor pairs include antibody-antigen; lectin-carbohydrate; peptide-cell membrane receptor; protein A-antibody; hapten-antihapten; digoxigenin-anti-digoxigenin; avidin and biotin, enzyme-cofactor and enzyme-substrate.

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[00533] In one embodiment, the receptor may comprise an antibody. As used herein, the term "antibody" refers to an immunoglobulin molecule or a fragment of an immunoglobulin molecule having the ability to specifically bind to a particular antigen. The antibody may be an anti-receptor antibody specific for the receptor used in the assay. Thus, the antibody may be capable of specifically binding the receptor as the antigen. Antibodies and methods for their manufacture are well known in the art of immunology. The antibody may be produced, for example, by hybridoma cell lines, by immunization to elicit a polyclonal antibody response, or by recombinant host cells that have been transformed with a recombinant DNA expression vector that encodes the antibody. Illustrative receptors include naturally occurring receptors, e.g., thyroxine binding globulin, antibodies, enzymes, Fab fragments, lectins, nucleic acids, repressors, protection enzymes, protein A, complement component C1q, DNA binding proteins or ligands and the like. In some embodiments, the receptor is an antibody, and the ligand is a molecule or portion of a molecule that is recognized by the antibody such as an epitope, or a hapten.

[00534] In some embodiments it is desirable that the ligand be a small organic molecule, for example, a compound of molecular weight less than about 1500 g/mol, generally between about 100 to about 1000 g/mol, often between about 300 to about 600 g/mol such as biotin or other haptens. The small organic molecule can provide a means for attachment of a nucleotide sequence to a label or to a support.

[00535] 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.

[00536] Attaching the receptors that are specific binders to the ligands to a support or surface may be accomplished by well-known techniques, commonly available in the literature. See, for example, "Immobilized Enzymes," Ichiro Chibata, Halsted Press, New York (1978) and Cuatrecasas, J. Biol. Chem., 245:3059 (1970).

[00537] The ligand and receptor can be members of capture pairs. For example, capture pairs can employ reversible (e. g. , cleavable) or irreversible linkages. Non-limiting examples of reversible linkages include thiol-thiol, digoxigenin/anti- digoxigenin, and linkages using VECTREX (E Avidin DLA (Vector Laboratories, Burlingame, CA), CaptAvidinTM, NeutrAvidinN, and D-desthiobiotin (Molecular Probes, Inc., Eugene, OR).

[00538] In some embodiments, the ligand-receptor pair can be pairs of reactive molecules that can react to form a covalent bond, thus binding the polynucleotide to the surface. For example, the ligand can comprise an amine group bound to the polynucleotide that can be reacted with a functional group attached to the solid surface to form a covalent bond. The amine on the polynucleotide can react, for example with an activated carbonyl group attached to the surface, e.g. an N-hydroxy succinimide (NHS) ester. Functional groups such as N-acylimidazole, 2- or 3-bromoacrylate, cyanuric chloride, disulfide, N- hydroxysuccinimide ester, hydrazide, iodoacetyl, imidoester, isocyanate, isothiocyanate, maleimide, succinimidyl carbonate, acyl chloride, and sulfonyl chloride can be used in order to form a covalent linkage to the solid surface.

DNA Polymerase, and an Agent Capable of Cleaving an RNA-DNA Hybrid

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[00539] The isothermal amplification methods of the invention employ the following enzymes: an RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase, and an agent capable of cleaving an RNA strand of an RNA-DNA hybrid (for example, a ribonuclease such as RNase H). 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. Many reverse transcriptases, such as those from avian myeloblastosis 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 or has reduced levels RNase H activity. Reverse transcriptase devoid of RNase H or with reduced levels 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. The RNA-dependent DNA polymerase activity and DNA-dependent DNA polymerase activity may be provided by the same enzyme (for example, Bst polymerase), or these activities may be provided in separate enzymes. DNA polymerases with strand displacement activity are also useful.

[00540] One aspect of the invention is the formation of a complex comprising an RNA/DNA partial heteroduplex. This process generally utilizes the enzymatic activities of an RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase. Generally, RNA in the RNA/DNA partial heteroduplex is cleaved by an agent (such as an enzyme, such as a ribonuclease) capable of cleaving RNA from an RNA/DNA hybrid, generating a 3' single stranded portion with sequences that are complementary to RNA in a composite primer (and thus forming a binding site for a composite primer).

[00541] 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 DNA 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 and, for example, a DNA polymerase that possesses both DNA-dependent and RNA-dependent DNA polymerase activity, such as Bst DNA polymerase.

[00542] DNA-dependent DNA polymerases for use in the methods and compositions of the invention are capable of effecting extension of the composite primer according to the methods of the 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 formation of the complex comprising the RNA/DNA partial heteroduplex can be carried out by a DNA polymerase which comprises both RNA-dependent DNA polymerase and DNA-dependent DNA polymerase activities (such as Bst DNA polymerase, or a reverse transcriptase). Amplification of an RNA sequence according to methods of the invention involves the use of a DNA polymerase

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that is 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 nucleic acid strand. Preferably, the DNA polymerase does not possess substantial nicking activity. Generally, the DNA polymerase preferably has little or no 5' to 3' exonuclease activity so as to minimize degradation of primer, or primer extension products. 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' to 3' exonuclease activity has been deleted, are known in the art and are suitable for the amplification methods described herein. Mutant DNA polymerases which lack both 5' to 3' nuclease and 3' to 5' nuclease activities have also been described, for example, exo $^{-/-}$-Klenow DNA polymerase. 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. 5,744,312 (and references cited therein). Generally, the DNA polymerase has little to no proofreading activity

[00543] Suitable DNA polymerases for use in the methods and compositions of the invention include those disclosed in U.S. Pat. Nos. 5,648,211 and 5,744,312, which include exo $^-$ Vent (New England Biolabs), exo $^-$ Deep Vent (New England Biolabs), Bst (BioRad), exo $^-$ Pfu (Stratagene), Bca (Panvera), sequencing grade Taq (Promega), exo $^{-/-}$Klenow DNA polymerase, and thermostable DNA polymerases from thermoanaerobacter thermohydrosulfuricus.

[00544] One of the ribonuclease for use in the methods and compositions of the invention is capable of cleaving ribonucleotides in an RNA/DNA hybrid or heteroduplex. Preferably, the ribonuclease cleaves ribonucleotides in an RNA/DNA hybrid 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 invention are well known in the art, including ribonuclease H (RNase H), e.g., Hybridase.

[00545] As is well known in the art, DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity, and the ability to cleave RNA from a RNA/DNA hybrid may be present in different enzymes, or two or more activities may be present in the same enzyme. Accordingly, in some embodiments, the same enzyme comprises RNA-dependent DNA polymerase activity and cleaves RNA from an RNA/DNA hybrid. In some embodiments, the same enzyme comprises DNA-dependent DNA polymerase activity and cleaves RNA from an RNA/DNA hybrid. In some embodiments, the same enzyme comprises DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity and cleaves RNA from an RNA/DNA hybrid. In some embodiments, different enzymes comprise RNA-dependent DNA polymerase activity and DNA-dependent DNA polymerase activity. In some embodiments, different enzymes comprise RNA -

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dependent DNA polymerase activity and cleave RNA from an RNA/DNA hybrid. In some embodiments, different enzymes comprise DNA-dependent DNA polymerase activity and cleave RNA from an RNA/DNA hybrid. In other embodiments the RNA targets are degraded by the use of other RNases such as RNase 1, for example, for removing the target RNA following extension of all DNA first primer along the RNA targets by transcription, or a combination of RNase H and other RNases.

Nucleic Acid Target

[00546] The DNA, RNA, or polynucleotide target is generally a polymeric nucleotide, which in the intact natural state can have about 30 to 5,000,000 or more nucleotides and in an isolated state can have about 20 to 50,000 or more nucleotides, usually about 100 to 20,000 nucleotides, more frequently 500 to 10,000 nucleotides. The polynucleotide target to be amplified includes nucleic acids from any source in purified or unpurified form, which can be DNA (dsDNA and ssDNA) or RNA, mitochondrial DNA and RNA, chloroplast DNA and RNA, DNA-RNA hybrids, or mixtures thereof, genes, chromosomes, plasmids, the genomes of biological material such as microorganisms, e.g., bacteria, yeasts, viruses, viroids, molds, fungi, plants, animals, humans, and fragments thereof. Exemplary RNAs include, but are not limited to, mRNAs, tRNAs, snRNAs, rRNAs, retroviruses, small non-coding RNAs, microRNAs, polysomal RNAs, pre-mRNAs, intronic RNA, viral RNA and fragments thereof. Exemplary DNAs include, but are not limited to, genomic DNA, plasmid DNA, phage DNA, nucleolar DNA, mitochondrial DNA, chloroplast DNA, cDNA, synthetic DNA, yeast artificial chromosomal DNA ("YAC"), bacterial artificial chromosome DNA ("BAC"), other extrachromosomal DNA, primer extension products and fragments thereof. Target polynucleotide includes DNA (e.g., genomic DNA, including human genomic DNA, and mammalian genomic DNA (such as mouse, rat,)) and RNA (e.g., mRNA, ribosomal RNA, and total RNA). It should be understood that template RNA includes coding and non-coding RNA. The sequences can be naturally occurring or recombinant nucleic acid targets, including cloned nucleic fragments of interest. The terms "polynucleotide target", "nucleic acid target", "target polynucleotide" and "polynucleotide target" are used interchangeably. The term "DNA target" is interchangeable with the term "target DNA", and the term "RNA target" is interchangeable with the term "target RNA". The target nucleic acid may be a mixture of DNA and RNA targets.

[00547] The target polynucleotide 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. Polynucleotides can be obtained from sources containing very small quantities of nucleic acid, such a single cells, small numbers of cells, patient samples, forensic samples, and archeological samples. Obtaining and purifying nucleic acids use standard techniques in the art, including methods designed to isolate one or a very small number of cells, such a cell sorting or laser capture micro-dissection. The methods of the invention are particularly suited for use with genomic DNA (e.g., human and other mammalian genomic DNA), as well as RNA (e.g., total RNA or mRNA samples) or fragments thereof.

[00548] The target polynucleotide(s) 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

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each other. If the target polynucleotide is double stranded (e.g., double stranded DNA or a double stranded DNA/RNA hybrid, such as is produced by first strand cDNA synthesis), the target may first be treated to render it single stranded (e.g., by denaturation or by cleavage of the RNA portion of a DNA/RNA hybrid). Denaturation may also be carried out to remove secondary structure present in a single stranded target molecule (e.g., RNA). In some cases, double stranded DNA target polynucleotide may be first cleaved by one or more restriction endonuclease enzymes.

[00549] When the target polynucleotide is DNA, the initial step of the amplification of a target nucleic acid sequence can be rendering the target single stranded. If the target nucleic acid is a double stranded (ds) DNA, the initial step can be target denaturation. The denaturation step may be thermal denaturation or any other method known in the art, such as alkali treatment. If the target nucleic acid is present in a DNA-RNA hybrid, the initial step can be denaturation of the hybrid to obtain a DNA, or removal of the RNA strand using other means known in the art, such as thermal treatment, digestion with an enzyme that cleaves RNA from an RNA/DNA hybrid (such as RNase H) or alkali treatment, to generate single stranded DNA. When 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, and include reverse transcription of RNA strand using a primer that binds to a specific target, such as the poly-A tail of eukaryotic mRNAs or other specific or consensus sequences. In addition, reverse transcription can be primed by a population of degenerate or partially degenerate primers. First strand cDNA can be separated from the complex of RNA and first strand cDNA as described herein.

[00550] RNAs can be 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. It is understood that the RNA can be coding or noncoding RNA (such as untranslated small RNAs). RNAs can be obtained and purified using standard techniques in the art. Use of a DNA target (including genomic DNA target) can involve initial transcription of the DNA target into RNA form, which can be achieved using methods disclosed in Kurn, U.S. Pat. No. 6,251,639 B1, and by other techniques (such as expression systems) known in the art. Thus, RNA template can be itself generated from a DNA source (such as genomic DNA), using methods known in the art, including Kurn, U.S. Pat. No. 6,251,639. RNA copies of genomic DNA would generally include untranscribed sequences generally not found in mRNA, such as introns, regulatory and control elements, etc. RNA targets may also be generated from cloned genomic DNA sequences that can be subjected to in vitro transcription. Use of a DNA-RNA hybrid can involve denaturation of the hybrid to obtain a single stranded RNA, denaturation followed by transcription of the DNA strand to obtain an RNA, or other methods known in the art such as digestion with an RNase H to generate single stranded DNA.

Reaction Conditions and Detection

[00551] Appropriate reaction media and conditions for carrying out the methods of the invention include those that permit nucleic acid extension, copying, and amplification according to the methods of the invention. Such media and conditions are known to persons of skill in the art, and are

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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.sup.2+, or Mn.sup.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. 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. In some embodiments, the amplification reactions (particularly, primer extension and transcription; and generally not the step of denaturing) are performed isothermally, which substantially avoids the thermocycling process. The isothermal amplification reaction is carried out at a temperature that permits hybridization of the oligonucleotides (primer) 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.

[00552] 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.

[00553] The oligonucleotide components of the 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} times the amount of target nucleic acid.

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Primers can 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.

[00554] In the methods of the invention, the steps may be carried out in the order listed or, in some cases, may be carried out in a different order. In some methods a later step depends on the formation of a product from an earlier step, in which case such steps must be carried out in the order listed. One of ordinary skill in the art will understand which steps should be carried out in the order listed, and which steps can be carried out in a different order.

[00555] In some embodiments, the foregoing components are added simultaneously at the initiation of the isothermal 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 a denaturation step, following the denaturation step, or following hybridization of a primer to a polynucleotide template, as determined by their thermal stability and/or other considerations known to the person of skill in the art. The first primer extension product and the second primer extension product synthesis reactions can be performed consecutively, followed by an amplification steps. In these embodiments, the reaction conditions and components may be varied between the different reactions.

[00556] In some embodiments, 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 a first primer extension product synthesis. Another timepoint is at the end of a second primer extension product 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 (or depleted) enzyme. 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.

[00557] In some embodiments 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 primer extension product synthesis. Another timepoint is at the end of second primer extension synthesis. In some embodiments, the removal of primers and/or target at the end of a defined step by enzymes with appropriate nuclease activities are also useful, for example, cleavage of the RNA portion of free composite tailed primer prior to the isothermal amplification step by treatment with RNase 1.

[00558] The detection of the amplification product can be indicative of the presence of a target sequence. Quantitative analysis is also an aspect of the instant invention. Direct and indirect detection methods (including quantitation) are well known in the art. For example, by comparing

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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. The global amplification by the methods of the invention and/or the amplification of selected targets, when present in the sample, are useful for various methods which enable highly parallel nucleic acid interrogations.

Characterization of Nucleic Acids

[00559] The methods of the invention are amenable to quantitative analysis, as in some embodiments, amplification can yield sufficient single stranded polynucleotide (generally, DNA and RNA) products which accurately reflect the representation of the various DNA or RNA sequences (e.g. genomic DNA or 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. Pat. 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.

[00560] In another embodiment, the amplification methods of the invention are utilized to generate multiple copies of single stranded polynucleotide products from RNA or DNA targets that are labeled by the incorporation of labeled nucleotides during DNA 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 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 RNA or 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.

[00561] The labeled amplified products are 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) a DNA or RNA sequence of interest by generating labeled polynucleotide (generally, 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

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immobilized on blots (such as a membrane), for example arrays. 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 DNA or 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 DNA or 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 or to detect the presence or absence of defined target DNA or RNA in a sample. The measurement is also useful for measuring the relative amounts of various DNA sequences corresponding, for example, to genetic regions in the sample. 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 of the corresponding target DNA or target RNA species in the sample.

Sequencing of the Polynucleotide Products of the Invention

[00562] As described above, the methods can be used to obtain sequence information about a target RNA or target DNA of interest. The sequencing can be carried out on the primer extension products or amplification products produced by the methods herein. In some embodiments the sequencing is performed on the polynucleotides attached to solid surfaces as described herein. In one embodiment sequencing is performed on polynucleotides that are attached to the beads through oligonucleotides attached to the beads which capture amplified product, and are extended to produce a polynucleotide attached to the surface comprising a defined sequence at its 3' end.

[00563] The methods of the invention are useful, for example, for sequencing of a polynucleotide sequence of interest. The sequencing process is carried out as described for the methods described herein.

[00564] Known methods for sequencing include, for example, those described in:, Sanger, F. et al. , Proc. Natl. Acad. Sci. U. S. A. 75,5463-5467 (1977); Maxam, A. M. & Gilbert, W. Proc Natl Acad Sci USA 74,560-564 (1977); Ronaghi, M. et al. , Science 281, 363,365 (1998); Lysov, 1. et al., Dokl Akad Nauk SSSR 303, 1508-1511 (1988); Bains W. & Smith G. C. J. Theor Biol 135,303-307 (1988); Drnanac, R. et al. , Genomics 4,114-128 (1989); Khrapko, K. R. et al. , FEBS Lett 256.118-122 (1989); Pevzner P. A. J Biomol Struct Dyn 7, 63-73 (1989); and Southern, E. M. et al., Genomics 13, 1008-1017 (1992). Pyrophosphate-based sequencing reaction as described, e. g., in US patent 6,274, 320,6258, 568 and 6,210, 891), may also be used. In some cases, the methods above require that the nucleic acid attached to the solid surface be single stranded. In such cases, the unbound strand may be melted away using any number of commonly known methods such as addition of NaOH, application of low ionic (e. g., salt) strength, enzymatic degradation or displacement of the second strand, or heat processing. Where the solid surface comprises a plurality of beads, following this strand removal step, the beads can be pelleted and the supernatant discarded. The beads can then be resuspended in a buffer, and a sequencing primer or other non-amplification

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primer can be added. The primer is annealed to the single stranded amplification product. This can be accomplished by using an appropriate annealing buffer and temperature conditions, e. g., as according to standard procedures in the art.

[00565] The methods of the invention are useful, for example, for sequencing of an RNA sequence of interest. The sequencing process can be carried out by processing and amplifying a target RNA containing the sequence of interest by any of the methods described herein. Addition of nucleotides during primer extension can be analyzed using methods known in the art, for example, incorporation of a terminator nucleotide or sequencing by synthesis (e.g. pyrosequencing).

[00566] In embodiments wherein the end product is in the form of 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. 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.

[00567] Suitable dNTP analogs 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.

[00568] 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, 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

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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.

The Solid Surface

[00569] In various exemplary embodiments, a solid surface may have a wide variety of forms, including membranes, slides, plates, micromachined chips, microparticles, beads and the like. Solid surfaces may comprise a wide variety of compositions including, but not limited to, glass, plastic, silicon, alkanethiolate derivatized gold, cellulose, low cross linked and high cross linked polystyrene, silica gel, polyamide, and the like, and can have various shapes and features (e.g., wells, indentations, channels, etc.). As used herein, the terms "solid surface" and "solid substrate" are used interchangeably. In some cases these will be referred to as the surface or the support. The surface can be hydrophilic or capable of being rendered hydrophilic and may comprise inorganic powders such as silica, magnesium sulfate, and alumina; natural polymeric materials, particularly cellulosic materials and materials derived from cellulose, such as fiber containing papers, e.g., filter paper, chromatographic paper, etc.; synthetic or modified naturally occurring polymers, such as nitrocellulose, cellulose acetate, poly (vinyl chloride), polyacrylamide, cross linked dextran, agarose, polyacrylate, polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), nylon, poly(vinyl butyrate), etc.; either used by themselves or in conjunction with other materials; glass available as Bioglass, ceramics, metals, and the like. Natural or synthetic assemblies such as liposomes, phospholipid vesicles, and cells can also be employed. The surface can have any one of a number of shapes, such as strip, rod, particle, including bead, and the like.

[00570] In some embodiments, the solid surface comprises a bead or plurality of beads. The beads may be of any convenient size and fabricated from any number of known materials. Example of such materials include: inorganics, natural polymers, and synthetic polymers. Specific examples of these materials include: cellulose, cellulose derivatives, acrylic resins, glass, silica gels, polystyrene, gelatin, polyvinyl pyrrolidone, co- polymers of vinyl and acrylamide, polystyrene cross-linked with divinylbenzene or the like (as described, e. g, in Merrifield, Biochemistry 1964,3, 1385-1390), polyacrylamides, latex gels, polystyrene, dextran, rubber, silicon, plastics, nitrocellulose, natural sponges, silica gels, control pore glass, metals, cross-linked dextrans (e. g., Sephadex) agarose gel (SepharoseTM), and other solid phase supports known to those of skill in the art. The beads are generally about 2 to about 100 um in diameter, or about 5 to about 80 pm in diameter, in some cases, about 10 to about 40, um in diameter. In some embodiments the beads can be magnetic. Having magnetic beads can be useful for isolation and purification of the beads comprising nucleic acids described herein. Other methods to separate beads can also be used. For example, the capture beads

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may be labeled with a fluorescent moiety which would make the nucleic acid- bead complex fluorescent. The target capture bead complex may be separated, for example, by flow cytometry or fluorescence cell sorter.

Attachment of Oligonucleotides to the Solid Surfaces

[00571] One aspect of the invention involves attaching oligonucleotides to solid surfaces such that the oligonucleotides can hybridize with polynucleotides produced by the methods of the invention. Many methods of attaching oligonucleotides to surfaces are known. In some embodiments, the oligonucleotide is attached covalently to the solid surface. In some embodiments such attached oligonucleotides act to capture nucleic acids such as amplification products, and in some cases such attached oligonucleotides also act as primers. Methods of attaching oligonucleotides and primers to a surface are known in the art (see, e.g., Beier et al., 1999, Nucleic Acids Res. 27(9):1970-1977; Brison et al., 1982, Molecular and Cellular Biology 2:578 587; Cheung et al., 1999, Nat. Genet 21(1 Suppl):15-19; Chrisey et al., 1996, Nucleic Acids Res. 24(15):3031-3039; Cohen et. al., 1997, Nucleic Acids Res. 1997 Feb. 15; 25(4):911-912; Devivar et al., 1999, Bioorg. Med. Chem. Lett. 9(9):1239-1242; Heme et al., 1997. J. Am. Chem. Soc. 119:8916-8920; Kumar et al., 2000, Nucleic Acis Res. 28(14):e71; Lipshutz et al., 1999, Nat. Genet. 21(1 Suppl):20-24; Milner et al., 1997, Nat. Biotechnol. June;15(6):537-541; Morozov et al., 1999, Anal. Chem. 71(15):3110-3117; Proudnikov et al., 1998, Anal Biochem. 259(1):34-41; Rasmussen et al., 1991, Anal Biochem. 198(1):138-142; Rogers et al., 1999, Anal. Biochem. 266(1):23-30; Salo et al., 1999, Bioconjug Chem. 10(5):815-823; Singh-Gasson et al., 1999, Nat. Biotechnol. 17(10):974-978, and Pierce Chemical Company Catalog 1994, pp. 155-200), incorporated herein by reference).

Solid Surfaces in Microreactors or Emulsions

[00572] One aspect of the invention comprises methods and libraries relating to the attachment of nucleic acids on beads in an microreactors such as in an emulsion. As used herein, a microreactor is a small volume of fluid, generally in the volume range of microliters to nanoliters. The small volumes of the microreactors are isolated from one another allowing reactions to occur within each microreactor without significant contamination from or mixing with other microreactors unless desired. Emulsions or microemulsions comprise one approach to microreactors, in which the individual drops each can represent a microreactor. Microreactors such as emulsions can allow for the clonal amplification from a single molecule of a nucleic acid to produce a population of identical nucleic acids within the microreactor. Generally water-in-oil emulsions, also referred to as inverse emulsions are used. These emulsions have small water droplets dispersed in a hydrophobic medium. As used herein, the term oil is used broadly to refer to a hydrophobic fluid in which aqueous droplets can be dispersed. various exemplary embodiments a hydrophobic medium can be can include an oil (e.g., mineral oil, light mineral oil, silicon oil) or a hydrocarbon (e.g., hexane, heptane, octane, nonane, decane, etc.) and the like. Fluorinated hydrocarbons can also be used.

[00573] In some embodiments, solid surfaces can be included within each microreactor. For example, where a bead comprises the solid surface, an emulsion can be produced such that the solid surfaces are included within the emulsion. In some embodiments, an emulsion is produced such that

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a plurality of microreactors within the emulsion have one or fewer beads. Related methods are described, for example in WO/2004/069849, and WO/05010145A2. In some aspects of the invention, the nucleic acids are attached to the beads by binding of a ligand attached to the nucleic acid to a receptor bound to the solid surface.

[00574] The emulsion can be formed either in bulk, for example, by mixing, or can be formed by injection, for example using a microfluidic device. For use with the present invention, beads with or without attached nucleic acid template are suspended in a water-in-oil emulsion. Where the amplification reaction requires thermal cycling, such as a PCR reaction, a heat stable emulsion must be used. Where isothermal amplification is employed, the need for thermal stability may be reduced, expanding the types of emulsions that can be used. In some embodiments, a plurality of the microreactors include only one template nucleic acid species and one bead. There may be many droplets that do not contain a template nucleic acid or which do not contain a bead. Likewise there may be droplets that contain more than one copy of a template. The emulsion may be formed according to any suitable method known in the art. One method of creating emulsion is described below but any method for making an emulsion may be used. These methods are known in the art and include adjuvant methods, counter-flow methods, cross-current methods, rotating drum methods, and membrane methods. Furthermore, the size of the microcapsules may be adjusted by varying the flow rate and speed of the components. For example, in dropwise addition, the size of the drops and the total time of delivery may be varied. In some embodiments, the emulsion contains a density of about 3,000 beads encapsulated per microliter.

[00575] Various emulsions that are suitable for biologic reactions are referred to in Griffiths and Tawfik, EMBO, 22, pp. 24-35 (2003); Ghadessy et al. , Proc. Natl. Acad. Sci. USA 98, pp. 4552-4557 (2001); United States Patent No. 6,489, 103 and WO 02/22869, each fully incorporated herein by reference.

[00576] The microreactors should be sufficiently large to encompass sufficient amplification reagents for the degree of amplification required. However, the microreactors should be sufficiently small so that a population of microreactors, each containing a member of a DNA library, can be amplified by conventional laboratory equipment. The use of microreactors as described herein allows amplification of complex mixtures of templates (e. g. , genomic DNA samples or whole cell RNA) without intermixing of sequences, or domination by one or more templates (e. g. , PCR selection bias; see, Wagner et al. , 1994, Suzuki and Giovannoni, 1996; Chandler et al., 1997, Polz and Cavanaugh, 1998).

[00577] In some embodiments the micoreactors are produced such that a plurality of microreactors have one bead and one copy of a nucleic acid template. In some embodiments, limiting dilution can be used to isolate polynucleotides in a manner that is suitable for clonal amplification. In some embodiments the beads have the one copy of a nucleic acid bound to the bead. Producing a plurality of microreactors having only one bead and one nucleic acid template can be performed by diluting the bead and the nucleic acid template or the bead with the bound nucleic acid template to a dilution level at which, on average, each microreactor will contain one or fewer beads and/or one or fewer nucleic acid templates per microreactor. Determining the level of dilution at which this condition occurs can be done by calculation, and by experiment. Dilution to single bead and/or single

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molecule is described, for example in WO/2004/069849. For example, a sample comprising a plurality of beads, each comprising, on average one or fewer nucleic acids can be diluted to a concentration such that aliquots of the diluted sample that can be divided into individual microreactors (e.g., wells of a multi-well plate) can be predicted to comprise on average >0 and <1 nucleic acid molecule. Therefore, a percentage of reaction vessels can be predicted on a statistical basis (e.g., Poisson distribution) to comprise an isolated polynucleotide suitable for clonal amplification. Once isolated within the reaction vessels, polynucleotides can be amplified by various methods as described herein to yield clonal amplification.

[00578] Subsequent to amplification, the microreactors can be accessed in order to remove the contents, for example, the beads, for later analysis such as by sequencing. One method of accessing the microreactors comprises breaking the emulsion.

[00579] In one embodiment, following amplification of the nucleic acid template and the attachment of amplification copies to the bead, the emulsion is "broken" (also referred to as "demulsification" in the art). There are many methods of breaking an emulsion (see, e. g., U. S. Patent No. 5,989, 892 and references cited therein) and one of skill in the art would be able to select an appropriate method. In the present invention, one preferred method of breaking the emulsion uses additional oil to cause the emulsion to separate into two phases. The oil phase can then be removed, and a suitable organic solvent (e. g. , hexanes) is added. After mixing, the oil/organic solvent phase is removed. Subsequently, the aqueous layers above the beads are removed. The beads are then washed, for example, with a mixture of an organic solvent and buffer. Suitable organic solvents include alcohols such as methanol, ethanol, and the like. The beads bound to amplification products may then be resuspended in aqueous solution for use, for example, in a sequencing reaction according to known technologies.

[00580] In some embodiments, because the clonal amplicons that are produced are isolated as discrete populations, clonal amplification products can be carried analyzed, for example sequenced, in a parallel manner. Therefore, in some embodiments, at least at least 100, 500, 1000, 10000, 50000, 100000, 300000, 500000, or 1000000 populations of clonal amplification products can be analyzed in parallel. The skilled artisan will appreciate that various methods can be suitable for parallel analysis of clonal amplicons. Generally, such methods can produce a discrete detectable signal that can be associated or linked to individual populations of clonal amplicons.

Determination of Gene Expression Profile

[00581] The amplification methods of the invention can be used 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 whole transcriptome or total RNAs, 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.

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[00582] The methods of the present invention allow for the storage and subsequent analysis of samples, allowing for a sample to be bound to a solid substrate for archiving, then later to be analyzed by the methods described herein to determine a gene expression profile. In some embodiments, the sample can be analyzed multiple times, and stored between analyses.

[00583] 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 the 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. In some cases, the methods further provide for clonal amplification of the target RNA or a subset of the target RNA.

[00584] 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) sample to a test sample, including test samples collected at different times, including before, after, and/or during development, a treatment, and the like.

Libraries

[00585] In another embodiment, the invention encompasses a library comprising a plurality of nucleic acid molecules, wherein each nucleic acid molecule is separately immobilized to a different bead. In another embodiment, the invention encompasses a library comprising a plurality of nucleic acid molecules, wherein each nucleic acid molecule is separately immobilized to a different bead and wherein each bead comprises over 100, 000 clonal amplification copies of each nucleic acid molecule, wherein the library is contained in a single vessel. As examples, the nucleic acid molecules may be genomic DNA, cDNA, episomal DNA, BAC DNA, or YAC DNA. The genomic DNA may be animal, plant, viral, bacterial, or fungal genomic DNA. Preferably, the genomic DNA is human genomic DNA or human cDNA.

Kits

[00586] One aspect of the invention comprises kits useful for carrying out the methods of the invention.

[00587] In one aspect, the kit comprises (a) a first primer comprising a 3'-DNA portion and a 5'-RNA portion, wherein the 3'-DNA portion comprises a random sequence or a specific sequence, and

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the 5' RNA portion further comprises sequence (A), (b) a second primer comprising a 5'-ligand. In some embodiments, the kit may further comprise (c), an RNA dependent DNA polymerase, (d) a DNA dependent DNA polymerase with strand displacement activity, (e) RNase H, (f) an amplification chimeric primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the sequence of the amplification primer is the substantial the same sequence as the (A) sequence, or a combination thereof. In some cases, the kit may further comprise instructions for the use of said kit.

[00588] In one aspect, the kit comprises (a) a first primer comprising a 3'-DNA portion and a 5'-RNA portion, wherein the 3'-DNA portion comprises a random sequence and the 5' RNA portion further comprises sequence (A), (b) a second primer comprising a 5'-ligand, and (c) a chimeric oligonucleotide comprising a 3'-DNA portion substantially comprising sequence (A) and a 5'-RNA sequence (C). In some cases, the kit may further comprise (d) RNase H, (e) an RNA dependent DNA polymerase, (f) a DNA dependent DNA polymerase with strand displacement activity, (g) a chimeric amplification primer comprising a 3'-DNA portion and a 5'-RNA portion, wherein the chimeric amplification primer comprises a sequence which is substantially the same as sequence (C), or a combination thereof. In some cases, the kit may further comprise instructions for the use of said kit.

[00589] In some embodiments the second primer further comprises a sequence (B) at or near the 5'-end. In some embodiments the kit further comprises solid support with immobilized ligand binding component on it surface.

[00590] In some embodiments the kit further comprises solid surface with an oligonucleotide attached to the surface by the 5'-end and comprising a sequence (B). In other embodiment the oligonucleotide attached to the solid surface comprises a sequence hybridizable to sequence (A) and the oligonucleotide is attached by the 5'-end.

[00591] In some embodiments the kit comprises (a) a first primer that is a tailed DNA primer comprising a 5'-tail sequence (D), (b) a second primer that is a chimeric primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the 5'-end comprises a tail sequence (E), (c) a third primer which is a tailed primer comprising a 3'-sequence that comprises a sequence substantially the same as sequence (D), optionally a 5'-tail sequence (F), and 5'-ligand. In some cases, the kit may further comprise (d) an RNA dependent DNA polymerase, (e) a DNA dependent DNA polymerase with strand displacement activity, (f) RNase H, (g) a chimeric amplification primer comprising a 3'-DNA portion and a 5'-.RNA portion wherein the chimeric amplification primer comprises a sequence which is substantially the same a sequence (E), or a combination thereof. In some cases, the kit may further comprise instructions for the use of said kit.

[00592] In some embodiments, the kit comprises (a) reagents for forming an emulsion and (b) a DNA polymerase with substantial strand displacement activity. Reagents for forming a suitable water in oil emulsion are known and commercially available for example in emPCR kits II and III (454/Roche LifeSciences). Said emulsion forming reagents may include for example decamethylcyclopentasiloxane, polyphenylmethylsiloxane, water and/or buffer. In some cases, the kit may further comprise (c) one or more RNA-DNA chimeric primers, (d) an all DNA primer, (e) a solid surface such as a bead or set of beads, a substantially planar array, a well or wells in a plate, or

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an isolated surface or set of isolated surfaces, (f) RNase H, (g) a chimeric oligonucleotide, or a combination thereof. In some cases, the kit may further comprise instructions for the use of said kit.

[00593] In some embodiments the kit comprises (a) a first primer that is a tailed DNA primer comprising a 5'-tail sequence (D), (b) a second primer that is a chimeric primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the 5'-end comprises a tail sequence (E), (c) a third primer which is a tailed primer comprising a 3'-sequence that comprises a sequence substantially the same as sequence (D), optionally a 5'-tail sequence (F), and 5'-ligand. In some cases, the kit may further comprise (d) an RNA dependent DNA polymerase, (e) a DNA dependent DNA polymerase with strand displacement activity, (f) RNase H, (g) a chimeric oligonucleotide comprising a 3'-DNA sequence (E) and a 5'-RNA sequence (G), (h) a chimeric amplification primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the chimeric amplification primer comprises a sequence which is substantially the same as sequence (G), or a combination thereof. In some cases, the kit may further comprise instructions for the use of said kit.

[00594] The 3'-end sequence of the first and second primer may comprise a specific sequence or a random sequence. In some embodiments the kits useful for carrying out the methods of the invention may further comprise an inhibitor of the DNA dependent DNA polymerase, such as Actinomycin.

[00595] The components of the kits may comprise the same aspects and embodiments as described above for the components in the description of methods. For example, the ligands and receptors, the primers, the enzymes and the oligonucleotides can be those described herein to carry out the methods of the invention.

Example 1: Clonal Expansion of RNA

Step 1: Synthesis of first primer extension product

[00596] 100ng of an RNA template is provided. The provided RNA template is produced from a biological specimen using a commercially available kit (i.e. Qiagen RNeasy) according to the manufacturer's instructions. A first primer extension reaction mixture is assembled comprising a first primer consisting of a 3' annealing sequence, a portion of which is DNA, and a 5' tail sequence (A), a portion of which is RNA and the following reagents in a total volume of 10 µl:

100ng of RNA template
20 pmol of primer
0.5 µl dNTPs (25 mM)
0.1 µl RNasin
0.1 µl DTT
2 µl 5× AMV reverse transcriptase reaction buffer
DEPC treated water to 10 µl total volume

[00597] The reaction mixture is incubated for 2 min at 75° C., and then cooled to 37° C. 1 µl AMV reverse transcriptase (USB 70041Y, 15U/µl) is added to each reaction and the reaction mixture is further incubated at this temperature for 60 min. The resulting product is a first primer extension

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product of DNA with a 5' RNA tail sequence (A) annealed to an RNA template. The reaction mixture is heated to 90°C for 3 min. for enzyme inactivation.

Step 2: Synthesis of Second Primer Extension Product (Formation of (A)/(A') RNA/DNA heteroduplex)

[00598] The first primer extension product is mixed with 10 µl of the second primer extension mixture containing the following:

1 µl 10× Klenow reaction buffer

0.1 µl dNTPs (25 mM)

0.5 µl Klenow Exonuclease free (USB 70057Y 10U/µl)) DNA polymerase

8.4 µl water

[00599] The reaction mixture is incubated for 30 min at 37° C., followed by heating to 75° C. for 5 min to stop the reaction by inactivating the enzymes. The resulting primer extension products comprise a double stranded DNA product with a DNA/RNA heteroduplex at one end of sequence (A')/(A) and a partial duplex with a tailed B sequence at the other end.

Step 3: Cleavage of DNA/RNA Hybrid

[00600] To the second primer extension product reaction mixture is added 0.02 U Hybridase (RNase H) and the reaction mixture is incubated at 50° C. for 60 min and cooled to 4° C. The resulting RNase H digested primer extension product reaction mixture comprises a first primer extension product of DNA without a 5' RNA tail sequence (A) annealed to the second primer extension product of DNA with a 5' tail sequence (B) and a 3' annealing sequence (A').

Step 4: Annealing of Chimeric Oligonucleotide

[00601] Next, a chimeric oligonucleotide comprising a 3' annealing sequence (A), a portion of which is DNA, and a 5' tail sequence (C), a portion of which is RNA is added to the reaction mixture and annealed to the complementary sequence (A'). The annealing step is carried out at 50°C for 10 min. and the reaction is cooled to 4° C.

Step 5: Extension of the Second Primer Extension Product along the Chimeric Oligonucleotide

[00602] To the reaction mixture is added 2.5 units of Exonuclease Free Klenow polymerase and the reaction mixture is incubated for 30 min at 37° C., followed by heating to 75° C. for 5 min to stop the reaction by inactivating the enzymes. The resulting first primer extension product comprises a sequence (A), a 5' tail sequence (C), a portion of which is RNA, and a 3' tail sequence (B') of DNA. The product is a double stranded DNA with a DNA-RNA heteroduplex at one end (C-C').

Step 6: Attachment to a Solid Support

[00603] To the reaction mixture is added a solid support in the form of Polystyrene beads which are derivatized with a short DNA oligonucleotide comprised of sequence B. The reaction mixture is heated to 98°C for 2 minutes and cooled to room temperature to allow for annealing of the first primer extension product to the solid support. The beads are washed in 1x SSC to remove the

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second primer extension product. To the beads is added 1x Thermopole buffer (NEB), dNTPs, 1x NEB BSA, Bst DNA polymerase, and water in amounts known in the art to promote DNA polymerase activity. The reaction mixture is incubated in a thermocycler at 25°C for 5 minutes, 50°C for 30 min, 95°C for 5 min, and then cooled to 4°C. The resulting double stranded product, illustrated as the final product in **Figure 17,** is a SPIA substrate suitable for clonal expansion via SPIA amplification. Enzymes, buffers and salts are removed by washing the solid-support-bound SPIA substrate and resuspending in a suitable volume of water.

Step 7: Clonal Expansion

[00604] SPIA amplification is carried out using buffer and enzyme mixtures from NuGEN's WT-Ovation Pico RNA amplification system (NuGen Technologies Inc, San Carlos CA). In addition, the SPIA amplification uses a chimeric amplification primer that has a 5' RNA sequence and a short 3' DNA sequence (approximately 7 base pairs) and is complementary to the sequence (C') of the SPIA substrate produced in step 6. The SPIA amplification is set up as follows:

3µl Amplification primer (50µM stock)
10µl Solid-support-bound SPIA substrate
17µl water
40µl amplification buffer (WT-Ovation Pico System)
20µl Amplification Enzyme Mix (WT-Ovation Pico System)

[00605] The reactions are incubated in a thermocycler at 50°C for 60 min, followed by 95°C for 5 min, and cooled down. The amplification products are generated in close proximity to the remaining unhybridized B-sequence oligonucleotides on the solid support on which the parent SPIA substrate is bound and are therefore also immobilized on the same solid support as they are generated. This process ultimately provides a plurality of solid supports (e.g. beads), each bead with a clonally expanded sequence hybridized therein, such that the hybridized sequence has a known sequence (B') and (C) at each end. The product of this example is useful for such methods as archiving of nucleic acid sequences, reducing the complexity of nucleic acid samples, and providing a set of clonally expanded sequences on beads suitable for use in next generation sequencing platforms such as the SOLiD system by Applied Biosystems.

Example 2: Diagnosis and Prognosis of Cancer

[00606] A suggested course of treatment can be determined by RNA expression analysis of a tumor biopsy. A needle biopsy is performed on a subject to obtain tissue from the suspicious mass for further analysis. The biopsied tissue recovered from the subject is processed to extract and purify total RNA using a commercially available Qiagen RNeasy kit according to the manufacturer's instructions.

[00607] 500pg of total RNA representing at least a portion of the transcriptome of the biopsied material is amplified by the methods of the present invention as described briefly herein. To the RNA in a reaction mixture is added: 100 pmol of a first primer comprising random first primer and a poly dT first primer, a 5' segment and a 3' segment, a portion of the 5' segment comprising RNA,

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and a portion of the 3' segment comprising DNA. The 3' DNA segment of the random first primer further comprises an annealing sequence that comprises random hexamers. The 5' RNA segment of the random first primer comprises a tag sequence (A).

[00608] To the reaction mixture 10pmol of a poly-T first primer is also added comprising a 5' segment and a 3' segment, a portion of the 5' segment comprising RNA, and a portion of the 3' segment comprising DNA in the reaction mixture. The 3' DNA segment of this poly-T first primer further comprises an annealing sequence that hybridizes to and is complementary to a portion of the poly A tail of mRNA transcripts present in the total RNA of the reaction mixture, and the 5' RNA segment comprises a tag sequence (A).

[00609] The volume of the reaction mixture is adjusted to 10µl with DEPC treated water, and then the reaction mixture is heated to 75°C for 2 minutes and cooled. First strand synthesis is carried out using the buffer and enzyme reagents provided with the WT-Ovation Pico RNA Amplification kit, and incubation conditions are as described in the User Guide and Quick Protocol: (http://www.nugeninc.com/tasks/sites/nugen/assets/File/user_guides/userguide_wt_ov_pico.pdf and http://www.nugeninc.com/tasks/sites/nugen/assets/File/quick_protocols/qp_wt_ov_pico.pdf.).

[00610] The first primer extension product is mixed with 10µl of the second primer extension mixture containing the following:
second strand cDNA synthesis enzyme mixture and second strand buffer mixture from the NuGEN's WT-Ovation Pico amplification system (as above)
20pmol of a second primer comprising a 5' segment and a 3' segment, a portion of the 5' segment comprising a tag sequence (B), and a portion of the 5' segment comprising an annealing sequence.

[00611] The reaction mixture is incubated under the conditions described in the use Guide for the WT-Ovation Pico RNA Amplification system, followed by heating to 75°C for 5 minutes to stop the reaction by inactivating the enzymes. The resulting primer extension products comprise a partial double stranded DNA product with a DNA/RNA heteroduplex at one end of a sequence (A')/(A). The first primer extension product in the reaction mixture is then digested using exonuclease 1 (0.5 µl at 37°C for 30 min.) followed by inactivation of the enzymes (80°C for 20 minutes). The first and second primer extension products are then purified using Agencourt magnetic beads as per the manufacturer's instructions (User Guide as above) .

[00612] Isothermal linear amplification (SPIA) is then carried out in a reaction mixture containing the above purified reaction products (10 µl), 2 µl chimeric amplification primer (100 µM stock solution), 18 µl water, 40 µl amplification buffer and 20 µl amplification enzyme mixture as provided in the WT-Ovation Pico RNA Amplification System (NuGen Technologies). The amplification is carried out according to the instructions provided for the WT-Ovation Pico RNA Amplification System.

[00613] The amplified product is then analyzed and quantitated by Real Time qPCR with SYBR Green, using an MJ Opticon thermocycler. Amplification reactions are diluted 1:100 in Tris-EDTA and 2 µl of the diluted DNA are analyzed using primer pairs specific for abl, ras, and her2 in three separate reactions.

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[00614] The results of the qPCR are analyzed to determine that abl is overexpressed in the cells of the suspicious mass. The results are combined with immunohistochemical and cytological analysis to determine a suggested course of therapy.

Altenatively, a gene expression profile of the sample can be obtained using microarrays such as GeneChip (Affymetrix). The amplified reaction products are subjected to fragmentation and labeling with Biotin using NuGEN's Ovation-F/L reagents and protocol. The fragmented and labeled products are used for hybridization to GeneChip according to the manufacturer protocol. The resulting hybridization data provides a gene expression profile of the sample.

Example 3: Personal Genomics

[00615] An individual is tested by a personal genomics business using the methods of the present invention for single nucleotide polymorphisms (SNPs) within the BRCA1, BRCA2, p53, MPO, NAT1, NAT2, and ras coding regions that are related to increased risks for specific types of cancer.

[00616] The individual supplies a small sample of tissue (i.e. a cheek swab) to the personal genomics business. Genomic DNA from the sample of tissue is isolated using a commercially available kit (i.e. Promega's Wizard ® Genomic DNA Purification Kit), according to the manufacturer's protocol.

[00617] 1 to 10ng of purified genomic DNA is used to clonally amplify the sequences corresponding to the genomic regions with known, cancer related, SNPs of the BRCA1, BRCA2, p53, MPO, NAT1, NAT2, and ras genes on a solid support (i.e. a bead). The target sequences are clonally amplified by isothermal linear amplification using the steps shown in Figure 13 steps I(b), II(b), and III(b); Figure 17 steps IV to X; and Figure 18 of the present application.

[00618] The set of beads comprising the clonally amplified sequences are loaded onto a SOLiD™ Analyzer and the sequences of the regions of interest are determined using the manufacturer's protocol using a primer complementary to the sequence (A) on the 5' end of the amplified product.

[00619] The resulting sequences are used to determine the presence or absence of SNPs related to cancer in the genes of interest. A report is generated that includes the SNPs identified, the impact of the SNPs on lifetime risk of developing specific diseases or conditions, and suggestions for prophylactic or therapeutic interventions.

Example 4: Analysis of Fetal DNA

[00620] A fetal sample is obtained by amniocentesis. DNA is extracted and purified from the sample using a commercially available kit (i.e. Promega's Wizard ® Genomic DNA Purification Kit). The DNA is attached to a solid support according to the method outlined in Figure 19 and clonally amplified according to the method outlined in Figure 18. The set of beads comprising the clonally amplified sequences are loaded onto a Genome Sequencer FLX Titanium Series from Roche/454 Life Sciences and the sequences of the regions of interest are determined using the manufacturer's protocol using a primer complementary to the sequence (A) on the 5' end of the amplified product.

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[00621] The resulting sequences are used to determine the presence or absence of SNPs related to prenatal diseases or conditions. A report is generated that includes the SNPs identified, the impact of the SNPs on lifetime risk of developing specific diseases or conditions, and suggestions for prophylactic or therapeutic interventions.

[00622] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

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CLAIMS

WHAT IS CLAIMED IS:

1. A method for amplifying a target nucleic acid sequence or its complement on a solid support to form a plurality of amplified products comprising clonally amplifying said target sequence or its complement by:

(i). linear amplification;

(ii). amplification using a single primer;

(iii). amplification from a double-stranded nucleic acid having a 3' overhang;

(iv). amplification using a DNA-RNA chimeric primer; or

(v). (i), (ii), (iii), and (iv);

wherein said target nucleic acid sequence is coupled to said solid support.

2. The method of claim 1 wherein said amplification is isothermal.

3. The method of claim 1 wherein said solid support is a bead or an array.

4. The method of claim 1 wherein said amplification results in at least 50, 100, 500, 1,000, 5,000 copies of the target nucleic acid sequence.

5. The method of claim 1, wherein said solid surface comprises a plurality of primers of substantially identical sequence.

6. The method of claim 1, wherein said target nucleic acid sequence is from a linear template.

7. The method of claim 1, wherein said target nucleic acid sequence is greater than 200 base pairs.

8. A method for clonally amplifying a target nucleic acid sequence or its complement comprising:

delivering the target nucleic acid sequence or its complement into an emulsion; and

performing linear amplification of said target nucleic acid sequence inside said emulsion.

9. The method of claim 8 wherein said amplification results in a plurality of non-multimerized amplified products.

10. The method of claim 1 or 8 wherein the amplified products have fewer than 2 errors for every 100,000 nucleotides.

11. A kit comprising a first primer, and an RNA-DNA chimeric oligonucleotide; the kit optionally further comprising a second primer, a polymerase having substantial strand displacement activity, RNase H, and instructions for use of said reagents.

12. A kit comprising a container containing reagents for forming a water in oil emulsion and a DNA polymerase with substantial strand-displacement activity; the kit optionally further comprising one or more RNA-DNA chimeric primers, an RNA-DNA chimeric oligonucleotide, RNase H, and instructions for use of said reagents.

13. A method comprising:

(a) extending a first primer or a set of first primers with a DNA polymerase comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer, sequence (P) is complementary to

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a target nucleic acid and a portion of the 5' RNA segment, sequence (A), is not complementary to the target nucleic acid, to form a first primer extension product or set of first primer extension products hybridized to the target nucleic acid;

(b) separating or removing the first primer extension product or the set of first primer extension products from the target nucleic acid;

(c) extending a second primer with a DNA polymerase comprising a 3' DNA segment and a 5' DNA segment, wherein a portion of the 3' DNA segment is complementary to the first primer extension product or set of first primer extension products and a 5' portion, sequence (B), of the of the second primer is not complementary to the first primer extension product or the set of first primer extension products, to produce a double-stranded DNA product or a set of double-stranded DNA products, each product comprising the first primer extension product hybridized to a second primer extension product, whereby the second primer extension product has a DNA sequence (A') that is complementary and hybridized to the RNA sequence (A) of the first primer extension product at its 3' end, thereby forming the double stranded nucleic acid product or products with an RNA/DNA heteroduplex at one end;

(d) cleaving the RNA from the RNA/DNA heteroduplex;

(e) annealing a chimeric oligonucleotide comprising a 5' end wherein the 5' end comprises sequence (C), and the 5' further end comprises RNA, and a 3' end wherein the 3' end comprises the DNA sequence (A), to the double stranded nucleic acid product or products; and

(f) extending the double stranded nucleic acid product or products with a DNA polymerase to produce a sequence (C)-(C') RNA-DNA heteroduplex.

14. The method of claim 13 wherein the second primer further comprises a ligand at a 5' end of the 5' DNA segment.

15. The method of claim 13 further comprising binding the ligand to a solid surface, whereby the first and second primer extension product or products are bound to the solid surface.

16. The method of claims 13 or 15 further comprising an amplification with the steps of:

(g) cleaving the RNA portion of the chimeric oligonucleotide in the DNA-RNA heteroduplex, whereby sequence (C') of step (f) is single stranded.

(h) annealing an amplification primer to the single stranded portion of the second primer extension product complementary to sequence (C), wherein the amplification primer has a DNA portion and a 5' RNA portion;

(i) extending the amplification primer with a DNA polymerase having strand displacement activity to produce an amplified product hybridized to the second primer extension product;

(j) cleaving the RNA from the amplified product hybridized to the second primer extension product in the RNA-DNA heteroduplex; and

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(k) repeating steps (h) to (j) to produce multiple copies of amplified product comprising a 5' end and a 3' end, wherein the 5' end comprises sequence (A) and the 3' end comprises sequence (B').

17. The method of claim 13 wherein the 3' portion of the first primer that is complementary to the target nucleic acid comprises a random nucleotide sequence.

18. The method of claim 13 using a set of first primers wherein the members of the set of first primers each comprises a distinct 3' DNA annealing sequence, each specific for a target or region of template nucleic acid.

19. The method of claim 13 wherein the 3' portion of the first primer that is complementary to the target nucleic acid comprises a degenerate or a random sequence for annealing to multiple target sequences.

20. The method of claim 13 wherein the target nucleic acid is RNA.

21. The method of claim 13 wherein the target nucleic acid is DNA.

22. The method of claim 13 wherein, in step (b), the target RNA is cleaved by heat, enzyme treatment, or chemical treatment or enzymes.

23. The method of claim 20 wherein the 3' portion of the first primer that is complementary to the target RNA comprises a sequence that is complementary to polyadenosine (poly-A).

24. The method of claim 20 wherein the RNA target is contained within a sample that also comprises DNA, and actinomycin is added prior to step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a).

25. The method of claim 13 wherein the 3' segment of the second primer complementary to a portion of the first primer extension product comprises one or more sequences selected from the group consisting of a random nucleotide sequence, a specific sequence complementary to a specific sequence of the first primer extension product, and a sequence common to multiple first primer extension products.

26. The method of claim 16 wherein the amplification is a clonal amplification.

27. The method of claim 16 wherein the solid surface is a bead or an isolated area on a surface.

28. The method of claim 27 wherein the bead or isolated area is the only bead or isolated area within isolated liquid volume such that the amplified product is contained within such liquid volume.

29. The method of claim 28 wherein the liquid volume is an aqueous droplet within a non-aqueous fluid.

30. The method of claim 29 wherein the droplet is part of a microemulsion.

31. The method of claim 28 wherein the liquid volume is a well in a plate.

32. The method of claim 28 wherein the bead or isolated area comprises covalently attached thereto multiple oligonucleotides comprising the sequence (B), at their 3' ends, whereby upon the amplification of step (k) multiple copies of amplified product comprising sequence (B') at their 5' end are hybridized to the bead or isolated area.

33. A method of producing a bead or isolated area with multiple copies of a nucleotide sequence covalently attached thereto by performing the method of claim 32 further comprising extending the oligonucleotide comprising sequence (B) to produce a multiple polynucleotides covalently attached to the bead or isolated area that

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are substantially complementary to the amplified product, wherein the multiple polynucleotides comprise sequence (A') near their 5' ends and sequence (B) near their 3' ends.

34. A sequencing method comprising performing the method of claim33, further comprising the steps of removing the amplified product to render the covalently attached polynucleotides single-stranded, and extending a sequencing primer, a portion of which is complementary to a portion of sequence (C') to produce detectable oligonucleotide fragments characteristic of the sequence of the polynucleotide bound to the bead or isolated area.

35. The method of claim 34 wherein the sequencing method comprises cleavable labeled terminators.

36. The method of claim 34 wherein the sequencing method comprises pyrophosphate detection.

37. The method of claim 34 wherein the sequencing method is an isothermal sequencing method.

38. The method of claim 34 wherein the sequencing method comprises cycle sequencing.

39. The method of claim 34 wherein the sequencing method comprises sequencing by ligation.

40. A method for generating an amplified nucleic acid of comprising:

(a) providing a first nucleic acid or a set of first nucleic acids comprising DNA corresponding to a region of a target nucleic acid and further comprising a 5' end and a 3' end, wherein said 5' end of the first nucleic or the set of first nucleic acids comprises a sequence (A) and a sequence (C), wherein said sequence (A) is 3' of sequence (C), and wherein sequence (C) comprises RNA, and wherein said 3' end of the first nucleic acid comprises sequence (B');

(b) hybridizing said first nucleic acid or set of first nucleic acids to a solid support comprising an oligonucleotide comprising a sequence (B) complementary to the sequence (B') of the first nucleic acid;

(c) extending the oligonucleotide to produce a double stranded product comprising a second nucleic acid hybridized to the first nucleic acid, wherein the second nucleic acid comprises a 3' segment complementary to a portion of the first nucleic acid and a 5' segment complementary to a portion of the first nucleic acid, whereby a portion of the 3' segment comprises a sequence (A') and a portion of the 3' segment comprises a sequence (C'), and wherein the sequence (A') is 5' of the sequence (C'), and whereby a portion of the 5' segment comprises a sequence (B).

41. The method of claim 40 wherein the solid support comprises a substantially planar array.

42. The method of claim 40 wherein the solid support comprises a bead.

43. The method of claim 42 wherein the bead comprises a magnetic bead.

44. The method of claim 42 wherein the bead comprises only one copy of the first and second nucleic acid.

45. The method of claim 40, wherein the target nucleic acid is RNA.

46. The method of claim 40, wherein the target nucleic acid is DNA.

47. The method of claim 40, wherein a plurality of first and second nucleic acids are provided corresponding to different sequences in the target nucleic acid.

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48. The method of claim 47 wherein the plurality of first and second nucleic acids are bound to one or a plurality of beads or a plurality of isolated areas on a surface.

49. The method of claim 48 wherein the plurality of first and second nucleic acids are bound to a plurality of beads under conditions such that generally, one or fewer first and second nucleic acid is bound to one bead or one isolated area on a surface.

50. The method of claim 49 further comprising amplification by treating the beads or isolated areas with reagents to produce multiple copies of an amplification product complementary to all or a portion of the second nucleic acid products.

51. The method of claim 50 wherein the plurality of beads or isolated areas are contained within a plurality of isolated volumes such that generally one or fewer beads or isolated area is associated with each isolated volume, and whereby the production of multiple copies of amplification product results in multiple copies of substantially one amplification product in each volume.

52. The method of claim 51 wherein the amplification is carried out with a reaction mixture comprising RNase H, an amplification primer comprising a DNA portion and a 5' RNA portion, and a DNA polymerase with strand displacement activity.

53. The method of claim 49 further comprising the step of storing the beads or isolated areas comprising generally one or fewer primer extension products per bead or isolated area.

54. The method of claim 50 further comprising clonally amplifying the primer extension product bound to the beads or isolated areas after storing them, wherein the amplification is carried out with a reaction mixture comprising RNase H, an amplification primer comprising a DNA portion and a 5' RNA portion, and a DNA polymerase with strand displacement activity.

55. A method for attaching a polynucleotide sequence that is representative of a sequence within a nucleic acid target molecule to a solid surface comprising:

(a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer, sequence (P), is complementary to a target nucleic acid and a 5' portion of the of the primer, sequence (A), is not complementary to the target nucleic acid, to form a first primer extension product hybridized to the target nucleic acid;

(b) separating or removing the first primer extension product from the target nucleic acid;

(c) extending a second primer to produce a double stranded product comprising a second primer extension product hybridized to the first primer extension product, wherein the second primer comprises a 3' segment complementary to a portion of the first primer extension product and a ligand, whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer; and

(d) binding the ligand to a receptor bound to a solid surface whereby the second primer extension product is attached to the solid surface.

56. A method comprising:

(a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA; to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid;

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(b) cleaving the target RNA from the RNA/DNA hybrid; and

(c) extending a second primer, comprising a ligand and a 3' segment complementary to a portion of the first primer extension product, to produce a double stranded product with a DNA/RNA heteroduplex at one end; wherein the double stranded product comprises a second primer extension product hybridized to the first primer extension product, and whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer.

57. The method of claim 56 wherein the 3' portion of the primer that is complementary to the target RNA comprises a random nucleotide sequence.

58. The method of claim 56 wherein the 3' portion of the primer that is complementary to the target RNA comprises a sequence that is complementary to polyadenosine (poly-A).

59. The method of claim 56 wherein the 3' portion of the primer that is complementary to the target RNA comprises a specific sequence that is complementary to a multiplicity of targets.

60. The method of claim 56 wherein, in step (b), the target RNA is cleaved by heat, enzyme treatment, or chemical treatment.

61. The method of claim 56 wherein the RNA target is in a sample that also comprises DNA, and wherein actinomycin is added prior to step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a).

62. The method of claim 56 wherein the 3' segment of the second primer complementary to a portion of the first primer extension product comprises a random nucleotide sequence, a specific sequence complementary to a specific sequence of the first primer extension product, or a sequence common to multiple first primer extension products.

63. The method of claim 56 wherein the second primer further comprises a nucleotide sequence (B) that is not complementary to the first primer extension product sequence.

64. The method of claim 56 further comprising step (d) of binding the ligand to a solid surface, whereby the second primer extension product becomes bound to the solid surface.

65. The method of claim 64 wherein the binding of the ligand results in the double stranded product being bound to the solid surface.

66. The method of claim 64 wherein the binding of the ligand results in the single stranded second primer extension product being bound to the solid surface.

67. The method of claims 64 further comprising treating the solid surface with reagents to produce multiple copies of an amplification product that are substantially complementary the second primer extension product.

68. The method of claim 64 wherein the solid surface comprises a substantially planar array.

69. The method of claim 64 wherein the solid surface comprises a bead.

70. The method of claim 69 wherein the bead comprises a magnetic bead.

71. The method of claim 69 wherein the bead comprises only one copy of second primer extension product.

72. The method of claim 71 wherein the second primer extension product is single-stranded.

73. The method of claim 71 wherein the second primer extension product is double-stranded.

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74. The method of claim 57 wherein a plurality of second primer extension products are produced corresponding to different sequences in the target RNA.

75. The method of claim 74 wherein the plurality of second primer extension products is bound to one or a plurality of beads or a plurality of isolated areas on a surface.

76. The method of claim 75 wherein the plurality of second primer extension products is bound to a plurality of beads under conditions such that generally, one or fewer second primer extension products is bound to one bead or one isolated area on a surface.

77. The method of claim 75 further comprising treating the beads or isolated areas with reagents to produce multiple copies of an amplification product complementary to all or a portion of the second primer extension products.

78. The method of claim 77 wherein the plurality of beads or isolated areas are contained within a plurality of isolated volumes such that generally one or fewer beads or isolated area is associated with each isolated volume, and whereby the production of multiple copies of amplification product results in multiple copies of substantially one amplification product in each volume.

79. The method of claim 78 wherein the amplification is carried out with a reaction mixture comprising RNase H, an amplification primer comprising a DNA portion and a 5' RNA portion, and a DNA polymerase with strand displacement activity.

80. The method of claim 76 further comprising the step of storing the beads or isolated areas comprising generally one or fewer primer extension products per bead or isolated area.

81. The method of claim 80 further comprising clonally amplifying the primer extension product bound to the beads or isolated areas after storing them.

82. A method for amplifying a nucleic acid representative of a target RNA comprising the method of claim 64 and further comprising the steps of:

(e) cleaving the RNA in the heteroduplex from the first primer extension product such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded;

(f) annealing an amplification primer to the single stranded portion of the second primer extension product complementary to sequence (A), wherein the amplification primer has a DNA portion and a 5' RNA portion;

(g) extending the amplification primer with a DNA polymerase having strand displacement activity to produce an amplified product hybridized to the second primer extension product;

(h) cleaving the RNA from the amplified product hybridized to the second primer extension product; and

(i) repeating steps (f) to (h) to produce multiple copies of amplified product.

83. The method of claim 82 wherein the second primer further comprises a sequence (B that is not complementary to the first primer extension product sequence, whereby the amplification product comprises a sequence at its 3' end which is complementary to (B).

84. The method of claim 56 wherein the target RNA comprises messenger RNA.

85. A method comprising:

(a) denaturing a double-stranded target DNA;

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(b) annealing to the target DNA and extending with a DNA polymerase comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the of the primer comprises sequence (A), which is not complementary to the target DNA; to form a plurality of first primer extension products, each with sequence (A) at its 5' end; and

(c) extending a second primer comprising a ligand and a 3' DNA region that comprises a random sequence, wherein the primer is optionally a tailed primer comprising a nucleic acid sequence (B) that is 5' of the random sequence, to form a plurality of double-stranded products each comprising a first primer extension product and a second primer extension product whereby the second primer extension product comprises a ligand.

86. The method of claim 85 wherein step (b) comprises a first incubation at a temperature below about 30 °C, and a second incubation at a temperature above about 40 °C.

87. The method of claim 86 wherein a DNA polymerase which is active at temperatures above about 45 °C is used to extend the first primer.

88. The method of claim 85 further comprising step (d) of binding the ligand to a solid surface, whereby the plurality of second primer extension products become bound to the solid surface.

89. The method of claim 88 wherein the binding of the ligand results in the double stranded product being bound to the solid surface.

90. The method of claim 88 wherein the binding of the ligand results in the single stranded second primer extension product being bound to the solid surface.

91. The method of claims 88 further comprising treating the solid surface with reagents to produce multiple copies of amplification products that are substantially complementary to the plurality of second primer extension products.

92. The method of claim 88 wherein the solid surface comprises a substantially planar array.

93. The method of claim 88 wherein the solid surface comprises a plurality of beads or a plurality of isolated areas on a surface.

94. The method of claim 93 wherein the plurality of second primer extension products is bound to a plurality of beads or isolated areas under conditions such that generally, one or fewer copies of a single second primer extension product is bound to one bead or one isolated area.

95. The method of claim 94 further comprising treating the beads or isolated areas with reagents to produce multiple copies of an amplification product substantially complementary to the second primer extension products.

96. The method of claim 95 wherein the plurality of beads or isolated areas are contained within a plurality of isolated volumes such that generally one or fewer beads or isolated area is associated with each isolated volume, and whereby the production of multiple copies of amplification product results in multiple copies of substantially one amplification product in each volume.

97. The method of claim 96 wherein the amplification is carried out with a reaction mixture comprising RNase H, an amplification primer with a DNA portion and a 5' RNA portion, and a DNA polymerase with strand displacement activity.

98. The method of claim 94 further comprising the step of storing the beads or isolated areas comprising generally one or fewer primer extension products.

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99. The method of claim 98 further comprising clonally amplifying the primer extension product bound to the beads or isolated areas after storing them.

100. A method for amplifying a nucleic acid representative of a target DNA comprising the method of claim 88, further comprising the steps of:

(e) cleaving the RNA from the first primer extension products such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded;

(f) annealing an amplification primer to the single stranded portion of the second primer extension products complementary to sequence (A), wherein the amplification primer has a DNA portion and a 5' RNA portion;

(g) extending the amplification primer with a DNA polymerase having strand displacement activity to produce an amplified product hybridized to the second primer extension product;

(h) cleaving the RNA from the amplified product hybridized to the second primer extension product; and

(i) repeating steps (f) to (h) to produce multiple copies of amplified product

101. The method of claim 100 wherein the second primer comprises a tailed primer comprising a nucleic acid sequence (B) that is 5' of the random sequence, whereby the amplified product comprises a portion complementary to sequence (B) at or near its 3' end.

102. The method of claim 85 wherein the target DNA is genomic DNA

103. .The method of claim 85 wherein the target DNA comprises multiple genomes.

104. A method for archiving and/or clonal expansion of a nucleotide sequence comprising the steps of:

(a) obtaining a plurality of partially double-stranded DNA products comprising a first polynucleotide and a second polynucleotide, wherein the second polynucleotide comprises a sequence (A') at its 3' end and a ligand, wherein the sequence (A') portion of the second polynucleotide is single-stranded, wherein optionally the second polynucleotide comprises a sequence (B) at or near its 5' end;

(b) attaching the partially double stranded DNA products to a plurality of beads or a plurality of isolated areas on a surface by binding the ligands to the bead or isolated area;

(c) annealing an amplification primer to the single stranded portion of the second polynucleotide complementary to sequence (A'), wherein the amplification primer has a DNA portion and a 5' RNA portion;

(d) extending the amplification primer with an enzyme having strand displacement activity to produce a plurality of amplified products hybridized to the second polynucleotide products;

(e) cleaving the RNA from the amplified product hybridized to the second polynucleotide products using RNase H; and

(f) repeating steps (c) to (e) to produce multiple copies of amplified products.

105. The method of claim 104 wherein the ligands of the DNA products are attached to beads or isolated areas, and on average, one DNA product is attached to one or fewer beads or isolated areas.

106. The method of claim 105 further comprising storing the plurality of beads or isolated areas for later analysis.

107. The method of claim 106 wherein the beads or isolated areas are stored after step (b), then later amplified with steps (c) through (f).

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108. The method of claim 107 wherein the amplification is a clonal amplification carried out in multiple isolated volumes wherein on average, one isolated volume has one or fewer beads or isolated areas.

109. The method of claim 108 wherein the multiple isolated volumes are droplets in a non-aqueous phase.

110. A method comprising:

(a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA; to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid;

(b) cleaving the target RNA from the RNA/DNA hybrid;

(c) extending a second primer, comprising a ligand and a 3' segment complementary to a portion of the first primer extension product, to produce a double stranded product with a DNA/RNA heteroduplex at one end; wherein the double stranded product comprises a second primer extension product hybridized to the first primer extension product, and wherein a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer;

(d) cleaving the RNA in the heteroduplex from the first primer extension product such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded;

(e) annealing to the second primer extension product an oligonucleotide comprising a 3'- DNA segment that is complementary to sequence (A') and a 5' RNA segment comprising sequence (C);

(f) optionally extending the oligonucleotide to form an oligonucleotide extension product hybridized to the second primer extension product;

(g) extending the second primer extension product to create a heteroduplex such that the second primer comprises a DNA sequence (C') that is complementary to sequence (C); and

(h) cleaving the RNA from the heteroduplex created in step (g) to produce a single-stranded portion of the second primer extension product corresponding to sequence (C').

111. The method of claim 110 wherein the 3' portion of the first primer that is complementary to the target RNA comprises a random nucleotide sequence.

112. The method of claim 110 wherein the 3' portion of the first primer that is complementary to the target RNA comprises a sequence that is complementary to polyadenosine (poly-A).

113. The method of claim 110 wherein the 3' portion of the primer that is complementary to the target RNA comprises a specific sequence that is complementary to a multiplicity of targets.

114. The method of claim 110 wherein the RNA target is contained within a sample that also comprises DNA, and actinomycin is added prior to step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a).

115. The method of claim 110 wherein, in step (b), the target RNA is cleaved by chemical heat, or enzyme treatment.

116. The method of claim 110 wherein the 3' segment of the second primer complementary to a portion of the first primer extension product comprises a random nucleotide sequence, a specific sequence complementary

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to a specific sequence of the first primer extension product, or a sequence common to multiple first primer extension products.

117. The method of claim 110 further comprising: (i) binding the ligand on the second primer extension product to a solid surface.

118. The method of claim 117 wherein step (i) of binding the ligand to the solid surface is performed before step (h).

119. The method of claim 117 wherein step (i) of binding the ligand to the solid surface is performed after step (h).

120. A method of amplifying a sequence representative of an sequence within an RNA target molecule comprising the method of claim 117, and further comprising the steps of:

(j) annealing an amplification primer, wherein the amplification primer has a DNA portion and a 5' RNA portion, to the single stranded portion of the second primer extension product complementary to sequence (C');

(k) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product;

(l) cleaving the RNA from the amplified product; and

(m) repeating steps (j) to (l) to produce multiple copies of amplified product wherein the 5' portion of the amplified product has a sequence complementary to sequence (A').

121. The method of claim 120 wherein the second primer further comprises a segment (B) that is not complementary to the first primer extension product sequence, whereby the amplified product comprises a sequence (B') at or near its 3' end that is substantially complementary to sequence (B), and a sequence (A) near its 5' end that is complementary to sequence (A').

122. The method of claim 121 wherein the amplification is a clonal amplification.

123. The method of claim 122 wherein the solid surface is a bead or an isolated area on a surface.

124. The method of claim 123 wherein the bead or isolated area is the only bead or isolated area associated with a isolated liquid volume such that the amplified product is contained within such liquid volume.

125. The method of claim 124 wherein the liquid volume is an aqueous droplet within a non-aqueous fluid.

126. The method of claim 125 wherein the solid surface is a bead and the droplet is part of a microemulsion.

127. The method of claim 124 wherein the liquid volume is a well in a plate.

128. The method of claim 117 wherein the solid surface is a substantially planar substrate.

129. The method of claim 124 wherein the bead or isolated area comprises covalently attached thereto multiple oligonucleotides comprising the sequence (B) at their 3' ends, whereby upon the amplification of step (m) multiple copies of amplified product comprising sequence (B') at their 5' end are hybridized to the bead or isolated area.

130. A method of producing a bead or isolated area with multiple copies of a nucleotide sequence covalently attached thereto by performing the method of claim 129, and further comprising extending the (B) sequences to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product and that comprise sequence (A') near their 3' ends.

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131. A sequencing method comprising performing the method of claim 130, further comprising the steps of removing the amplified product to render the covalently attached polynucleotides single-stranded, and extending a primer to sequence (A') to produce detectable oligonucleotide fragments characteristic of the sequence of the polynucleotide bound to the bead or isolated area.

132. The method of claim 131 wherein the sequencing method comprises cleavable labeled terminators.

133. The method of claim 131 wherein the sequencing method comprises pyrophosphate detection.

134. The method of claim 131 wherein the sequencing method is an isothermal sequencing method.

135. The method of claim 131 wherein the sequencing method comprises cycle sequencing.

136. A method of performing bridge PCR comprising performing the method of claim 121 further comprising the steps of exposing the amplified product to a solid substrate comprising oligonucleotide sequences attached thereto complementary to the A and B' sequences on the amplified product in the presence of components necessary for polymerase chain reaction, and thermal cycling the system to perform bridge PCR amplification.

137. A method of performing rolling circle amplification comprising performing the method of claim 121 further comprising the steps of:

(n) hybridizing the amplified product to a nucleic acid sequence comprising regions complementary to A and B' sequences in close proximity;

(o) optionally extending the gap with a DNA polymerase enzyme;

(p) ligating to form a circular nucleic acid comprising the amplified product, and performing rolling circle amplification by extending a primer that is complementary to a sequence in the circular nucleic acid.

138. The method of claim 137 wherein the primer is complementary to sequence (A), sequence (B'), or a sequence that was between sequences (A) and (B') in the amplified product.

139. The method of claim 137 wherein the primer is an oligonucleotide attached to a solid surface.

140. A method of PCR amplification comprising performing the method of claim 121 further comprising the steps of amplifying the amplified product using primers complementary to sequences (A) and (B), or using primers complementary to sequences (A') and (B').

141. A method of strand displacement amplification (SDA) comprising performing the method of claim 121 wherein sequences (A) and (B') in the amplified product are designed to be cleaved by a restriction enzyme, and performing strand displacement amplification on the amplified product.

142. A method comprising:

(a) denaturing a double-stranded target DNA;

(b) annealing to the target DNA and extending with and enzyme comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the of the primer comprises sequence (A), which is not complementary to the target DNA; to form a plurality of first primer extension products, each with sequence (A) at its 5' end;

(c) extending a second primer comprising a ligand and a 3' DNA region that comprises a random sequence, wherein the primer is optionally a tailed primer comprising a nucleic acid sequence (B) that is 5' of the random sequence, to form a plurality of double-stranded products each comprising a first primer extension product and a second primer extension product whereby the second primer extension product comprises a ligand;

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(d) cleaving the RNA from the first primer extension products such that a portion of the second primer extension products that are complementary to sequence (A) are single stranded;

(e) annealing to the second primer extension product an oligonucleotide comprising a 3'-DNA segment that is complementary to sequence (A') and a 5'-RNA segment comprising sequence (C);

(f) optionally extending the oligonucleotide to form a plurality of oligonucleotide extension products hybridized to the second primer extension products;

(g) extending the second primer extensions product to create a heteroduplex such that the second primer extension products comprise a DNA sequence (C') that is complementary to sequence (C); and

(h) cleaving the RNA from the heteroduplex created in step (g).

143. The method of claim 142 further comprising: (i) binding the ligand on the second primer extension products to a solid surface.

144. The method of claim 143 wherein step (i) of binding the ligand to the solid surface is performed before step (h).

145. The method of claim 143 wherein step (i) of binding the ligand to the solid surface is performed after step (h).

146. A method of amplifying a sequence representative of an sequence within a DNA target molecule comprising the method of claim 143, and further comprising the steps of:

(j) annealing an amplification primer, wherein the amplification primer has a DNA portion and a 5' RNA portion, to the single stranded portion of the second primer extension products complementary to sequence (C');

(k) extending the amplification primer with an enzyme having strand displacement activity to produce a amplified products;

(l) cleaving the RNA from the amplified products; and

(m) repeating steps (j) to (l) to produce multiple copies of amplified products wherein the 5' portion of the amplified product has a sequence complementary to sequence (A').

147. The method of claim 146 wherein the second primer comprises the segment (B) that is not complementary to the first primer extension product sequence, whereby the amplified products comprise a sequence (B') at or near their 3' ends that is substantially complementary to sequence (B), and a sequence (A) near their 5' ends that is complementary to sequence (A').

148. The method of claim 147 wherein the amplification is a clonal amplification.

149. The method of claim 148 wherein the solid surface is a bead or isolated area on a surface.

150. The method of claim 149 wherein the bead or isolated area is the only bead or isolated area within isolated liquid volume such that the amplified product is contained within such liquid volume.

151. The method of claim 150 wherein the liquid volume is an aqueous droplet within a non-aqueous fluid.

152. The method of claim 151 wherein solid surface is a bead and the droplet is part of a microemulsion.

153. The method of claim 150 wherein the liquid volume is a well in a plate.

154. The method of claim 143 wherein the solid surface is a substantially planar substrate.

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155. The method of claim 150 wherein the bead or isolated area comprises covalently attached multiple oligonucleotides comprising the sequence (B) at their 3' ends, whereby upon the amplification of step (m) multiple copies of amplified products comprising sequence (B') at their 5' end are hybridized to the bead or isolated area.

156. A method of producing a bead or isolated area with multiple copies of a nucleotide sequence covalently attached thereto by performing the method of claim 155 further comprising extending the (B) sequences to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product and that comprise sequence (A') near their 5' ends.

157. A sequencing method comprising performing the method of claim 155, further comprising the steps of removing the amplified product to render the covalently attached polynucleotides single-stranded, and extending a primer to sequence (A') to produce detectable oligonucleotide fragments characteristic of the sequence of the polynucleotide bound to the bead or isolated area.

158. The method of claim 157 wherein the sequencing method comprises cleavable labeled terminators.

159. The method of claim 157 wherein the sequencing method comprises pyrophosphate detection.

160. The method of claim 157 wherein the sequencing method is an isothermal sequencing method.

161. The method of claim 157 wherein the sequencing method comprises cycle sequencing.

162. A method of performing bridge PCR comprising performing the method of claim 147 further comprising the steps of exposing the amplified products to a solid substrate comprising oligonucleotide sequences attached thereto complementary to the A and B' sequences on the amplified products in the presence of components necessary for polymerase chain reaction, and thermal cycling the system to perform bridge PCR amplification.

163. A method of performing rolling circle amplification comprising performing the method of claim 147 further comprising the steps of:

(n) hybridizing the amplified products to a target nucleic acid comprising regions complementary to A and B' sequences in close proximity;

(o) optionally extending the gap with a polymerase enzyme;

(p) ligating to form a circular nucleic acid comprising the amplified product, and performing rolling circle amplification by extending a primer that is complementary to a sequence in the circular nucleic acid.

164. The method of claim 163 wherein the primer is complementary to sequence (A), sequence (B'), or a sequence that was between sequences (A) and (B') in the amplified product.

165. The method of claim 163 wherein the primer is an oligonucleotide attached to a solid surface.

166. A method of PCR amplification comprising performing the method of claim 147 further comprising the steps of amplifying the amplified product using primers complementary to sequences (A) and (B), or using primers complementary to sequences (A') and (B').

167. A method of strand displacement amplification (SDA) comprising performing the method of claim 147 wherein sequences (A) and (B') in the amplified product are designed to be cleaved by a restriction enzyme, and performing strand displacement amplification on the amplified product.

168. A method comprising:

(a) extending a first primer comprising a 3' portion complementary to a target RNA and a 5' portion, sequence (D), not complementary to the target RNA, to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid;

(b) cleaving the target RNA from the RNA/DNA hybrid;

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(c) extending a second primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to the first primer extension product and a 5' portion, sequence (E), of the of the second primer is not complementary to the first primer extension product, to produce a double-stranded DNA product comprising the first primer extension product hybridized to a second primer extension product, whereby the second primer extension product has a sequence (D') that is complementary to sequence (D) at its 3' end;

(d) denaturing the double-stranded DNA product;

(e) annealing to the second primer extension product and extending a third primer comprising, from its 5' end, a ligand, optionally a sequence (F), and a sequence (D), wherein sequence (D) is complementary to sequence (D') on the second primer extension product to produce a double-stranded DNA product comprising the second primer extension product hybridized to a third primer extension product, whereby the third primer extension product comprises a sequence (E') at its 3' end complementary to sequence (E).

169. The method of claim 168 further comprising binding the ligand to a solid surface, whereby the third primer extension product is bound to the solid surface.

170. The method of claim 168 further comprising an amplification with the steps of:

(f) cleaving the RNA portion of the second primer extension product in the DNA-RNA heteroduplex, whereby sequence (E') of the third primer extension product is single stranded.

(g) annealing an oligonucleotide comprising a 3' DNA segment (E) that is complementary to sequence (E') and a 5' RNA segment comprising sequence (G);

(h) extending the third primer extension product to produce a sequence (G') at its 3' end complementary to sequence (G);

(i) cleaving the RNA from the heteroduplex created in step (h) to produce a single-stranded portion of the third primer extension product corresponding to sequence (G').

171. The method of claim 170 further comprising binding the ligand to a solid surface, whereby the third primer extension product comprising sequence (G') is bound to the solid surface.

172. The method of claim 170 wherein the 3' portion of the first primer that is complementary to the target RNA comprises a random nucleotide sequence.

173. The method of claim 170 wherein the 3' portion of the first primer that is complementary to the target RNA comprises a sequence that is complementary to polyadenosine (poly-A).

174. The method of claim 170 wherein the RNA target is contained within a sample that also comprises DNA, and actinomycin is added prior to step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a).

175. The method of claim 170 wherein, in step (b), the target RNA is cleaved by heat, enzyme treatment, or chemical treatment or enzymes.

176. The method of claim 170 wherein the 3' segment of the second primer complementary to a portion of the first primer extension product comprises a random nucleotide sequence, a specific sequence complementary to a specific sequence of the first primer extension product, or a sequence common to multiple first primer extension products.

177. A method of amplifying a sequence representative of an sequence within an RNA target molecule comprising the method of claim 171 further comprising the steps of:

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(j) annealing an amplification primer, wherein the amplification primer has a DNA portion and a 5' RNA portion, to the single stranded portion of the third primer extension product complementary to sequence (G');

(k) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product;

(l) cleaving the RNA from the amplified product; and

(m) repeating steps (j) to (l) to produce multiple copies of amplified product wherein the 5' portion of the amplified product has a sequence (E) complementary to sequence (E') and the 3' end of the amplified product has sequence (D') complementary to sequence (D) and optionally sequence (F') complementary to sequence (F).

178. The method of claim 177 wherein the amplification is a clonal amplification.

179. The method of claim 177 wherein the solid surface is a bead or an isolated area on a surface.

180. The method of claim 179 wherein the bead or isolted area is the only bead or isolated area within isolated liquid volume such that the amplified product is contained within such liquid volume.

181. The method of claim 180 wherein the liquid volume is an aqueous droplet within a non-aqueous fluid.

182. The method of claim 181 wherein the droplet is part of a microemulsion.

183. The method of claim 180 wherein the liquid volume is a well in a plate.

184. The method of claim 178 wherein the solid surface is a substantially planar substrate.

185. The method of claim 180 wherein the bead or isolated area comprises covalently attached thereto multiple oligonucleotides comprising the sequence (D), and/or sequence (F) at their 3' ends, whereby upon the amplification of step (m) multiple copies of amplified product comprising sequence (D') (and/or F') at their 5' end are hybridized to the bead or isolated area.

186. A method of producing a bead or isolated area with multiple copies of a nucleotide sequence covalently attached thereto by performing the method of claim 185 further comprising extending the oligonucleotide at the (D), and/or (F) sequences to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product comprising sequence (E') near their 5' ends.

187. A sequencing method comprising performing the method of claim 186, further comprising the steps of removing the amplified product to render the covalently attached polynucleotides single-stranded, and extending a primer to sequence (E') to produce detectable oligonucleotide fragments characteristic of the sequence of the polynucleotide bound to the bead or isolated area.

188. The method of claim 187 wherein the sequencing method comprises cleavable labeled terminators.

189. The method of claim 187 wherein the sequencing method comprises pyrophosphate detection.

190. The method of claim 187 wherein the sequencing method is an isothermal sequencing method.

191. The method of claim 187 wherein the sequencing method comprises cycle sequencing.

192. A method comprising:

(a) denaturing a double-stranded target DNA;

(b) annealing to the target DNA and extending a first primer comprising a 3' portion comprising a random sequence and a 5' portion, sequence (D), which is not complementary to the target DNA, to form a plurality of first primer extension products, each comprising sequence (D) at its 3' end;

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(c) extending a second primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion comprises a random sequence, and a 5' portion, sequence (E), of the of the second primer is not complementary to the first primer extension products, to produce a plurality of double-stranded DNA products comprising a first primer extension product hybridized to a second primer extension product, whereby the second primer extension products have a sequence (D') that is complementary to sequence (D) at their 3' ends;

(d) denaturing the double-stranded DNA products;

(e) annealing to the second primer extension products and extending a third primer comprising, from its 5' end, a ligand, optionally a sequence (F), and a sequence (D), wherein sequence (D) is complementary to sequence (D') on the second primer extension products to produce double-stranded DNA products comprising second primer extension products hybridized to third primer extension products, whereby the third primer extension products comprise a sequence (E') at its 3' end complementary to sequence (E) in a DNA-RNA heteroduplex.

193. The method of claim 192 further comprising binding the ligand to a solid surface, whereby the third primer extension products are bound to the solid surface.

194. The method of claim 192 further comprising the steps of:

(f) cleaving the RNA portion of the second primer extension products in the DNA-RNA heteroduplex, whereby sequence (E') of the third primer extension products is single stranded.

(g) annealing an oligonucleotide comprising a 3' DNA segment (E) that is complementary to sequence (E') and a 5' RNA segment comprising sequence (G);

(h) extending the third primer extension products to produce a sequence (G') at their 3' ends complementary to sequence (G);

(i) cleaving the RNA from the heteroduplex created in step (h) to produce single-stranded portions of the third primer extension products corresponding to sequence (G').

195. The method of claim 194 further comprising binding the ligand to a solid surface, whereby the third primer extension products comprising sequence (G') are bound to the solid surface.

196. A method of amplifying a sequence representative of a sequence within a DNA target molecule comprising following the steps of claim 195 further comprising the steps of:

(j) annealing an amplification primer to the single stranded portion of the third primer extension products complementary to sequence (G'); wherein the amplification primer has a DNA portion and a 5' RNA portion,

(k) extending the amplification primer with an enzyme having strand displacement activity to produce a amplified products;

(l) cleaving the RNA from the amplified products; and

(m) repeating steps (j) to (l) to produce multiple copies of amplified products wherein the 5' portion of the amplified products have a sequence (E) complementary to sequence (E') and the 3' end of the amplified products have sequence (D') complementary to sequence (D) and optionally sequence (F') complementary to sequence (F).

197. The method of claim 196 wherein the amplification is a clonal amplification.

198. The method of claim 196 wherein the solid surface is a plurality of beads or a plurality of isolated areas on a surface.

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199. The method of claim 198 wherein each bead or isolated area in the plurality of beads or isolated areas is the only bead or isolated area within isolated liquid volume such that the amplified product corresponding to the sequence on that bead or isolated area is associated with such liquid volume.

200. The method of claim 199 wherein the liquid volume is an aqueous droplet within a non-aqueous fluid.

201. The method of claim 199 wherein the droplet is part of a microemulsion.

202. The method of claim 199 wherein the liquid volume is a well in a plate.

203. The method of claim 196 wherein the solid surface is a substantially planar substrate.

204. The method of claim 196 wherein the bead or isolated area comprises covalently attached thereto multiple oligonucleotides comprising the sequence (D), and/or sequence (F) at their 3' ends, whereby upon the amplification of step (m) multiple copies of amplified product comprising sequence (D') (and/or sequence (F') at their 5' end are hybridized to the bead or isolated area.

205. A method of producing a bead or isolated area with multiple copies of a nucleotide sequence covalently attached thereto by performing the method of claim 204 further comprising extending the (D), and/or (F) sequences to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product comprising sequence (E') near their 5' ends.

206. A sequencing method comprising performing the method of claim 205, further comprising the steps of removing the amplified product to render the covalently attached polynucleotides single-stranded, and extending a primer to sequence (E') to produce detectable oligonucleotide fragments characteristic of the sequence of the polynucleotide bound to the bead or isolated area.

207. The method of claim 206 wherein the sequencing method comprises cleavable labeled terminators.

208. The method of claim 206 wherein the sequencing method comprises pyrophosphate detection.

209. The method of claim 206 wherein the sequencing method is an isothermal sequencing method.

210. The method of claim 206 wherein the sequencing method comprises cycle sequencing.

211. A library comprising a plurality of double-stranded oligonucleotides each of the oligonucleotides comprising:

(a) a first strand comprising DNA which has, proceeding from its 5' end (i) a ligand, (ii) a specific sequence (B, D, or DF), (iii) a sequence corresponding to or complementary to a sequence within a nucleic acid target, (iv) a specific sequence (A' or E'); and a specific sequence (C' or G'); and

(b) a second strand having from its 5' end, (i) a specific RNA sequence (C or G) complementary to specific sequence C' or G', (ii) a specific sequence (A or E) complementary to sequence (A' or E'), (iii) a sequence complementary to or corresponding to a sequence within a target nucleic acid.

212. The library of claim 211 wherein the ligands are bound to a solid surface.

213. The library of claim 212 wherein the solid surface comprises a plurality of beads.

214. The library of claim 213 wherein each of the plurality of beads comprises a single molecule of double-stranded oligonucleotide.

215. The library of claim 212 wherein the solid surface comprises a plurality of isolated areas on a surface.

216. A kit comprising:

(a) a first primer comprising a 3'-DNA portion and a 5'-RNA portion, wherein the 5' RNA portion further comprises sequence (A);

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(b) a second primer comprising a 5'-ligand;

(c) an RNA dependent DNA polymerase;

(d) a DNA dependent DNA polymerase with strand displacement activity;

(e) RNase H; and

(f) an amplification chimeric primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the sequence of the amplification primer is the substantial the same sequence as the (A) sequence.

217. The kit of claim 216 wherein the 3'-DNA portion of the first primer comprises a random sequence.

218. The kit of claim 216 wherein the second primer is a DNA primer that comprises a random sequence at its 3' end.

219. The kit of claim 216 wherein the 3'-DNA portion of the first primer comprises a random sequence, and the second primer is a DNA primer that comprises a random sequence at its 3' end.

220. A kit comprising:

(a) a first primer comprising a 3'-DNA portion and a 5'-RNA portion, wherein the 5' RNA portion further comprises sequence (A);

(b) a second primer comprising a 5'-ligand;

(c) an RNA dependent DNA polymerase;

(d) a DNA dependent DNA polymerase with strand displacement activity;

(e) RNase H;

(f) a chimeric oligonucleotide comprising a 3'-DNA portion substantially comprising sequence (A) and a 5'-RNA sequence (C); and

(g) a chimeric amplification primer comprising a 3'-DNA portion and a 5'-RNA portion, wherein the chimeric amplification primer comprises a sequence which is substantially the same as sequence (C).

221. The kit of claim 220 wherein the 3'-DNA portion of the first primer comprises a random sequence.

222. The kit of claim 220 wherein the second primer is a DNA primer that comprises a random sequence at its 3' end.

223. The kit of claim 220 wherein the 3'-DNA portion of the first primer comprises a random sequence, and the second primer is a DNA primer that comprises a random sequence at its 3' end.

224. The kit of claim 220 wherein the second primer further comprises a sequence (B) at or near the 5'-end.

225. The kit of claim 220 wherein the kit further comprises solid support with immobilized receptor to the ligand on it surface.

226. The kit of claim 220 wherein the kit further comprises solid surface with an oligonucleotide attached to the surface by the 5'-end and comprising a sequence substantially the same as sequence (B).

227. The kit of claim 220 wherein the kit further comprises solid surface with an oligonucleotide attached to the surface by the 5'-end hybridizable to sequence (A).

228. The kit of claim 216 or 220 wherein the kit further comprises an inhibitor of the DNA dependent DNA polymerase,

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229. The kit of claim 228 wherein the inhibitor of the DNA dependent DNA polymerase is Actinomycin.

230. A kit comprising:

(a) a first primer that is a tailed DNA primer comprising a 5'-tail sequence (D);

(b) a second primer that is a chimeric primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the 5'-end comprises a tail sequence (E);

(c) a third primer which is a tailed primer comprising a 3'-sequence that comprises a sequence substantially the same as sequence (D), optionally a 5'-tail sequence (F), and 5'-ligand;

(d) an RNA dependent DNA polymerase;

(e) a DNA dependent DNA polymerase with strand displacement activity;

(f) RNase H; and

(g) a chimeric amplification primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the chimeric amplification primer comprises a sequence which is substantially the same a sequence (E).

231. The kit of claim 230 wherein the 3'-DNA portion of the first primer comprises a random sequence.

232. The kit of claim 230 wherein the second primer is a DNA primer that comprises a random sequence at its 3' end.

233. The kit of claim 230 wherein the 3'-DNA portion of the first primer comprises a random sequence, and the second primer is a DNA primer that comprises a random sequence at its 3' end.

234. A kit comprising:

(a) a first primer that is a tailed DNA primer comprising a 5'-tail sequence (D);

(b) a second primer that is a chimeric primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the 5'-end comprises a tail sequence (E);

(c) a third primer which is a tailed primer comprising a 3'-sequence that comprises a sequence substantially the same as sequence (D), optionally a 5'-tail sequence (F), and 5'-ligand;

(d) an RNA dependent DNA polymerase;

(e) a DNA dependent DNA polymerase with strand displacement activity;

(f) RNase H;

(g) a chimeric oligonucleotide comprising a 3'-DNA sequence (E) and a 5'-RNA sequence G; and

(h) a chimeric amplification primer comprising a 3'-DNA portion and a 5'-RNA portion wherein the chimeric amplification primer comprises a sequence which is substantially the same as sequence (G).

235. The kit of claim 234 wherein the 3'-DNA portion of the first primer comprises a random sequence.

236. The kit of claim 234 wherein the second primer is a DNA primer that comprises a random sequence at its 3' end.

237. The kit of claim 234 wherein the 3'-DNA portion of the first primer comprises a random sequence, and the second primer is a DNA primer that comprises a random sequence at its 3' end.

238. The kit of claim 230 or 234 wherein the kit further comprises an inhibitor of the DNA dependent DNA polymerase,

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239. The kit of claim 238 wherein the inhibitor of the DNA dependent DNA polymerase is Actinomycin.

240. A method for attaching a polynucleotide sequence that is representative of a sequence within a nucleic acid target molecule to a solid surface comprising:

(a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer, sequence (P), is complementary to a target nucleic acid and a 5' portion of the of the primer, sequence (A), is not complementary to the target nucleic acid, to form a first primer extension product hybridized to the target nucleic acid;

(b) separating or removing the first primer extension product from the target nucleic acid;

(c) extending a second primer to produce a double-stranded product comprising a second primer extension product hybridized to the first primer extension product, wherein the second primer comprises a 3' segment complementary to a portion of the first primer extension product and 5' segment non-complementary sequence (B) to the first primer extension product, whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer and a portion of the 5' end of the second primer extension product comprises non-complementary sequence (B);

(d) adding an exonuclease to the double-stranded DNA/RNA hybrid, whereby single stranded 3' nucleotides are removed from the first primer extension product;

(e) extending the first primer extension product to produce a sequence (B'), complementary to sequence (B) on the second primer extension product;

(f) denaturing the first and second primer extension products;

(g) binding the sequence (B') of the first primer extension product to a third primer comprising sequence (B) bound to a solid surface, whereby the first primer extension product is attached to the solid surface; and

(h) extending the sequence (B) of the third primer to produce a double-stranded product comprising a third primer extension product hybridized to the first primer extension product, wherein the 5'end of the third primer comprises a sequence (B) complementary to the sequence (B') of the first primer extension product, whereby a portion of the 3' end of the third primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer.

241. A method comprising:

(a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA; to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid;

(b) removing the target RNA from the RNA/DNA hybrid;

(c) extending a second primer, comprising a 3' segment complementary to a portion of the first primer extension product and a 5' segment non-complementary to the first primer extension product comprising sequence (B), to produce a double-stranded DNA product with a DNA/RNA heteroduplex at one end, wherein the double-stranded product comprises a second primer extension product hybridized to the first primer extension product and wherein a portion of the 3' end of the second

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primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer;

(d) adding an exonuclease to the double-stranded DNA product, whereby single stranded 3' nucleotides are removed from the 3' region of the first primer extension product that is not hybridized to the second primer extension product;

(e) extending the first primer extension product to produce a sequence (B'), complementary to sequence (B) on the second primer extension product;

(f) denaturing the double-stranded DNA product;

(g) attaching the single-stranded first primer extention product to a solid support by annealing sequence (B') to the solid support comprising an oligonucleotide attached thereto, comprising a sequence (B); and

(h) extending sequence (B) on the solid support to produce a third primer extension product, hybridized to the first extension product, wherein the third primer extension product comprises a 3' sequence (A'), whereby a DNA/RNA heteroduplex at one end is generated.

242. The method of claim 241 further comprising:

(i) cleaving the RNA region from the first polynucleotide product hybridized to the third primer extension product using RNase H;

(j) annealing an amplification primer to sequence (A') on the single-stranded portion of the third primer extension product, wherein the amplification primer has a DNA portion and a 5' RNA portion;

(k) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product hybridized to the third primer extension product on the solid support;

(l) repeating steps (i) to (k) to produce multiple copies of an amplified product wherein the amplified product comprises sequence (B') at its 3' end; and

(m) capturing the amplified product on the solid support wherein the solid support comprises sequence (B).

243. The method of claim 241 wherein the 3' portion of the primer that is complementary to the target RNA comprises a random nucleotide sequence.

244. The method of claim 241 wherein the 3' portion of the primer that is complementary to the target RNA comprises a sequence that is complementary to polyadenosine (poly-A).

245. The method of claim 241 wherein the 3' portion of the primer that is complementary to the target RNA comprises a specific sequence that is complementary to a multiplicity of targets.

246. The method of claim 241 wherein, in step (b), the target RNA is cleaved by heat, enzyme treatment, or chemical treatment.

247. The method of claim 241 wherein the RNA target is in a sample that also comprises DNA, and wherein actinomycin is added prior to step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a).

248. The method of claim 241 wherein the 3' portion of the second primer complementary to a portion of the first primer extension product comprises a random nucleotide sequence, a specific sequence complementary to a specific sequence of the first primer extension product, or a sequence common to multiple first primer extension products.

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249.The method of claim 241 wherein the solid support comprises a bead.

250.The method of claim 241 wherein the solid support comprises an isolated area.

251.The method of claim 241 wherein the solid support comprises a plurality of beads or a plurality of isolated areas on a surface.

252.The method of claim 241 wherein the solid support comprises a substantially planar array.

253.The method of claim 249 wherein the bead comprises a magnetic bead.

254.The method of claim 249 wherein the bead comprises only one copy of the first primer extension product.

255.The method of claim 254 wherein the first primer extension product is single-stranded.

256.The method of claim 243 wherein a plurality of first primer extension products are produced corresponding to different sequences in the target RNA.

257.The method of claim 256 wherein the plurality of first primer extension products is bound to a solid support comprising either one or a plurality of beads or a plurality of isolated areas on a surface.

258.The method of claim 257 wherein the plurality of first primer extension products is bound to a plurality of beads under conditions such that generally, one or fewer first primer extension products is bound to one bead or one isolated area on a surface.

259.The method of claim 257 wherein the plurality of beads or isolated areas are contained within a plurality of isolated volumes such that generally one or fewer beads or isolated area is associated with each isolated volume, and whereby the production of multiple copies of amplification product results in multiple copies of substantially one amplification product in each volume.

260.The method of claim 257 further comprising the step of storing the beads or isolated areas comprising generally one or fewer primer extension products per bead or isolated area.

261.The method of claim 260 further comprising clonally amplifying the primer extension product bound to the beads or isolated areas after storing them.

262.The method of claim 241 wherein the target RNA comprises messenger RNA.

263.A method comprising:

(a) denaturing a double-stranded target DNA;

(b) annealing to the target DNA and extending with a DNA polymerase comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the primer comprises sequence (A), which is not complementary to the target DNA; to form a a plurality of first primer extension product hybridized to the target DNA and comprising sequence (A) at its 5' end;

(c) separating the first primer extension product from the target DNA;

(d) annealing to the first primer extension product and extending a second primer comprising a 3' complementary DNA region that comprises a random sequence, wherein the second primer is a tailed primer comprising a 5' sequence (B), to form a double-stranded product comprising a first primer extension product and a second primer extension product, whereby a double-stranded product with a DNA/RNA heteroduplex at one end is generated;

(e) adding an exonuclease to the double-stranded DNA product, whereby single stranded 3' nucleotides are removed from the 3' region of the first primer extension product that is not hybridized to the second primer extension product;

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(f) extending the first primer extension product to produce a sequence (B'), complementary to sequence (B) on the second primer extension product;

(g) denaturing the double-stranded DNA product;

(h) attaching the single-stranded first primer extention product to a solid support by annealing sequence (B') to the solid support comprising an oligonucleotide attached thereto, comprising a sequence (B), whereby a plurality of first primer extension products become bound to the solid surface; and

(i) extending sequence (B) on the solid support to produce a third primer extension product, hybridized to the first primer extension product, comprising a 3' sequence (A'), whereby a DNA/RNA heteroduplex at one end is generated.

264.The method of claim 263 further comprising:

(j) cleaving the RNA from the first polynucleotide product hybridized to the amplified product using RNase H;

(k) annealing an amplification primer to the single-stranded portion of the amplified product complementary to sequence (A'), wherein the amplification primer has a DNA portion and a 5' RNA portion;

(l) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product hybridized to the third primer extension product on the bead or isolated area;

(m) repeating steps (j) to (l) to produce multiple copies of an amplified product wherein the amplified product comprises sequence (B') at its 3' end; and

(n) capturing the amplified product on the solid support comprising sequence (B).

265.The method of claim 263 wherein step (b) comprises a first incubation at a temperature below about 30 °C, and a second incubation at a temperature above about 40 °C.

266.The method of claim 263 wherein a DNA polymerase which is active at temperatures above about 45 °C is used to extend the first primer.

267.The method of claim 263 wherein the solid support comprises a bead.

268.The method of claim 263 wherein the solid support comprises an isolated area.

269.The method of claim 263 wherein the solid support comprises a plurality of beads or a plurality of isolated areas on a surface.

270.The method of claim 263 wherein the solid support comprises a substantially planar array.

271.The method of claim 267 wherein the bead comprises a magnetic bead.

272.The method of claim 269 wherein the plurality of first primer extension products is bound to a plurality of beads or isolated areas under conditions such that generally, one or fewer copies of a single first primer extension product is bound to one bead or one isolated area.

273.The method of claim 269 wherein the plurality of beads or isolated areas are contained within a plurality of isolated volumes such that generally one or fewer beads or isolated area is associated with each isolated volume, and whereby the production of multiple copies of amplification product results in multiple copies of substantially one amplification product in each volume.

IDT Exhibit 1017.0862

274. The method of claim 272 further comprising the step of storing the beads or isolated areas comprising generally one or fewer primer extension products.

275. The method of claim 274 further comprising clonally amplifying the primer extension product bound to the beads or isolated areas after storing them.

276. The method of claim 263 wherein the target DNA is genomic DNA.

277. The method of claim 263 wherein the target DNA comprises multiple genomes.

278. A method comprising:

(a) extending a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer is complementary to a target RNA and a 5' portion, sequence (A), of the of the primer is not complementary to the target RNA; to form a first primer extension product hybridized to the target RNA, forming an RNA/DNA hybrid;

(b) removing the target RNA from the RNA/DNA hybrid;

(c) extending a second primer, comprising a 3' segment complementary to a portion of the first primer extension product and a 5' segment non-complementary to the first primer extension product comprising sequence (B), to produce a double-stranded product with a DNA/RNA heteroduplex at one end; wherein the double-stranded product comprises a second primer extension product hybridized to the first primer extension product, and whereby a portion of the 3' end of the second primer extension product comprises a sequence (A') that is complementary to the sequence (A) of the of the first primer;

(d) cleaving the RNA in the heteroduplex from the first primer extension product such that a portion of the second primer extension product that is complementary to sequence (A) is single-stranded;

(e) annealing to the second primer extension product an oligonucleotide comprising a 3'- DNA sequence (A) that is complementary to sequence (A') and a 5'-RNA segment comprising sequence (C) that is non-complementary to the second primer extension product;

(f) extending the oligonucleotide at the 3' segment to form an oligonucleotide extension product hybridized to the second primer extension product;

(g) denaturing the double-stranded DNA product;

(h) attaching the single-stranded first primer extension product to a solid support by annealing sequence (B') to the solid support comprising a sequence (B); and

(i) extending sequence (B) on the solid support to produce a third primer extension product, comprising a 3' sequence (A') and (C'), whereby a DNA/RNA heteroduplex at one end is generated.

279. The method of claim 278 comprising:

(j) cleaving the RNA from the heteroduplex polynucleotide product hybridized to the amplified product using RNase H to produce a single-stranded portion of the third primer extension product corresponding to sequence (C');

(k) annealing an amplification primer to the single-stranded portion of the third primer extension product complementary to sequence (C'), wherein the amplification primer has a DNA portion and a 5' RNA portion;

(l) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product hybridized to the third primer extension product on the solid support;

IDT Exhibit 1017.0863

(m) repeating steps (j) to (l) to produce multiple copies of the amplified product comprising sequences (A) and (B'); and

(n) capturing the amplified product on the solid support wherein the solid support comprises sequence (B).

280. The method of claim 278 further comprising extending sequence (B), whereby multiple copies are bound to solid support through sequence (B) having sequence (A') at 3' end.

281. The method of claim 278 further comprising sequencing by synthesis using a sequence (A) complementary to (A') as the priming sequence.

282. The method of claim 278 wherein the 3' portion of the first primer that is complementary to the target RNA comprises a random nucleotide sequence.

283. The method of claim 278 wherein the 3' portion of the first primer that is complementary to the target RNA comprises a sequence that is complementary to polyadenosine (poly-A).

284. The method of claim 278 wherein the 3' portion of the primer that is complementary to the target RNA comprises a specific sequence that is complementary to a multiplicity of targets.

285. The method of claim 278 wherein the RNA target is contained within a sample that also comprises DNA, and actinomycin is added prior to step (a) to selectively inhibit the production of extension product complementary to the DNA during step (a).

286. The method of claim 278 wherein, in step (b), the target RNA is cleaved by chemical heat, or enzyme treatment.

287. The method of claim 278 wherein the 3' segment of the second primer complementary to a portion of the first primer extension product comprises a random nucleotide sequence, a specific sequence complementary to a specific sequence of the first primer extension product, or a sequence common to multiple first primer extension products.

288. The method of claim 278 wherein the amplification is a clonal amplification.

289. The method of claim 278 wherein the solid support is a bead.

290. The method of claim 278 wherein the solid support is an isolated area on a surface.

291. The method of claim 278 wherein the bead or isolated area is the only bead or isolated area associated with an isolated liquid volume such that the amplified product is contained within such liquid volume.

292. The method of claim 291 wherein the liquid volume is an aqueous droplet within a non-aqueous fluid.

293. The method of claim 291 wherein the solid surface is a bead and the droplet is part of a microemulsion.

IDT Exhibit 1017.0864

294. The method of claim 291 wherein the liquid volume is a well in a plate.

295. The method of claim 278 wherein the solid support is a substantially planar substrate.

296. A method of producing a solid support with multiple copies of a nucleotide sequence covalently attached thereto by performing the method of claim 278, and further comprising extending the (B) sequences to produce multiple polynucleotides covalently attached to the solid support that are substantially complementary to the amplified product and that comprise sequence (A') near their 3' ends.

297. A sequencing method comprising performing the method of claim 296, further comprising the steps of removing the third primer extension product to render the covalently attached polynucleotides single-stranded, and extending a primer to sequence (A) to produce detectable oligonucleotide fragments characteristic of the sequence of the polynucleotide bound to the bead or isolated area.

298. The method of claim 297 wherein the sequencing method comprises cleavable labeled terminators.

299. The method of claim 297 wherein the sequencing method comprises pyrophosphate detection.

300. The method of claim 297 wherein the sequencing method is an isothermal sequencing method.

301. The method of claim 297 wherein the sequencing method comprises cycle sequencing.

302. A method of performing bridge PCR comprising performing the method of claim 278 further comprising the steps of exposing the amplified product to a solid substrate comprising oligonucleotide sequences attached thereto complementary to the A and B' sequences on the amplified product in the presence of components necessary for polymerase chain reaction, and thermal cycling the system to perform bridge PCR amplification.

303. A method of performing rolling circle amplification comprising performing the method of claim 278 further comprising the steps of:

(o) hybridizing the amplified product to a nucleic acid sequence comprising regions complementary to A and B' sequences in close proximity;

(p) optionally extending the gap with a DNA polymerase enzyme;

(q) ligating to form a circular nucleic acid comprising the amplified product, and performing rolling circle amplification by extending a primer that is complementary to a sequence in the circular nucleic acid.

304. The method of claim 303 wherein the primer is complementary to sequence (A), sequence (B'), or a sequence that was between sequences (A) and (B') in the amplified product.

305. The method of claim 303 wherein the primer is an oligonucleotide attached to a solid surface.

306. A method of PCR amplification comprising performing the method of claim 278 further comprising the steps of amplifying the amplified product using primers complementary to sequences (A) and (B), or using primers complementary to sequences (A') and (B').

IDT Exhibit 1017.0865

307.A method of strand displacement amplification (SDA) comprising performing the method of claim 278 wherein sequences (A) and (B') in the amplified product are designed to be cleaved by a restriction enzyme, and performing strand displacement amplification on the amplified product.

308.A method comprising:

(a) denaturing a double-stranded target DNA;

(b) annealing to the target DNA and extending with a DNA polymerase comprising strand displacement activity, a first primer comprising a DNA segment and a 5' RNA segment, wherein a 3' portion of the primer comprises a random sequence, and a 5' portion of the of the primer comprises sequence (A), which is not complementary to the target DNA; to form a first primer extension product hybridized to the target DNA and comprising sequence (A) at its 5' end;

(c) separating the first primer extension product from the target DNA;

(d) annealing to the first primer extension product and extending a second primer comprising a 3' complementary DNA region that comprises a random sequence, wherein the second primer is a tailed primer comprising a 5' sequence (B), to form a double-stranded product comprising a first primer extension product and a second primer extension product, whereby a double-stranded product with a DNA/RNA heteroduplex at one end is generated.

(e) cleaving the RNA in the heteroduplex from the first primer extension product such that a portion of the second primer extension product that is complementary to sequence (A) is single stranded;

(f) annealing to the second primer extension product an oligonucleotide comprising a 3'- DNA segment that is complementary to sequence (A') and a 5' RNA segment comprising sequence (C);

(g) extending the oligonucleotide along the second primer extension product to form an oligonucleotide extension product comprising a sequence (B'), complementary to sequence (B) on the second primer extension product;

(h) denaturing the double-stranded DNA product;

(i) attaching the single-stranded first primer extension product to solid support by annealing sequence (B') to the bead or isolated area comprising a sequence (B); and

(j) extending sequence (B) on the solid support to produce a third primer extension product, hybridized to the oligonucleotide extension product, comprising a 3' sequence (A') and (C'), whereby a DNA/RNA heteroduplex at one end is generated.

309.The method of claim 308 further comprising:

(k) cleaving the RNA from the heteroduplex polynucleotide product hybridized to the amplified product using RNase H to produce a single-stranded portion of the second primer extension product corresponding to sequence (C');

(l) annealing an amplification primer to the single-stranded portion of the amplified product complementary to sequence (C'), wherein the amplification primer has a DNA portion and a 5' RNA portion;

(m) extending the amplification primer with an enzyme having strand displacement activity to produce an amplified product hybridized to the amplified product on the bead or isolated area; and

(n) repeating steps (k) to (m) to produce multiple copies of the second polynucleotide product comprising sequences (A) and (B').

IDT Exhibit 1017.0866

310.The method of claim 308 wherein the amplification is a clonal amplification.

311.The method of claim 308 wherein the solid support is a bead or isolated area on a surface.

312.The method of claim 311 wherein the bead or isolated area is the only bead or isolated area within isolated liquid volume such that the amplified product is contained within such liquid volume.

313.The method of claim 312 wherein the liquid volume is an aqueous droplet within a non-aqueous fluid.

314.The method of claim 313 wherein solid surface is a bead and the droplet is part of a microemulsion.

315.The method of claim 312 wherein the liquid volume is a well in a plate.

316.The method of claim 308 wherein the solid support is a substantially planar substrate.

317.The method of claim 309 wherein the bead or isolated area comprises covalently attached multiple oligonucleotides comprising the sequence (B) at their 3' ends, whereby upon the amplification of step (m), multiple copies of amplified products comprising sequence (B') at their 5' end are hybridized to the bead or isolated area.

318.A method of producing a bead or isolated area with multiple copies of a nucleotide sequence covalently attached thereto by performing the method of claim 317 further comprising extending the (B) sequences to produce a multiple polynucleotides covalently attached to the bead or isolated area that are substantially complementary to the amplified product and that comprise sequence (A') near their 5' ends.

319.A sequencing method comprising performing the method of claim 317, further comprising the steps of removing the amplified product to render the covalently attached polynucleotides single-stranded, and extending a primer to sequence (A') to produce detectable oligonucleotide fragments characteristic of the sequence of the polynucleotide bound to the bead or isolated area.

320.The method of claim 319 wherein the sequencing method comprises cleavable labeled terminators.

321.The method of claim 319 wherein the sequencing method comprises pyrophosphate detection.

322.The method of claim 319 wherein the sequencing method is an isothermal sequencing method.

323.The method of claim 319 wherein the sequencing method comprises cycle sequencing.

324.A method of performing bridge PCR comprising performing the method of claim 308 further comprising the steps of exposing the amplified products to a solid substrate comprising oligonucleotide sequences attached thereto complementary to the A and B' sequences on the amplified products in the presence of components necessary for polymerase chain reaction, and thermal cycling the system to perform bridge PCR amplification.

325.A method of performing rolling circle amplification comprising performing the method of claim 308 further comprising the steps of:

(o) hybridizing the amplified products to a target nucleic acid comprising regions complementary to A and B' sequences in close proximity;

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(p) optionally extending the gap with a polymerase enzyme;

(q) ligating to form a circular nucleic acid comprising the amplified product, and performing rolling circle amplification by extending a primer that is complementary to a sequence in the circular nucleic acid.

326. The method of claim 325 wherein the primer is complementary to sequence (A), sequence (B'), or a sequence that was between sequences (A) and (B') in the amplified product.

327. The method of claim 325 wherein the primer is an oligonucleotide attached to a solid surface.

328. A method of PCR amplification comprising performing the method of claim 308 further comprising the steps of amplifying the amplified product using primers complementary to sequences (A) and (B), or using primers complementary to sequences (A') and (B').

329. A method of strand displacement amplification (SDA) comprising performing the method of claim 308 wherein sequences (A) and (B') in the amplified product are designed to be cleaved by a restriction enzyme, and performing strand displacement amplification on the amplified product.

IDT Exhibit 1017.0868

Figure 1

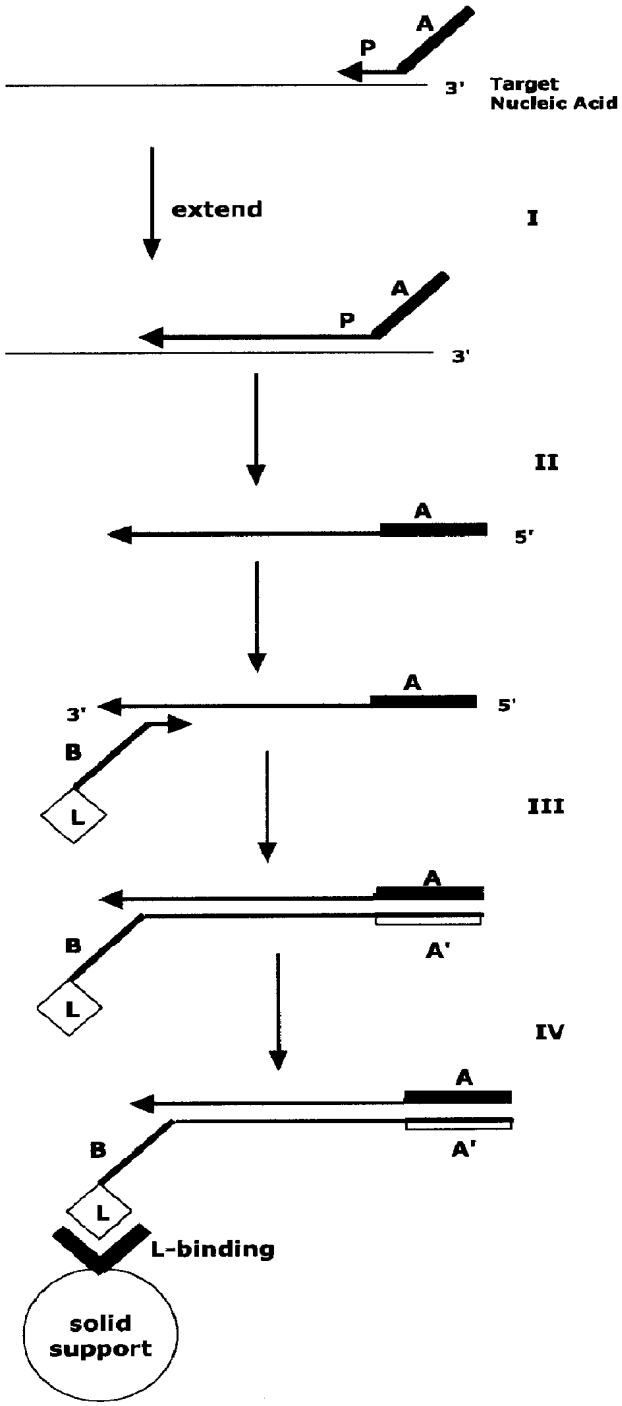

A
P
3'
Target Nucleic Acid
extend
I
A
P
3'
II
A
5'
A
3'
5'
B
L
III
A
B
A'
L
IV
A
B
A'
L
L-binding
solid support

Figure 2

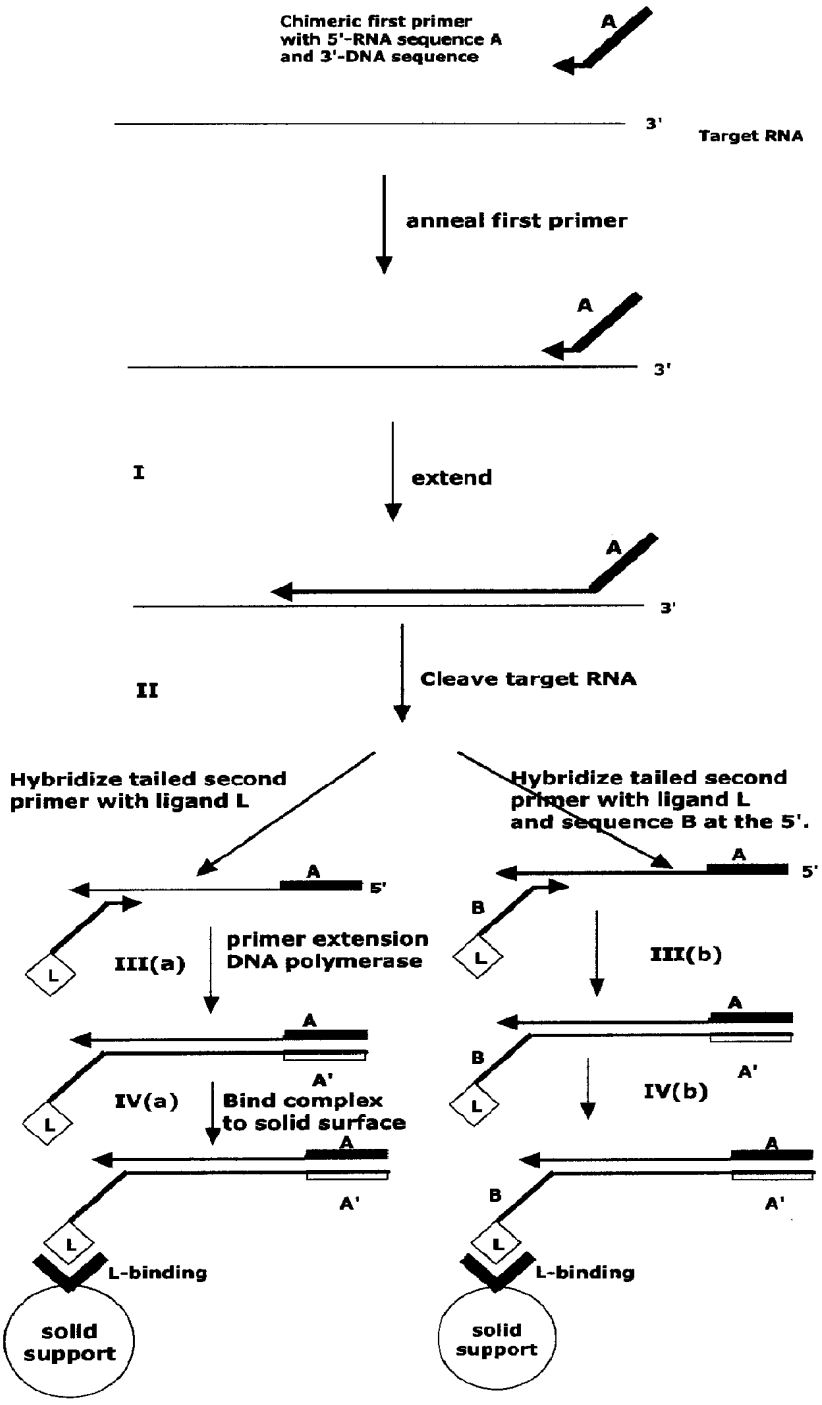

Chimeric first primer with 5'-RNA sequence A and 3'-DNA sequence
A
3'
Target RNA
anneal first primer
A
3'
I
extend
A
3'
II
Cleave target RNA
Hybridize tailed second primer with ligand L
A
5'
L
III(a)
primer extension DNA polymerase
A
A'
L
IV(a)
Bind complex to solid surface
A
A'
L
L-binding
solid support
Hybridize tailed second primer with ligand L and sequence B at the 5'.
A
5'
B
L
III(b)
A
A'
B
L
IV(b)
A
A'
B
L
L-binding
solid support

Figure 3

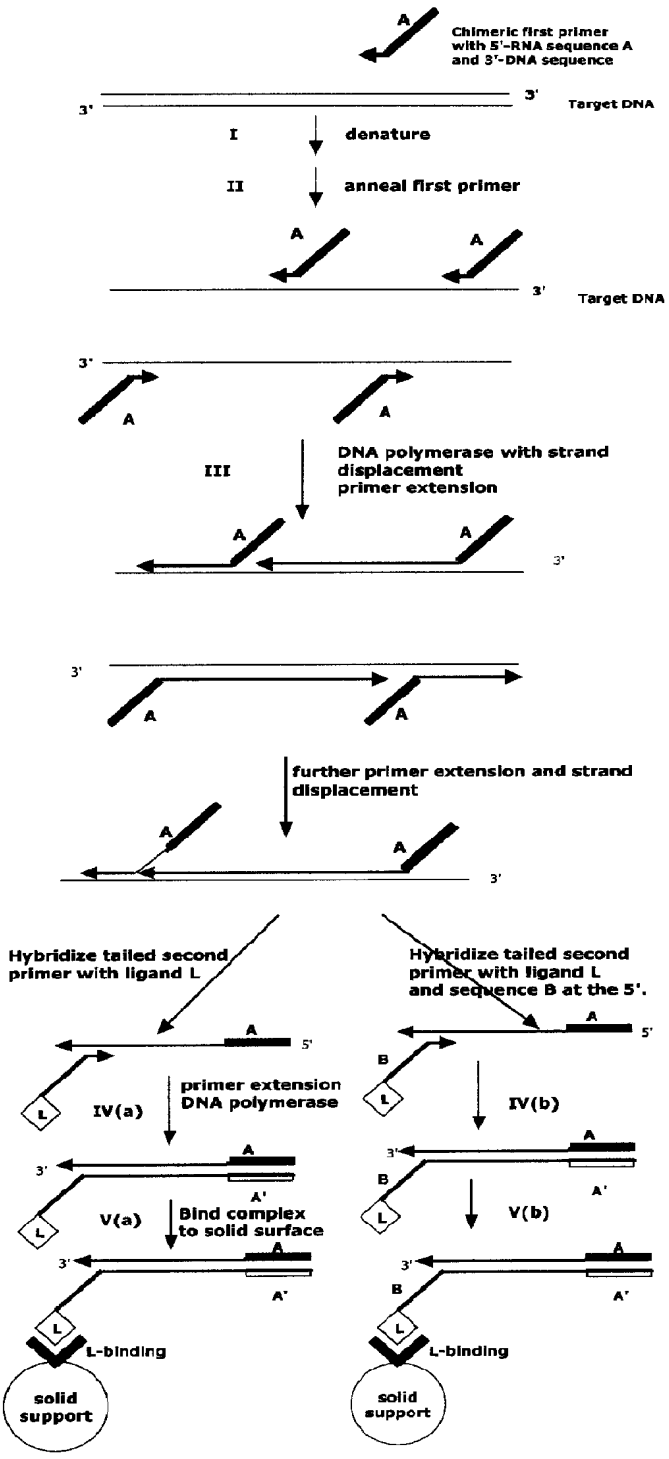

Chimeric first primer with 5'-RNA sequence A and 3'-DNA sequence
Target DNA
I denature
II anneal first primer
Target DNA
III DNA polymerase with strand displacement primer extension
further primer extension and strand displacement
Hybridize tailed second primer with ligand L
IV(a) primer extension DNA polymerase
V(a) Bind complex to solid surface
L-binding
solid support
Hybridize tailed second primer with ligand L and sequence B at the 5'.
IV(b)
V(b)
L-binding
solid support

Figure 4

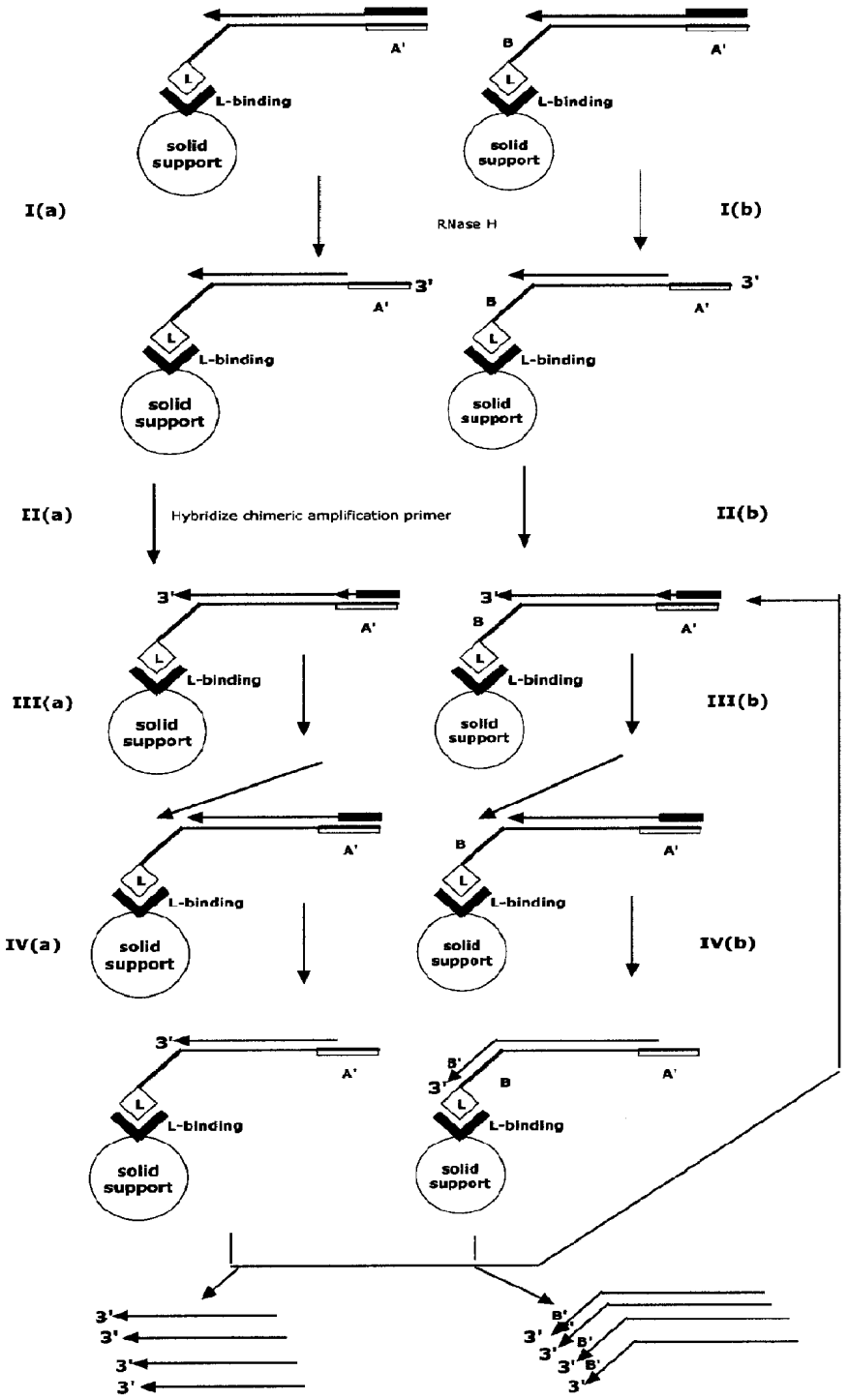

I(a)
I(b)
RNase H
L
L-binding
solid support
A'
B
3'
II(a)
Hybridize chimeric amplification primer
II(b)
III(a)
III(b)
IV(a)
IV(b)
B'

Figure 5A

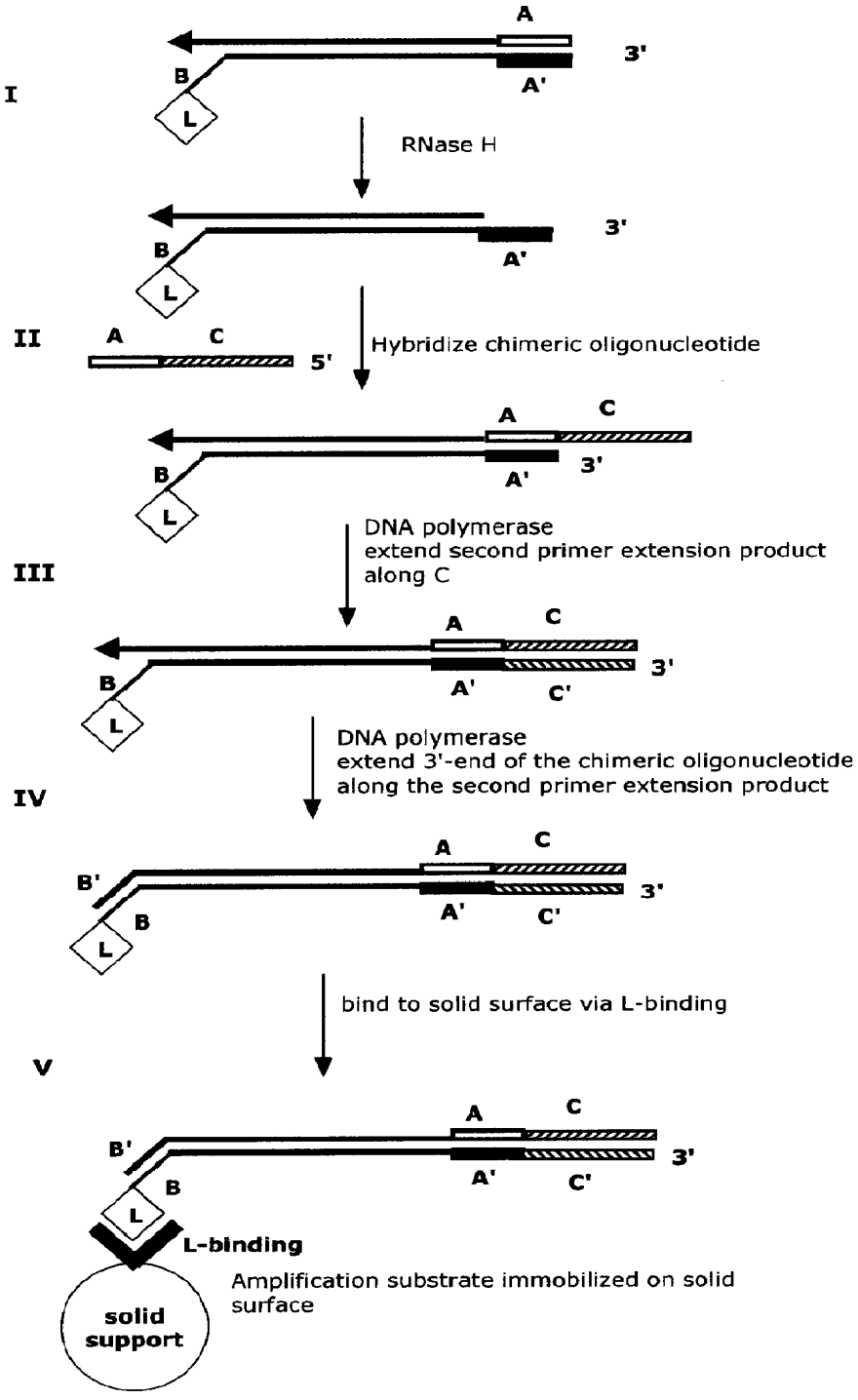

I
A
3'
B
A'
L
RNase H
3'
B
A'
L
II
A
C
5'
Hybridize chimeric oligonucleotide
A
C
3'
B
A'
L
III
DNA polymerase
extend second primer extension product
along C
A
C
3'
B
A'
C'
L
IV
DNA polymerase
extend 3'-end of the chimeric oligonucleotide
along the second primer extension product
A
C
B'
3'
B
A'
C'
L
bind to solid surface via L-binding
V
A
C
B'
3'
B
A'
C'
L
L-binding
Amplification substrate immobilized on solid
surface
solid
support

Figure 5B

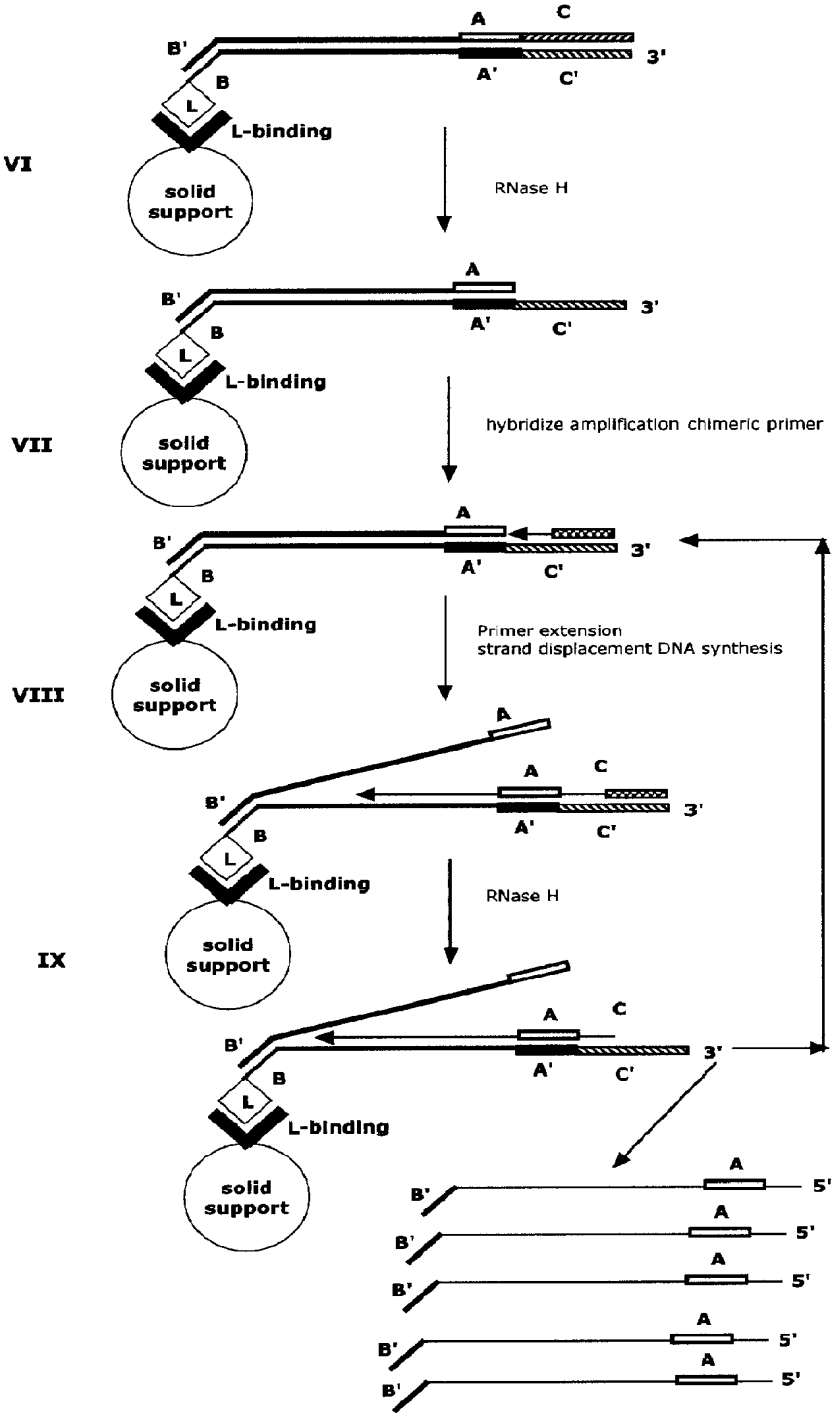

VI
VII
VIII
IX
A
C
B'
3'
A'
C'
B
L
L-binding
solid support
RNase H
hybridize amplification chimeric primer
Primer extension
strand displacement DNA synthesis
5'

Figure 6

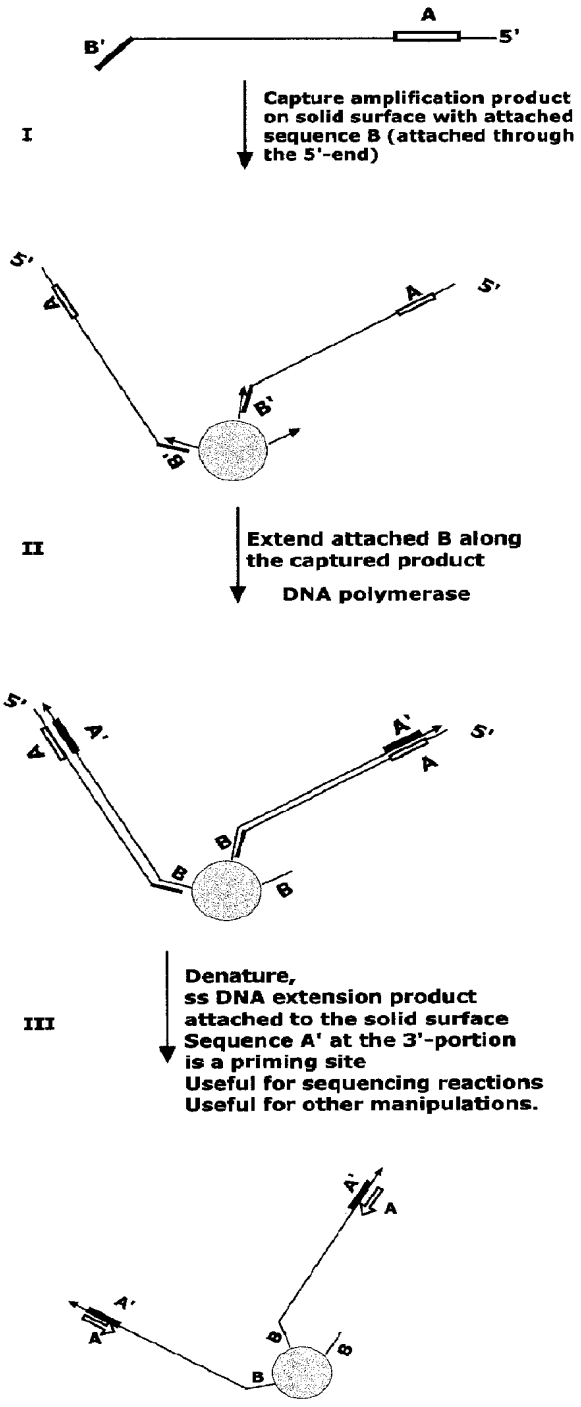

A
B'
5'
I
Capture amplification product on solid surface with attached sequence B (attached through the 5'-end)
5'
A'
A
5'
B'
B
II
Extend attached B along the captured product
DNA polymerase
5'
A'
A
A'
5'
A
B
B
B
III
Denature,
ss DNA extension product attached to the solid surface
Sequence A' at the 3'-portion is a priming site
Useful for sequencing reactions
Useful for other manipulations.
A'
A
A'
A
B
B
B

Figure 7

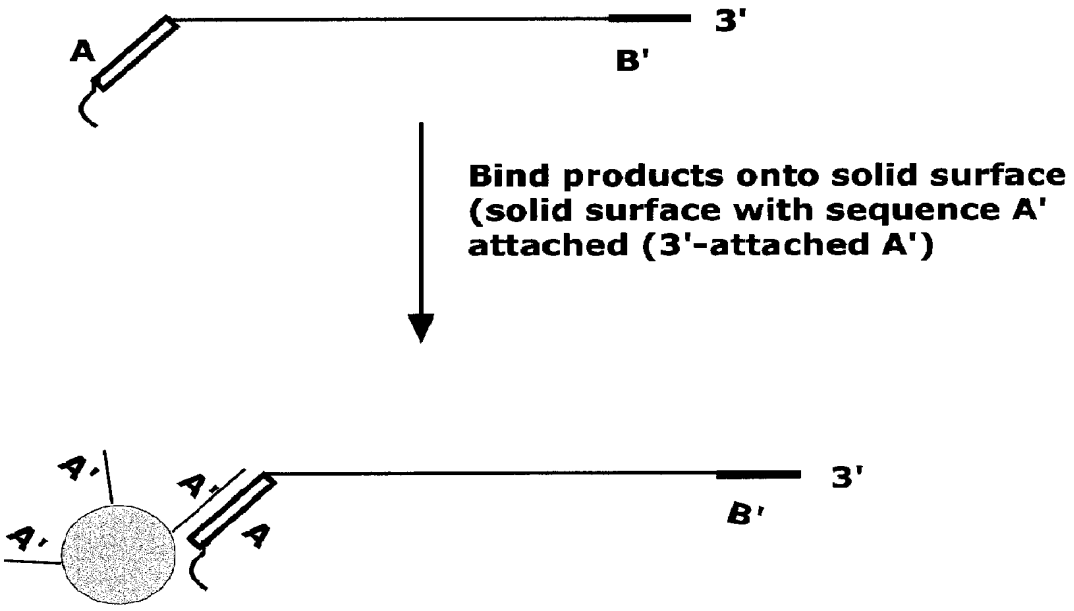

A
3'
B'
Bind products onto solid surface
(solid surface with sequence A'
attached (3'-attached A')
A'
A'
A'
A
3'
B'

Figure 8

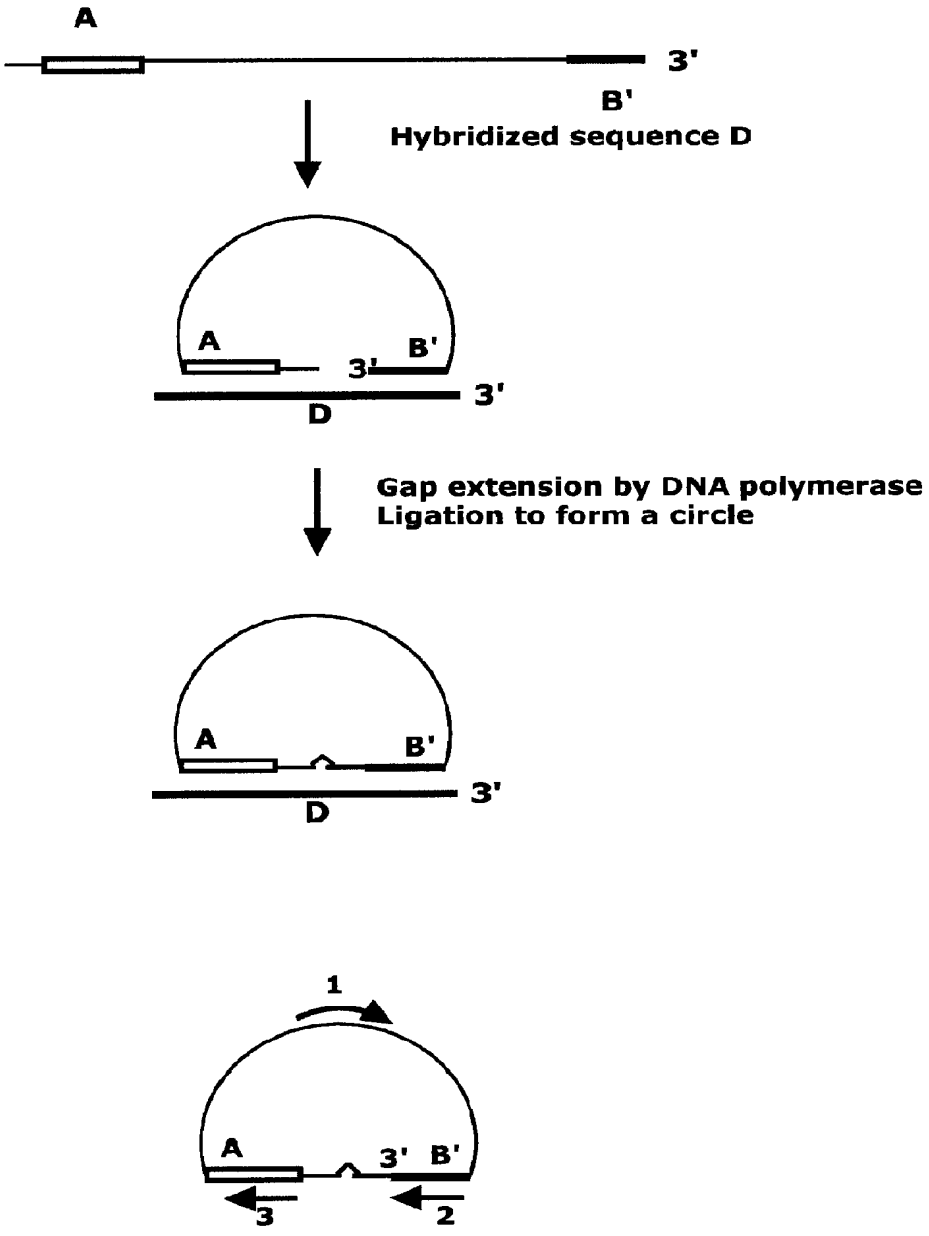

A
3'
B'
Hybridized sequence D
A
B'
3'
3'
D
Gap extension by DNA polymerase
Ligation to form a circle
A
B'
3'
D
1
A
3'
B'
3
2

Figure 9

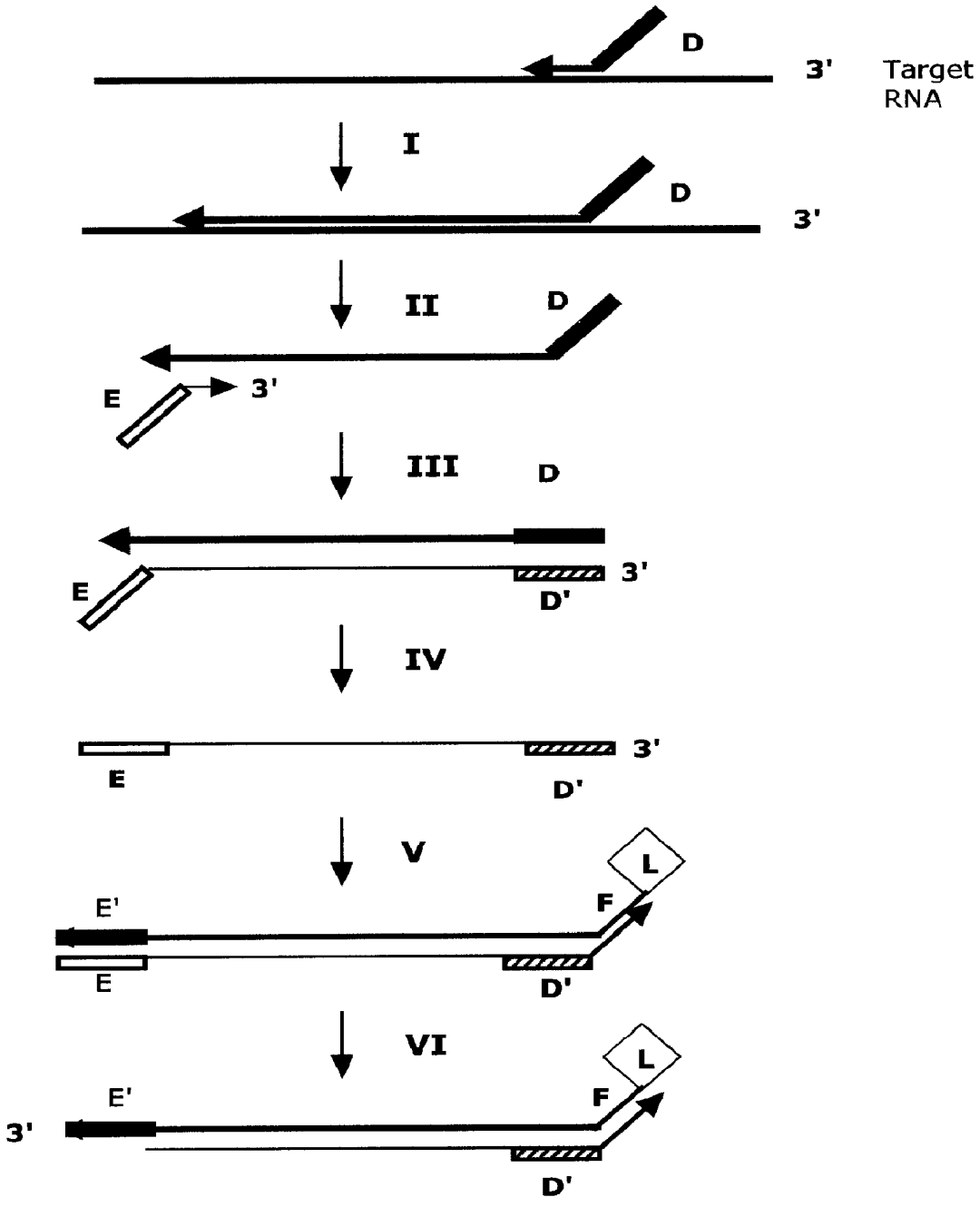


Figure 10

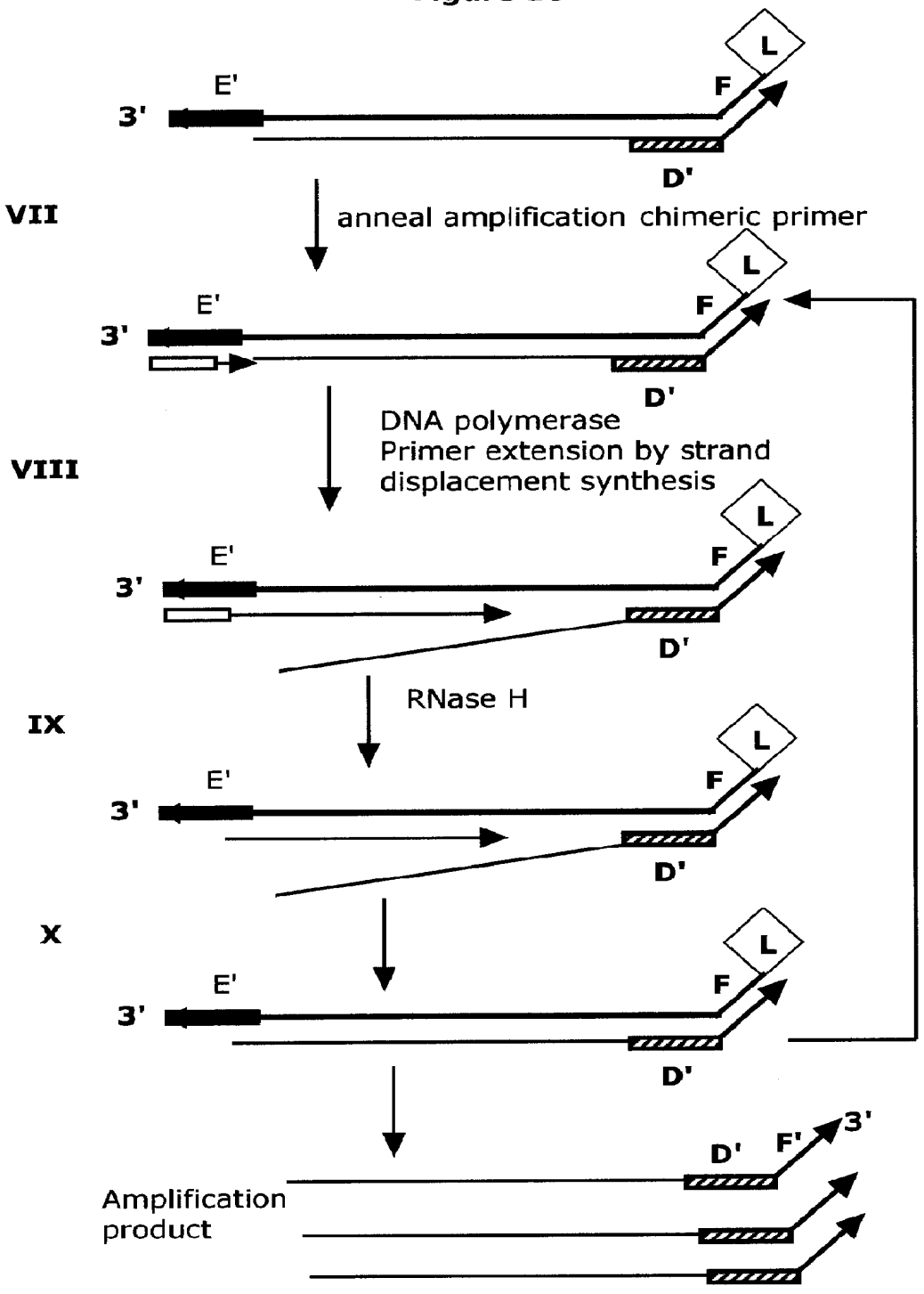

L
E'
F
3'
D'
VII
anneal amplification chimeric primer
DNA polymerase
Primer extension by strand
displacement synthesis
VIII
RNase H
IX
X
Amplification
product
F'

Figure 11

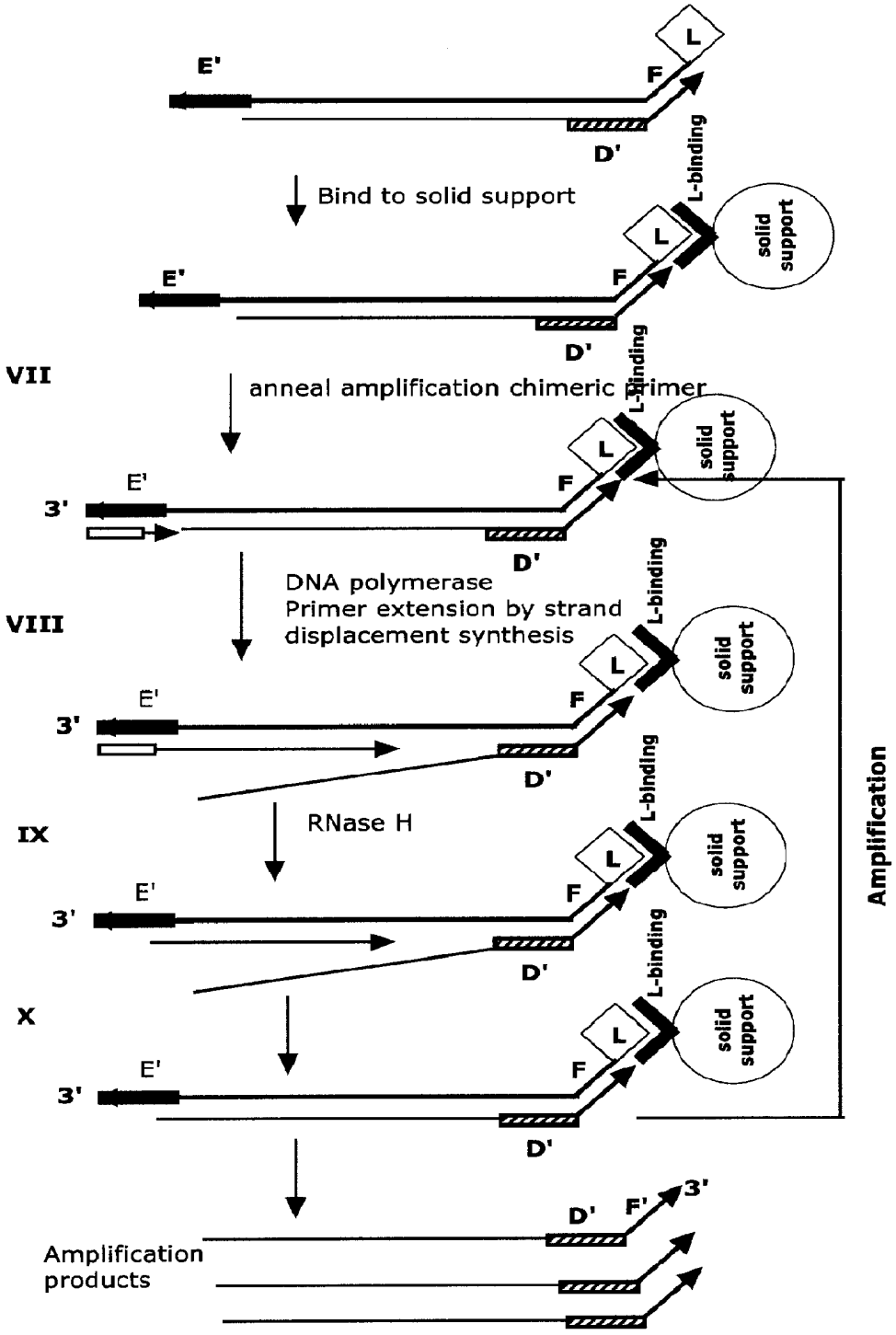

E'
L
F
D'
L-binding
Bind to solid support
solid support
VII
anneal amplification chimeric primer
3'
DNA polymerase
Primer extension by strand displacement synthesis
VIII
RNase H
IX
X
Amplification
Amplification products
F'

Figure 12

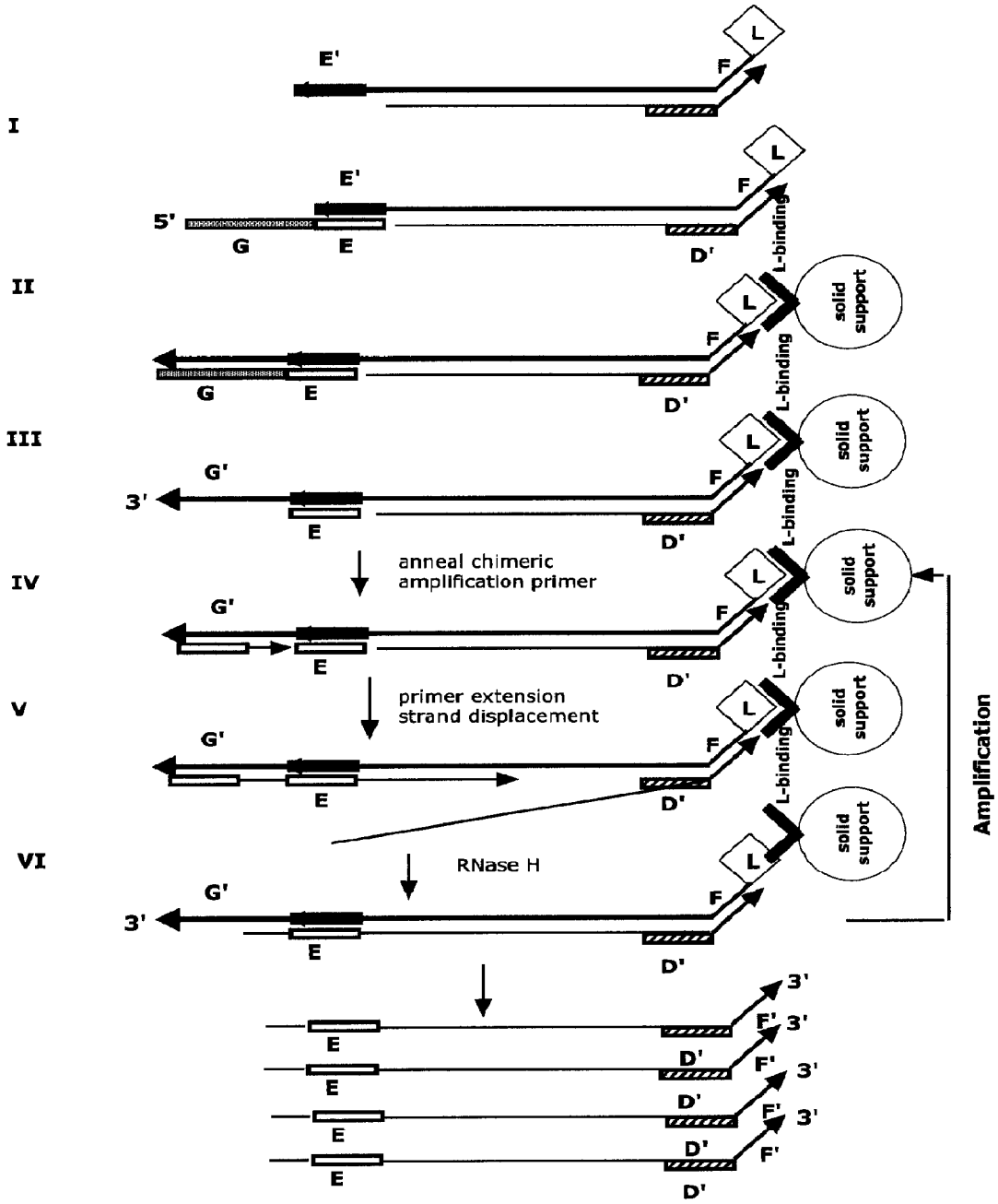

I
II
III
IV
V
VI
E'
F
L
5'
G
E
D'
L-binding
solid support
G'
3'
anneal chimeric amplification primer
primer extension strand displacement
RNase H
F'
Amplification

Figure 13

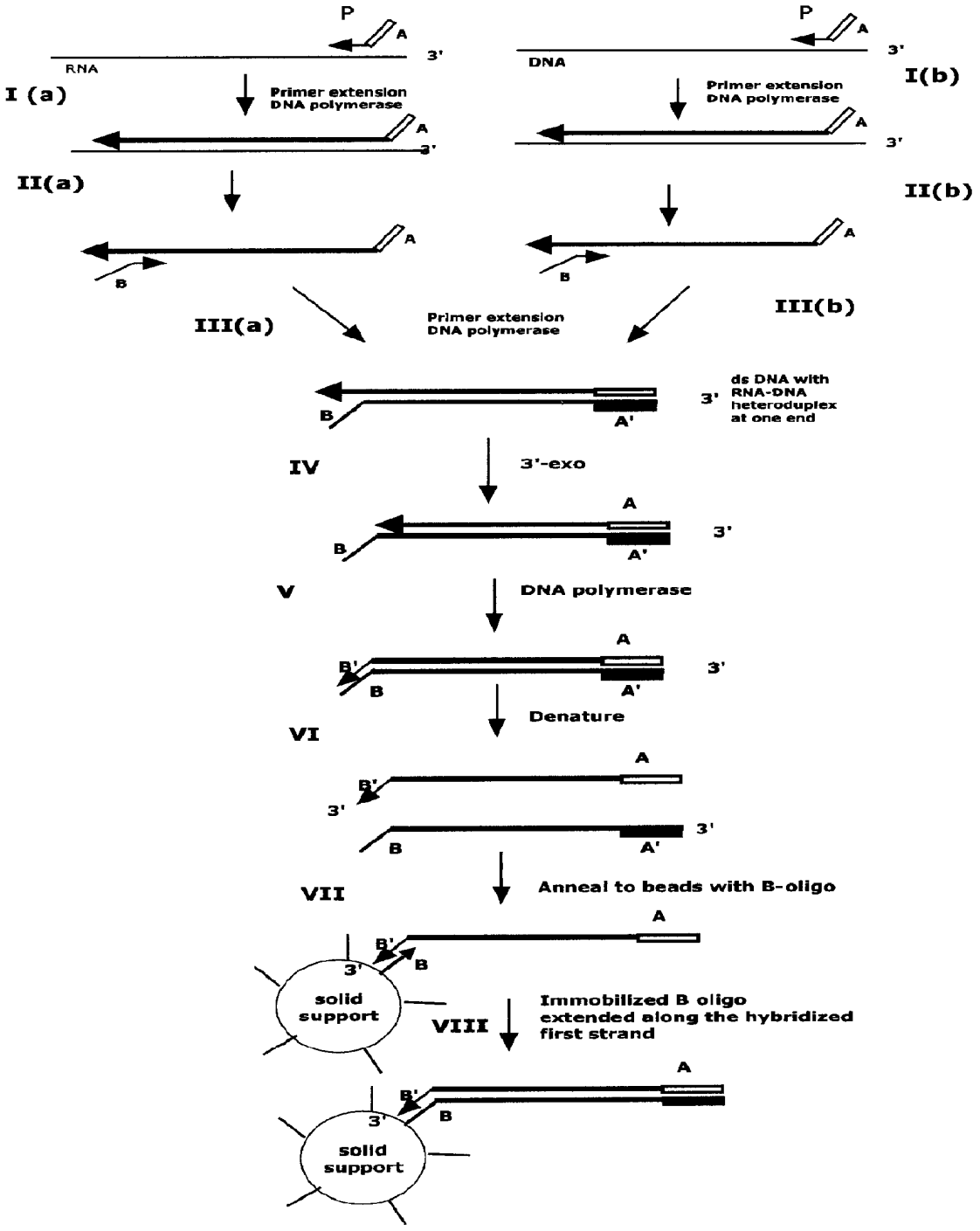

P
A
RNA
3'
I (a)
Primer extension
DNA polymerase
A
3'
II(a)
A
B
III(a)
DNA
I(b)
II(b)
III(b)
Primer extension
DNA polymerase
B
A'
ds DNA with
RNA-DNA
heteroduplex
at one end
IV
3'-exo
V
DNA polymerase
B'
VI
Denature
VII
Anneal to beads with B-oligo
solid
support
VIII
Immobilized B oligo
extended along the hybridized
first strand

Figure 14

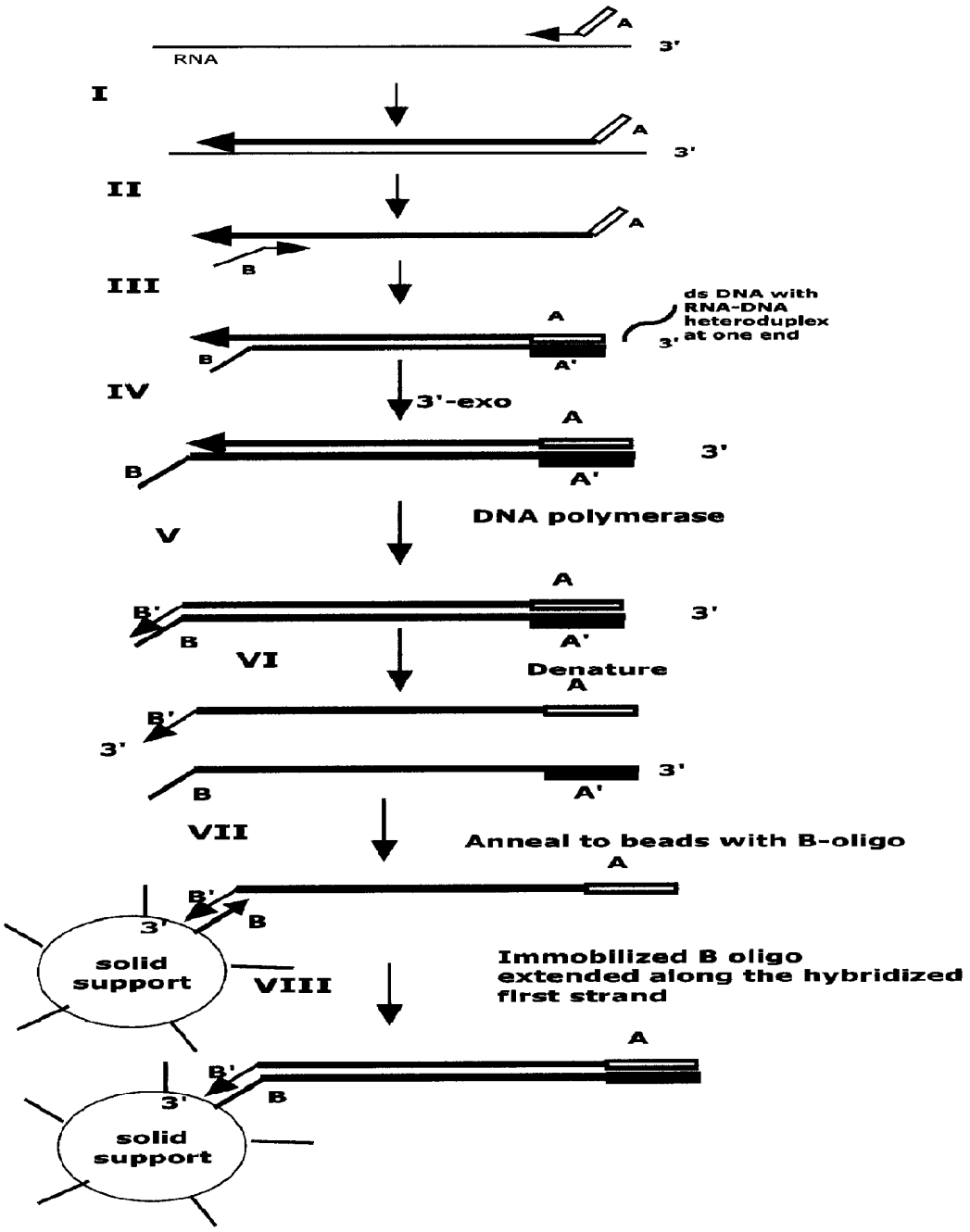

RNA
A
3'
I
II
III
B
ds DNA with RNA-DNA heteroduplex at one end
A'
IV
3'-exo
V
DNA polymerase
VI
Denature
VII
Anneal to beads with B-oligo
B'
solid support
VIII
Immobilized B oligo extended along the hybridized first strand

Figure 15

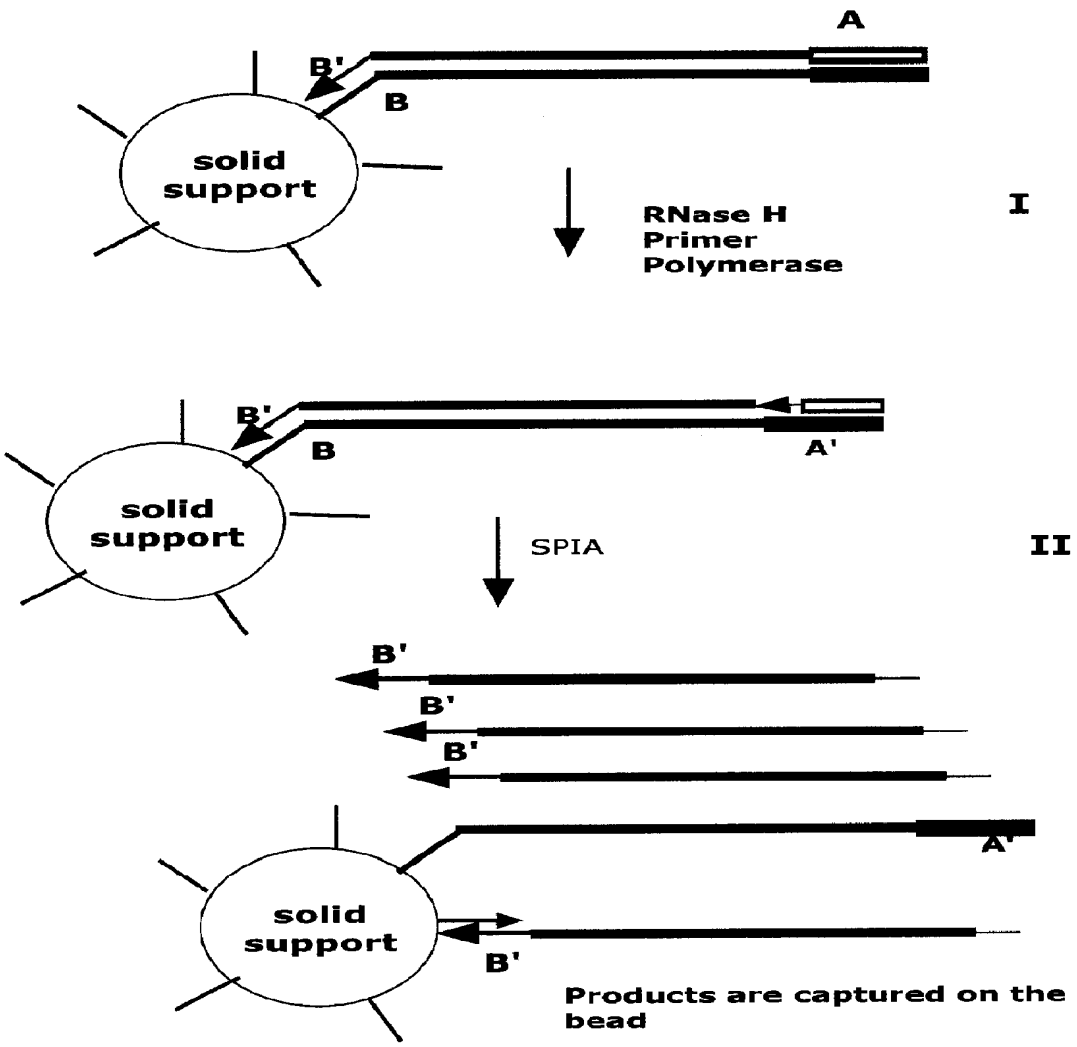

A
B'
B
solid support
RNase H
Primer
Polymerase
I
B'
B
A'
solid support
SPIA
II
B'
B'
B'
A'
solid support
B'
Products are captured on the bead

Figure 16

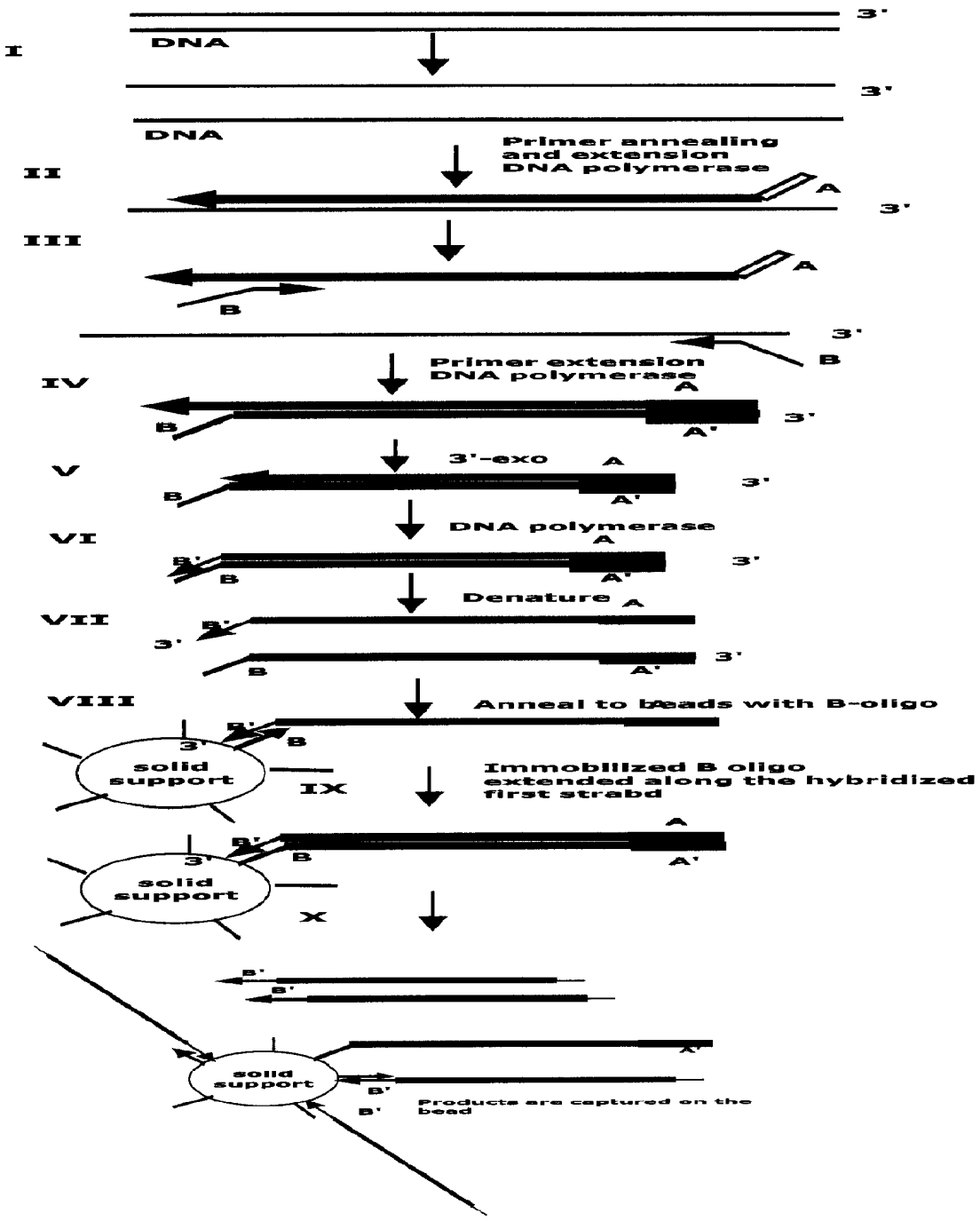

I
DNA
3'
3'
DNA
II
Primer annealing
and extension
DNA polymerase
A
3'
III
A
B
3'
B
IV
Primer extension
DNA polymerase
A
B
A'
3'
V
3'-exo
A
B
A'
3'
VI
DNA polymerase
A
B'
B
A'
3'
VII
Denature
A
B'
3'
B
A'
3'
VIII
Anneal to beads with B-oligo
B'
3'
B
solid
support
IX
Immobilized B oligo
extended along the hybridized
first strabd
A
B'
3'
B
A'
solid
support
X
B'
B'
A'
solid
support
B'
B'
Products are captured on the
bead

Figure 17

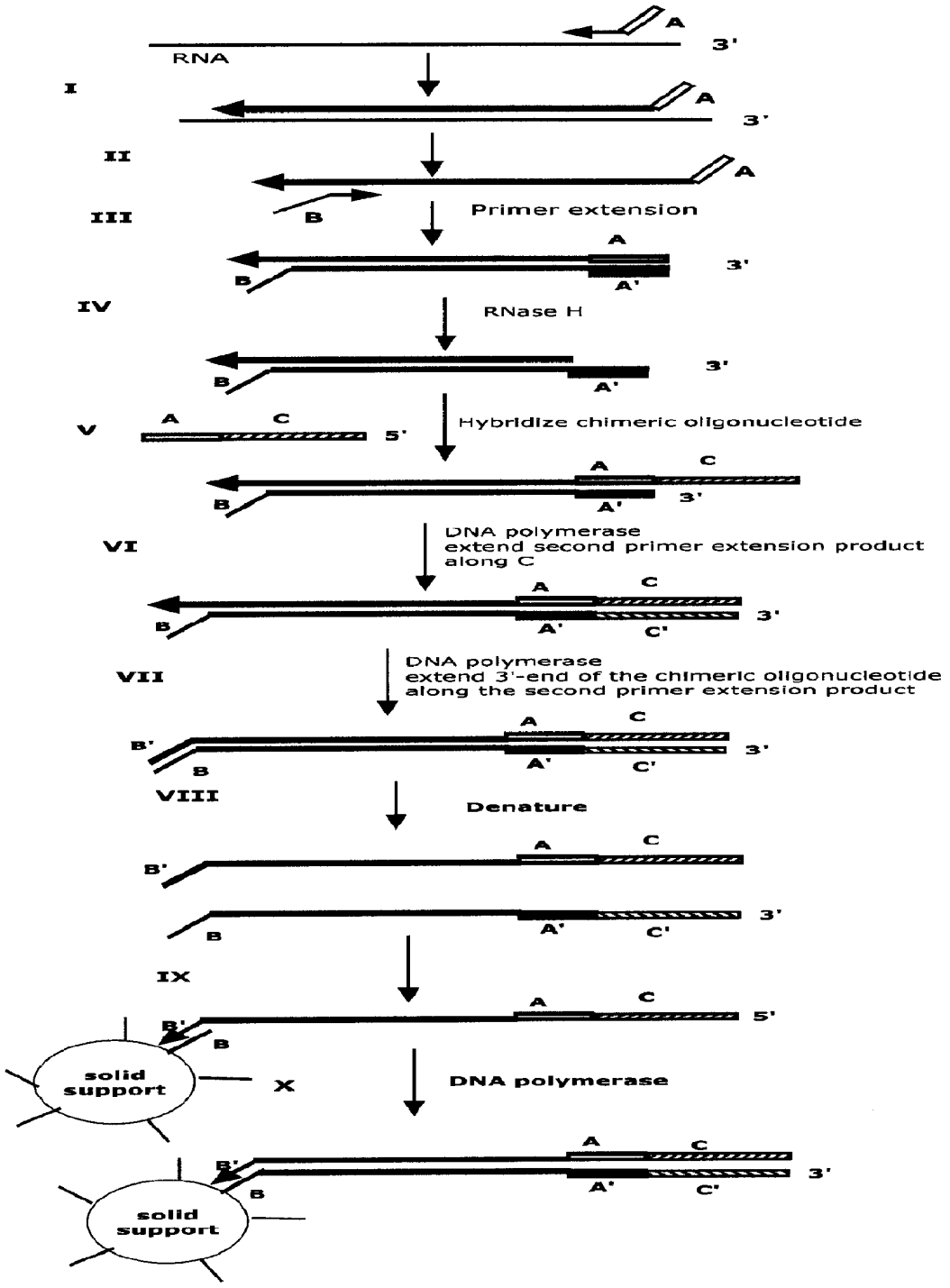

A
3'
RNA
I
A
3'
II
A
III
B
Primer extension
A
3'
B
A'
IV
RNase H
3'
B
A'
V
A
C
5'
Hybridize chimeric oligonucleotide
A
C
3'
B
A'
VI
DNA polymerase
extend second primer extension product
along C
A
C
3'
B
A'
C'
VII
DNA polymerase
extend 3'-end of the chimeric oligonucleotide
along the second primer extension product
A
C
B'
3'
B
A'
C'
VIII
Denature
A
C
B'
3'
B
A'
C'
IX
A
C
5'
B'
B
solid
support
X
DNA polymerase
A
C
B'
3'
B
A'
C'
solid
support

Figure 18

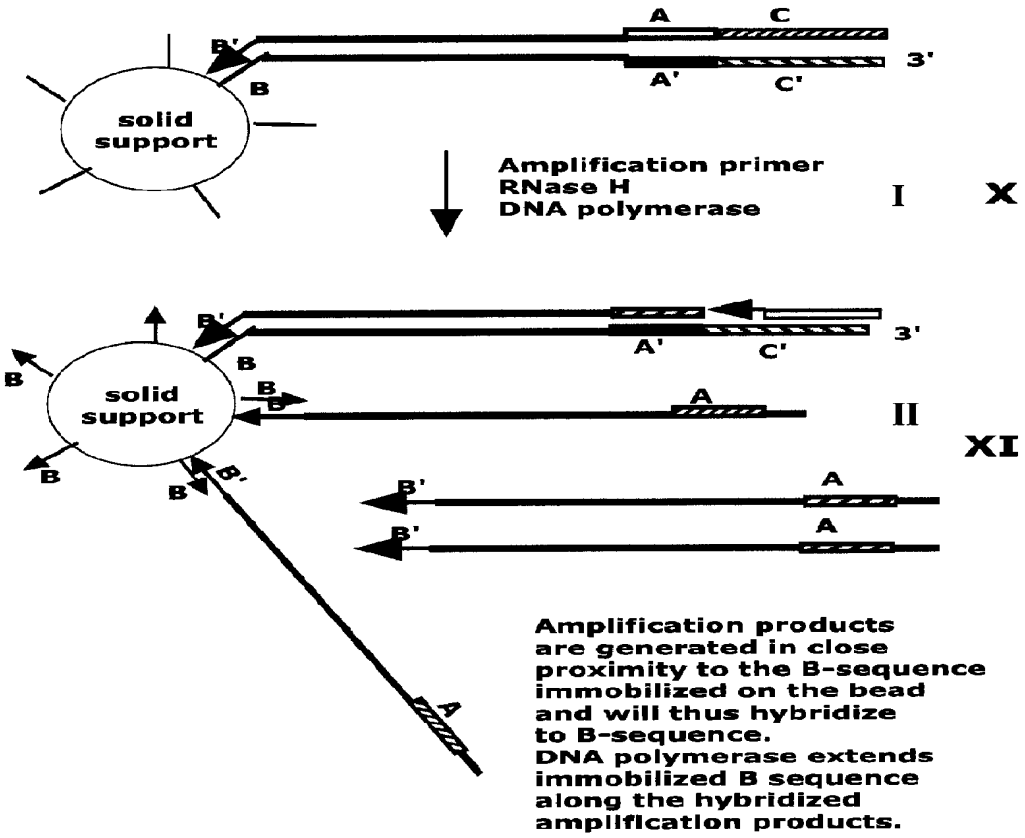
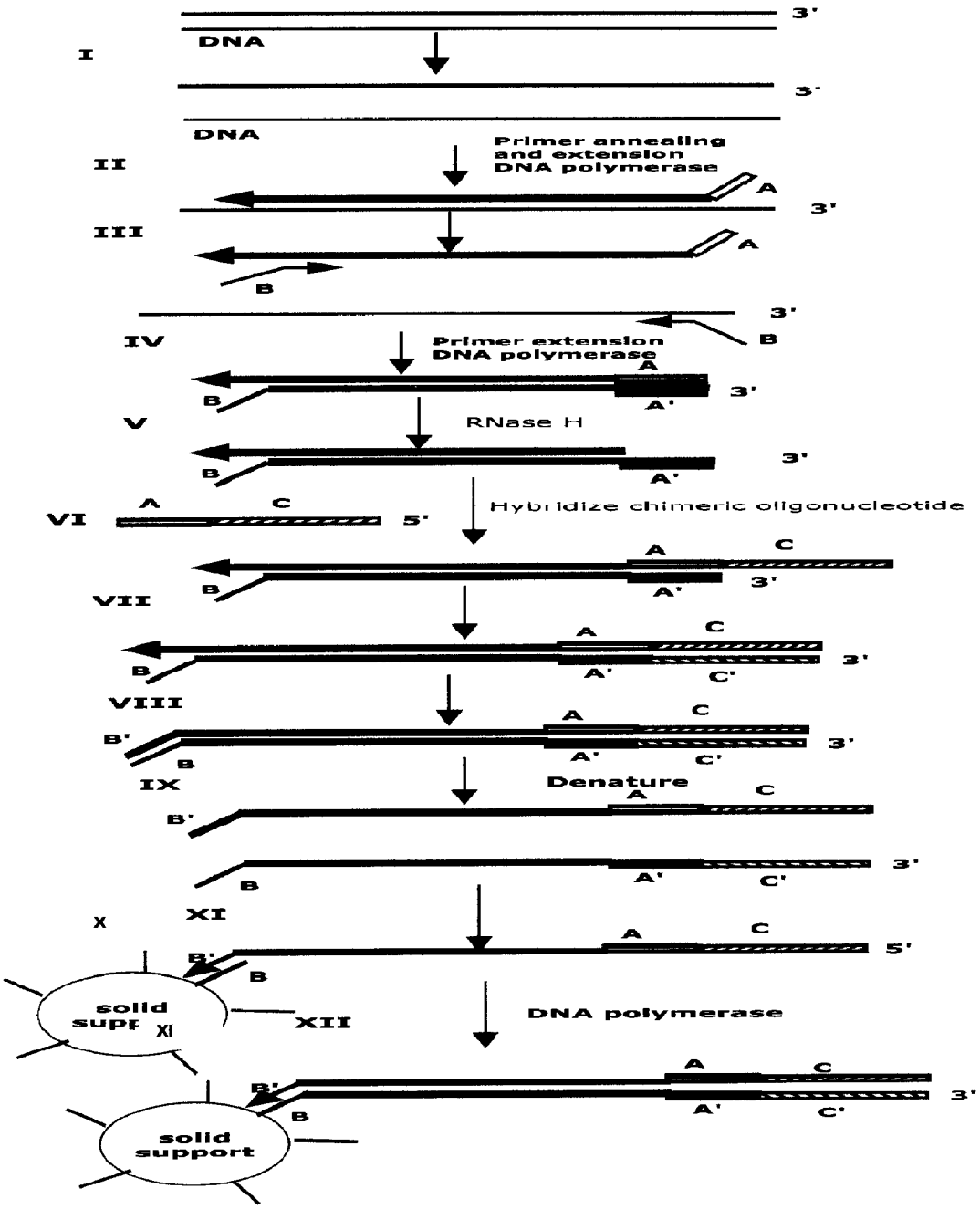

A
C
B'
3'
B
A'
C'
solid support
Amplification primer
RNase H
DNA polymerase
I
X
B'
3'
A'
C'
B
B
solid support
B
A
II
XI
B
B
B'
B'
A
B'
A
A
Amplification products are generated in close proximity to the B-sequence immobilized on the bead and will thus hybridize to B-sequence.
DNA polymerase extends immobilized B sequence along the hybridized amplification products.

Figure 19


I
DNA
3'
3'
II
DNA
Primer annealing
and extension
DNA polymerase
A
3'
III
A
B
IV
3'
B
Primer extension
DNA polymerase
A
B
A'
3'
V
RNase H
B
A'
3'
VI
A
C
5'
Hybridize chimeric oligonucleotide
VII
A
C
B
A'
3'
VIII
A
C
B'
B
A'
C'
3'
IX
Denature
A
C
B'
B
A'
C'
3'
X
XI
A
C
B'
B
5'
solid
supp
XI
XII
DNA polymerase
A
C
B'
B
A'
C'
3'
solid
support

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(54) Title: GLOBAL AMPLIFICATION USING A RANDOMLY PRIMED COMPOSITE PRIMER

(57) Abstract: The invention relates to the field of polynucleotide amplification. More particularly, the invention provides methods, compositions and kits for amplification of (i.e., making multiple copies of) a multiplicity of different polynucleotide template sequences using a randomly primed RNA/DNA composite primer.

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GLOBAL AMPLIFICATION USING A RANDOMLY PRIMED COMPOSITE PRIMER

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Applications Nos. 60/462,962, filed April 14, 2003, and 60/462,965, filed April 14, 2003, each of which is incorporated by reference in its entirety.

TECHNICAL FIELD

[0002] 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 of) a multiplicity of different polynucleotide template sequences using a randomly primed RNA/DNA composite primer.

BACKGROUND ART

[0003] The quality and quantity of nucleic acid (e.g. genomic DNA) sample is important for many studies. High-throughput genomic analysis requires large amounts of template for testing, yet typically the yield of nucleic acids from individual patient samples is limited. Forensic and paleoarcheology work also can be severely limited by nucleic acid sample size. The limitation of starting material impacts the ability to carry out large scale analysis of multiple parameters, as is required for, for example, the genotyping of multiple loci in the study of complex diseases. Moreover, it is well accepted that molecular analysis determination of genomic instability in various pathological condition such as cancer, is most precisely carried out in well defined cell populations, such as that obtained by laser capture micro-dissection or cell sorting. Nucleic acid amplification technologies that provide global amplification of very small polynucleotide samples, for example, from one or a very few cells, may provide a solution to the limited starting materials generally available for analysis.

[0004] Likewise, the ability to amplify ribonucleic acid (RNA) is an important aspect of efforts to elucidate biological processes. Total cellular mRNA represents gene expression activity at a defined time. Gene expression is affected by cell cycle progression,

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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. Non-coding RNAs have been shown to be of great importance in regulation of various cellular functions and in certain disease pathologies. Such RNAs are often present in very low levels. Thus, amplification methods capable of amplifying low abundance RNAs, including RNAs that are not polyadenylated, are of great importance.

[0005] Various methods for global amplification of DNA target molecules (e.g., whole genome amplification) have been described, including methods based on the polymerase chain reaction (PCR). *See, e.g.*, U.S. Patent Nos. 5,731,171; 6,365,375; Daigo et al., (2001) Am. J. Pathol. 158 (5):1623-1631; Wang et al, (2001); Cancer Res. 61:4169-4174; Zheng et al, (2001) Cancer Epidemiol. 10:697-700; Dietmaier et al (1999) Am. J. Pathol. 154 (1) 83-95; Stoecklein et al (2002) Am. J. Pathol. 161 (1):43-51; U.S. Patent Nos. 6,124,120; 6,280,949; Dean et al (2002) PNAS 99 (8):5261-5266. However, PCR-based global amplification methods, such as whole genome amplification (WGA), may generate non-specific amplification artifacts, give incomplete coverage of loci, or generate DNA of insufficient length that cannot be used in many applications. PCR-based methods also suffer from the propensity of the PCR reaction to generate products that are preferentially amplified, and thus resulting in biased representation of genomic sequences in the products of the amplification reaction.

[0006] Additionally, a number of methods for the analysis of gene expression have been developed in recent years. See, for example, U.S. Pat. Nos. 6,251,639, 6,692,918, 6,686,156, 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; and U.S. published patent applications nos. 2002/0115088, 2003/0186234, 2003/0087251, and 2004/0023271. 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. RNA 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

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require thermocycling; b) the products are double stranded, thus rendering them less accessible to binding to probes; and c) the reactions are prone to contamination with products of prior amplification, thus requiring strict containment of reaction mixtures. Other current RNA amplification methods use initiation of replication of mRNA from the poly-A tail at their 3' ends. However, not all RNA transcripts have a mRNA tail (for example, prokaryotic RNAs and non-coding RNAs). In addition, due to sample preparation procedures, the RNA transcript structural integrity is compromised. Thus, it may be desirable in certain circumstances to use RNA amplification methods that do not require initiation of replication at the defined poly-A tail. Although analysis of non-amplified RNA is feasible, a significant amount of starting RNA would be required. However, the total amount of sample RNA 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 RNA species is not equal; some species are more abundant than others are, and these are more likely and easier, to analyze. The ability to amplify RNA sequences enables the analysis of less abundant, rare RNA species. The ability to analyze small samples, by means of nucleic acid amplification, is also advantageous for design parameters of large scale screening 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.

[0007] Therefore, there is a need for improved amplification methods, particularly methods which can globally amplify DNA or RNA polynucleotide targets. The invention described herein fulfills this need.

[0008] All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

DISCLOSURE OF THE INVENTION

[0009] The invention provides methods, compositions, and kits for isothermal global amplification using a randomly hybridized RNA/DNA composite primer, as well as applications of the amplification methods.

[0010] Accordingly, in one aspect, the invention provides methods for amplification of a template polynucleotide, said methods comprising: (a) incubating a

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reaction mixture, said reaction mixture comprising: (i) a template polynucleotide; (ii) a first primer, wherein the first primer is a composite primer that is hybridizable to a multiplicity of template polynucleotide sites, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (iii) a DNA-dependent DNA polymerase ; and (iv) an RNA-dependent DNA polymerase (which may be present as a separate enzyme or as an enzyme comprising both DNA-dependent DNA polymerase and RNA-dependent DNA polymerase activities); wherein the incubation is under conditions that permit composite primer random hybridization, primer extension and, in some embodiments, displacement of the primer extension product from template polynucleotide, whereby a complex comprising an RNA/DNA partial heteroduplex is generated; and (b) incubating a reaction mixture, said reaction mixture comprising (i) the reaction products generated according to step (a) (or an aliquot thereof); (ii) a composite primer (which may be the same as the first primer, or may be a different primer), wherein the composite primer comprises an RNA portion and a 3' DNA portion; (iii) an DNA-dependent DNA polymerase; and (iv) an agent (such as an enzyme) that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit RNA cleavage from an RNA/DNA heteroduplex, primer hybridization, primer extension, and displacement of the primer extension product from the complex of (a) when its RNA is cleaved and another composite primer binds to the template and is extended, whereby multiple copies of a polynucleotide (generally, DNA) amplification product are generated. In embodiments wherein the template polynucleotide is RNA, the reaction mixture of step (a) further comprises (v) an agent (such as an enzyme)that cleaves RNA from an RNA/DNA hybrid, whereby template RNA is cleaved form the complex comprising template RNA and first primer extension product. In some embodiments, the reaction mixture of step (b) comprises the reaction mixture according to step (a) (or an aliquot thereof). In other embodiments, step (b) is initiated by the addition of an agent that cleaves RNA from a partial RNA/DNA heteroduplex (such as RNase H), and optionally, a DNA-dependent DNA polymerase, to the reaction mixture of step (a). In some embodiments, the reaction mixture of step (a) and/or (b) further comprises auxiliary primers. In some embodiments (generally embodiments in which the template polynucleotide is DNA), the RNA-dependent DNA polymerase may be omitted from reaction mixture (a).

[0011] In another aspect, the invention provides methods for amplification of a template polynucleotide by incubating a reaction mixture, said reaction mixture

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comprising: (a) a complex comprising a RNA/DNA partial heteroduplex, wherein the complex is generated by incubating a first reaction mixture, said first reaction mixture comprising: (i) a polynucleotide template; (ii) a first primer; wherein the first primer is a composite primer, the composite primer comprising a RNA portion and a 3' DNA portion; and wherein the composite primer is capable of hybridizing to a multiplicity of template polynucleotide sites; (iii) a DNA-dependent DNA polymerase; and (iv) an RNA-dependent DNA polymerase; wherein the incubation is under conditions that permit composite primer random hybridization, primer extension and displacement of the primer extension product from template polynucleotide, whereby a complex comprising an RNA/DNA partial heteroduplex is generated; (b) a composite primer, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (c) a DNA-dependent DNA polymerase; and (d) an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of composite primer from the complex of step (a) when its RNA is cleaved and another composite primer binds and is extended, whereby multiple copies of a polynucleotide amplification product are generated. In some embodiments, the reaction mixture and/or first reaction mixture further comprises auxiliary primers. In some embodiments wherein the template polynucleotide is RNA, template RNA in step (a) is cleaved following primer extension via conditions or agents promoting cleavage. In some embodiments, the first reaction mixture further comprises: (v) an agent (such as an enzyme) that cleaves RNA from an RNA/DNA hybrid, whereby template RNA is cleaved from the complex comprising template RNA and composite primer extension product. In some embodiments (generally those in which the template polynucleotide is DNA), the complex comprising a RNA/DNA partial heteroduplex may be generated without the use of RNA-dependent DNA polymerase.

[0012] In another aspect, the invention provides methods for amplification of a template polynucleotide by incubating a reaction mixture, said reaction mixture comprising: (a) a complex of a first primer extension product and a second primer extension product, wherein the first primer extension product is generated by extension of a randomly primed first primer hybridized to target polynucleotide with a DNA polymerase, wherein the first primer is a composite primer comprising an RNA portion and a 3' DNA portion, wherein the first primer is capable of hybridizing to a multiplicity of template polynucleotide sites, and wherein the second primer extension product is generated by

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extension of a second primer hybridized to the first primer extension product; (b) a composite primer that is hybridizable to the second primer extension product, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (c) a DNA-dependent DNA polymerase; and (d) an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of composite primer from the complex of step (a) when its RNA is cleaved and another composite primer binds and is extended, whereby multiple copies of a polynucleotide amplification product are generated. In some embodiments wherein the template polynucleotide is RNA, the first primer extension product is generated by extension of a randomly primed first primer hybridized to target RNA with a RNA-dependent DNA polymerase. In some embodiments, the first primer extension product and/or the second primer extension product are generated in the presence of auxiliary primers.

[0013] In another aspect, the invention provides methods for amplification of a polynucleotide template comprising: (a) random priming of polynucleotide template strand with a composite primer; wherein the composite primer comprises an RNA portion and a 3' DNA portion, and wherein the composite primer is capable of hybridizing to a multiplicity of template polynucleotide sites; and (b) incubating template strand in the presence of a DNA-dependent DNA polymerase, an RNA-dependent DNA polymerase, and an agent that cleaves RNA from an RNA/DNA, whereby multiple copies of polynucleotide amplification product are generated via primer extension and strand displacement. In some embodiments, random priming occurs in the presence of a DNA polymerase. In some embodiments, auxiliary primers are included in step (a) and/or step (b). In some embodiments (generally those in which the polynucleotide template is DNA), the RNA-dependent DNA polymerase is omitted from the incubation.

[0014] In another aspect, the invention provides methods for amplification of RNA template polynucleotides which operate as follows: a multiplicity of template polynucleotide sequences are amplified by incubating a reaction mixture, the reaction mixture comprising: (a) a complex of a first primer extension product and a second primer extension product, wherein the first primer extension product is generated by extension of a first primer hybridized to template RNA strand with an RNA-dependent DNA polymerase, wherein the first primer is a composite primer comprising an RNA portion and a 3' DNA portion, wherein the first primer is capable of hybridizing to a multiplicity of sites on

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template RNA; wherein the second primer extension product is generated by extension of a second primer hybridized to the first primer extension product; and wherein RNA from the complex of first and second primer extension product is cleaved (using e.g., an enzyme that cleaves RNA from an RNA/DNA hybrid or conditions permitting cleavage, such as heat and/or alkaline conditions); (b) a composite primer that is hybridizable to the second primer extension product, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (c) a DNA-dependent DNA polymerase; and (d) an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of composite primer from the complex of step (a) when its RNA is cleaved and another composite primer binds to the second primer extension product and is extended, whereby multiple copies of polynucleotide amplification product are generated. In some embodiments, the complex of step (a) is generated in the presence of auxiliary primers. In some embodiments, the second primer comprises fragment(s) of cleaved RNA template.

[0015] In another aspect, the invention provides methods for amplification of a template polynucleotide by (a) randomly priming a template polynucleotide with a first primer, wherein said first primer is a composite primer that is hybridizable to a multiplicity of template polynucleotide sites, wherein the composite primer comprises a RNA portion and a 3' DNA portion; (b) extending the first primer with a DNA polymerase; (c) cleaving RNA from the first primer with an agent that cleaves RNA from a RNA/DNA heteroduplex; (d) hybridizing an amplification primer to the template polynucleotide, wherein said amplification primer is a composite primer comprising a RNA portion and a 3' DNA portion; (e) extending the hybridized amplification primer by strand displacement DNA synthesis; and (f) cleaving RNA from the amplification primer with an agent that cleaves RNA from a RNA/DNA heteroduplex, such that another amplification primer can hybridize and be extended, whereby multiple copies of a polynucleotide amplification product are generated.

[0016] In another aspect, the invention provides methods for amplification of a template polynucleotide by incubating a reaction mixture including: (a) a polynucleotide template strand; (b) a first primer, wherein said first primer is a composite primer comprising a RNA portion and a 3' DNA portion, and wherein the first primer is capable of hybridizing to a multiplicity of template polynucleotide sites; (c) a DNA-dependent DNA

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polymerase; (d) a RNA-dependent DNA polymerase; and (e) an agent that cleaves RNA from a RNA/DNA heteroduplex, whereby multiple copies of polynucleotide amplification product are generated by primer extension and strand displacement. In some embodiments (generally those embodiments in which the polynucleotide template is DNA), the RNA-dependent DNA polymerase is omitted from the reaction mixture.

[0017] As is clear to one skilled in the art, reference to production of copies of a polynucleotide (*e.g.*, DNA or RNA) template or copies of a polynucleotide sequence complementary to a polynucleotide template 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.

[0018] Various embodiments of the composite primer(s) used in the methods of the invention are described herein. For example, 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 RNA portion of the composite primer consists of 7 to about 20 nucleotides and the DNA portion of the composite primer consists of about 5 to about 20 nucleotides. In still other embodiments, the RNA portion of the composite primer consists of about 10 to about 20 nucleotides and the DNA portion of the composite primer consists of about 7 to about 20 nucleotides. In some embodiments the composite primer is selected from the following composite primers: 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

[0019] In some embodiments, the composite primer comprises random sequence or partially randomized sequence, although certain embodiments (such as certain embodiments wherein the template polynucleotide is RNA) exclude the use of primers comprising random or partially random sequence. In embodiments utilizing a composite primer with random or partially random sequence, the composite primer may be a population or pool of different primers comprising at least 2, at least 3, at least 4, at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, or at least 100 different sequences. In other embodiments, the composite primer contains one or more "degenerate"

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nucleotides that is able to hybridize to multiple different nucleotide bases (*e.g.*, inosine, which is able to hybridize to all four canonical bases).

[0020] In some embodiments, the composite primer that hybridizes to target polynucleotide (such as mRNA or genomic DNA) and the composite primer used during single primer isothermal amplification (i.e., phase (b) of the methods) are the same. In some embodiments, the composite primer that hybridizes to target polynucleotide (such as mRNA or genomic DNA) and the composite primer used during single primer isothermal amplification are different. In some embodiments, two (or more) different composite primers that hybridize to target polynucleotide are used in the methods of the invention.

[0021] The methods are applicable to amplifying any target polynucleotide, including, for example, DNA (such as genomic DNA, including human and other mammalian genomic DNA) and RNA (such as total RNA, mRNA, noncoding RNA 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), and, as is evident, the invention includes various combinations of the steps described herein. 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 random hybridization of composite primer. Methods of the invention encompass embodiments in which later, "downstream" steps are an initial step.

[0022] The enzymes which may be used in the methods and compositions are described herein. For example, the agent (such as an enzyme) that cleaves RNA may be an RNase H, and the RNA-dependent DNA polymerase may be reverse transcriptase. The RNA-dependent DNA polymerase may comprise an RNase H enzyme activity, or separate enzymes may be used. Similarly, a DNA polymerase may comprise both RNA-dependent and DNA-dependent DNA polymerase enzyme activities, or separate enzymes may be used. A DNA-dependent DNA polymerase, an RNA-dependent DNA polymerase, and the enzyme that cleaves RNA can also be the same enzyme, or separate enzymes comprising each of these activities may be used.

[0023] In some embodiments, methods of the invention are used to generate labeled polynucleotide products (generally DNA products). In some embodiments of methods for generating labeled DNA products, at least one type of dNTP used is a labeled dNTP. In

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other embodiments of methods for generating labeled DNA products, a labeled composite primer is used.

[0024] The invention also provides methods which employ (usually, analyze) the products of the amplification methods of the invention, such as detection of sequence alteration(s) (*e.g.*, genotyping, nucleic acid mutation detection, analysis of splice variants, and the like); determining presence or absence of a sequence of interest; quantifying a sequence of interest; gene expression profiling; subtractive hybridization; preparation of subtractive hybridization probe; differential amplification; preparation of libraries (including genomic, cDNA and differential expression libraries); preparation of an immobilized nucleic acid (which can be a nucleic acid immobilized on a microarray, preparing labeled probes for analysis on arrays (including high density arrays) for the detection and quantification of sequences of interest, including, for example, sequence determination, detecting sequence variation and genotyping; comparative genome hybridization; detection and/or identification of novel RNAs; and characterizing nucleic acids using the amplification nucleic acid products generated by the methods of the invention.

[0025] Any of the methods of the invention can be used to generate polynucleotide products that are suitable for characterization of a polynucleotide sequence of interest in a sample. In one embodiment, the invention provides methods for characterizing (for example, detecting (presence or absence) and/or quantifying) a polynucleotide sequence of interest comprising: (a) amplifying a target polynucleotide 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 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 (such as DNA) products that are labeled by incorporating labeled nucleotides and/or labeled composite primers into appropriate step(s) 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. In one aspect, the invention provides a method of characterizing a polynucleotide sequence of interest, comprising (a) amplifying a target polynucleotide by a method described herein to generate labeled polynucleotide products; and (b) analyzing the labeled polynucleotide products. In

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some embodiments, the step of analyzing polynucleotide products comprises determining amount of said products, whereby the amount of the polynucleotide sequence of interest present in a sample is quantified.

[0026] The amplification products can also serve as template for further analysis such as sequence analysis, polymorphism detection (including multiplex SNP detection) using, e.g., oligonucleotide ligation-based assays, analysis using Invader, Cleavase or limited primer extension, and other methods known in the art. For methods that generally require larger volumes of input material, the methods of the invention may be used to "pre" amplify a pool of polynucleotides to generate sufficient input material for subsequent analysis.

[0027] In another embodiment, 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, 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.

[0028] In another aspect, the invention provides methods of determining a gene expression profile in a sample, the methods comprising (a) amplifying RNA template in a sample using any of the methods described herein; and (b) determining an amount of amplification products of each RNA sequence of interest in the sample, whereby the gene expression profile of the sample is determined. The invention further provides methods of determining a gene expression profile by determining an amount of amplification products of each RNA sequence of interest in a sample, the sample comprising multiple copies of RNA template amplified by any of the methods described herein, whereby the gene expression profile of the sample is determined.

[0029] Additionally, the invention also provides methods for archiving polynucleotide templates. Because the amplification methods of the invention provide representative amplification of the sequences of the template polynucleotide, amplified product produced by the instant methods may be used as an archival source for the original template polynucleotide. Accordingly, the invention provides methods for archiving a

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polynucleotide template by storing the amplification products produced by the methods of the invention. The archived amplification products may be analyzed as described herein, or may be subjected to further amplification in accordance with the methods of the invention.

[0030] In another aspect, the invention provides products (*e.g.*, multiple copies of a template polynucleotide) produced by the methods disclosed herein.

[0031] 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.

[0032] 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.

[0033] 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.

[0034] In another aspect, the invention provides reaction mixtures (or compositions comprising reaction mixtures) which contain various combinations of components described herein.

[0035] 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.

[0036] In another aspect, the invention provides systems for effecting the amplification methods described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037] Figure 1 illustrates one embodiment of a composite primer useful in the methods of the present invention. As illustrated in the Figure, 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.

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[0038] Figure 2 illustrates a composite primer that hybridizes to multiple sites on a template polynucleotide where differing portions of the composite primer are hybridized to template polynucleotide depending on the site at which it is hybridized.

[0039] Figure 3 illustrates primer extension from composite primers that are hybridized at multiple sites on a template strand, where a composite primer extension products is being displaced by primer extension from a composite primer hybridized at a downstream site on the template strand.

[0040] Figure 4 shows a collection of composite primer extension products comprising composite primer 1 linked (via extension) to sequences corresponding to a multiplicity of target polynucleotide sequences.

[0041] Figure 5 shows generation of second primer extension product that is randomly primed by the composite primer.

[0042] Figure 6 shows single primer isothermal amplification using the complex comprising a RNA/DNA partial heteroduplex as a template for further composite-primer dependent amplification.

[0043] Figure 7 illustrates primer extension from composite primers and auxiliary primers that are hybridized at multiple sites on a template strand.

[0044] Figure 8 illustrates generation of a second primer extension product primed by auxiliary primers hybridized to composite primer extension product.

[0045] Figure 9 shows a photograph of a gel showing amplified reaction product generated using a single randomly primed composite primer to amplify a multiplicity of template polynucleotide sequences from human genomic DNA.

MODES FOR CARRYING OUT THE INVENTION

Overview of the Invention and Its Advantages

[0046] The invention discloses novel methods, compositions and kits for global amplification. The methods provide for amplification using a composite primer that is capable of binding to multiple sites within template polynucleotide (e.g., mRNA or genomic DNA), whereby a large multiplicity of template polynucleotide sequences (for example, essentially all genomic DNA) is amplified. The methods are suitable for use with either DNA or RNA template. They generate polynucleotide (generally, DNA) products, which are readily suitable for a variety of uses including comparative genome

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hybridization, expression profiling, and multiple genotype determinations, e.g., by multiplex analysis by microarray technologies. The methods are amenable to automation and do not require thermal cycling. Thus, one of the major advantages of the methods of the invention is the ability to amplify an entire pool of sequences (or a subset thereof, depending on the desired extent of amplification), which is essential for application such as comparative genome hybridization, generation of cDNA libraries, generation of subtractive hybridization probes, and array based assays, including multiple genotype determinations.

[0047] The amplification methods of the invention involve composite primer random hybridization, primer extension and displacement of composite primer extension product by strand displacement, whereby a complex comprising a RNA-DNA heteroduplex is generated, followed by composite primer-dependent isothermal amplification using the complex as a substrate for further amplification, an aspect that permits rapid amplification and distinguishes the invention from other strand displacement amplification methods, such as MDA.

[0048] In one aspect, the methods of the invention involve two phases: (a) composite primer random hybridization to template polynucleotide, primer extension and displacement of composite primer extension product by strand displacement DNA synthesis, whereby a complex comprising a RNA/DNA partial heteroduplex is generated, and (b) composite primer-dependent single primer isothermal amplification using the complex comprising a RNA/DNA partial heteroduplex as a substrate for further amplification.

[0049] The methods generally comprise using specially-designed primers, generally a RNA/DNA composite primer, to randomly prime template polynucleotide (such as genomic DNA template, mRNA or noncoding RNA). By "randomly prime" or "random hybridization", as used herein, it is meant that the composite primer hybridizes to multiple sites within template polynucleotide. In some embodiments, an aspect of the invention is displacement of primer extension product from template polynucleotide(s) during primer extension by strand displacement DNA synthesis (*e.g.*, by primer extension with a DNA polymerase having strand displacement activity) of primers hybridized at a downstream position(s) on the template.

[0050] Thus, the invention provides methods of incubating a reaction mixture, said reaction mixture comprising: (a) a polynucleotide template; (b) a composite primer; wherein the composite primer comprises an RNA portion and a 3' DNA portion, and

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wherein the composite primer is capable of hybridizing to a multiplicity of template polynucleotide sites; (c) a DNA-dependent DNA polymerase with strand displacement activity; and (d) an RNA-dependent DNA polymerase; wherein the incubation is under conditions that permit composite primer random hybridization, primer extension and displacement of the primer extension product from template polynucleotide, whereby a population of intermediate complexes are generated that generally includes (a) copies of template polynucleotide and/or copies of the complement of polynucleotide sequence appended (via extension) to composite primer sequences; and (b) copies of template polynucleotide and copies of the complement of template polynucleotide appended (via extension) to the complement of composite primer sequences. The intermediate complexes may be double-stranded or may be partially double stranded. By virtue of the presence of composite primer sequences in the intermediate complexes, the complexes comprise a RNA/DNA heteroduplex partial heteroduplex. The RNA portion of the RNA/DNA partial heteroduplex generally is introduced by the RNA portion of the composite primer, and the DNA portion of the heteroduplex is made of the complement of the RNA portion of the composite primer. For simplicity, this population of intermediate complexes is termed "a complex comprising an RNA/DNA partial heteroduplex."

[0051] Generally, the composite primer comprises at least a 3' DNA portion that is capable of randomly priming template polynucleotide. Thus, and as the description makes clear, reference to a primer that hybridizes to a sequence encompasses embodiments in which at least a portion of the primer is hybridized, embodiments in which two (or more portions) of the primer are hybridized (separated by unhybridized (looped out) portions of the primer), and embodiments in which the entire primer is hybridized.

[0052] The complex comprising a RNA/DNA partial heteroduplex is substrate for further amplification as follows: an enzyme which cleaves RNA from an RNA/DNA hybrid (such as RNase H) cleaves RNA sequence from the hybrid, leaving a 3' single stranded DNA sequence available for binding by a composite primer (which may or may not be the same as the first composite primer). Extension of a bound composite primer by DNA-dependent DNA polymerase produces a primer extension product, which displaces the previously bound cleaved first primer extension product, whereby polynucleotide (generally, DNA) product accumulates. It is understood that amplified product generally is a mixture of sense and antisense copies of a given template polynucleotide. For example, if the template polynucleotide is double stranded DNA, the amplification product will

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correspond to each strand. If the template polynucleotide is single stranded, amplification product will generally be produced that is the copy of template polynucleotide (sense copy) and the complement of the template polynucleotide (antisense copy).

[0053] The methods disclosed herein are applicable to the amplification of any target polynucleotide, including both DNA (*e.g.*, genomic DNA) and RNA (*e.g.*, mRNA and ribosomal RNA) targets. As is evident from the description and shown in the example, the methods of the invention are composite-primer dependent. That is, amplification is not observed in the absence of the composite primer.

[0054] In another aspect of the invention, auxiliary primers are present in the reaction mixture comprising template polynucleotide, composite primer, DNA-dependent DNA polymerase and RNA-dependent DNA polymerase. As used herein, "auxiliary primers" refers to a population of random and/or partially randomized primers. Inclusion of a population of random primers during the amplification is believed to enhance the efficiency of production of and/or global (template-wide, *e.g.*, providing representative amplification of the template, whether the template is DNA or RNA) coverage of the amplification product.

[0055] In certain aspects, global amplification of genomic DNA is exemplified herein. It is understood, however, that the amplification methods of the invention are suitable for amplification of any pool or subset (or polynucleotides representing a significant proportion of a pool or subset, depending on desired extent of amplification) of polynucleotides.

[0056] Accordingly, in one aspect, the invention provides methods for amplification of a template polynucleotide, said methods comprising: (a) incubating a reaction mixture, said reaction mixture comprising: (i) a template polynucleotide; (ii) a first primer, wherein the first primer is a composite primer that is hybridizable to a multiplicity of template polynucleotide sites, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (iii) a DNA-dependent DNA polymerase ; and (iv) an RNA-dependent DNA polymerase (which may be present as a separate enzyme or as an enzyme comprising both DNA-dependent DNA polymerase and RNA-dependent DNA polymerase activities); wherein the incubation is under conditions that permit composite primer random hybridization, primer extension and displacement of the primer extension product from template polynucleotide, whereby a complex comprising an RNA/DNA partial heteroduplex is generated; and (b) incubating a reaction mixture, said reaction mixture

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comprising (i) the reaction products generated according to step (a) (or an aliquot thereof); (ii) a composite primer (which may be the same as the first primer, or may be a different primer), wherein the composite primer comprises an RNA portion and a 3' DNA portion; (iii) an DNA-dependent DNA polymerase; and (iv) an agent (such as an enzyme) that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of the primer extension product (*i.e.*, strand displacement DNA synthesis) from the complex of (a) when its RNA is cleaved and another composite primer binds to the template and is extended, whereby multiple copies of a polynucleotide (generally, DNA) amplification product are generated. In some embodiments, the reaction mixture of step (b) comprises the reaction mixture according to step (a) (or an aliquot thereof). In other embodiments, step (b) is initiated by the addition of an agent that cleaves RNA from an RNA/DNA heteroduplex (such as RNase H), and optionally, a DNA-dependent DNA polymerase, to the reaction mixture of step (a). In some embodiments, the reaction mixture of step (a) and/or (b) further comprises auxiliary primers.

[0057] In another aspect, the invention provides methods for amplification of a template polynucleotide by incubating a reaction mixture, said reaction mixture comprising: (a) a complex of a first primer extension product and a second primer extension product, wherein the first primer extension product is generated by extension of a randomly primed first primer hybridized to target polynucleotide with a DNA polymerase, wherein the first primer is a composite primer comprising an RNA portion and a 3' DNA portion, wherein the first primer is capable of hybridizing to a multiplicity of template polynucleotide sites, and wherein the second primer extension product is generated by extension of a second primer hybridized to the first primer extension product; (b) a composite primer that is hybridizable to the second primer extension product, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (c) a DNA-dependent DNA polymerase; and (d) an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of composite primer from the complex of step (a) when its RNA is cleaved and another composite primer binds and is extended, whereby multiple copies of a polynucleotide amplification product are generated. In some embodiments, the first primer extension product and/or the second primer extension product are generated in the presence of auxiliary primers.

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[0058] In another aspect-of the invention wherein the template polynucleotide is RNA, template RNA is cleaved following random composite primer hybridization and primer extension. Template RNA can be cleaved using methods well-known in the art, including cleavage with an agent (such as an enzyme, such as RNase H) that cleaves RNA from an RNA/DNA hybrid, cleavage resulting from heat treatment, and cleavage due to chemical treatment (e.g., treatment under alkaline conditions). In some embodiments, the invention provides methods of incubating a reaction mixture, said reaction mixture comprising: (a) an RNA template; (b) a composite primer; wherein the composite primer comprises an RNA portion and a 3' DNA portion; and wherein the composite primer is capable of hybridizing to a multiplicity of sites in template RNA; (c) a DNA-dependent DNA polymerase; (d) an RNA-dependent DNA polymerase; and (e) an enzyme capable of cleaving RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit composite primer random hybridization, primer extension, template RNA cleavage from an RNA/DNA heteroduplex, whereby a complex comprising an RNA/DNA partial heteroduplex is generated. The complex comprising an RNA/DNA partial heteroduplex is the substrate for further amplification as described above (i.e., single primer isothermal amplification). In some embodiments, an auxiliary primer is included in the reaction mixture.

[0059] Template polynucleotide may also be prepared from RNA template by synthesis of cDNA. cDNA synthesis may be accomplished using standard methods, such as priming with random primers (*e.g.*, random hexamer deoxyoligonucleotides) and primer extension with a RNA-dependent DNA polymerase (*e.g.*, reverse transcripatase) in the presence of dNTPs and appropriate reaction conditions (*e.g.*, temperature, pH and ionic conditions). Only the first strand cDNA synthesis need be performed, as first strand cDNA synthesis will produce a DNA polynucleotide that can be amplified in accordance with the methods of the invention.

[0060] The methods of the invention include methods using the amplification products (so-called "applications"). The invention also provides methods which employ (usually, analyze) the products of the amplification methods of the invention, such as methods of nucleic acid mutation detection (including methods of genotyping), determining the presence or absence of a sequence of interest, quantitating a sequence of interest, preparation of an immobilized nucleic acid, comparative genomic hybridization, discovery

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of novel nucleic acid sequences, and characterizing nucleic acids using the amplified nucleic acid products generated by the methods of the invention.

[0061] Any of the methods of the invention can be used to generate polynucleotide products that are suitable for characterization of a polynucleotide sequence of interest in a sample. In one embodiment, the invention provides methods for characterizing (for example, detecting (presence or absence) and/or quantifying) a polynucleotide sequence of interest comprising: (a) amplifying a target polynucleotide 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 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 (such as DNA) products that are labeled by incorporating labeled nucleotides and/or labeled composite primers into appropriate step(s) 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.

[0062] The invention provides methods to characterize (for example, detect presence or absence of and/or quantify) an polynucleotide sequence of interest by generating polynucleotide 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. These amplification products may be labeled or unlabeled.

[0063] The methods of the invention may be used to amplify a pool of polynucleotides (or polynucleotides representing a significant proportion of a pool, depending on desired extent of amplification) to generate sufficient input material for subsequent analysis. Thus, and as is described herein, amplification products can also serve as template for further analysis such as sequence, polymorphism detection (including multiplex SNP detection) using, e.g., oligonucleotide ligation-based assays, analysis using Invader, Cleavase or limited primer extension, and the like. Amplification product may also serve as template for further amplification by the methods of the invention or other amplification method known in the art.

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[0064] In yet another embodiment, the invention provides methods for immobilizing nucleic acids, including methods for generating microarrays of nucleic acids, using amplification products of the amplification methods of the invention.

[0065] In another embodiment, the invention provides methods of generating cDNA libraries, methods of generating subtractive hybridization probes, and methods of generating subtractive hybridization libraries.

Advantages of the invention

[0066] Various methods for global amplification of nucleic acids have been developed. PCR-based methods, such as PEP, may generate non-specific amplification artifacts, give incomplete coverage of loci, or generate DNA product of insufficient length that cannot be used in many applications. PCR-based methods also suffer from the propensity of the PCR reaction to generate products that are preferentially amplified, and thus resulting in biased representation of genomic sequences in the products of the amplification reaction. Also, PCR-based methods require cumbersome thermal cycling.

[0067] Additionally, a number of 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, enable the detection and/or quantification of the expression of a multitude of genes in a given specimen. Using these previously known methods to detect presence of absence of and/or quantify multiple RNA species in a sample, which in turn is used as a measure of gene expression profiling, generally requires direct labeling of cDNA, which requires a large amount of input total RNA. Thus, when using total RNA preparations from a given cell or tissue sample to analyze mRNA, the analysis of gene expression in the sample using methods such as arrays requires a substantial amount of input RNA, which generally 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

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molecules. Also, previous transcription-based methods of amplification of mRNA (described in, for example, Lockhart et al, Nature Biotechnology (1996), 14, 1675-1680); van Gelder et al., U.S. Patent No. 5,716,785), are limited to the amplification efficiency of DNA-dependent RNA polymerases and some of these methods require multiple steps. Moreover, the process by which the polymerase promoter sequence is incorporated is prone to result in non-specific amplification.

[0068] The methods of the invention offer the ability to efficiently amplify template polynucleotides (including both RNA and DNA). Thus, the utility of detection/quantification methods which ca be used with the amplification products of the invention, such as those based on array technology, real time PCR, quantitative TaqMan, quantitative PCR using molecular beacons, and the like, should be greatly enhanced.

[0069] The methods of the invention do not require thermocycling and all of the steps can be performed isothermally, although the various steps may be carried out a different temperatures. This feature provides numerous advantages, including facilitating automation and adaptation for high through-put procedures. The isothermal reaction is generally faster than that afforded by thermal cycling, and is suitable for performing the methods of the invention in miniaturized devices.

[0070] The ability to efficiently amplify pools of template polynucleotide sequences (such as genomic DNA) under conditions that will generally not alter the representation of the nucleic acid sequences in the starting sample, will greatly enhance the utility of the detection/quantification methods such as those based on the powerful array technology.

[0071] The ability to efficiently amplify RNA using the random initiation of replication according the methods of the invention provides means for representing all sequences in the pool of sequences (or representing a significant proportion of a pool depending on desired extent of the amplification) in the sample, for example, sequences representing the full length of mRNA species in a sample. The methods of the invention do not rely on oligo-dT primers (to bind the poly-A tail of mRNA) to initiate amplification; thus, the methods may be used to amplify non-poly-A tailed RNAs, such as noncoding RNAs and RNAs of non-eukaryotic species. The methods of the invention do not require prior knowledge of the sequences in the sample and are thus suitable for discovery of novel transcripts, even when present in low abundance and/or representing non-coding transcripts. The ability to efficiently amplify RNA under conditions that will generally not

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alter the representation of the nucleic acid sequences in the preparation, will greatly enhance the utility of the detection/quantification methods such as those based on the powerful array technology.

General Techniques

[0072] 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).

[0073] Primers, oligonucleotides and polynucleotides employed in the invention can be generated using standard techniques known in the art.

Definitions

[0074] A "template," "template strand," "template polynucleotide," "template DNA," target sequence," "target nucleic acid," or "target DNA," "target polynucleotide," "template RNA," or "target RNA," as used herein, is a polynucleotide for which amplification is desired. The template polynucleotide can comprise DNA or RNA. The template sequence may be known or not known, in terms of its actual sequence. Generally, the terms "target," "template ," and variations thereof, are used interchangeably.

[0075] "Amplification," or "amplifying", 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,

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copying does not necessarily mean perfect sequence complementarity or identity with respect to the template sequence.

[0076] "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, carbamates, 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, poly-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, 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

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("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.

[0077] 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 ^{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.

[0078] The "type" of dNTP or rNTP, as used herein, refers to the particular base of a nucleotide, namely adenine, cytosine, thymine, uridine, or guanine.

[0079] "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 composite primer and auxiliary primer. The terms "oligonucleotide" and "polynucleotide" are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides.

[0080] A "primer," as used herein, refers to a nucleotide sequence, generally with a free 3'-OH group, that hybridizes with a template sequence (such as a target polynucleotide, target DNA, or a primer extension product) and is capable of promoting

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polymerization of a polynucleotide complementary to the template. A "primer" can be, for example, an oligonucleotide.

[0081] "Auxiliary primers" as used herein, are a population of primers comprising randomized and/or partially-randomized sequences. Auxiliary primers are a polynucleotide as described herein, though generally, auxiliary primers are made of DNA. Random primers are known in the art and are commercially available. An example of auxiliary primers is the population of randomized hexamer primers shown in Example 1.

[0082] To "inhibit" is to decrease or reduce an activity, function, and/or amount as compared to a reference.

[0083] By "randomly prime" or "random hybridization", as used herein, it is meant that the composite primer hybridizes to multiple sites within the template polynucleotide.

[0084] As used herein, "complex comprising an RNA/DNA partial heteroduplex" generally refers to a population of intermediate complexes that generally includes (a) copies of template polynucleotide and/or copies of the complement of template polynucleotide sequence appended to composite primer sequences; and (b) copies of template polynucleotide and/or copies of the complement of template polynucleotide appended to the complement of composite primer sequences. By virtue of the presence of composite primer sequence in the intermediate complexes, the complexes comprise **at least** a RNA/DNA partial heteroduplex. The RNA portion of the partial heteroduplex generally is introduced (via extension) by the RNA portion of the composite primer, and the DNA portion of the partial heteroduplex comprises the complement of the RNA portion of the composite primer. As discussed herein, the complex comprising an RNA/DNA partial heteroduplex functions as a substrate for further amplification during the single primer isothermal amplification phase of the methods. Generally, RNA in the RNA/DNA partial heteroduplex is cleaved, generating a 3' single stranded portion with sequences that are complementary to RNA in a composite primer (and thus forming a binding site for a composite primer). Thus, reference to "a complex comprising a 3' single stranded portion" generally refers to the complex comprising an RNA/DNA partial heteroduplex when its RNA is cleaved.

[0085] 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

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intermediate with respect to the final amplification product(s). An example of a complex is a complex of composite primer extension product and second composite primer extension product, as described herein.

[0086] 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 or more contiguous nucleotides.

[0087] 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.

[0088] 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).

[0089] "A", "an" and "the", and the like, unless otherwise indicated include plural forms. "A" fragment means one or more fragments.

[0090] 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, and/or strand extension.

[0091] 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, transversion, deletion or insertion. Single nucleotide polymorphism (SNP) is also a sequence mutation as used herein.

[0092] "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 locations on a substrate. Arrays are formed

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on substrates fabricated with materials such as paper, glass, plastic (e.g., polypropylene, nylon, polystyrene), polyacrylamide, nitrocellulose, silicon, 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), 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.

[0093] 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.

[0094] 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.

[0095] 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

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1 to about 50, more preferably from about 10 to about 40, even more preferably from about 20 to about 30 nucleotides.

[0096] 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 1 to about 50, more preferably from about 10 to about 40, even more preferably from about 20 to about 30 nucleotides.

[0097] "Absent" or "absence" of product, and "lack of detection of product" as used herein includes insignificant, or *de minimus* levels, generally due to lack of significant accumulation of product.

[0098] In accordance with well established principles of patent law "comprising" means "including."

Amplification methods of the invention

[0099] 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. For example, reference to using a composite primer means that any of the composite primers described herein may be used.

Amplification using a composite primer that hybridizes to a multiplicity *of template polynucleotide sites*

[0100] The invention provides methods for global amplification using a composite primer that is capable of binding to multiple sites within template polynucleotide, including DNA and RNA template polynucleotides.

[0101] Generally, the methods of the invention involve two phases. (a) composite primer random hybridization, primer extension and displacement of composite primer extension product by strand displacement, whereby a complex comprising a RNA/DNA partial heteroduplex is generated, and (b) composite-primer dependent single primer isothermal amplification.

[0102] Thus, the methods of the invention work as follows: (a) incubating a reaction mixture, said reaction mixture comprising: (i) a template polynucleotide; (ii) a first

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primer, wherein the first primer is a composite primer that is hybridizable to a multiplicity of template polynucleotide sites, wherein the composite primer comprises an RNA portion and a 3' DNA portion; (iii) a DNA-dependent DNA polymerase ; and (iv) an RNA-dependent DNA polymerase (which may be present as a separate enzyme or as an enzyme comprising both DNA-dependent DNA polymerase and RNA-dependent DNA polymerase activities); wherein the incubation is under conditions that permit composite primer random hybridization, primer extension and, in some embodiments, displacement of the primer extension product from template polynucleotide, whereby a complex comprising an RNA/DNA partial heteroduplex is generated; and (b) incubating a reaction mixture, said reaction mixture comprising (i) the reaction products generated according to step (a) (or an aliquot thereof); (ii) a composite primer (which may be the same as the first primer or may different from the first primer); (iii) an DNA-dependent DNA polymerase; and (iv) an agent (such as an enzyme) that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of the primer extension product when its RNA is cleaved and another composite primer binds to the template and is extended, whereby multiple copies of a polynucleotide (generally, DNA) amplification product are generated. In some embodiments, the reaction mixture of step (b) comprises the reaction mixture according to step (a) (or an aliquot thereof). In other embodiments, step (b) is initiated by the addition of an agent that cleaves RNA from an RNA/DNA heteroduplex (such as RNase H) to the reaction mixture of step (a). In embodiments in which the template polynucleotide is RNA, the reaction mixture of step (a) further comprises an agent (such as an enzyme) that that cleaves RNA from an RNA/DNA heteroduplex.

(a) Composite primer random hybridization, primer extension and displacement of composite primer extension product by strand displacement

[0103] The methods generally comprise using specially-designed primers, generally a RNA/DNA composite primer. In a first phase of the amplification methods, composite primer is used to randomly prime template polynucleotide (such as genomic DNA). By "randomly prime" or "random hybridization", as used herein, it is meant that the composite primer hybridizes to multiple sites within template polynucleotide. We have discovered that certain composite primers bind a multiplicity of sites within template polynucleotide

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(generally, under conditions promoting random primer hybridization) and are thus particularly suitable for use in the methods of the invention. Generally, suitable composite primers show partial homology to a multiplicity of template nucleic acid sequences, particularly in the 3' sequences of the composite primer, and thus, the composite primer comprises at least a 3' DNA portion that is capable of randomly priming template polynucleotide (particularly under conditions permitting random primer hybridization). Selection and design of composite primers is described further below.

[0104] Various embodiments of the composite primer and used in the methods of the invention are described herein. For example, Figure 1 illustrates one embodiment of a composite primer useful in the methods of the present invention. As illustrated in the Figure, 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. In some embodiments, the composite primer that hybridizes to target polynucleotide and the composite primer used during single primer isothermal amplification are the same. In some embodiments, the composite primer that hybridizes to target polynucleotide and the composite primer used during single primer isothermal amplification are different.

[0105] Reference to a primer that binds (hybridizes to) a sequence (or template) encompasses embodiments in which at least a portion of the primer is hybridized, as well as those embodiments in which an entire primer is hybridized. Thus, and as the description makes clear, reference to a primer that hybridizes to a sequence encompasses embodiments in which at least a portion of the primer is hybridized as well as embodiments in which two (or more portions) of the primer are hybridized, separated by unhybridized (looped out) portions of the primer, and embodiments in which the entire primer is hybridized. For example, Figure 2 illustrates a single composite primer that hybridizes to multiple positions on a template polynucleotide where differing portions of the composite primer are hybridized to template polynucleotide depending on the site (sequence) at which it is hybridized. Thus, according to the methods of the invention, only a portion of the 3'-end of the composite primer must be hybridized in order for initiation of primer extension by DNA polymerase. In some embodiments, for example, only 2, 3, 4, 5, 6, 7, 8 or more nucleotides of the 3' end of the primer need to hybridize in order for primer extension to be

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initiated. It is understood that hybridization of the 3'-most portion of the composite primer may be stabilized to various extents by further hybridization of another portion of the primer (with or without looping out of intervening primer portions). A DNA polymerase can be included during primer hybridization to enhance (e.g., stabilize) hybridization of composite primer by initiation of primer extension, and thus, stabilization of primer hybridization.

[0106] Random hybridization of the composite primer to template polynucleotide generally occurs under conditions permitting random (nonspecific) primer hybridization. Such conditions are well known in the art and include: decreased stringency during primer hybridization and/or primer extension (including reduced temperature and/or buffer conditions of reduced stringency); composite primer selection and/or design (discussed further herein); and composite primer and template concentration. It is understood that stringency of hybridization, composite primer selection and/or primer concentration may be used to control the frequency of composite primer hybridization, and thus to control coverage and/or representation of template sequences in amplification product. As noted above, an aspect of the invention is displacement of intervening primer extension product during primer extension of composite primers hybridized at downstream position(s) on the template, whereby primer extension products are displaced from template polynucleotide. Preferably, a DNA polymerase is used that possesses stand displacement activity.

[0107] Composite primer random hybridization, primer extension and displacement of primer extension product by strand displacement results in generation of a multiplicity of complexes comprising a RNA/DNA partial heteroduplex. The complexes comprise (a) copies of template polynucleotide and/or copies of the complement of polynucleotide sequence appended (via extension) to composite primer sequences; and (b) copies of template polynucleotide and copies of the complement of template polynucleotide appended (via extension) to the complement of composite primer sequences. Generally, the RNA portion of the complex is introduced by the composite primer.

[0108] In some embodiments, generation of complexes comprising a RNA/DNA partial heteroduplex involves the following steps: (i) formation of a composite primer extension product; and (ii) formation of a second primer extension product by primer extension along the first primer extension product. For example, in some embodiments, complex comprising an RNA/DNA partial heteroduplex is generated as follows: following random hybridization of the composite primer at multiple sites on template polynucleotide

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strands, a DNA polymerase extends the composite primer along the template strand generating a composite primer extension product that is complementary to the polynucleotide template strand. Primer extension extends to and displaces strands being extended from primers hybridized at upstream sites on the template. Thus, as noted above, an aspect of the invention is displacement of intervening primer extension product during primer extension of composite primers hybridized at downstream site(s) on the template, whereby composite primer extension products are displaced from template polynucleotide. Figure 3 illustrates primer extension from composite primers that are hybridized at multiple sites on a template strand, where a composite primer extension products is being displaced by primer extension from a composite primer hybridized at a downstream site(s) on template strand.

[0109] Displaced composite primer product comprises the composite primer sequence at the 5' end, including the 5' RNA portion. Although for convenience, reference is made only to "a" composite primer extension product, it is understood that a multiplicity of composite primer extension products are generated the complement of a multiplicity of template polynucleotide sequences appended (via extension) to the sequence of the composite primer. Figure 4 shows a collection of composite primer extension products comprising composite primer 1 linked (by extension) to sequences comprising the complement of a multiplicity of target polynucleotide sequences.

[0110] Using displaced composite primer extension product as a template, a second primer extension product complementary to the first primer extension product is generated by extension by a DNA-dependent DNA polymerase along the DNA portion of the composite primer extension product, and extension by a RNA-dependent DNA polymerase along the 5' RNA portion of the composite primer extension product, generating a double stranded complex comprising a RNA/DNA complex at the end. Generation of second primer extension product may be random primed using the composite primer, as depicted in Figure 5. Alternatively, second primer extension product may be primed by the 3' end of a different composite primer extension product. Additional embodiments in which second strand production is primed by exogenous (added) primers and/or by fragments of template RNA (endogenous primers) are described below.

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(b) Single primer isothermal amplification using a complex comprising an RNA/DNA partial heteroduplex as a template

[0111] In a second phase of the methods, termed single primer isothermal amplification, the complex comprising an RNA/DNA partial heteroduplex is a substrate for further amplification as follows: an enzyme which cleaves RNA sequence from an RNA/DNA hybrid (such as RNase H) cleaves RNA from the partial heteroduplex, leaving a partially double stranded polynucleotide complex comprising a 3' single stranded DNA sequence. The 3' single stranded sequence (formed by cleavage of RNA in the complex comprising an RNA/DNA partial heteroduplex) is generally the complement of the RNA in the composite primer, and thus forms a specific binding site for a composite primer (which may or may not be the same as the first composite primer). Extension of a bound composite primer by a DNA-dependent DNA polymerase produces a primer extension product, which displaces the previously bound cleaved primer extension product, whereby polynucleotide (generally, DNA) product accumulates. See, for example, U.S. Patents Nos. 6,251,639 and 6,692,918. Figure 6 shows amplification of DNA product using a composite primer and a complex comprising a RNA/DNA partial heteroduplex as a template for further amplification.

[0112] Amplification using a complex comprising an RNA/DNA partial heteroduplex as a template for further amplification (also termed single primer isothermal amplification) generally occurs under conditions permitting composite primer hybridization, primer extension, cleavage of RNA from an RNA/DNA hybrid and strand displacement. In so far as the composite primer hybridizes to the 3' single stranded portion (of the partially double stranded polynucleotide which is formed by cleaving RNA in the complex comprising an RNA/DNA partial heteroduplex) comprising, generally, the complement of at least a portion of the composite primer sequence, composite primer hybridization may be under conditions permitting specific hybridization. Thus, in some embodiments, the reactions conditions permit stringent hybridization (i.e., hybridization of sequences that are generally complementary). As is evident from the description herein, in other embodiments, the reaction conditions are less stringent (i.e., permit hybridization of sequences that are less than fully complementary).

[0113] Generally, the methods of the invention result in amplification of a multiplicity, a large multiplicity, or a very large multiplicity of template polynucleotide sequences. In some embodiments, essentially all of the template polynucleotide present in

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the initial sample (*e.g.*, all of the mRNA or all of the genomic DNA) is amplified. In other embodiments, at least 50, at least 100, at least 200, at least 300, or more distinct sequences (such as a gene or other subsegment of a polynucleotide, a marker (such as a SNP or other polymorphism) are amplified, as assessed, e.g., by analysis of marker sequences known to be present in the template sample under analysis, using methods known in the art. Template polynucleotide sequences that are amplified may be present on the same polynucleotide (e.g., a chromosome or portion of a chromosome for genomic DNA template or on the same RNA for RNA template) or on different template polynucleotides (e.g., different chromosome or portions of chromosomes for DNA template, or different RNAs for RNA template). Although, amplification of genomic DNA is exemplified herein, it will be understood by those of skill in the art, however, that the global amplification methods of the invention are suitable for amplification of any pool or subset of polynucleotides.

[0114] For convenience, reference is made to a polynucleotide (generally, DNA) product. It is understood that amplified product generally is a mixture of sense and antisense copies of a given template polynucleotide. For example, if the template polynucleotide is double stranded DNA, the amplification product will correspond to each strand. If the template polynucleotide is single stranded (*e.g.*, RNA or single stranded DNA), amplification product will generally be produced that is the copy of template polynucleotide (sense copy) and the complement of the template polynucleotide (antisense copy). The amplification product of different senses can be annealed to form a double stranded (or partially double stranded) complex, or can be prevented from annealing (or subsequently denatured) to produce a mixture of single stranded amplification products. The amplified products may be of differing lengths.

[0115] As is evident from the description and shown in the example, the methods of the invention are composite-primer dependent. That is, amplification is not observed in the absence of the composite primer.

[0116] As illustrated in these embodiments, all steps are isothermal (in the sense that thermal cycling is not required), although the temperatures for each of the steps may or may not be the same. It is understood that various other embodiments may be practiced, given the general description provided above. For example, as described and exemplified herein, certain steps may be performed as temperature is changed (e.g., raised, or lowered).

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[0117] For simplicity, the methods of the invention are described as two distinct steps or phases, above. It is understood that the two phases may occur simultaneously in some embodiments (for example, if the enzyme that cleaves RNA from RNA/DNA hybrid is included in the first reaction mixture). In other embodiments, step (b) may be initiated by addition of an enzyme that cleaves RNA from an RNA/DNA hybrid (e.g., ribonuclease, such as RNase H), and optionally, a DNA-dependent DNA polymerase, as shown in Example 1. In this embodiment, addition of an enzyme that cleaves RNA from an RNA/DNA hybrid permits further amplification using the complex comprising an RNA/DNA partial heteroduplex as a template (i.e., step (b), above). It is understood, however, that primer extension (and strand displacement) along template polynucleotide strand from random primed composite primer(s) may continue during single primer isothermal amplification.

[0118] Although generally only one composite primer is described above, it is further understood that the amplification methods may be performed in the presence of two or more different composite primers that randomly prime template polynucleotide. In addition, the amplification polynucleotide products of two or more separate amplification reactions conducted using two or more different composite primers that randomly prime template polynucleotide can be combined. In addition, it is understood that different composite primers can be used in step (a) (i.e., random priming of template polynucleotide) and step (b) (i.e., single primer isothermal amplification). In this instance, the different composite primer comprises sequences hybridizable to the 3' single stranded DNA portion of the partially double stranded complex (which is generated by cleaving RNA from the complex comprising a RNA/DNA partial heteroduplex). Generally, the second composite primer comprises sequences overlapping with the first composite primer.

Amplification using a composite primer that hybridizes to a multiplicity of template polynucleotide sites and auxiliary primers

[0119] In another aspect of the invention, auxiliary primers are present in the reaction mixture comprising template polynucleotide, composite primer, DNA-dependent DNA polymerase and RNA-dependent DNA polymerase. As used herein, "auxiliary primers" refers to a population of random or partially randomized primers. An example of auxiliary primers is the random hexamer primers used in Example 1. Inclusion of auxiliary primers (i.e., population of different random primers) during the amplification is believed

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to enhance the efficiency of production of and/or target coverage of the amplification product.

[0120] In some embodiments, the methods of the invention work as follows: (a) incubating a reaction mixture, said reaction mixture comprising a composite primer as described herein; auxiliary primers; a template polynucleotide, DNA-dependent DNA polymerase, and RNA-dependent DNA polymerase (which may be present as a single enzyme comprising both activities), wherein the incubation is under conditions suitable for random composite primer hybridization, auxiliary primer hybridization, primer extension, and strand displacement, whereby a complex comprising an RNA/DNA partial heteroduplex is generated; and (b) incubating a reaction mixture, said reaction mixture comprising the reaction products from step (a) (or an aliquot thereof); a composite primer (which may be the same as the composite primer of step (a) or may be a different composite primer); a DNA-dependent DNA polymerase; optionally, auxiliary primers; and an enzyme that cleaves RNA from a RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of the primer extension product from the complex when its RNA is cleaved and another composite primer binds to the template and is extended, whereby multiple copies of a polynucleotide template sequence are generated.

[0121] Inclusion of auxiliary primers (i.e., a population of different random primers) during the amplification is believed to enhance the efficiency of production of and/or coverage of template polynucleotide. Without being bound by theory, it is believed that primer extension of auxiliary primers increases displacement of composite primer extension product from template polynucleotide and/or primes generation of second primer extension product. Figure 7 illustrates primer extension from composite primers and auxiliary primers that are hybridized at multiple sites on a template strand. Figure 8 illustrates generation of a second primer extension product primed by auxiliary primers hybridized to composite primer extension product.

[0122] Although for simplicity, use of auxiliary primers is described only in the first phase, random composite primer hybridization (i.e., step (a)), it is evident that auxiliary primers may be present in the reaction mixture for the second phase of the methods, single primer isothermal amplification (i.e., step (b)).

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[0123] As is evident from the description and shown in the example, the methods of the invention are composite-primer dependent. That is, amplification is not observed in the absence of the composite primer.

Amplification using a composite primer that hybridizes to a multiplicity of template RNA sites and auxiliary primers

[0124] In another aspect of the invention, auxiliary primers are present in the reaction mixture comprising template RNA, composite primer, DNA-dependent DNA polymerase and RNA-dependent DNA polymerase. As used herein, "auxiliary primers" refers to a population of random or partially randomized primers. Inclusion of auxiliary primers (i.e., population of different random primers) during the amplification is believed to enhance the efficiency of production of and/or target coverage of the amplification product.

[0125] In some embodiments, the methods of the invention operate as follows: (a) incubating a reaction mixture, said reaction mixture comprising a composite primer as described herein; auxiliary primers; a template RNA, DNA-dependent DNA polymerase, and RNA-dependent DNA polymerase (which may be present as a single enzyme comprising both activities), wherein the incubation is under conditions suitable for random composite primer hybridization, auxiliary primer hybridization, primer extension, and strand displacement, whereby a complex comprising a RNA/DNA partial heteroduplex is generated; and (b) incubating a reaction mixture, said reaction mixture comprising the reaction products from step (a) (or an aliquot thereof); a composite primer (which may be the same as the composite primer of step (a) or may be a different composite primer); a DNA-dependent DNA polymerase; optionally, auxiliary primers; and an enzyme that cleaves RNA from an RNA/DNA hybrid; wherein the incubation is under conditions that permit primer hybridization, primer extension, RNA cleavage from an RNA/DNA heteroduplex, and displacement of the primer extension product from the complex when its RNA is cleaved and another composite primer binds to the template and is extended, whereby multiple copies of a polynucleotide template sequence are generated.

[0126] Inclusion of auxiliary primers (i.e., a population of different random primers) during the amplification is believed to enhance the efficiency of production of and/or coverage of template RNA. Without being bound by theory, it is believed that primer extension of auxiliary primers increases displacement of composite primer

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extension product from template RNA and/or primes generation of second primer extension product. Figure 7 illustrates primer extension from composite primers and auxiliary primers that are hybridized at multiple sites on a template strand. Figure 8 illustrates generation of a second primer extension product primed by auxiliary primers hybridized to composite primer extension product.

[0127] Although for simplicity, use of auxiliary primers is described only in the first phase, random composite primer hybridization (i.e., step (a)), it is evident that auxiliary primers may be present in the reaction mixture for the second phase of the methods, single primer isothermal amplification (i.e., step (b)).

[0128] As is evident from the description and shown in the example, the methods of the invention are composite-primer dependent. That is, amplification is not observed in the absence of the composite primer.

Components and reaction conditions used in the methods of the invention

Template nucleic acid

[0129] 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. Preferred target polynucleotide includes DNA (*e.g.*, genomic DNA, including human genomic DNA, and mammalian genomic DNA (such as mouse, rat,))and RNA (*e.g.*, mRNA, ribosomal RNA, and total RNA). It should be understood that template RNA includes coding and non-coding RNA. The sequences can be naturally occurring or recombinant nucleic acid targets, including cloned nucleic fragments of interest.

[0130] 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. Nucleic acid can be obtained from sources containing very small quantities of nucleic acid, such a single cells, small numbers of cells, patient samples, forensic samples, and archeological samples. Obtaining and purifying nucleic acids use standard techniques in the art, including methods designed to isolate one or a very

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small number of cells, such a cell sorting or laser capture micro-dissection. The methods of the invention are particularly suited for use with genomic DNA (*e.g.*, human and other mammalian genomic DNA), as well as RNA (*e.g.*, total RNA or mRNA samples). Amplification of an RNA target may be accomplished by initial cDNA synthesis, as known in the art, followed by amplification from the cDNA template.

[0131] The target polynucleotide(s) 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. If the target polynucleotide is double stranded (*e.g.*, double stranded DNA or a double stranded DNA/RNA hybrid, such as is produced by first strand cDNA synthesis), the target may first be treated to render it single stranded (*e.g.*, by denaturation or by cleavage of the RNA portion of a DNA/RNA hybrid). Denaturation may also be carried out to remove secondary structure present in a single stranded target molecule (*e.g.*, RNA). In some cases, double stranded DNA target polynucleotide may be first cleaved by one or more restriction endonuclease enzymes.

[0132] When the target polynucleotide is DNA, 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 can be target denaturation. The denaturation step may be thermal denaturation or any other method known in the art, such as alkali treatment. If the target nucleic acid is present in an DNA-RNA hybrid, the initial step can be denaturation of the hybrid to obtain a DNA, or removal of the RNA strand using other means known in the art, such as thermal treatment, digestion with an enzyme that cleaves RNA from an RNA/DNA hybrid (such as RNase H) or alkali treatment, to generate single stranded DNA. When 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, and include reverse transcription of RNA strand using a primer that binds to a specific target, such as the poly-A tail of eukaryotic mRNAs or other specific or consensus sequences. In addition, reverse transcription can be primed by a population of degenerate or partially degenerate primers. First strand cDNA can be separated from the complex of RNA and first strand cDNA as described herein.

[0133] RNAs can be 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,

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plants, and fragments thereof. It is understood that the RNA can be coding or noncoding RNA (such as untranslated small RNAs). RNAs can be obtained and purified using standard techniques in the art. Use of a DNA target (including genomic DNA target) would require initial transcription of the DNA target into RNA form, which can be achieved using methods disclosed in Kurn, U.S. Patent No. 6,251,639 B1, and by other techniques (such as expression systems) known in the art. Thus, RNA template can be itself generated from a DNA source (such as genomic DNA), using methods known in the art, including Kurn, U.S. Patent No. 6,251,639. RNA copies of genomic DNA would generally include untranscribed sequences generally not found in mRNA, such as introns, regulatory and control elements, etc. RNA targets may also be generated from cloned genomic DNA sequences that can be subjected to in vitro transcription. Use of a DNA-RNA hybrid would require denaturation of the hybrid to obtain a single stranded RNA, denaturation followed by transcription of the DNA strand to obtain an RNA, or other methods known in the art such as digestion with an RNAse H to generate single stranded DNA.

Composite primer

[0134] The methods of the invention employ a composite primer that is composed of RNA and DNA portions. We have observed that suitable composite primers show partial nucleic acid sequence homology to a multiplicity of genomic DNA sequences, particularly in the 3' sequences of the composite primer, when analyzed using standard nucleic acid comparison algorithms. For example, composite primer sequence can be used as a query sequence in Blast, to search the human genomic DNA database (or other suitable database, such as a mammalian genomic DNA database). Generally, the search is performed using search parameters suitable for identification of partial or "low stringency" alignments, generally the least stringent conditions provided by the program. Such parameters are known in the art and include use of the NCBI Blast program for searching "short, nearly exact matches", with word size = 7 (conditions permitting as few as 7 consecutive nucleotide perfect matches at any position in the primer sequence). *See, e.g.,* http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?ALIGNMENTS=50&ALIGNMENT_VIEW=Pairwise&AUTO_FORMAT=Semiauto&CLIENT=web&DATABASE=nr&DESCRIPTIONS=100&ENTREZ_QUERY=>(none)&EXPECT=1000&FORMAT_BLOCK_ON_RESPAGE=None&FORMAT_ENTREZ_QUERY=(none)&FORMAT_OBJECT=Alignment&F

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ORMAT_TYPE=HTML&LAYOUT=TwoWindows&NCBI_GI=on&PAGE=Nucleotides &PROGRAM=blastn&SERVICE=plain&SET_DEFAULTS.x=16&SET_DEFAULTS.y=8 &SHOW_OVERVIEW=on&WORD_SIZE=7&END_OF_HTTPGET=Yes. Composite primers useful in the methods of the invention (i.e., that randomly hybridize to template polynucleotide) generally exhibit high partial homology rate with genomic DNA sequences, for example homology of stretches of 7 nucleotides with about 100 genomic DNA sequences, with about 70% of the hits located at the 3' end of the composite primer. A composite primer with a very unique sequence (i.e., low levels of homology with target genomic DNA sequences) did not function efficiently in the methods of the invention when used with genomic DNA template.

[0135] As is evident from the discussion above, reference to a primer that binds (hybridizes to) a sequence (or template) encompasses embodiments in which at least a portion of the primer is hybridized, embodiments in which two (or more portions) of the primer are hybridized (separated by unhybridized (looped out) portions of the primer), and embodiments in which the entire primer is hybridized. In certain embodiments, a 5'-portion, commonly the 5'-most portion, of the composite primer is designed such that the particular 5'-portion it is not expected to randomly hybridize to template polynucleotide (composite primers of this configuration are referred to as "tailed" primers, in reference to the 'tail' of unhybridized primer). In some embodiments, the tail portion of the composite primer is the entire 5' RNA portion of the composite primer. Thus, according to the methods of the invention, only a portion of the 3'-end of the composite primer must be hybridized in order for initiation of primer extension by DNA polymerase. In some embodiments, for example, only 2, 3, 4, 5, 6, 7 or more nucleotides of the 3' end of the primer must hybridize in order for primer extension to be initiated. It is understood that hybridization of the 3'-most portion of the composite primer may be stabilized to various extents by further hybridization of another portion of the primer (with or without looping out of intervening primer portions). A DNA polymerase can be included during primer hybridization to enhance (e.g., stabilize) hybridization of composite primer by initiation of primer extension, and thus, stabilization of primer hybridization.

[0136] We have also observed that composite primers that are suitable for use in the present methods can be identified by conducting single primer isothermal amplification as described in Kurn, U.S. Patent No. 6,251,639, using the composite primer under high stringency conditions using a genomic DNA template, and observing the presence of a

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smear of reaction products as visualized, for example, on a gel. Preferably, the genomic DNA does not contain a sequence(s) that is complementary to the composite primer. Production of a "smear" of reaction products, i.e., generation of a complex mixture of product of multiple molecular weights, visible on a gel as a smear, indicates that the composite primer is randomly priming genomic DNA amplification.

[0137] In another example, single primer isothermal amplification of a specific synthetic target oligonucleotide (e.g., a target oligonucleotide comprising a specific target for composite primer hybridization) is conducted at high stringency in the presence or absence of genomic DNA template (e.g., 1-100 ng of human genomic DNA). Composite primers that are suitable for the methods of the invention will demonstrate a strong effect of genomic DNA on the efficiency of the amplification of the specific synthetic target, resulting in about a 100-fold or greater reduction of amplification efficiency as compared with amplification efficiency conducted in the absence of genomic DNA.

[0138] Random composite primer hybridization and/or generation of composite primer extension product is promoted by use of conditions designed to permit random (non-specific) primer hybridization. Such conditions are well known in the art, and are further discussed below, and include: decreased stringency during primer hybridization and/or first strand synthesis (including reduced temperature and/or buffer conditions of reduced stringency, such as reduced ionic strength); composite primer selection and/or design (discussed further herein); composite primer and template concentration, presence or absence of an agent that stabilizes a 3' hybridized primer (such as a DNA polymerase), and presence or absence of agents such as DMSO that lower the temperature requirements for stable hybridization. It is understood that the selection of reaction conditions may be used to control the frequency of composite primer hybridization, and thus control coverage and/or representation of template polynucleotide sequences in amplification product.

[0139] Generally, the composite primer is also designed so that there is no primer-dimer formation capability, as determined using software routinely available to one of ordinary skill in the art, e.g. Oligo Primer Analysis Software from Molecular Biology Insight, and references therein. One of skill in the art will understand that other factors affect nucleic acid hybridization affinities. For example, any and all of the guanosine-cytosine content of the primer-target and primer-primer duplexes, minor groove binders, O-methylation or other modification of nucleotides, temperature, and salt are potentially important factors in constructing primers with the requisite differences in binding energies.

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Another factor in designing and constructing primers is the free energy parameters of hybridization of given sequences under a given set of hybridization conditions. The free energy parameters for the formation of a given hybrid may be calculated by methods known in the art (see, e.g., Tinoco et al. *Nature* (1973) 246:40-41 and Freier et al., *Proc. Natl. Acad. Sci. USA* (1986) 83:9373-9377; computer programs, e.g., Oligo Primer Analysis Software from Molecular Biology Insight, and references therein), and it is possible to predict, for a given oligonucleotide template, primer sequences for which the required free energy changes for formation of various complexes will be met.

[0140] The primers should be extendable by DNA polymerase. 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). Generally, the primer should permit high efficiency of amplification of a synthetic target that contains a specific primer target binding site (e.g., the complementary sequence to the primer), for example, permitting amplification of about 10^6 to 10^9 using methods described in Kurn, U.S. Patent 6,251,639. 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. It is understood that, in the following section that generally describes aspects of the composite primers used in the methods of the invention, characteristics described may be applicable to the primers if used for hybridizing and initiating the polynucleotide amplification (production of composite extension product) and/or for single primer isothermal amplification as described herein.

[0141] In some embodiments, a first composite primer is used in the methods of the invention, including those steps which involve single primer isothermal amplification (i.e., phase (b)). In other embodiments, a first and second, different, composite primer are used in the methods of the invention. The second composite primer is used for the single primer isothermal amplification step, and may comprise some or all of the sequence of the first composite primer, and the first composite primer may comprise some or all of the sequence of the second composite primer. In some embodiments, the second composite primer comprises a different sequence than the first composite primer.

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[0142] For use in single primer isothermal amplification and/or composite primer extension product formation, a composite primer comprises at least one RNA portion that is capable of (a) binding (hybridizing) to a sequence on the single stranded portion of the complex (formed by cleavage of RNA in the complex comprising a RNA/DNA partial heteroduplex) (in some embodiments, on second primer extension product) independent of hybridization of the DNA portion(s) to a sequence on the same single stranded portion; and (b) being cleaved with an agent such as a ribonuclease when hybridized to the single stranded portion. The composite primers bind to the single stranded portion, and are extended by DNA polymerase to form a RNA/DNA partial heteroduplex in which only the RNA portion of the primer is cleaved upon contact with an agent which cleaves RNA in an RNA/DNA hybrid, such as an enzyme, such as a ribonuclease (such as RNase H), while the composite primer extension product remains intact, thus enabling annealing of another composite primer.

[0143] When used for the single primer isothermal amplification described herein, the composite primers also comprise a 3' DNA portion that is capable of hybridization to a sequence on the 3' single stranded portion of the complex such that its hybridization to the 3' single stranded portion is favored over that of the nucleic acid strand that is displaced from the complex 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 complex (e.g., in the second primer extension product) favored over the hybridization of the homologous sequence in the 5' end of the displaced strand to the composite primer extension product.

[0144] 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

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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).

[0145] 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 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, 14, 15, 20, 25, 3, 50 nucleotides. In certain embodiments, the composite primer has an RNA portion of about 14 or about 20 nucleotides.

[0146] 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 14, 15, 17, 18, 20, 50 nucleotides. In certain embodiments, the composite primer has an RNA portion of about 14 or about 20 nucleotides.

[0147] 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.

[0148] 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

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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 14, 15, 17, 18, 20, 50 nucleotides. In certain embodiments, the composite primer has an RNA portion of about 14 or about 20 nucleotides.

[0149] 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, 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.

[0150] 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

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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 one embodiment, the composite primer has a 3'-DNA portion of about 7 nucleotides.

[0151] 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 one embodiment, the composite primer has a 3'-DNA portion of about 7 nucleotides.

[0152] 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.

[0153] 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. In one embodiment, the composite primer has a 3'-DNA portion of about 7 nucleotides.

[0154] 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'-

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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.

[0155] 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. In one embodiment, the composite primer has a 3'-DNA portion of about 7 nucleotides.

[0156] 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. In one embodiment, the composite primer has a 3'-DNA portion of about 7 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.

[0157] 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

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3' most nucleotide of the primer (and the 5'-RNA portion includes the 5' most nucleotide of the primer).

[0158] 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, 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. In certain embodiments, the composite primer is about 21 or about 27 nucleotides in length. It is understood that the length can be greater or less, as appropriate under the reaction conditions of the methods of this invention.

[0159] As described herein, one or more different composite primers may be used in an amplification reaction.

Auxiliary primers

[0160] "Auxiliary primer" as used herein, are a population of primers comprising randomized and/or partially-randomized sequences. Auxiliary primers are a polynucleotide as described herein, though generally, auxiliary primers are made of DNA. Such random primers are known in the art. An example of auxiliary primers is the population of randomized hexamer primers shown in Example 1. In some embodiments, the random primers may contain natural or non-natural nucleotide(s) that permit non-specific hybridization in order to increase the number of sequences to which the random primers may bind. Similarly, abasic sites can be introduced randomly within the population of random primers, which can permit non-specific hybridization by stabilizing mismatches between primer and template. The primers should be extendable by DNA polymerase. Generation of primers suitable for extension by polymerization is well known in the art, such as described in PCT Pub. No. WO 99/42618 (and references cited therein).

[0161] In some embodiments, auxiliary primers can be at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 15, at least 18, at least 20, or more nucleotides in length. In some embodiments, a population of primers of differing lengths is used.

DNA polymerase, and an agent capable of cleaving an RNA-DNA hybrid

[0162] The amplification methods of the invention employ the following enzymes: an RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase, and an agent

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capable of cleaving an RNA strand of an RNA-DNA hybrid (for example, a ribonuclease such as RNase H). 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. Many reverse transcriptases, such as those from avian myeloblastosis 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. The RNA-dependent DNA polymerase activity and DNA-dependent DNA polymerase activity may be provided by the same enzyme (for example, Bst polymerase), or these activities may be provided in separate enzymes.

[0163] One aspect of the invention is the formation of a complex comprising an RNA/DNA partial heteroduplex. This process generally utilizes the enzymatic activities of an RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase. Generally, RNA in the RNA/DNA partial heteroduplex is cleaved by an agent (such as an enzyme, such as a ribonuclease) capable of cleaving RNA from an RNA/DNA hybrid, generating a 3' single stranded portion with sequences that are complementary to RNA in a composite primer (and thus forming a binding site for a composite primer).

[0164] 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 DNA 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 and, for example, a DNA polymerase that possesses both DNA-dependent and RNA-dependent DNA polymerase activity, such as Bst DNA polymerase.

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[0165] DNA-dependent DNA polymerases for use in the methods and compositions of the invention are capable of effecting extension of the composite primer according to the methods of the 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 formation of the complex comprising the RNA/DNA partial heteroduplex can be carried out by a DNA polymerase which comprises both RNA-dependent DNA polymerase and DNA-dependent DNA polymerase activities (such as Bst DNA polymerase, or a reverse transcriptase). Amplification of an RNA sequence according to methods of the invention involves the use of a DNA polymerase that is 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 nucleic acid strand. Preferably, the DNA polymerase does not possess substantial nicking activity. Generally, the DNA polymerase preferably 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. Mutant DNA polymerases which lack both 5' to 3' nuclease and 3' to 5' nuclease activities have also been described , for example, exo$^{-/-}$Klenow DNA polymerase. 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

[0166] 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

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exo⁻ Vent (New England Biolabs), exo⁻ Deep Vent (New England Biolabs), Bst (BioRad), exo⁻ Pfu (Stratagene), Bca (Panvera), sequencing grade Taq (Promega), exo$^{-/-}$Klenow DNA polymerase, and thermostable DNA polymerases from thermoanaerobacter thermohydrosulfuricus.

[0167] The ribonuclease for use in the methods and compositions of the invention is capable of cleaving ribonucleotides in an RNA/DNA hybrid. Preferably, the ribonuclease cleaves ribonucleotides in an RNA/DNA hybrid 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 invention are well known in the art, including ribonuclease H (RNase H), e.g., Hybridase.

[0168] As is well known in the art, DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity, and the ability to cleave RNA from a RNA/DNA hybrid may be present in different enzymes, or two or more activities may be present in the same enzyme. Accordingly, in some embodiments, the same enzyme comprises RNA-dependent DNA polymerase activity and cleaves RNA from an RNA/DNA hybrid. In some embodiments, the same enzyme comprises DNA-dependent DNA polymerase activity and cleaves RNA from an RNA/DNA hybrid. In some embodiments, the same enzyme comprises DNA-dependent DNA polymerase activity, RNA-dependent DNA polymerase activity and cleaves RNA from an RNA/DNA hybrid. In some embodiments, different enzymes comprise RNA-dependent DNA polymerase activity and DNA-dependent DNA polymerase activity. In some embodiments, different enzymes comprise RNA -dependent DNA polymerase activity and cleave RNA from an RNA/DNA hybrid. In some embodiments, different enzymes comprise DNA-dependent DNA polymerase activity and cleave RNA from an RNA/DNA hybrid.

[0169] In general, the enzymes used 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

[0170] 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

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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. Such additives include proteins such as BSA or acetylated BSA, single strand binding proteins (for e.g., 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 other than the composite and second primer extension product synthesis steps, and strand displacement) are performed isothermally, which avoids the cumbersome thermocycling process. The amplification reaction is carried out at a temperature that permits hybridization of the oligonucleotides (primer) of the invention to the template polynucleotide and primer extension products, and that does not substantially inhibit the activity of the enzymes employed. The temperature can be in the range of 0° C to about 85° C, about 25° C to about 85° C, about 30°C to about 80°C, and about 37°C to about 75°C.

[0171] Random priming and/or primer extension and/or isothermal amplification can be conducted under conditions of reduced stringency (i.e., permitting hybridization of sequences that are not fully complementary). For a given set of reaction conditions, the ability of two nucleotide sequences to hybridize with each other is based on the degree of

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complementarity of the two nucleotide sequences, which in turn is based on the fraction of matched complementary nucleotide pairs. The more nucleotides in a given sequence that are complementary to another sequence, the more stringent the conditions can be for hybridization and the more specific will be the binding of the two sequences. Conversely, the lower the stringency of the conditions for hybridization, the lower the complementarity necessary for binding between the hybridizing and/or partially hybridizing composite primer and template polynucleotide. Decreased stringency is achieved by any one or more of the following: reducing the temperature, decreasing the ratio of cosolvents, lowering the salt concentration, and the like. Conditions that increase or reduce the stringency of a hybridization reaction are widely known and published in the art. See, for example, Sambrook et al. (1989), and in Ausubel (1987), supra. Useful hybridization conditions are also provided in, e.g., Tijessen, 1993, Hybridization With Nucleic Acid Probes, Elsevier Science Publishers B.V. and Kricka, 1992, Nonisotopic DNA Probe Techniques, Academic Press San Diego, Calif. The hybridization conditions chosen depend on a variety of factors known in the art, for example the length and type (e.g., RNA, DNA, PNA) of primer and primer binding region of the oligonucleotide template, as well as the concentration of primer and template polynucleotides.

[0172] Insofar as it is convenient to use buffer conditions that are compatible with DNA polymerase activity and/or ribonuclease activity, stringency of hybridization of composite primers can be controlled by altering temperature of the reaction. Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of approximately 15°C, 20°C, 25°C, 30°C, 37°C, 40°C, 45°C, 50°C, 60°C, or more. Accordingly, in some embodiments, composite primer random hybridization occurs at a reduced temperature, for example at 25°C - 37°C, followed at incubation at increased temperature(s) suitable for the isothermal amplification phase of the methods (such as about 50°C). In some embodiments, temperature is increased at 5°C increments. In other embodiments, temperature is shifted from low to high temperature.

[0173] 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. In some embodiments, a nucleotide or nucleotide analog whose presence in the primer extension strand enhances

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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.

[0174] 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 can each be provided at about or at least about any of the following concentrations: 50 nM, 100 nM, 500 nM, 1 uM, 2.5 uM , 5uM, 10 uM. Composite primer concentration also impacts frequency and/or position of composite primer hybridization. Generally, increased primer concentrations increased frequency of primer hybridization. Auxiliary primers can be provided at about or at least about any of the following concentrations: about 25 nM, about 50nM, about 100 nM, about 500 nM, about 1 uM, about 2.5 uM, about 5 uM, about 10 uM, or more.

[0175] 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 polynucleotide, as determined by their thermal stability and/or other considerations known to the person of skill in the art. In these embodiments, the reaction conditions and components may be varied between the different reactions.

[0176] The amplification process 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 random composite primer hybridization. Another timepoint is at the end of random composite primer hybridization and composite primer extension product synthesis. Another timepoint (in some embodiments) is following cleavage of template RNA. Another timepoint is immediately prior to initiation of single primer isothermal amplification (which in some embodiments, may be initiated by addition of an enzyme (such as RNase H) that cleaves RNA from RNA/DNA heteroduplex, and

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optionally, DNA polymerase). Another timepoint is at the end of second primer extension product 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, replenishing a destroyed (depleted) enzyme, or adding reagent(s) necessary for initiation of a step (for example, addition of RNase H and/or DNA polymerase to initiate the single primer isothermal amplification phase of the methods). In some embodiments, one or more of the components of the reactions is replenished prior to, at, or following the resumption of the reactions. For example, it may be necessary to replenish the composite primer prior to beginning the single primer isothermal amplification reaction if the same composite primer is being used. Alternatively, the reaction can be allowed to proceed (i.e., from start to finish) without interruption.

[0177] 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 formation of the complex comprising an RNA/DNA partial heteroduplex. Another timepoint is at the end of random composite primer hybridization.

[0178] 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.

Compositions and kits of the invention

[0179] 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.

[0180] In one embodiment, the invention provides a composition comprising a composite primer as described herein. In some embodiments, the composite primer

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comprises an RNA portion adjacent to the DNA portion. In another embodiment, the composite primer comprises 5'- and 3'-DNA portions with at least one intervening RNA portion. In other embodiments, the RNA portion of the composite primer consists of 7 to about 20 nucleotides and the DNA portion of the composite primer consists of about 5 to about 20 nucleotides. In still other embodiments, the RNA portion of the composite primer consists of about 10 to about 20 nucleotides and the DNA portion of the composite primer consists of about 7 to about 20 nucleotides. In some embodiments, the composite primer is selected from the following composite primers: 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

[0181] In other examples, the invention provides a composition comprising a composite primer as described herein, and auxiliary primers (for example, a population of randomized hexamer primers). In some embodiments, the composite primer is selected from the following composite primers: 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

[0182] In other examples, the invention provides a composition comprising a composite primer that is 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 by attachment of a positively charged moiety such as an amine. In other embodiments, the composite primer is labeled, for example by derivatizing the composite primer with a detectable moiety, such as a label, or a moiety that can be covalently or non-covalently attached to a label. Labeled composite primers are further described herein.

[0183] In other examples, the invention provides composition comprising a composite primer and one or more of: a DNA polymerase; an enzyme that cleaves RNA from an RNA/DNA duplex; and auxiliary primers (for example, a population of random hexamer primers). In some embodiments, the composition further comprises a labeled dNTP. In still other embodiments, the composition comprises a non-canonical nucleotide (such as dUTP), and reagents suitable for labeling and/or fragmenting abasic sites, as described in U.S. Patent Application Publication No. 2004/0005614 and Kurn et al, co-pending U.S. patent application No. 60/533,381.

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[0184] The compositions are generally in lyophilized or aqueous form, preferably in a suitable buffer.

[0185] The invention also provides compositions comprising the amplification products described herein. Accordingly, the invention provides a population of DNA which are copies or the complement of a target sequence, which are produced by any of the methods described herein (or compositions comprising the products). The invention also includes compositions and various configurations (such as arrays) of these populations, which may be homogeneous (same sequence) or heterogeneous (different sequence). These populations may be any assembly of sequences obtained from the methods described herein.

[0186] 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).

[0187] 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: methods of amplification; genotyping, nucleic acid mutation detection (including methods of genotyping), determining the presence or absence of a sequence of interest, quantitating a sequence of interest, preparation of an immobilized nucleic acid (which can be a nucleic acid immobilized on a microarray), comparative genomic hybridization, and characterizing nucleic acids using the amplified nucleic acid products generated by the methods of the invention, methods of expression profiling, subtractive hybridization and the preparation of probes for subtractive hybridization, and methods of preparing libraries (which may be cDNA and/or differential hybridization libraries).

[0188] 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, the kit comprises one or more composite primer selected from the following composite primers: 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides. In some embodiments, a kit further comprises auxiliary primers, which may or may not be

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separately packaged. The composite primer may be labeled or unlabeled. Kits may also optionally further include any of one or more of the enzymes described herein (for example, DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, a DNA polymerase that provides both DNA-dependent and RNA-dependent DNA polymerase activities, and an enzyme capable of cleaving RNA from an RNA/DNA hybrid, such as RNase H), as well as deoxynucleoside triphosphates (labeled or unlabeled or derivatized). Kits may also include one or more suitable buffers (for example, as described herein). Kits may also include a labeled dNTP(s) and/or a non-canonical nucleotide (such as dUTP), as described in Kurn et al, co-pending U.S. patent application No. 60/381,457.

[0189] 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.

[0190] 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 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.

[0191] In another example, the kits of the invention comprise a complex of composite primer extension product and second primer extension product. In yet another example, any of these kits further comprises one or more controls (which can be, for example, template polynucleotide (*e.g.*, DNA template such as genomic DNA or RNA template such as total RNA or mRNA), composite primers, and/or auxiliary primer(s).

[0192] 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.

[0193] 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

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reactions that need to occur to practice the methods disclosed herein and/or to further optimize the sensitivity of any assay.

[0194] The invention also provides systems for effecting the methods described herein. These systems comprise various combinations of the components discussed above.

[0195] Any of the systems embodiments may also comprise a template (target) sequence, as described herein. 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 methods of the invention are particularly suitable for use with miniaturized devices, as thermal cycling is not required for any of the steps. A non-limiting example of suitable devices includes the BioAnalyzer (Agilant and Caliper) and the eSensor.

[0196] The invention also provides reaction mixtures (or compositions comprising reaction mixtures) which contain various combinations of components described herein. Examples of reaction mixtures have been described. In some embodiments, the invention provides reaction mixtures comprising (a) a target polynucleotide; (b) a composite primer comprising a 3' DNA portion and an RNA portion; (c) auxiliary primers; and (d) 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 that 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. In some embodiments, the composite primer is selected from the following composite primers: 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

[0197] Other reaction mixtures are described herein and are encompassed by the invention.

[0198] The invention also includes compositions comprising any of the complexes (which are intermediates in the methods described herein) described herein. Examples of such complexes are schematically depicted in Figures 1-8. As an example, one complex of the invention is a complex comprising: (a) a target polynucleotide 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

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3" DNA portion. As another example, a complex of the invention is a complex comprising: (a) a composite primer extension product; and (b) a target polynucleotide.

[0199] In yet another example, a complex of the invention is a complex comprising a RNA/DNA partial heteroduplex, prepared by any of the methods described herein. In some embodiments, the complex further comprises a second RNA/DNA partial heteroduplex at a second end. In yet another example, the complex of the invention is a complex comprising a 3' single stranded DNA portion produced by any of the methods described herein. In some embodiments, the complex further comprises a second 3' single stranded region. In another example, the complex of the invention is (a) a complex comprising a 3' single stranded DNA portion, and (b) a composite primer hybridized to the 3' single stranded portion. .

Methods using the amplification methods and compositions of the invention

[0200] The methods and compositions of the invention can be used for a variety of purposes. For purposes of illustration, methods of nucleic acid mutation detection (including methods of genotyping), determining the presence or absence of a sequence of interest, quantitating a sequence of interest, preparation of an immobilized nucleic acid (which can be a nucleic acid immobilized on a microarray), comparative genomic hybridization, and characterizing nucleic acids using the amplified nucleic acid products generated by the methods of the invention, detecting and/or identifying novel nucleic acid sequences (such as novel coding or non-coding transcripts), and characterization of splice variant sequences, 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.

Method of preparing nucleic acids immoblized to a substrate, including a microarray of nucleic acids

[0201] The products of some of the amplification methods of the invention are suitable for immobilizing to a surface. In so far as the amplification products of the methods of the invention generally comprises a mixture of sequences corresponding to sense and antisense copies of template polynucleotide, it is useful to immobilize a population of sequences generated by amplification of template polynucleotide from a

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defined source (*e.g.*, DNA or RNA from a defined cell population or a single cell; organism-specific template (for example, the DNA or RNA of specific viruses or other pathogen(s) sufficient to identify the organism); or a disease-specific template. Immobilized amplification product may then be probed with different probes and the hybridization signals can be compared. For example, an immobilized array of genomic polynucleotides (DNA or RNA) from a known pathogen or non-pathogen(such as a virus, or group of viruses) may be used for assessment of the presence or identity of a pathogen within a sample of genetic material. Such arrays would be of use in disease surveillance and in identification of a pathogenic agent in the event of a disease outbreak. Polynucleotides may be isolated from a suspected sample, labeled using any method known in the art, and hybridized to such an array. The detection of signal due to hybridization to the array provides information as to the presence or identity of a pathogen present in sample polynucleotide.

[0202] Amplification products can be attached to a solid or semi-solid support or surface, which may be made, e.g., from glass, plastic (e.g., polystyrene, polypropylene, nylon), polyacrylamide, nitrocellulose, or other materials.

[0203] 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 amplification 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 amplification product and become covalently attached to the glass slide. Microarrays comprising the amplification 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.* (1995) 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

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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/.

[0204] 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.

[0205] The amplified polynucleotides may be spotted as a matrix on substrates comprising paper, glass, plastic, polystyrene, polypropylene, nylon, polyacrylamide, nitrocellulose, silicon, optical fiber or any other suitable solid or semi-solid (e.g., thin layer of polyacrylamide gel (Khrapko, et al., *DNA Sequence* (1991), 1:375-388) surface.

[0206] 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.

[0207] Arrays may also be composed of particles, such as beads. The beads may be labeled with the amplified products alone, or may be labeled with both the amplified products and an additional label, such as defined dyes or other labels.

Characterization of nucleic acids

[0208] The amplification products obtained by the methods of the invention are amenable to further characterization. The products of the methods of the invention are particularly amenable to quantitative analysis, as sufficient DNA is produced which generally accurately reflect the representation of the various polynucleotides in the starting material.

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[0209] The amplified polynucleotide products (i.e., products of any of the amplification methods described herein), can be analyzed using, for example, probe hybridization techniques known in the art, such as Southern and Northern blotting, and hybridizing to probe arrays. They can also be analyzed by electrophoresis-based methods, such as differential display and size characterization, which are known in the art. In addition, the polynucleotide products may serve as 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 U.S. Patents Nos. 6,251,639, 6,686,156, and 6,692,918; U.S. Patent Publication Nos. 2002/0115088 A1, 2003/0186234 A1, 2003/0087251A1, 2002/0164628, and 2003/0215926, and International Patent Application Publication WO 03/08343. Thus, the invention includes those further analytical and/or quantification methods as applied to any of the products of the methods herein.

[0210] In one embodiment, the amplification methods of the invention are utilized to generate multiple copies of polynucleotide products, and products are analyzed by contact with a probe.

[0211] In some embodiments, the amplification methods of the invention are utilized to generate multiple copies of single stranded polynucleotide (generally, DNA) products that are labeled by using composite primers that are labeled (in the portion(s) that is not cleaved). For example, the primer can be labeled with an aminoallyl labeled nucleotide. In other embodiments, the amplification methods of the invention are utilized to generate multiple copies of polynucleotide (generally, DNA) products that are labeled by the incorporation of labeled nucleotides during DNA. For example, amplification according to the methods of the invention can be carried out with suitable labeled dNTPs. These labeled nucleotides can be directly attached to a label, or can 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 template polynucleotide 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. Amplification can also be in the presence of an

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aminoallyl-derivatized nucleotide, such as aminoallyl dUTP. Amplification product comprising aminoallyl dUTP can be coupled to a label, such as Cy3 or Cy5.

[0212] In other embodiments, the methods of the amplification are performed in he present of a non-canonical nucleotide, e.g., dUTP, and amplification comprising a non-canonical nucleotide is labeled and/or fragmented according to the methods disclosed in U.S. Patent Application Publication No. 2004/0005614. Briefly, non-canonical nucleotide (when incorporated into amplification product) is cleaved, generating an abasic site. The abasic site is then labeled by contacting with a reagent capable of labeling an abasic site. The polynucleotide comprising an abasic site can also be cleaved at the abasic site, generating fragments suitable for further analysis, e.g., hybridization to an array. The fragments can also be labeled as described above.

[0213] The labeled amplification 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 target polynucleotide of interest by generating labeled polynucleotide (generally, 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 amplification products, and thus by extrapolation the identity of the target polynucleotide 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 target polynucleotide of interest.

[0214] 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

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indicative of the detection and/or quantification of the corresponding target polynucleotide species in the sample.

[0215] Methods of characterization include sequencing by hybridization (see, e.g., Dramanac, U.S. Patent No. 6,270,961) and global genomic hybridization (also termed comparative genome hybridization) (*see, e.g.*,.Pinkel, U.S. Patent No. 6,159,685; Daigo et al (2001) Am. J. Pathol. 158 (5):1623-1631. Briefly, comparative genome hybridization comprises preparing a first population of labeled polynucleotides (which can be polynucleotide fragments) according to any of the methods described herein, wherein the template from which the first population is synthesized is total genomic DNA. A second population of labeled polynucleotides (to which the first population is desired to be compared) is prepared from a second genomic DNA template. The first and second populations are labeled with different labels. The hybridized first and second populations are mixed, and hybridized to an array or chromosomal spread. The different labels are detected and compared.

[0216] In another aspect, the invention provides a method of quantitating labeled and/or fragmented nucleic acids comprising use of an oligonucleotide (probe) of defined sequence (which may be immobilized, for example, on a microarray).

[0217] The 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. When the target polynucleotide is genomic DNA or RNA, the sequence alterations may be sequence alterations present in the genomic sequence or may be sequence alterations which are not reflected in the genomic sequence, for example, alterations due to post transcriptional alterations, and/or mRNA processing, including splice variants. Sequence alterations (interchangeably called "mutations") include deletion, substitution, insertion and/or transversion of one or more nucleotide.

[0218] 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 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

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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; WO 92/15712; 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 a target polynucleotide comprising a mutation (which can be a single nucleotide polymorphism), comprising: (a) amplifying a target polynucleotide using any of the methods described herein; and (b) analyzing the amplification products for presence of an alteration (mutation) as compared to a reference polynucleotide.

[0219] It is understood that the amplification products can also serve as template for further analysis such as sequence, polymorphism detection (including multiplex SNP detection) using, e.g., oligonucleotide ligation-based assays, analysis using Invader, Cleavase or limited primer extension, and the like. For methods that generally require larger volumes of input material, the methods of the invention may be used to "pre" amplify a pool of polynucleotides to generate sufficient input material for subsequent analysis.

Determination of gene expression profile

[0220] The amplification methods of the invention are particularly suitable for use in determining the levels of expression of one or more genes in a sample since the methods described herein are capable of amplifying a multiplicity, including a large multiplicity of 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.

[0221] Accordingly, the invention provides methods of determining gene expression profile in a sample, said method comprising: amplifying single stranded product from template RNA s in the sample, using any of the methods described herein ; and determining amount of amplification products of each RNA, wherein each said amount is indicative of amount of each RNA in the sample, whereby the expression profile in the

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sample is determined. Generally, labeled products are generated. In certain embodiments, 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.

[0222] 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) 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

[0223] The DNA 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 DNA 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.

Methods of subtractive hybridization

[0224] The amplification methods of the invention are particularly suitable for use in subtractive hybridization methods, in which (at least) a first and second target polynucleotide population is compared, since the methods described herein are capable of amplifying multiple target polynucleotides in the same sample, and the methods of the invention are suitable for producing large amounts of single stranded antisense nucleic acid suitable for use as "driver" in subtractive hybridization. For example, two nucleic acid

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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. The methods of subtractive hybridization provided herein are particularly suited for subtractive hybridization using amplified target RNAs.

[0225] Accordingly, the invention provides methods for performing subtractive hybridization, said methods comprising: (a) preparing multiple DNA copies of the complement of target polynucleotide from a first polynucleotide population using any of the amplification methods described herein; and (b) hybridizing the multiple copies to a second polynucleotide population, whereby a subpopulation of the second polynucleotide population forms a complex with DNA copies of the first polynucleotide population. The invention also provides methods for performing subtractive hybridization, said methods comprising: hybridizing multiple copies of the complement of at least one polynucleotide from a first polynucleotide population using any of the amplification methods described herein to a second polynucleotide population, whereby a subpopulation of the second population forms a complex with a copy from the copies of the first polynucleotide population. In preferred embodiments, the polynucleotide populations utilized in subtractive hybridization are RNA populations. In some embodiments, "driver" single stranded anti-sense DNA product of the methods of the invention is combined with tester (sense) RNA species. In some embodiments, "driver" single stranded antisense nucleic acid (generally, DNA) product is produced using the methods of the invention described herein.

[0226] In another aspect, the invention provides methods of differential amplification in which single stranded driver (antisense) DNA sequences that hybridize with tester RNA sequence are subjected to cleavage by an agent that cleaves RNA present in a DNA/RNA hybrid, such as RNase H. Cleavage of the RNA results in the inability to generate single stranded DNA product from the test RNA strands. Conversely, non-cleaved tester (i.e., tester RNA 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

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expression libraries. Accordingly, the invention provides methods for differential amplification of one or more RNA template sequence, said method comprising: (a) preparing multiple polynucleotide (generally, DNA) copies of the complement of RNA from a first RNA population using any of the amplification methods described herein; (b) hybridizing the multiple copies to a second RNA population, whereby a subpopulation of the second RNA 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 RNA population, whereby multiple copies of single stranded DNA complementary to the unhybridized subpopulation of the second RNA population are generated. In some embodiments, step (d) is performed using any of the amplification methods described herein. In some embodiments, the methods comprise 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 RNA population, whereby a subpopulation of the second RNA 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 RNA population, whereby multiple copies of single stranded DNA complementary to the unhybridized subpopulation of the second RNA population are generated.

[0227] The following Examples are provided to illustrate, but not limit, the invention.

EXAMPLES

Example 1: Global amplification of human genomic DNA using a composite primer and random hexamer primers

[0228] Global amplification reactions were performed using composite primer IA20 and human genomic DNA as a template. The sequence of composite primer IA20 is as follows: IA20: 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3' (SEQ ID NO:1), where italics denote ribonucleotides, and "d" denotes deoxyribonucleotides.

[0229] Human genomic DNA (Clontech, Cat. No. 6550-1) was diluted in TE buffer and denatured by heating to 99°C. In some samples, DNA and primers were mixed in amplification buffer and heated at 96°C for 2-4 minutes.

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[0230] The following reaction mixture was used.

[0231] 2 ng of pre-denatured human genomic DNA (approximately 600 copies)

[0232] 2 µl of random hexamer N6 (final concentration 2.5 µM) (Qiagen-Operon; item No.: PolyN (6-mer));

[0233] 0.5 µl (100 µM) composite primer IA20 (final concentration: 2.5 µM),

[0234] 0.1 µl Bst DNA polymerase (0.04 U/µl) (New England BioLabs, Catalog No. M0275);

[0235] 10 µl buffer (final concentration: 20 mM Tris-HCl, pH 8.5; 5 mM $MgCl_2$; RNasin, 0.3 U/µl; DTT, 0.5 mM; acetylated BSA, 0.1 µg/µl; T4 gp32 protein, 0.15 µg/µl)), and

[0236] RNase-free water to final volume of 15 µl.

[0237] The mixture was incubated at 30°C for 5 minutes, followed by 5 minutes at 40°C, and 2 minutes at 50°C.

[0238] 5 µl of enzyme mixture (RNase H, final concentration of 0.025 U/µl; and Bst DNA polymerase (large fragment), final concentration 0.2U/µl) was added, and the reaction was incubated at 50°C for 30 – 40 minutes. The reaction was stopped by incubation at 80°C for 5 minutes to inactivate the enzymes.

[0239] Control reactions were prepared in which either composite primer, N6 random primers, or RNase H were omitted. In control reactions, the corresponding volume (of the omitted reagent) was replaced by water.

[0240] Amplification reaction product was analyzed as follows. 0.5 µl of reaction mixture was loaded onto 4-20% gradient acrylamide gel and electrophoresed at 200-220 V constant voltage for 30 min. The gel was stained in 0.005 mg/ml Ethidium Bromide for 2 minutes and washed in water for 1 minute. The gel was then visualized and photographed on the AlphaImager 2200 system. The results are shown in Figure 9. Lanes correspond to the reaction mixtures containing the following components:

[0241] Lanes #1-2: Complete reaction mixture

[0242] Lanes #3-4: Reaction lacking N6 random primer.

[0243] Lanes #5-6: Reaction lacking composite primer.

[0244] Lanes #7-8: Reaction lacking RNase H.

[0245] Figure 9 shows that amplification product of varying molecular weight was produced in reactions containing the complete reaction mixture (described above), and in

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reaction lacking N6 random primers. However, reactions in which composite primer or RNase H were omitted did not show detectable reaction product.

[0246] Amplification product was quantified using the following procedure: reactions were prepared and processed as described above. Reaction mixture was diluted 100-fold and 2 µl of the diluted samples were used in Real Time PCR quantification with the following primer pairs that amplify a single copy sequence on Chromosome 7:

[0247] 221PF2 (5'-AGTATCTGGCACATCTT-3' (SEQ ID NO:_____)) and

[0248] 221PR2 (5'-GGGAGATATTATTTGGC-3' (SEQ ID NO:____)).

[0249] Amplification with primers 221PF2 and 221 PR2 was expected to yield a 62 base pair PCR product. The PCR reaction mixture contained: 1 µl of 10 µM of each primer, 6 µl of water, 10 µl of 2x SYBR Green PCR Master Mix (Applied Biosystems), and 2 µl diluted reaction mixture (diluted as described above). A control reaction was conducted using 2 µl human genomic DNA, instead of amplification reaction product as a template.

[0250] The thermal cycling program used was: one cycle of 94^{0}C for 10 min., followed by 45 cycles of 94^{0}C for 30 sec., 55^{0}C for 30 sec., and 72^{0}C for 30 sec. The Real Time PCR quantification data were presented as Ct values (threshold cycle). The values obtained for quantification of amplification products were compared with that obtained for 2 µl human genomic DNA (labeled "non-amplified Genomic DNA" in Table 1). The dilution factor between the diluted global amplification products and the original human genomic DNA input into the amplification reactions is 1000 fold. The data were summarized in Table 1.

Table 1: Quantification of amplification products and target human genomic DNA employing Real Time PCR with SYBR Green. A single copy sequence on Chromosome 7 of human genomic DNA (221) was used for determination of amplification efficiency. Amplification efficiency is expressed as the relative amount of this single copy sequence in starting genomic DNA sample and following global amplification.

Reaction components	**Ct**	**Efficiency**
Complete	29	4000 fold
No N6 random primers	36	40 fold
No Composite primer	None	None
No RNase H	None	None
None (non amplified Genomic DNA)	31	None

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[0251] In a second experiment, human genomic DNA was amplified using composite primer IA20 essentially as described above. Following amplification, several target sequences were quantified using Real Time PCR essentially as described above. Chromosomal location of target sequences and PCR primer pairs are shown in Table 2. Table 2 shows the relative amount of target sequence following global amplification, and the amplification efficiency. Real Time PCR quantification data were presented as Ct values (threshold cycle).

Table 2:

Target location	Forward PCR primer	Reverse PCR primer	Real time Delta C(t)	Amplification fold
chromosome #6	GGACGTGTGTTCCTGTTAA (SEQ ID NO___)	CACTTTGATCCTGAAAGACT (SEQ ID NO__)	3.5	2000
chromosome #7	AGTATCTGGCACATCTT (SEQ ID NO:__)	GGGAGATATTATTTGGC (SEQ ID NO:__)	4	3000
chromosome #11	AGGTTCCCAGCCTTGGTCC (SEQ ID NO__)	TGAGGCCATGTGTGTGGAAT (SEQ ID NO:__)	2	800
chromosome #12	AATAATGTCCAGATATCTTGGT (SEQ ID NO:__)	TCCCTACTCCAGCTACTTCT (SEQ ID NO:__)	2.5	1000
chromosome #16	CAGCAAGAACACAAGGGAC (SEQ ID NO__)	TCTTGAGAGCGAGGGCA (SEQ ID NO__)	2.5	1000

[0252] In a third experiment, human genomic DNA was amplified using composite primer BSCA-128F essentially as described above. The sequence of composite primer BSCA-128F is:

[0253] 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2) where italics denote ribonucleotides, and "d" denotes deoxyribonucleotides.

[0254] Amplification reaction product was analyzed as described above. Amplification product of varying molecular weights was generated, suggesting that the composite primer permitted amplification from a multiplicity of template sequences.

[0255] 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.

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CLAIMS

We claim:

1. A method for amplification of a template polynucleotide, comprising:

(a) incubating a reaction mixture, said reaction mixture comprising:

(i) a template polynucleotide;

(ii) a first primer, wherein the first primer is a composite primer that is hybridizable to a multiplicity of template polynucleotide sites, wherein the composite primer comprises an RNA portion and a 3' DNA portion;

(iii) a DNA-dependent DNA polymerase; and

(iv) an RNA-dependent DNA polymerase;

wherein the incubation is under conditions that permit first primer random hybridization to the template polynucleotide, and primer extension, whereby a complex comprising a RNA/DNA heteroduplex is generated; and

(b) incubating a reaction mixture, said reaction mixture comprising

(i) at least a portion of the reaction products generated according to step (a);

(ii) an amplification primer, wherein said amplification primer is a composite primer comprising an RNA portion and a 3' DNA portion;

(iii) auxiliary primers;

(iv) an DNA-dependent DNA polymerase; and

(v) an agent that cleaves RNA from an RNA/DNA hybrid;

wherein the incubation is under conditions that permit RNA cleavage, primer hybridization, primer extension, and displacement of the primer extension product when its RNA is cleaved and another amplification primer binds to the template and is extended by strand displacement, whereby multiple copies of a polynucleotide amplification product are generated.

2. The method of claim 1, wherein said DNA-dependent DNA polymerase said RNA-dependent DNA polymerase of step (a) are the same enzyme.

3. The method of claim 1, wherein said DNA-dependent DNA polymerase said RNA-dependent DNA polymerase of step (a) are different enzymes.

4. The method of claim 1, wherein said first primer and said amplification primer are the same primer.

5. The method of claim 1, wherein said first primer and said amplification primer are different primers.

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6. The method of claim 1, wherein step (b) is initiated by the addition of an agent that cleaves RNA from an RNA/DNA heteroduplex to the reaction mixture of step (a).

7. The method of claim 6, wherein said agent that cleaves RNA from an RNA/DNA heteroduplex is RNase H.

8. The method of claim 1, wherein the reaction mixture of step (a) further comprises auxiliary primers.

9. The method of claim 1, wherein said template polynucleotide is DNA.

10. The method of claim 9, wherein said first primer comprises a random sequence.

11. The method of claim 10, wherein said first primer is at least two different primers.

12. The method of claim 11, wherein said first primer is a plurality of different primers.

13. The method of claim 1, wherein said template polynucleotide is RNA.

14. The method of claim 13, wherein said template RNA is mRNA.

15. The method of claim 13, wherein the reaction mixture of step (a) further comprises an agent that cleaves RNA from a RNA/DNA heteroduplex to the reaction mixture of step (a).

16. The method of claim 15, wherein the agent in the reaction mixture of step (a) that cleaves RNA in a RNA/DNA heteroduplex is an enzyme.

17. The method of claim 16, wherein the enzyme that cleaves RNA in a RNA/DNA heteroduplex in the reaction mixture of step (a) is RNase H.

18. The method of claim 15, wherein said first primer and said amplification primer are different primers.

19. The method of claim 18, wherein step (b) is initiated by addition of the amplification primer to the reaction mixture of step (a).

20. The method of claim 15, wherein said DNA-dependent DNA polymerase said RNA-dependent DNA polymerase of step (a) are the same enzyme.

21. The method of claim 15, wherein said DNA-dependent DNA polymerase said RNA-dependent DNA polymerase of step (a) are different enzymes.

22. The method of claim 15, wherein the agent that cleaves RNA from an RNA/DNA heteroduplex is RNase H.

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23. The method of claim 15, wherein the reaction mixture of step (a) further comprises auxiliary primers.

24. The method of claim 15, wherein the reaction mixture of step (b) further comprises auxiliary primers.

25. The method of claim 1, wherein the RNA portion of the first primer is 5' with respect to the 3'-DNA portion.

26. The method of claim 25, wherein the RNA portion of the amplification primer is 5' with respect to the 3'-DNA portion.

27. The method of claim 26, wherein the 5' RNA portion of the amplification primer is adjacent to the 3' DNA portion.

28. The method of claim 25, wherein the 5' RNA portion of the first primer is adjacent to the 3' DNA portion.

29. The method of claim 28, wherein the RNA portion of the amplification primer is 5' with respect to the 3'-DNA portion.

30. The method of claim 29, wherein the 5' RNA portion of the amplification primer is adjacent to the 3' DNA portion.

31. The method of claim 1, wherein the RNA portion of the amplification primer is 5' with respect to the 3'-DNA portion.

32. The method of claim 31, wherein the 5' RNA portion of the amplification primer is adjacent to the 3' DNA portion.

33. The method of claim 1, wherein the RNA portion of the first primer consists of 7 to about 20 nucleotides.

34. The method of claim 33, wherein the DNA portion of the first primer consists of about 5 to about 20 nucleotides.

35. The method of claim 34, wherein the RNA portion of the first primer consists of about 10 to about 20 nucleotides.

36. The method of claim 35, wherein the DNA portion of the first primer consists of about 7 to about 20 nucleotides.

37. The method of claim 1, wherein the RNA portion of the amplification primer consists of 7 to about 20 nucleotides.

38. The method of claim 37, wherein the DNA portion of the amplification primer consists of about 5 to about 20 nucleotides.

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39. The method of claim 38, wherein the RNA portion of the amplification primer consists of about 10 to about 20 nucleotides.

40. The method of claim 39, wherein the DNA portion of the amplification primer consists of about 7 to about 20 nucleotides.

41. The method of claim 1, wherein the first primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

42. The method of claim 41, wherein the amplification primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

43. The method of claim 1, wherein the amplification primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

44. The method of claim 1, wherein the reaction mixture of step (b) further comprises a non-canonical nucleotide.

45. The method of claim 44, wherein the non-canonical nucleotide is dUTP.

46. The method of claim 1, wherein the reaction mixture of step (b) further comprises a labeled nucleotide.

47. A method for amplification of a template polynucleotide, comprising:

incubating a reaction mixture, said reaction mixture comprising:

(a) a complex comprising a RNA/DNA partial heteroduplex, wherein the complex is generated by incubating a first reaction mixture, said first reaction mixture comprising:

(i) a polynucleotide template;

(ii) a first primer; wherein the first primer is a composite primer, the composite primer comprises an RNA portion and a 3' DNA portion; and wherein the composite primer is capable of hybridizing to a multiplicity of template polynucleotide sites;

(iii) a DNA-dependent DNA polymerase; and

(iv) an RNA-dependent DNA polymerase;

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wherein the incubation is under conditions that permit first primer random hybridization, and primer extension, whereby a complex comprising an RNA/DNA partial heteroduplex is generated;

(b) an amplification primer, wherein the amplification primer is a composite primer comprising an RNA portion and a 3' DNA portion;

(c) auxiliary primers;

(d) a DNA-dependent DNA polymerase; and

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

wherein the incubation is under conditions that permit RNA cleavage, primer hybridization, primer extension, and displacement of primer extension product when its RNA is cleaved and another amplification primer binds and is extended, whereby multiple copies of a polynucleotide amplification product are generated.

48. The method of claim 47, wherein the agent that cleaves RNA from an RNA/DNA hybrid is an enzyme.

49. The method of claim 48, wherein the enzyme that cleaves RNA from an RNA/DNA hybrid is RNase H.

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

51. The method of claim 50, wherein said DNA-dependent DNA polymerase and said enzyme that cleaves RNA from an RNA/DNA hybrid are different enzymes.

52. The method of claim 47, wherein the RNA portion of the amplification primer is 5' with respect to the 3'-DNA portion.

53. The method of claim 52, wherein the 5' RNA portion of the amplification primer is adjacent to the 3' DNA portion.

54. The method of claim 47, wherein the RNA portion of the amplification primer consists of 7 to about 20 nucleotides.

55. The method of claim 54, wherein the DNA portion of the amplification primer consists of about 5 to about 20 nucleotides.

56. The method of claim 55, wherein the RNA portion of the amplification primer consists of about 10 to about 20 nucleotides.

57. The method of claim 56, wherein the DNA portion of the amplification primer consists of about 7 to about 20 nucleotides.

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58. The method of claim 47, wherein the amplification primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

59. The method of claim 47, wherein the reaction mixture further comprises a non-canonical nucleotide.

60. The method of claim 59, wherein the non-canonical nucleotide is dUTP.

61. The method of claim 47, wherein the reaction mixture further comprises a labeled nucleotide.

62. A method for amplification of a template polynucleotide, comprising:

incubating a reaction mixture, said reaction mixture comprising:

(a) a complex of a first primer extension product and a second primer extension product, wherein the first primer extension product is generated by extension of a randomly primed first primer hybridized to target polynucleotide with a DNA polymerase, wherein the first primer is a composite primer comprising an RNA portion and a 3' DNA portion, wherein the first primer is capable of hybridizing to a multiplicity of template polynucleotide sites, and wherein the second primer extension product is generated by extension of a second primer hybridized to the first primer extension product;

(b) an amplification primer, wherein the amplification is a composite primer comprises an RNA portion and a 3' DNA portion and is hybridizable to the second primer extension product;

(c) auxiliary primers;

(d) a DNA-dependent DNA polymerase; and

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

wherein the incubation is under conditions that permit RNA cleavage, primer hybridization, primer extension, and displacement of composite primer extension product from the second primer extension product when the RNA portion of the composite primer is cleaved and another composite primer binds and is extended, whereby multiple copies of a polynucleotide amplification product are generated.

63. The method of claim 62, wherein said agent that cleaves RNA from a RNA/DNA hybrid is an enzyme.

64. The method of claim 63, wherein the enzyme that cleaves RNA from a RNA/DNA hybrid RNase H.

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65. The method of claim 63, wherein said DNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA hybrid are the same enzyme.

66. The method of claim 62, wherein said DNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA hybrid are different enzymes.

67. The method of claim 62, wherein said template polynucleotide is RNA.

68. The method of claim 62, wherein said template polynucleotide is DNA.

69. The method of claim 62, wherein said reaction mixture further comprises auxiliary primers.

70. The method of claim 62, wherein the RNA portion of the amplification primer is 5' with respect to the 3'-DNA portion.

71. The method of claim 70, wherein the 5' RNA portion of the amplification primer is adjacent to the 3' DNA portion.

72. The method of claim 62, wherein the RNA portion of the amplification primer consists of 7 to about 20 nucleotides.

73. The method of claim 72, wherein the DNA portion of the amplification primer consists of about 5 to about 20 nucleotides.

74. The method of claim 73, wherein the RNA portion of the amplification primer consists of about 10 to about 20 nucleotides.

75. The method of claim 74, wherein the DNA portion of the amplification primer consists of about 7 to about 20 nucleotides.

76. The method of claim 62, wherein the amplification primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

77. The method of claim 62, wherein the reaction mixture further comprises a non-canonical nucleotide.

78. The method of claim 77, wherein the non-canonical nucleotide is dUTP.

79. The method of claim 62, wherein the reaction mixture further comprises a labeled nucleotide.

80. A method for amplification of a polynucleotide template, comprising:

(a) random priming of polynucleotide template strand with a composite primer; wherein the composite primer comprises an RNA portion and a 3' DNA portion, and wherein the composite primer is capable of hybridizing to a multiplicity of template

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polynucleotide sites, thereby producing a complex comprising a composite primer randomly hybridized to polynucleotide template; and

(b) incubating the complex in the presence of auxiliary primers, a DNA-dependent DNA polymerase, an RNA-dependent DNA polymerase, and an agent that cleaves RNA from a RNA/DNA heteroduplex, whereby multiple copies of polynucleotide amplification product are generated by primer extension and strand displacement.

81. The method of claim 80, wherein step (a) further comprises auxiliary primers.

82. The method of claim 80, wherein step (a) further comprises a DNA-dependent DNA polymerase.

83. The method of claim 82, wherein step (a) further comprises a RNA-dependent DNA polymerase.

84. The method of claim 80, wherein the agent that cleaves RNA from a RNA/DNA heteroduplex is an enzyme.

85. The method of claim 84, wherein the enzyme that cleaves RNA from a RNA/DNA heteroduplex is RNase H.

86. The method of claim 84, wherein the RNA-dependent DNA polymerase, and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are the same enzyme.

87. The method of claim 86, wherein the DNA-dependent DNA polymerase, the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are the same enzyme.

88. The method of claim 84, wherein the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are different enzymes.

89. The method of claim 88, wherein the DNA-dependent DNA polymerase, the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are all different enzymes.

90. The method of claim 80, wherein the DNA-dependent DNA polymerase and the RNA-dependent DNA polymerase are the same enzyme.

91. The method of claim 80, wherein the DNA-dependent DNA polymerase and the RNA-dependent DNA polymerase are different enzymes.

92. The method of claim 80, wherein the RNA portion of the composite primer is 5' with respect to the 3'-DNA portion.

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93. The method of claim 92, wherein the 5' RNA portion of the composite primer is adjacent to the 3' DNA portion.

94. The method of claim 80, wherein the RNA portion of the composite primer consists of 7 to about 20 nucleotides.

95. The method of claim 94, wherein the DNA portion of the composite primer consists of about 5 to about 20 nucleotides.

96. The method of claim 95, wherein the RNA portion of the composite primer consists of about 10 to about 20 nucleotides.

97. The method of claim 96, wherein the DNA portion of the composite primer consists of about 7 to about 20 nucleotides.

98. The method of claim 80, wherein the composite primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

99. The method of claim 80, wherein the step of incubating is further in the presence of a non-canonical nucleotide.

100. The method of claim 99, wherein the non-canonical nucleotide is dUTP.

101. The method of claim 80, wherein the step of incubating is further in the presence of a labeled nucleotide.

102. A method for amplification of a polynucleotide template, comprising:

incubating a reaction mixture comprising:

(a) a polynucleotide template strand;

(b) a first primer, wherein said first primer is a composite primer comprising a RNA portion and a 3' DNA portion, and wherein the first primer is capable of hybridizing to a multiplicity of template polynucleotide sites;

(c) auxiliary primers;

(d) a DNA-dependent DNA polymerase;

(e) a RNA-dependent DNA polymerase; and

(f) an agent that cleaves RNA from a RNA/DNA heteroduplex,

whereby multiple copies of polynucleotide amplification product are generated by primer extension and strand displacement.

103. The method of claim 102, wherein the agent that cleaves RNA from a RNA/DNA heteroduplex is an enzyme.

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104. The method of claim 103, wherein the enzyme that cleaves RNA from a RNA/DNA heteroduplex is RNase H.

105. The method of claim 103, wherein the RNA-dependent DNA polymerase, and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are the same enzyme.

106. The method of claim 105, wherein the DNA-dependent DNA polymerase, the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are the same enzyme.

107. The method of claim 103, wherein the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are different enzymes.

108. The method of claim 107, wherein the DNA-dependent DNA polymerase, the RNA-dependent DNA polymerase and the enzyme that cleaves RNA from an RNA/DNA heteroduplex are all different enzymes.

109. The method of claim 102, wherein the DNA-dependent DNA polymerase and the RNA-dependent DNA polymerase are the same enzyme.

110. The method of claim 102, wherein the DNA-dependent DNA polymerase and the RNA-dependent DNA polymerase are different enzymes.

111. The method of claim 102, wherein said reaction mixture further comprises an amplification primer, wherein said amplification primer is a composite primer comprising a RNA portion and a 3' DNA portion.

112. The method of claim 111, wherein the RNA portion of the amplification primer is 5' with respect to the 3'-DNA portion.

113. The method of claim 112, wherein the 5' RNA portion of the amplification primer is adjacent to the 3' DNA portion.

114. The method of claim 102, wherein the RNA portion of the first primer consists of 7 to about 20 nucleotides.

115. The method of claim 114, wherein the DNA portion of the first primer consists of about 5 to about 20 nucleotides.

116. The method of claim 115, wherein the RNA portion of the first primer consists of about 10 to about 20 nucleotides.

117. The method of claim 116, wherein the DNA portion of the first primer consists of about 7 to about 20 nucleotides.

118. The method of claim 102, wherein the first primer is selected from the group consisting of 5'-*GACGGAUGCGGUCU*dCdCdAdGdTdGdT-3 (SEQ ID NO:1); and 5'-

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*CGUAUUCUGACGACGUACUC*dTdCdAdGdCdCdT-3' (SEQ ID NO:2), wherein italics denote ribonucleotides and "d" denotes deoxyribonucleotides.

119. The method of claim 102, wherein the reaction mixture further comprises a non-canonical nucleotide.

120. The method of claim 119, wherein the non-canonical nucleotide is dUTP.

121. The method of claim 102, wherein the reaction mixture further comprises a labeled nucleotide.

122. A method of making a polynucleotide array, comprising:

immobilizing polynucleotide amplification product onto a substrate, said polynucleotide amplification product produced according to any of methods 1, 47, 62, 80, or 102.

123. The method of claim 122, wherein said polynucleotide amplification products are generated by amplification of template polynucleotide from a defined source.

124. The method of claim 123, wherein the defined source is a defined cell population.

125. The method of claim 122, wherein said substrate is selected from the group consisting of paper, glass, plastic, nitrocellulose, silicon, and optical fiber.

126. The method of claim 122, wherein the substrate is a particle.

127. The method of claim 126, wherein the particle is a bead.

128. The method of claim 127, wherein the bead is labeled with a dye.

129. A method of characterizing a nucleic acid, comprising:

analyzing polynucleotide amplification product, said amplification product produced by the method of any of claims 1, 47, 62, 80, or 102.

130. The method of claim 129, wherein the analyzing is carried out by contacting the amplification product with a probe.

131. The method of claim 129, wherein the analyzing is carried out by quantifying a sequence of interest in the amplification product.

132. The method of claim 129, wherein the analyzing is carried out by sequencing the amplification product.

133. The method of claim 129, wherein the analyzing is carried out by detecting any alteration in a target nucleic acid sequence in the amplification product, as compared to a reference nucleic acid sequence.

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134. The method of claim 133, wherein detection of an alteration in a target nucleic acid sequence is carried out y a method selected from the group consisting of allele specific primer extension, allele specific probe ligation, differential probe hybridization, and limited primer extension.

135. A method for determining a gene expression profile, comprising:

quantifying amplification products of sequences of interest, wherein said amplification products are produced by any of the methods of claims 1, 47, 62, 80, or 102.

136. The method of claim 135, wherein said amplification products are amplified from RNA template.

137. A method of preparing a library, comprising:

preparing a library of polynucleotide amplification products, said amplification products produced by any of the methods of claims 1, 47, 62, 80, or 102.

138. A method for performing subtractive hybridization, comprising:

hybridizing a polynucleotide amplification product comprising multiple copies of target polynucleotide to a polynucleotide population, whereby a subpopulation of the polynucleotide population forms a complex with the polynucleotide amplification product, wherein said polynucleotide amplification product is produced according to the method of any of claims 1, 47, 62, 80, or 102.

139. A method for differential amplification, comprising:

hybridizing a DNA driver polynucleotide population to a RNA population, whereby a subpopulation of the RNA population forms a complex with the driver population;

cleaving RNA with an agent that cleaves RNA in an RNA/DNA heteroduplex; and

amplifying the remaining RNA population according to the method of any of claims 1, 47, 62, 80, or 102.

140. A method for archiving polynucleotide templates, comprising:

storing polynucleotide amplification product, wherein said polynucleotide amplification product is produced according to the method of any of claims 1, 47, 62, 80, or 102.

141. A kit for amplifying template polynucleotide, said kit comprising:

a composite primer that is capable of binding to multiple sites within template polynucleotide; and

instructions for carrying out the method according to any of claims 1, 47, 62, 80, or 102.

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142. The kit of claim 141, further comprising auxiliary primers.

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Figure 1

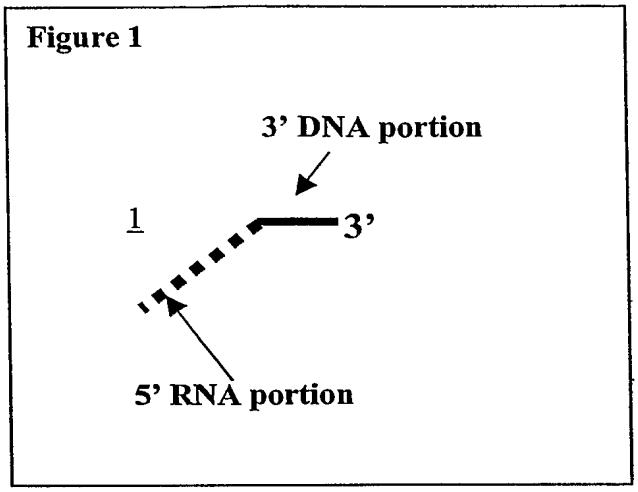


Figure 2

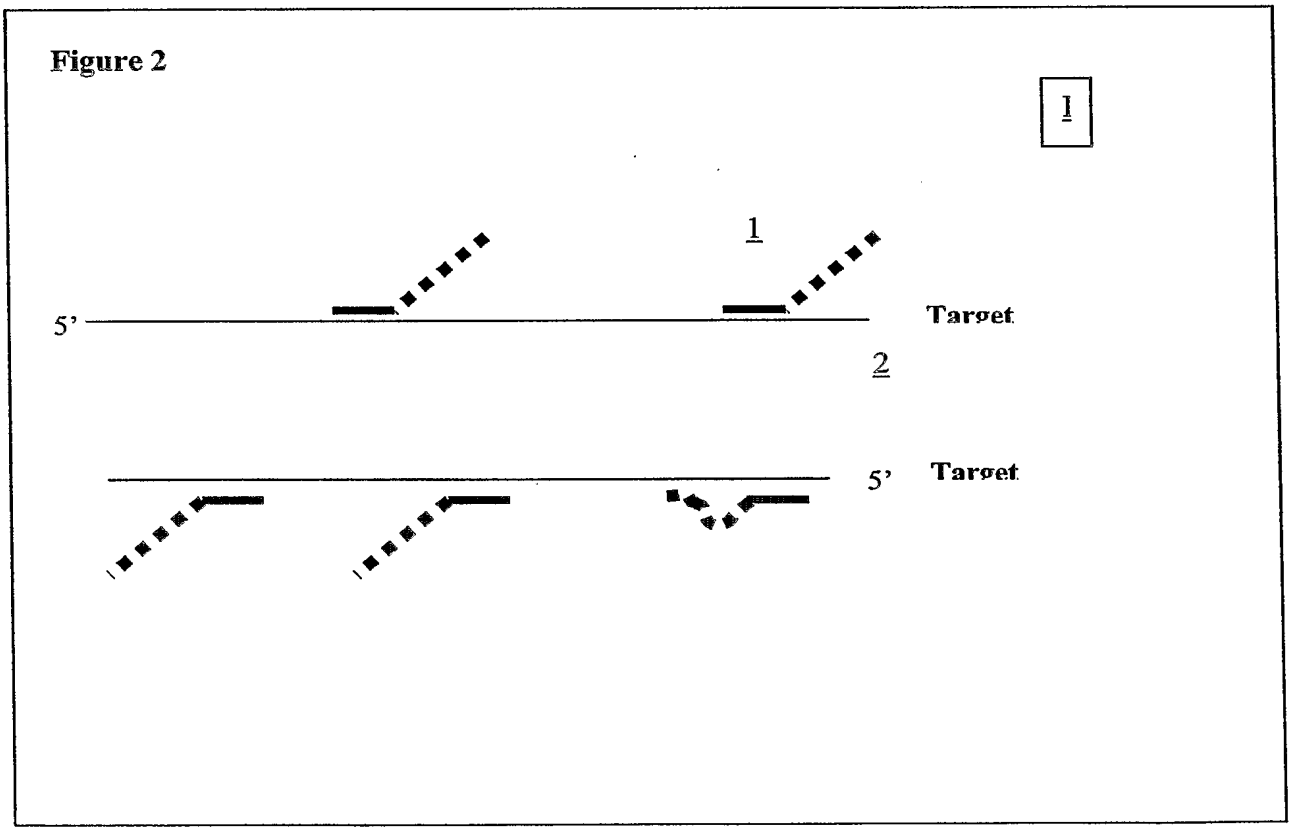


Figure 3

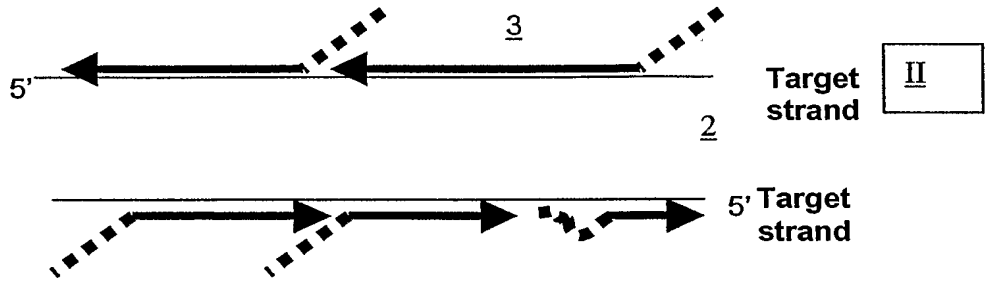


Figure 4

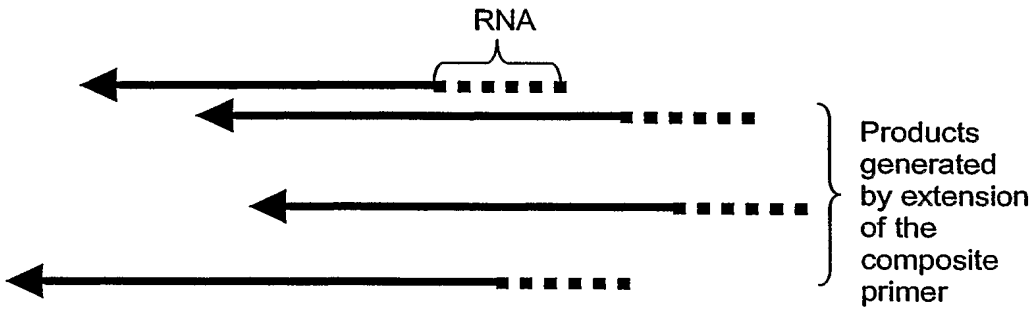

RNA
Products generated by extension of the composite primer

Figure 5

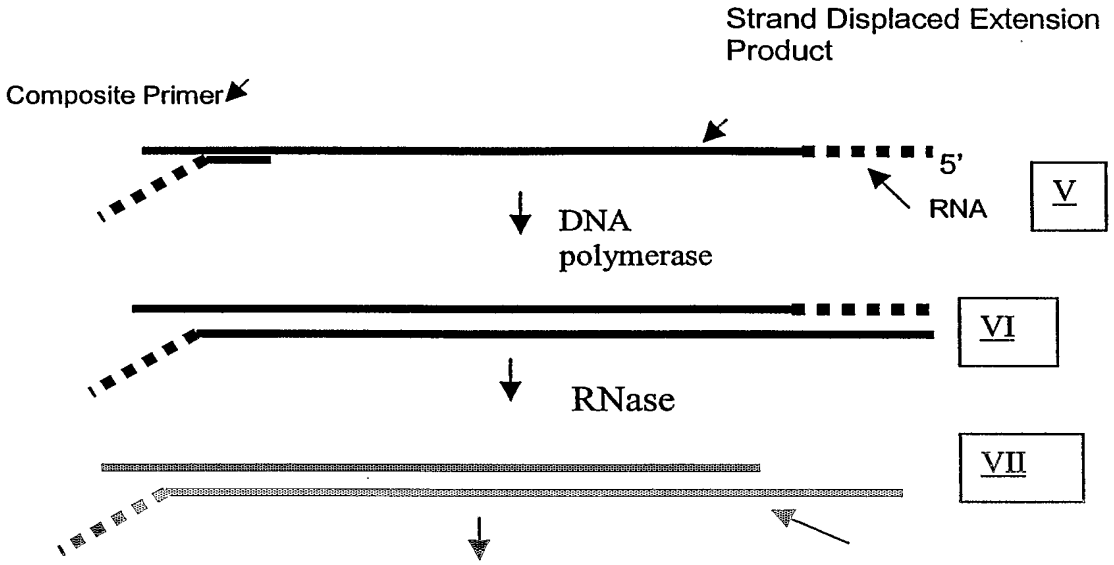

Strand Displaced Extension Product
Composite Primer
5'
RNA
V
DNA polymerase
VI
RNase
VII

Figure 6

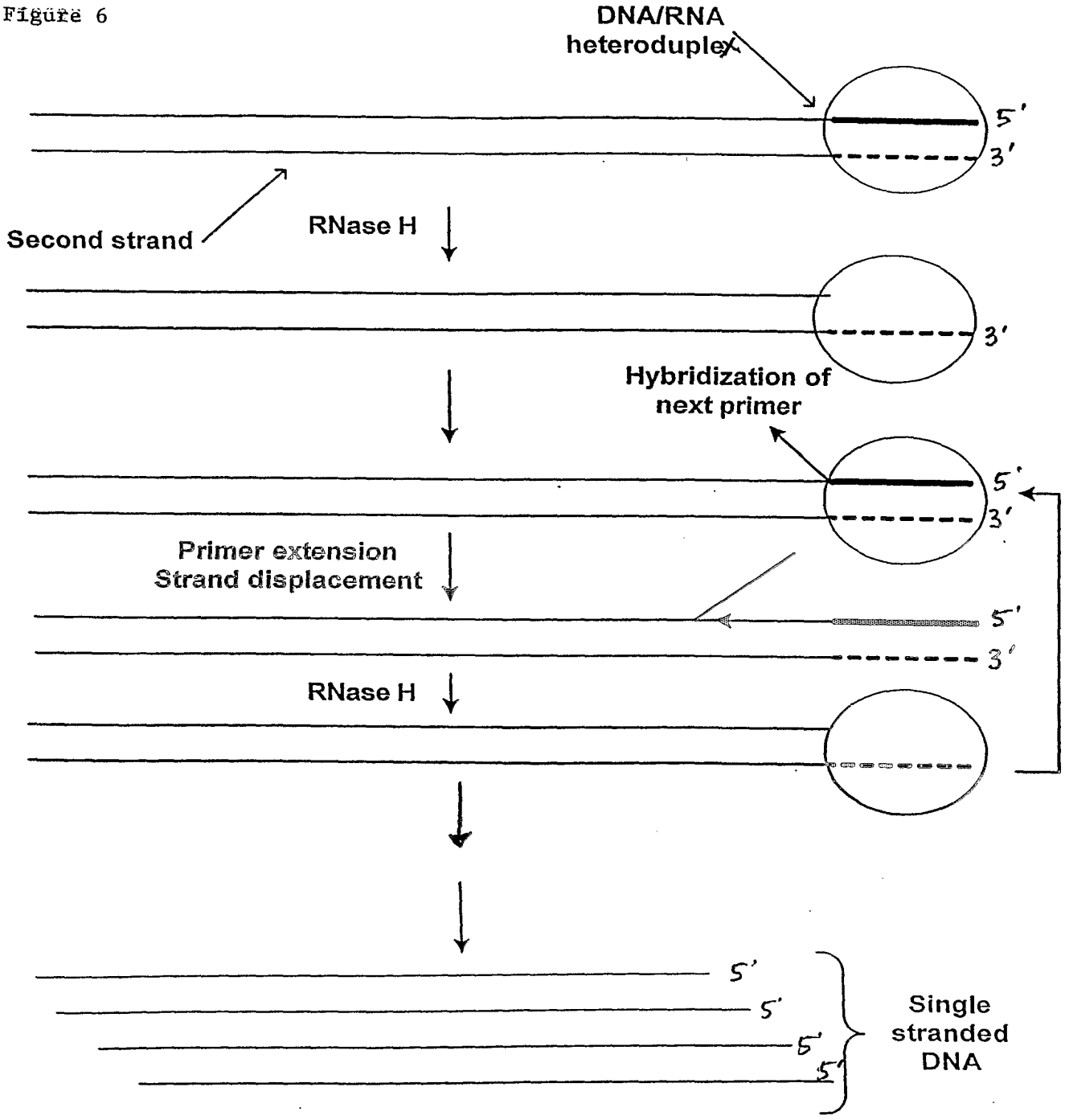
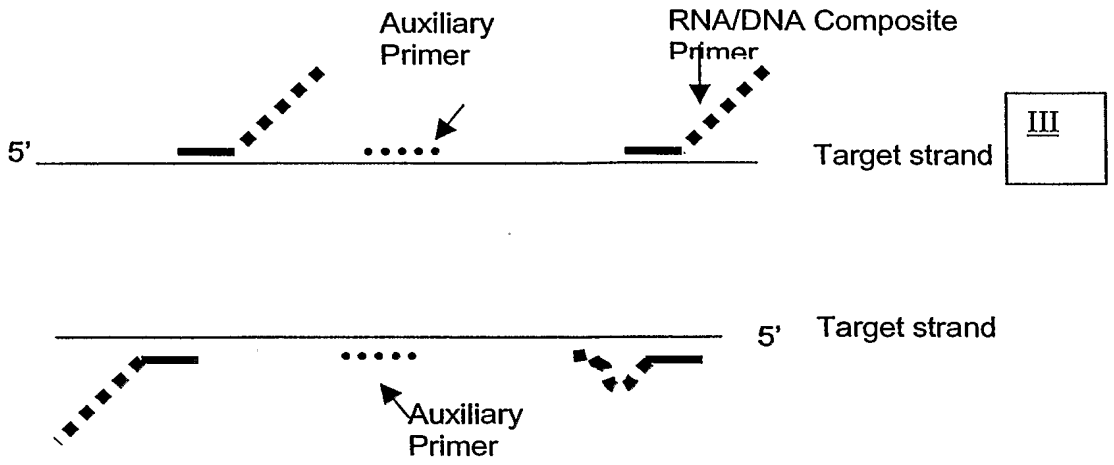

DNA/RNA heteroduplex
5'
3'
Second strand
RNase H
3'
Hybridization of next primer
5'
3'
Primer extension Strand displacement
5'
3'
RNase H
5'
5'
5'
5'
Single stranded DNA

Figure 7A



5

Figure 7B

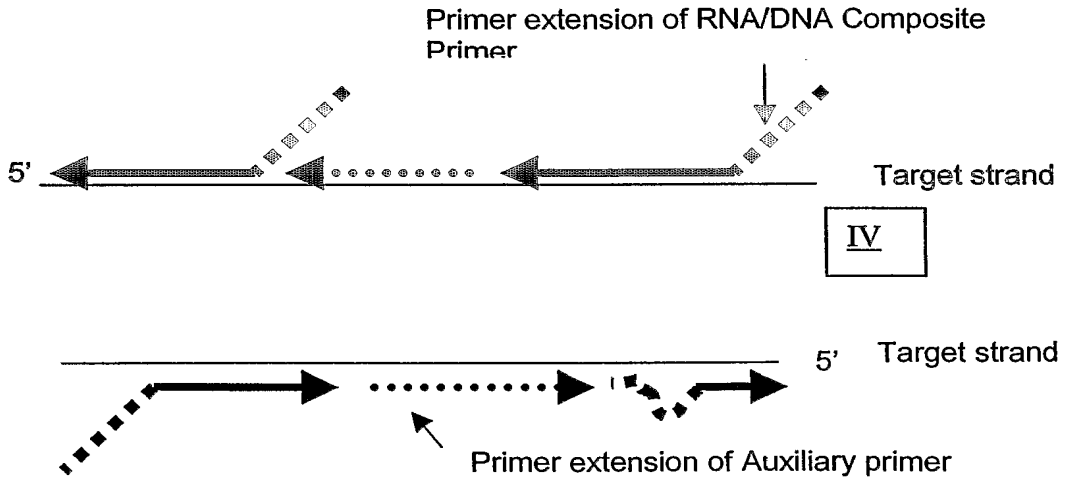


Figure 8

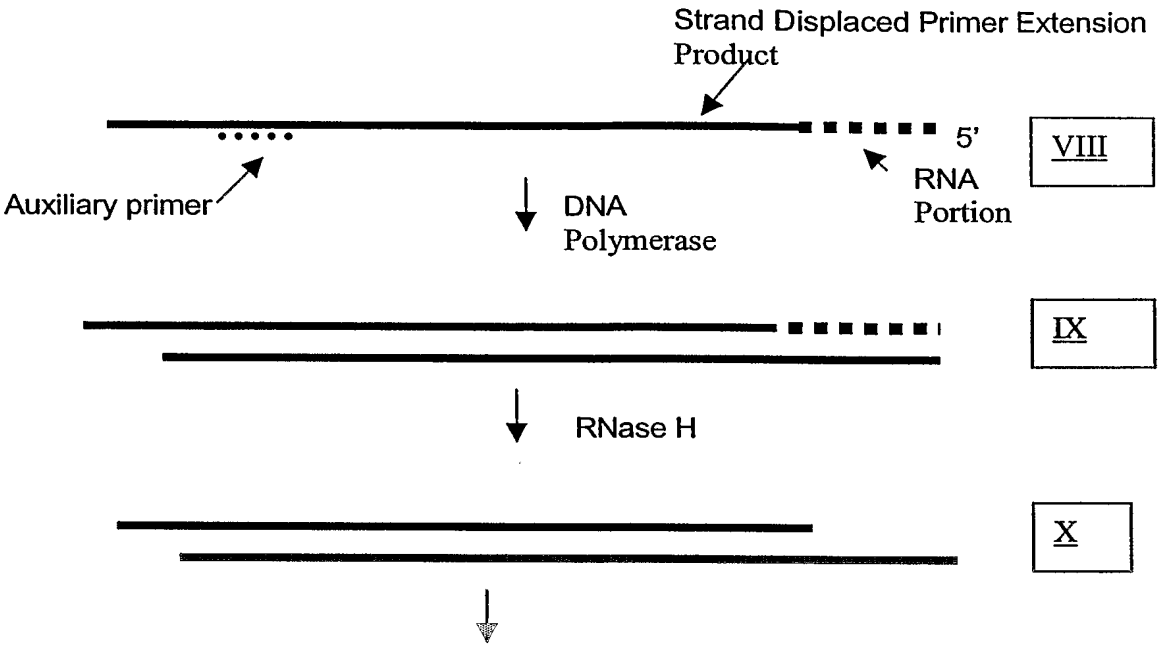
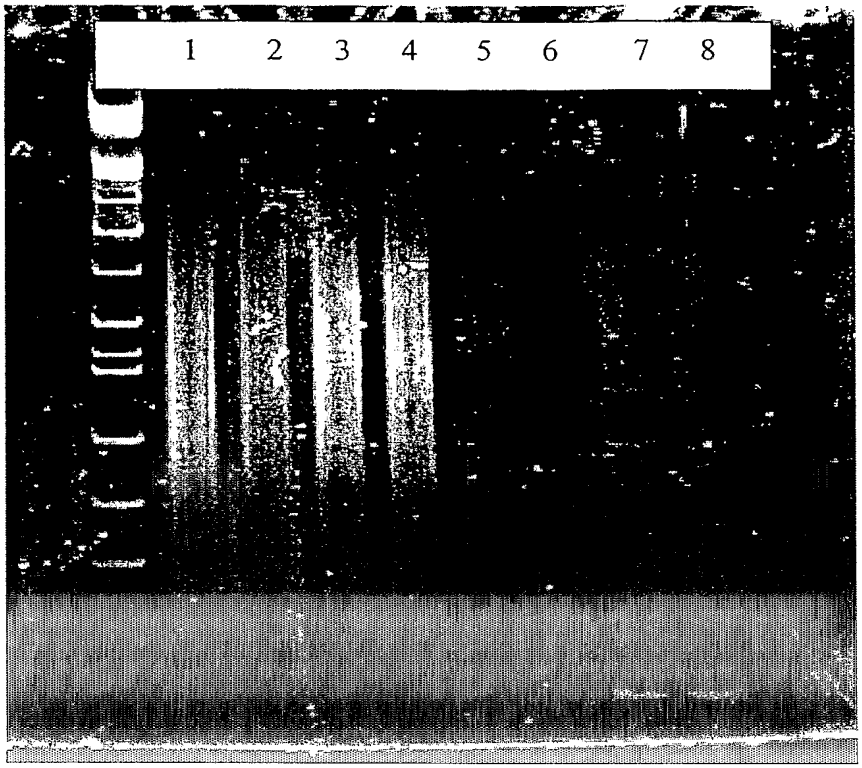

Strand Displaced Primer Extension Product
5'
VIII
RNA Portion
Auxiliary primer
DNA Polymerase
IX
RNase H
X

FIGURE 9



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(54) **Title**: ISOTHERMAL NUCLEIC ACID AMPLIFICATION METHODS AND COMPOSITIONS

(57) **Abstract**: Methods and compositions are provided related to the amplification of target polynucleotide sequences as well as total RNA and total DNA amplification. In some embodiments, the methods and compositions also allow for the immobilization and capture of target polynucleotides with defined 3' and or 5' sequences to solid surfaces. The polynucleotides attached to the solid surfaces can be amplified or eluted for downstream processing. In some cases, nucleotides attached to solid surfaces can be used for high throughput sequencing of nucleotide sequences related to target DNA or target RNA.

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ISOTHERMAL NUCLEIC ACID AMPLIFICATION METHODS AND COMPOSITIONS

CROSS REFERENCE

[0001] This application claims the benefit of U.S. Provisional Application Nos. 61/028,146, filed February 12, 2008, 61/074,991, filed June 23, 2008, and 61/085,811, filed August 1, 2008, which applications are incorporated herein by reference in their entirety. This application is also related to the co-pending patent application [Attorney Docket No 25115-726.201] filed February 12, 2009, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The quality and quantity of nucleic acid sample is important for many studies. High-throughput genomic analysis requires large amounts of template for testing, yet typically the yield of nucleic acids from individual patient samples is limited. Forensic and paleoarcheology work also can be severely limited by nucleic acid sample size. The limitation of starting material impacts the ability to carry out large scale analysis of multiple parameters, as is required for, for example, the genotyping of multiple loci in the study of complex diseases, detecting the presence or absence of specific nucleic acid species in a sample, large scale sequencing and the like. Moreover, it is well accepted that molecular analysis determination of genomic instability in various pathological condition such as cancer, is most precisely carried out in well defined cell populations, such as that obtained by laser capture micro-dissection or cell sorting. Nucleic acid amplification technologies that provide global amplification of very small polynucleotide samples, for example, from one or a very few cells, may provide a solution to the limited starting materials generally available for analysis.

[0003] Likewise, the ability to amplify ribonucleic acid (RNA) is an important aspect of efforts to elucidate biological processes. 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. Non-coding RNAs have been shown to be of great importance in regulation of various cellular functions and in certain disease pathologies. Such RNAs are often present in very low levels. Thus, amplification methods capable of amplifying low abundance RNAs, are of great importance.

[0004] Various methods for global amplification of DNA target molecules (e.g., whole genome amplification) have been described, including methods based on the polymerase chain reaction (PCR). See, e.g., U.S. Pat. Nos. 5,731,171; 6,365,375; Daigo et al., (2001) Am. J. Pathol. 158 (5):1623-1631; Wang et al, (2001); Cancer Res. 61:4169-4174; Zheng et al, (2001) Cancer Epidemiol. 10:697-700; Dietmaier et al (1999) Am. J. Pathol. 154 (1) 83-95; Stoecklein et al (2002) Am. J. Pathol. 161 (1):43-51; U.S. Pat. Nos. 6,124,120; 6,280,949; Dean et al (2002) PNAS 99 (8):5261-5266. However, PCR-based global amplification methods, such as whole genome amplification (WGA), may generate non-specific amplification artifacts, give incomplete coverage of loci, or generate DNA of insufficient length that cannot be used in many applications. PCR-based methods also suffer from the propensity of the PCR reaction to generate products that are preferentially amplified, and thus resulting in biased representation of genomic sequences in the products of the amplification reaction. Methods of global amplification of DNA using composite primers have been described. See e.g. U. S. Patent application 10/824,829.

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[0005] Additionally, a number of methods for the analysis of gene expression have been developed in recent years. See, for example, U.S. Pat. Nos. 6,251,639, 6,692,918, 6,686,156, 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; and U.S. published patent applications nos. 2002/0115088, 2003/0186234, 2003/0087251, and 2004/0023271. 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. RNA 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. However, the total amount of sample RNA 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 RNA species is not equal; some species are more abundant than others are, and these are more likely and easier, to analyze. The ability to amplify RNA sequences enables the analysis of less abundant, rare RNA species. The ability to analyze small samples, by means of nucleic acid amplification, is also advantageous for design parameters of large scale screening 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. Methods of amplification from RNA templates have been described, for example in U. S. Patent 6,946,251.

[0006] Sequencing of nucleic acids continues to be one of the most important and useful ways to analyze DNA and RNA samples. Recent developments have made possible highly parallel high throughput sequencing. Many of these approaches use an in vitro cloning step to generate many copies of each individual molecule. Emulsion PCR is one method, isolating individual DNA molecules along with primer-coated beads in aqueous bubbles within an oil phase. A polymerase chain reaction (PCR) then coats each bead with clonal copies of the isolated library molecule and these beads are subsequently immobilized for later sequencing. See, e.g. WO04069849A2, WO05010145A2. In other cases, surface methods of clonal amplification have been developed, for example, by the use of bridge PCR where fragments are amplified upon primers attached to a solid surface. These methods produce many physically isolated locations which each contain many copies of a single fragment. While these methods have provided improvements in sequencing throughput, there is a continuing need to improve the methods of obtaining samples appropriate for sequencing, and of handling, storing, and amplifying such samples. In particular, there is a need to improve methods for obtaining high throughput sequencing data for a specific set of genes or gene products from whole genome or transcriptome samples.

[0007] Therefore, there is a need for improved methods of obtaining, storing, amplifying, and analyzing DNA and RNA samples, including methods which can globally or specifically amplify DNA or RNA polynucleotide targets. The invention described herein fulfills this need.

SUMMARY OF THE INVENTION

[0008] In one aspect the present invention relates to a method for creating a partial RNA-DNA heteroduplex comprising: providing a template nucleic acid in a reaction mixture; adding an RNA-DNA chimeric primer;

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adding a DNA polymerase; and adding an exonuclease; wherein said DNA polymerase comprises RNA-dependent polymerase activity.

[0009] The present invention also describes a kit comprising a container containing reagents for forming a water in oil emulsion and a DNA polymerase with substantial strand-displacement activity; the kit optionally further comprising one or more all DNA primers, an RNA-DNA chimeric oligonucleotide, an exonuclease, RNA-DNA chimeric primer, RNase H, and instructions for use of said reagents. The DNA polymerase may have RNA-dependent polymerase activity. The invention also includes a kit comprising an all DNA first primer, an RNA-DNA chimeric oligonucleotide; the kit optionally further comprising a second primer, a polymerase having substantial strand-displacement activity, and RNase H.

[0010] In another aspect the invention relates to a method for producing amplified nucleic acid comprising: providing a template nucleic acid; annealing a first primer or set of first DNA primers comprising a 3' end whereby a portion of the 3' end comprises a template annealing sequence and a 5' tail sequence whereby a portion of the tail sequence comprises a sequence (A); extending said first primer or set of first primers with a DNA polymerase to create a first primer extension product or first set of DNA polymerase products; dissociating the primer extension product or products of said DNA polymerase from the template nucleic acid; annealing a second primer comprising a 3' DNA sequence and a 5' RNA sequence, whereby a portion of the 3' end comprises a randomized annealing sequence and a portion of the 5' end comprises sequence (A) or sequence (B), to said primer extension product or products; and extending said second primer with a DNA polymerase to create a second primer extension product or products to produce a double stranded DNA product or products comprising an A-A' DNA duplex at one end.

[0011] The template nucleic acid may be selected from the group consisting of DNA and RNA. The template nucleic acid may be RNA and the DNA polymerase of step (c) is an RNA dependant DNA polymerase. The dissociation step of step (d) is performed by degrading the RNA. The template nucleic acid is DNA and the DNA polymerase of step (c) can be a DNA dependant DNA polymerase. The dissociation step of step (d) may be performed by heat or chemical denaturation. The DNA polymerase may be one that does not exhibit substantial strand displacement activity. The methods herein may have each template annealing sequence of the set of primers is specific for a target or region of template nucleic acid.

[0012] The methods herein can have the template annealing sequence of the first primer comprises a random sequence. In some instances, the members of the set of first primers can each comprise a distinct 3' DNA annealing sequence, each specific for a target or region of template nucleic acid. The first primer tail sequence may not be complementary to the template nucleic acid. The 5' tail sequence of the second primer can comprise sequence (A). The 5' tail sequence of the second primer may be one that does not comprise tail sequence (A).

[0013] The method may further comprise step (g), degradation of single stranded 3' end of said first primer extension product or products in the complex of first and second primer extension products with a single stranded DNA specific 3' exonuclease. The method may further comprise step (h), the addition of a DNA polymerase with reverse transcriptase activity to create a double stranded nucleic acid with an A-A' DNA duplex at one end and an B-B' DNA-RNA heteroduplex at the other end.

[0014] The method may further comprise performing SPIA with a reaction mixture comprising RNAse H, a composite amplification primer comprising a 3' DNA portion and a 5' end whereby a portion of the 5' end comprises RNA wherein the RNase H reacts with the heteroduplex of step (h) to produce a single stranded

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end, and the amplification primer hybridizes to the single stranded end produced by RNaseH, and a DNA polymerase with strand displacement activity.

[0015] The first primer or set of primers described in any of the inventions herein can comprise a ligand portion at the 5'end.

[0016] Any of the methods herein can further comprise cleaving of the RNA from the DNA-RNA heteroduplex with RNase H; annealing a chimeric oligonucleotide comprising a 5' end whereby the 5' end comprises sequence (C) and a 3' end whereby the 3' end comprises the DNA sequence (B) to the double stranded nucleic acid product; extending the double stranded nucleic acid product with a DNA polymerase to produce a C-C' DNA-RNA heteroduplex; and amplifying the second primer extension product with a reaction mixture comprising RNase H, an amplification primer comprising a 3' DNA portion and a 5' end whereby a portion of the 5' end comprises RNA that hybridizes to a portion of the single stranded end produced by RNase H, and a DNA polymerase with strand displacement activity to produce an amplification product comprising sequence (B) near its 3' end and sequence (A') at the 5' end.

[0017] In another aspect the present invention relates to a method for producing amplified nucleic acid comprising: providing a template nucleic acid; annealing a first DNA primer or set of first DNA primers comprising a 3' end whereby a portion of the 3' end comprises a template annealing sequence and a 5' end whereby a portion of the 5' end comprises a DNA tail sequence (A); extending said first primer or set of first primers with a DNA polymerase to create a primer extension product or set of primer extension products; dissociation of the primer extension product or products from the template nucleic acid; annealing a second primer comprising a 3' DNA end, whereby a portion of the 3' end comprises a random template annealing sequence, and a 5' RNA end, whereby a portion of the 5' end comprises the tail sequence (A), to said primer extension product or products or to said template nucleic acid; extending said second primer with a DNA polymerase to create a second primer extension product or set of second primer extension products; and denaturing and annealing the second primer extension product or set of second primer extension products to form a stem loop structure comprising an A-A' RNA-DNA heteroduplex. The DNA polymerase may not exhibit substantial strand displacement activity. Each DNA annealing sequence in the set of first DNA primers can be specific for a target or region of the template nucleic acid. A portion of the 5' tail sequence of the first primer can be sequence (A) and a portion of the 5' tail sequence of the second primer can be sequence (A). The tail sequence may not be complimentary to the template nucleic acid.

[0018] The method can comprise the addition of a 3' single stranded DNA specific exonuclease and single strand specific RNase to degrade unincorporated primers and template nucleic acid. The methods can further comprising amplification with a reaction mixture comprising RNase H, an amplification primer comprising sequence (A'), and a DNA polymerase with strand displacement activity, wherein the amplification primer comprises a 3'-DNA sequence and a 5'-RNA sequence.

[0019] The template nucleic acid can be selected from the group consisting of RNA and DNA. In certain embodiments the template nucleic acid can be RNA and the DNA polymerase can be an RNA dependent DNA polymerase. In other embodiments the template nucleic acid can be DNA and the DNA polymerase can be a DNA dependent DNA polymerase.

[0020] In the methods described herein that include a dissociation step, such a step can be performed by degradation of the RNA. Dissociation can be carrier out or performed by chemical or heat denaturation.

[0021] In one aspect the present invention relates to a method of capturing and amplifying a target sequence or sequences comprising: providing a surface wherein a first primer or set of first primers comprising a 5' tail

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sequence comprising sequence (A) and a 3' template annealing sequence comprising sequence (P) are immobilized on said surface; annealing a target nucleic acid to said first primer or set of primers; extending the first primer or set of primers with a DNA polymerase to produce an immobilized primer extension product or set of immobilized primer extension products complimentary to and hybridized with the target nucleic acid to form a complex or set of complexes; dissociating the target nucleic acid-primer extension product complex or set of primer extension product complexes; annealing a second primer comprising a 3' DNA template annealing sequence (P) or set of template annealing sequences and a 5' RNA tail sequence comprising sequence (A) or sequence (B) to the immobilized DNA polymerase product or DNA polymerase products; and extending said second primer with a DNA polymerase to create a second primer extension product or set of primer extension products comprising a double stranded nucleic acid comprising a 3' DNA-DNA duplex A-A'.

[0022] The method can further comprise the step of eluting of the second primer extension product by heat or chemical denaturation. The method can also further comprise annealing an amplification primer comprising a 5' RNA sequence and a 3'DNA sequence; extending said annealed primer with a DNA polymerase; and amplifying the eluted DNA polymerase product with a reaction mixture comprising RNAse H, an amplification primer comprising a DNA portion and a 5' RNA portion, and a DNA polymerase with strand displacement activity to produce an amplified DNA product or products having a defined 5' sequence (A') or (B').

[0023] The DNA polymerase does not need to exhibit strand displacement activity.

[0024] The template nucleic acid can be selected from the group consisting of RNA and DNA. The first primer can include a spacer element between the immobilizing surface and the remaining portion of the primer, a 3' DNA end comprising a 3' DNA annealing sequence (P), and a 5' comprising a common 5' DNA sequence tail (A) or sequence tail (B) between said spacer element and annealing sequence.

[0025] The set of first primers can include a spacer element between the immobilizing surface and the remaining portion of the primer; a 3' DNA end wherein members of the set of first primers comprise unique target specific 3' DNA annealing sequences (P); and a 5' DNA end comprising a common 5' DNA sequence tail (A) or DNA sequence tail (B) between said spacer element and annealing sequence.

[0026] The annealing sequences (P) of set of first primers can each be specific to a target or region of the template nucleic acid. The first primers can be specific to one or more of the following gene targets: kinases, protein kinases, lipid kinases, phosphatases, G-protein coupled receptors, proteases, serine proteases, metalloproteases, cysteine proteases, aspartyl proteases, or cytokines.

[0027] The 5' tail sequence of the first primer can be sequence (A) and the 5' tail sequence of the second primer can be sequence (B). Alternatively, the 5' tail sequence of the first primer can be sequence (A) and the 5' tail sequence of the second primer can be sequence (A). The alternative method can further comprise separating the second primer extension product or products from the first primer extension product or products by heat or chemical denaturation; annealing the second primer extension product in solution to create a stem loop structure with a DNA sequence (A')-RNA sequence (A) heteroduplex end; degrading RNA in the heteroduplex with RNase H; annealing an amplification primer to the single stranded portion of the second primer extension product wherein the amplification primer has a DNA portion and a 5' RNA portion, wherein the RNA portion of the amplification primer forms a heteroduplex with a DNA portion of the second primer extension product; extending the amplification primer with a DNA polymerase having strand displacement activity to produce an amplified product hybridized to the second primer extension

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product to form a double stranded nucleic acid, said amplified product forming an RNA-DNA heteroduplex at one end of the double stranded nucleic acid; and repeating the steps to produce amplified DNA. A single stranded DNA specific 3' exonuclease can be added to degrade unincorporated primers.

[0028] The surface can be selected from the group consisting of a bead, a magnetic particle, a microarray, a gene chip, and an array.

[0029] In some embodiments the method can further include degradation of the single stranded 3' end of said first primer extension product and unextended immobilized first primers with a single stranded DNA specific 3' exonuclease; cleavage of spacer element with light or chemical cleavage to release the immobilized double stranded nucleic acid from the surface; and amplifying the released double stranded nucleic acid in solution with a reaction mixture comprising RNase H, an amplification primer comprising a DNA portion and a 5' RNA portion, and a DNA polymerase with strand displacement activity to produce an amplified DNA product or products having a defined 3' sequence comprising sequence (A').

[0030] In other embodiments the method can further include degrading the single stranded 3' end of said first primer extension product or products and unextended immobilized first primers with a single stranded DNA specific 3' exonuclease; extending said first primer extension product with a DNA polymerase; and degrading RNA in the heteroduplex from the first primer extension product and the RNA portion of the second primer with RNAse H; annealing an amplification primer to the single stranded portion of the second primer extension product wherein the amplification primer has a DNA portion and a 5' RNA portion; extending the amplification primer with a DNA polymerase having strand displacement activity to produce an amplified product hybridized to the second primer extension product; and repeating these steps to produce multiple copies of amplified product or products having a defined 3' sequence comprising sequence (A').

[0031] The present invention also describes a kit comprising a container or containers comprising a first primer comprising a 3' DNA portion and a 5' DNA portion, wherein the 5' DNA portion further comprises sequence (A) and the 3' portion further comprises an annealing sequence (P); a second primer comprising a 3' DNA portion and a 5' RNA portion, wherein the 5' RNA portion further comprises sequence (A) or (B) and the 3' portion further comprises an annealing sequence (P); a DNA polymerase; a DNA dependent DNA polymerase with strand displacing activity; RNase H; and a chimeric amplification primer comprising a 3' DNA portion and a 5' RNA portion wherein the sequence of the amplification primer is substantially the same as the (A) sequence.

[0032] The present invention also describes a kit comprising a container or containers comprising a first primer comprising a 3' DNA portion and a 5' DNA portion, wherein the 5' DNA portion further comprises sequence (A) and the 3' DNA portion further comprises annealing sequence (P); a second primer comprising a 3' DNA portion and a 5' RNA portion, wherein the 5' RNA portion further comprises sequence (A) or (B) and the 3' DNA portion further comprises annealing sequence (P); a DNA polymerase; a DNA dependent DNA polymerase with strand displacing activity; a chimeric oligonucleotide comprising a 3' DNA portion substantially comprising sequence (A) or (B) and a 5' RNA portion comprising sequence (C)' RNase H; and a chimeric amplification primer comprising a 3' DNA portion and a 5' RNA portion wherein the sequence of the amplification primer is substantially the same as the (A) sequence.

[0033] The present invention also describes a kit comprising a container or containers comprising a first primer comprising a DNA portion wherein the DNA portion further comprises an annealing sequence (P); a second primer comprising a 3' DNA portion and a 5' RNA portion, wherein the 5' RNA portion further

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comprises sequence (A) or (B) and the 3' portion further comprises an annealing sequence (P); a DNA polymerase; a DNA dependent DNA polymerase with strand displacing activity; RNase H; and a chimeric amplification primer comprising a 3' DNA portion and a 5' RNA portion wherein the sequence of the amplification primer is substantially the same as the (A) sequence.

[0034] The present invention also describes a kit comprising an immobilized first primer comprising a 3' DNA portion and a 5' DNA portion, wherein the 5' DNA portion further comprises sequence (A), and the 3' DNA portion further comprises annealing sequence (P); a second primer comprising a 3' DNA portion and a 5' RNA portion, wherein the 5' RNA portion further comprises sequence (A) or (B); a DNA polymerase; a DNA dependent DNA polymerase with strand displacing activity; RNase H; and a chimeric amplification primer comprising a 3' DNA portion and a 5' RNA portion wherein the sequence of the amplification primer is substantially the same as the (A) sequence.

[0035] The kits of the invention can have the DNA polymerase that is an RNA dependant DNA polymerase. The kits can have a DNA dependant DNA polymerase. The kits can have a DNA polymerase that does or does not possess substantial strand displacing activity.

[0036] The kits can comprise a first primer where the 3' DNA portion of the first primer comprises a random sequence. The kits can comprise a first primer where the 3' DNA portion of the first primer comprises a specific sequence. The kits can comprise a first primer where the 3' DNA portion of the first primer comprises a set of specific sequences. The kits can comprise a first primer which further comprises a ligand.

[0037] The kits can comprise a second primer where the 3' DNA portion of the second primer comprises a random sequence. The kits can comprise a second primer where the 3' DNA portion of the second primer comprises a specific sequence. The kits can comprise a second primer where the 3' DNA portion of the second primer comprises a set of specific sequences.

[0038] The kits can further comprise a single stranded DNA specific 3' exonuclease. The kits can further comprise an RNase specific for single and double stranded RNA.

[0039] In another aspect an amplification method comprises forming a polynucleotide strand comprising a sequence (A) at its 5' end and complementary sequence (A') at its 3' end wherein sequence (A) comprises RNA within a mixture comprising other polynucleotides that are single stranded; treating said polynucleotide so as to intramolecularly anneal the (A) sequence to the (A') sequence; purifying the polynucleotide by degrading the other polynucleotides by treating the mixture with one or more enzymes that degrade single stranded polynucleotides; and performing SPIA amplification with the purified polynucleotide from step (c) using sequence (A') as a priming site for the SPIA amplification.

[0040] In yet another one aspect an amplification method comprises: obtaining a polynucleotide strand comprising sequence (B') at its 3' end; annealing a chimeric oligonucleotide comprising a 3' DNA portion and a 5' RNA portion, wherein the 5'portion comprises sequence (C), and the 3' portion comprises sequence (B); extending the polynucleotide strand with a RNA dependent DNA polymerase to add a DNA sequence (C') to the polynucleotide strand; and performing SPIA amplification using the product of step (c) as the SPIA substrate using sequence (C') as a priming site for SPIA amplification.

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INCORPORATION BY REFERENCE

[0041] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0042] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention may be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0043] **Figure 1** illustrates the initial steps of a method for producing amplified DNA by way of generating a single primer isothermal linear amplification (SPIA) substrate using an all DNA first primer with a sequence tail (A) and an annealing sequence (P), a DNA-RNA chimeric second primer with a sequence tail (A) or (B), and a DNA template.

[0044] **Figure 2** illustrates methods for producing a SPIA substrate for producing amplified DNA with a defined sequence tail (A') from the product of **Figure 1.**

[0045] **Figure 3** illustrates an alternative method of producing amplified DNA from the product of **Figure 1.**

[0046] **Figure 4** illustrates methods for producing captured and immobilized DNA using immobilized all DNA first primers with sequence tails (A) and annealing sequences (P), a template nucleic acid, and a DNA-RNA chimeric second primer with a sequence tail (A) or (B).

[0047] **Figure 5** illustrates methods for generating amplified DNA from immobilized DNA of **Figure 4** having a defined 3' sequence (A').

[0048] **Figure 6** illustrates an alternative method for generating amplified DNA from immobilized DNA of **Figure 4** having a defined 3' sequence (A') in which the SPIA substrate is cleaved from the surface prior to amplification.

[0049] **Figure 7** illustrates the initial steps of a method for producing a SPIA substrate using an immobilized all DNA first primer with sequence tail (A), a DNA-RNA chimeric second primer with the same sequence tail (A), and a template nucleic acid.

[0050] **Figure 8** illustrates a method for producing a stem loop SPIA substrate from the product of **Figure 7.**

[0051] **Figure 9** illustrates a method of producing a SPIA substrate with a defined sequence (A) at its 5' end using an all DNA first primer with a sequence tail (A), a DNA-RNA chimeric second primer with a sequence tail (B), an RNA template, and a chimeric DNA/RNA amplification primer.

[0052] **Figure 10** illustrates a method of producing amplified DNA with a defined sequence (A') at its 3' end and a defined sequence (B) at its 5' end using a chimeric oligonucleotide.

[0053] **Figure 11** illustrates a method of producing a SPIA substrate with a 5' ligand for immobilization, functionalization, or capture and a defined 5'sequence (A) using a ligand attached all DNA first primer with a sequence tail (A), a DNA-RNA chimeric second primer with sequence tail (B) and an RNA template.

[0054] **Figure 12** illustrates a method of producing a SPIA substrate with a 5' ligand, a defined sequence (A) at its 5' end, and a defined sequence (B') at its 3' end using a ligand attached all DNA first primer with tail sequence (A), a DNA-RNA chimeric second primer with a sequence tail (B), a DNA-RNA chimeric

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oligonucleotide with a DNA sequence (B) at its 3' end and an RNA sequence (C) at its 5' end, and an RNA template.

[0055] **Figure 13** illustrates a method of producing a SPIA substrate from a DNA template using an all DNA first primer with a randomized annealing sequence (P) and without a sequence tail, and a DNA-RNA chimeric second primer with a sequence tail (B).

[0056] **Figure 14** illustrates a method of producing a SPIA substrate from a DNA template using an all DNA first primer with a target specific annealing sequence (P) and lacking a sequence tail, and a DNA-RNA chimeric second primer with a sequence tail (B).

[0057] **Figure 15** illustrates a method of producing a SPIA substrate with a 5' ligand for immobilization, functionalization, or capture and a defined 5' sequence (A) using a ligand attached all DNA first primer with a sequence tail (A) and a randomized annealing sequence (P), a DNA-RNA chimeric second primer with sequence tail (B) and an DNA template.

[0058] **Figure 16** illustrates a method of producing a SPIA substrate from a target nucleic acid for generation of amplified DNA having a defined 5' sequence (A) using an all DNA first primer with a sequence tail (A) and a randomized or target specific annealing sequence (P), an all DNA second primer, and an RNA-DNA chimeric oligonucleotide with DNA sequence (A) at its 3' end and RNA sequence (C) at its 5' end.

[0059] **Figure 17** illustrates a method of producing a SPIA substrate from a target nucleic acid for generation of amplified DNA having a defined 5' sequence (A) and a defined 3' sequence (B) using an all DNA first primer with a sequence tail (A) and a randomized or target specific annealing sequence (P), an all DNA second primer having a tail sequence (B), and an RNA-DNA chimeric oligonucleotide with DNA sequence (A) at its 3' end and RNA sequence (C) at its 5' end.

[0060] **Figure 18** illustrates a method of producing amplified DNA from the SPIA substrate of **Figure 17.**

[0061] **Figure 19** illustrates the results of SPIA-based target specific amplification of DNA using a tailed all-DNA target specific first primer.

[0062] **Figure 20** illustrates normalized results of SPIA-based target specific amplification of DNA using a tailed all-DNA target specific first primer.

[0063] **Figure 21** illustrates results of sequence-specific single and multiplex amplification of genes from yeast genomic DNA using a tailed all-DNA target specific first primer.

[0064] **Figure 22** illustrates results the results of using the Taq polymerase enzyme for extension of the tailed all-DNA sequence specific first primer and an RNA-DNA chimeric second primer.

DETAILED DESCRIPTION OF THE INVENTION

Introduction

[0065] This invention provides new methods of amplifying nucleic acids. The invention also provides for systems reaction mixtures, and kits relating to these methods. The methods of the invention generally incorporate single primer isothermal amplification (SPIA). SPIA is an isothermal amplification method described, for example, in U. S. Patents 6,692,918, 6,251,639, 6,946,251 and 7,354,717. In some instances SPIA amplification generally involves a chimeric RNA-DNA amplification primer, RNase H, and a DNA polymerase enzyme to produce amplified DNA product. The SPIA amplification generally amplifies the sequences downstream of the hybridization site of the SPIA primer on the nucleic acid to which the SPIA primer binds. SPIA is a rapid and effective method of isothermal, linear amplification that can be used to

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produce amplified DNA that is representative of DNA or RNA from a variety of sources, for example, genomic DNA or the messenger RNA transcripts.

[0066] The methods of the present invention generally utilize all-DNA first primers that are designed to hybridize to a nucleic acid target, often referred to as a template, that has sequences to be amplified. Prior methods have described how to generate a substrate for SPIA from a target nucleic acid by using chimeric RNA-DNA first primers. The methods of the present invention can be useful, for example, for amplifying specific sequences within the target nucleic acid. The methods of the invention are also useful for multiplex amplification, performing the simultaneous amplification of multiple specific sequences. The number of specific sequences can be, for example, 2 – 10, 10 – 100, 100 – 1,000, 1,000 -10,000, 10,000 – 100,000 or more than 100,000. In some cases, the number of specific sequences can be for example at least 2, 20, 200, 2,000, 20,000, 50,000, 100,000, 200,000 or more.

[0067] In some embodiments of the invention the all-DNA first primer is a tailed primer, for example, having a tailed sequence (A) that generally does not hybridize to the target nucleic acid. The use of a tailed all-DNA first primer having, for example, sequence (A) allows, for example, the preparation of amplified product having either sequence (A) or its complement (A') at one end.

[0068] In some embodiments of the invention, the all-DNA first primer is attached to a solid surface, or solid substrate such as a microarray or a bead. The all-DNA first primer can be attached, for example, to a microarray, such as an oligonucleotide microarray, such as a microarray described by Affymetrix, Agilent, or NimbleGen. The different regions of the microarray can each have different all-DNA primer sequences that can hybridize to different regions of a target nucleic acid sample. These primer sequences can then be extended by the methods of the invention to produce substrates for SPIA amplification. The use of solid phase-bound all-DNA primers to produce SPIA substrates is useful for multiplex amplification of a subset of sequences within a target DNA population. For example, the solid phase or microarray may comprise primers that can hybridize to a subset of genes in a sample that represents the genome of an organism or a population of organisms. This method results in the SPIA amplification of only that subset of genes or factions thereof. The SPIA amplified subset of genes or fraction thereof, can then be analyzed, for example, by sequencing, for example by the newly developed massively parallel high throughput next generation sequencing methods and instruments such as the sequencing methods described by Illumina, 454 (Roche), and Applied Biosystems (ABI) and in US Patent Nos. 7,211,390; 6,355,431; 5,750,341; 5,969,119; 6,274,320; 6,258, 568; 6,210, 891; and 6,306,597 herein incorporated by reference in their entirety. These same methods can be applied to RNA, for example to SPIA amplification of a subset of the messenger RNA transcripts from the transcriptome of an organism. The same type of parallel multiplex amplification described for microarrays can be carried out on beads by having a plurality of beads containing all-DNA primers. The beads can be used in the same manner as above, and in some cases may be useful for clonal amplification and/or archiving of sequences in the sample.

[0069] When the all-DNA primers are bound to a solid surface, in some embodiments, the primers have a tail sequences, for example, sequence (A), that are 5' to the primer sequences. By incorporating a tailed sequence, substrates for SPIA amplification and SPIA amplification products having either an (A) sequence, or the complementary (A') sequence can be produced. The generation of amplified product comprising as sequence (A) or (A'), e.g. a universal sequence, can be useful for the further processing and/or analysis of the sample. For example, the universal sequence can be used for sequencing, e.g. as the priming site for sequencing by polymerization and pyrosequencing.

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[0070] In some cases, the primer bound to the solid surface is bound covalently. In some cases, the primer is bound via a spacer or a linker. In some cases, the primer is bound by a spacer having a cleavable group, where the group can be cleaved, for example chemically, by heat, or by light.

[0071] One aspect of the invention comprises using an all-DNA first DNA primer having a 5' tail sequence (A), and using a second chimeric RNA-DNA primer having a 5' RNA tail sequence (A). Extension of the second primer along the first primer extension product produces a second primer extension product having a 5' RNA portion with sequence (A), and a 3' DNA end comprising sequence (A') complementary to (A). Denaturing and annealing can then result in the formation of stem-loop structures by intramolecular hybridization of the (A) and (A') sequences. The reaction mixture thus produced can then be treated with an exonuclease specific for single stranded DNA, and with an RNase that is specific for single and or double stranded RNA such as, for example, RNase 1. This process may result in the degradation of the primers and other products that do not form the stem-loop structure. The products that do form stem-loop structures may not be degraded by the enzymes. The stem-loop products can act as SPIA substrates by using a chimeric SPIA amplification primer that anneals to the (A') DNA sequence of the stem-loop product. Cleavage of the RNA portion of the RNA-DNA heteroduplex stem structure of the stem-loop products are cleaved by RNase H thus freeing the priming site for binding of the SPIA amplification chimeric primer and enabling isothermal linear SPIA amplification. Thus, this procedure results in the clean-up of the reaction mixture from the undesired products as well as target nucleic acid and unincorporated primers, for subsequent amplification, which can result in a purer amplification products and or a better yield of amplification products. The method of using the stem-loop can also be used with embodiments that employ a solid substrate as described herein.

Exemplary Methods of the Invention

Initial steps for generation of SPIA substrate using all DNA first primer

[0072] In one aspect of the present invention, target nucleic acids are amplified by SPIA using an all DNA first primer. The all-DNA first primer may comprise a 5'-tail sequence, e.g. sequence (A), and a 3'-annealing sequence (P) as described previously. In other cases, the primer comprises an annealing sequence and no tail sequence. The annealing sequence may comprise a random sequence suitable for binding to any or substantially any sequence portion of the target nucleic acid such as for example random hexamers or random decamers. In other cases, the annealing sequence may be designed to hybridize to a specific region of the target nucleic acid.

[0073] In some embodiments the target nucleic acid comprises DNA. In some cases, the DNA comprises genomic DNA such as for example the human genomic DNA from a subject or set of subjects. In other cases, the DNA comprises a subset of a genome. In still other cases, the target DNA comprises cDNA generated from an RNA source. In some cases, the target nucleic acid comprises RNA. In some cases, the RNA comprises messenger RNA or total RNA. In some cases, the RNA comprises a transcriptome or a subset of a transcriptome.

[0074] In some embodiments, a set or sets of more than one unique non-random all DNA first primer is used such that multiple regions of the target nucleic acid are amplified. This is often referred to in the art as multiplex amplification because multiple different amplification products are produced in a single reaction. The set or sets of target-specific primers of the invention may comprise a set of related sequences for hybridizing to a set of related genes, introns, exons, splice variants and the like. For example, the annealing sequences may

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hybridize to nucleic acid encoding serine/threonine kinases or their complement. In some cases, the annealing sequences may be randomized at certain positions and specific at other positions, such that they may hybridize to more than one member of a set of homologous target nucleic acids.

[0075] The all-DNA first primer may be annealed to a target nucleic acid and extended using a polymerase to generate a first primer extension product. In some cases, the polymerase may comprise no, or substantially no, strand displacement activity, for example to avoid whole genome or transcriptome amplification. In other cases, a polymerase may be chosen that possesses a substantial amount of strand displacement activity, for example to enable whole genome or transcriptome amplification. In some cases, a substantially thermostable polymerase that is maximally active above about 60°C such as Taq polymerase for example may be used, for example to enable higher processivity or avoid problems with secondary structure within target nucleic acid. In other cases, a polymerase that is slightly thermostable that is maximally active between 40°C and 60°C may be used. In still other cases, a mesophilic polymerase that is maximally active below 40°C may be used to extend the first and or second primer. In some cases, an RNA-dependant DNA polymerase may be used, while in other cases a DNA dependant DNA polymerase may be used. In some cases, it may be advantageous to use a polymerase that does not exhibit either 3'-5' or 5'-3' exonuclease activity. In other cases 3'-5' or 5'-3' exonuclease activity may be advantageous. In some cases, the polymerase used to generate the first primer extension product may be a reverse transcriptase. In some cases, the target nucleic acid may be removed after generation of the first primer extension product by heat or chemical denaturation and or degradation. In other cases, the target nucleic acid may be removed by enzymatic methods. In some cases the DNA polymerase comprises both DNA-dependent DNA polymerase activity and RNA-dependent RNA polymerase.

[0076] In some embodiments of the present invention, target nucleic acids are amplified using a chimeric DNA-RNA second primer. The chimeric second primer may comprise an RNA segment and a DNA segment. In some cases, the RNA segment is generally at the 5' end and the DNA segment is generally at the 3' end of the chimeric primer. In some cases, a portion of the RNA segment comprises a tail sequence that may comprise the same or substantially the same sequence (A) as the tail sequence of the all DNA first primer. In some cases, the tail sequence comprises a different tail sequence (B) than the tail sequence (A) of the all DNA first primer.

[0077] In some cases, a portion of the DNA segment of the chimeric second primer comprises a target nucleic acid annealing sequence. In some cases, the target nucleic acid annealing sequence is random such that it may hybridize with any, or substantially any, region of the target nucleic acid. In other embodiments, the annealing sequence is specific for a region of the target nucleic acid. In still other embodiments, a set of chimeric DNA-RNA second primers comprising a set of specific annealing sequences may be utilized to amplify a set of target nucleic acid regions in a multiplex fashion.

[0078] In some cases the chimeric DNA-RNA second primer may be annealed to a target nucleic acid or to the first primer extension product and extended using a polymerase to generate a second primer extension product. The polymerase may be a thermostable or mesophilic DNA dependant DNA polymerase as described above. Additionally, the polymerase may or may not exhibit substantial strand displacement or exonuclease activity.

[0079] In one aspect of the present invention, methods are provided for creating an RNA-DNA heteroduplex, for example on a nucleic acid, such as a target nucleic acid. The RNA-DNA heteroduplex may be useful, for example in providing a substrate for SPIA amplification, or for providing a defined 3', 5' or 3' and 5' end

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to the nucleic acid. The method may comprise providing a template nucleic acid in a suitable reaction mixture as provided herein, adding an RNA-DNA chimeric primer, adding a DNA polymerase or allowing a DNA polymerase previously present in the reaction mixture to extend the RNA-DNA chimeric primer, and adding an exonuclease. In some cases, the method may further comprise purifying the resulting product or products by for example standard nucleic acid purification techniques known in the art such as Agencourt magnetic beads. In some cases, the method may further comprise amplifying the product by adding an RNA-DNA chimeric amplification primer, RNase H, and a DNA-polymerase exhibiting substantial strand displacement activity.

[0080] A schematic example of an embodiment of the invention relating to the initial steps of a method for generating amplified DNA by way of producing a substrate for single primer isothermal linear amplification (SPIA) is shown in **Figure 1**. Step I shows the annealing of an all DNA first primer comprising a 5'-tail of sequence (A) and a 3'-target specific, or randomized, sequence for annealing to a target nucleic acid (P). In some embodiments it may be advantageous for tail sequence (A) to be designed to minimize hybridization with the target nucleic acid. Further, in some embodiments, the tail sequence of the first primer comprises only a portion of the 5' end of the primer. Likewise, in some embodiments, the sequence (P) comprises only a portion of the 3' end of the first primer.

[0081] In one aspect, a single first primer with a specific annealing sequence (P) may be used to amplify a target sequence. In other embodiments, a set of first primers each with a different specific annealing sequence may be used to amplify multiple target sequences in the same reaction. It is anticipated that the methods of the present invention may be use to amplify many specific target sequences in the same reaction in a multiplexed fashion. In some embodiments the target sequences to be amplified represent a set of genes or gene expression products (e.g. RNA, or mRNA), or fragments thereof, of interest. Such sets of genes or gene expression products may include but are not limited to kinase, phosphatases, G-protein coupled receptors, proteases, cytokines, genes or gene expression products related to a specific metabolic or signaling pathway, genes or gene expression products related to multiple metabolic or signaling pathways, genes or gene expression products related to cancer, hereditary disease, metabolic disorder, ageing and age related disorders, normal or pathogenic specific developmental stages, specific differentiation stages, tissue specific, or any gene, gene expression product, or fragment thereof; or combination of genes, gene expression products, or fragments thereof of interest. It is anticipated that the number of target sequences amplified in this fashion can be as few as 1 to as many as tens of thousands including any number between one and 99,999, or more than tens of thousands of target sequences. In some embodiments 2, 3, 4, 5, 6, 7, 8, 9, 10 – 20, 20-30, 30-50, 50-80, 80-100, 100-200, 200-400, 400-500, 500 – 1,000, 1,000 – 2,000, 2,000-4,000, 4,000-5,000, 5,000-10,000, 10,000-20,000, 20,000-50,000 or more than 50,000 target sequences can be amplified in multiplex fashion. In some cases, at least 2; 20; 200; 2,000; 20,000; 200,000; 2,000,000 or more sequences may be amplified in multiplex fashion using the methods of the present invention.

[0082] The annealing sequence (P) can act as a primer for a DNA polymerase to extend the first primer as also depicted in step I to create a first primer extension product. In some embodiments, the target nucleic acid comprises DNA and the DNA polymerase is a DNA dependant DNA polymerase. In other embodiments, the target nucleic acid comprises RNA and the DNA polymerase is an RNA dependant DNA polymerase or a reverse transcriptase. In some embodiments, especially wherein the annealing sequence (P) of the first primer is randomized, it may be advantageous to use a DNA polymerase that does not exhibit strand displacement activity to avoid whole or substantially whole genome or transcriptome amplification. In

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other embodiments, especially wherein randomized annealing sequences are used, it may be advantageous to use a DNA polymerase that exhibits strand displacement activity to provide whole or substantially whole genome or transcriptome amplification. In some cases, it may be advantageous to use a thermostable polymerase, in other cases it may be advantageous to use a thermolabile polymerase. In some cases it is advantageous to use polymerases devoid of 5'-exonuclease activity. In some cases, a polymerase devoid of 5'-exonuclease activity may be useful to avoid cleaving or degrading the 5'-tail of a first or second primer or the displaced primer extension product or products.

[0083] Step II shows the separation of the target nucleic acid from the newly synthesized primer extension product followed by annealing of a second primer. In the case of a DNA target, target nucleic acid may be separated from the first primer extension product by heat or chemical denaturation. In the case of an RNA target, target nucleic acid may be degraded by heat, an enzyme, or an enzyme combination such as RNaseH and RNase 1. Alternatively, RNA target nucleic acid may be removed by heat or chemical denaturation.

[0084] The second primer as shown in step II may contain a 5' tail sequence (A) that is the same or substantially the same as the tail sequence of the first primer, or may contain a tail sequence (B) that is substantially different than the tail sequence of the second primer. The term substantially the same and substantially different as used herein describes two or more nucleic acid sequences that are substantially the same if each are capable of hybridizing to the other's complimentary sequence, or different if each do not show significant hybridization to the other's complimentary sequence. For example, a sequence substantially similar to sequence (A) would be capable of hybridizing to the complement of sequence (A) also known as (A'). In some cases, it may be advantageous to design the tail sequence such that it does not hybridize to the target nucleic acid or to the first primer extension product.

[0085] The second primer may also contain an annealing sequence (P) that is either random or target specific. In some embodiments the use of a set of second primers with multiple target specific annealing sequences is anticipated. As described previously for sequence (P) of the first primer, the simultaneous amplification of multiple genes or gene expression products or fragments thereof is anticipated by this method using multiple target specific annealing sequences or a random annealing sequence.

[0086] In the embodiment shown in **Figure 1**, the second primer is a DNA-RNA chimeric primer in which the 5'-end is RNA and the 3'-end is DNA. In some cases, the portion of the 5'- end of the second primer that is RNA corresponds to the portion of the 5'-end that comprises the tail sequence (A) or (B). In other cases, a portion of the tail sequence of the second primer is RNA and a portion of the tail sequence is not RNA, for example a portion of the tail sequence may be DNA. Likewise, in some cases the 3'-end of the second primer that is DNA corresponds to the annealing sequence (P). In other cases, a portion of the annealing sequence may be RNA.

[0087] The annealed second primer may then be extended, as depicted in step III, by the use of a DNA polymerase to create a second primer extension product. In some cases it may be advantageous to use a polymerase that does not exhibit strand displacement activity to avoid whole or substantially whole genome or transcriptome amplification. In other cases, it may be advantageous to use a polymerase that does exhibit strand displacement activity to enable whole or substantially whole genome or transcriptome amplification. The double stranded nucleic acid product such as that depicted in **Figure 1** can be a useful product or, in the case of multiplexed primers or random primers, can be a useful set of products. The products have defined double stranded region (A)/(A') at one end, when the all-DNA first primer comprises a tail sequence (A), and the second primer extension product strand has a tailed region comprising sequence (A)

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or (B), and comprises an RNA tailed sequence. The double stranded nucleic acid product or products can, for example, be amplified as described below. The products can be analyzed before or after amplification, for example, to determine the sequence of portions of the template nucleic acid.

Amplification of target nucleic acid using all-DNA first primer

[0088] The double stranded nucleic acid product of the method depicted in **Figure 1** may be further manipulated and amplified by single primer isothermal linear amplification. In some embodiments, a 3'-exonuclease specific for single stranded DNA such as exonuclease 1 or a polymerase that exhibits substantial single stranded DNA 3'-exonuclease activity such as T4 DNA polymerase can be used to degrade any remaining non hybridized 3'-DNA of the first primer extension product. The first primer extension product may then be extended with a polymerase that exhibits substantial reverse transcriptase activity such as for example Bst polymerase such that the first primer extension product is extended along the tail of the second primer and the first primer extension product is substantially hybridized to the tail sequence of the chimeric DNA-RNA second primer. The first and second primer extension products may form a double stranded nucleic acid with a chimeric DNA-RNA heteroduplex at one end. The double stranded nucleic acid with a chimeric DNA-RNA heteroduplex may comprise a SPIA substrate. The SPIA substrate may be amplified using the SPIA method. By way of example, the double stranded nucleic acid may be contacted with a chimeric amplification primer, an RNAse such as RNAse H that can degrade the RNA portion of a DNA-RNA heteroduplex, and a polymerase with strand displacement activity. The chimeric amplification primer may comprise a DNA portion and a 5'-RNA portion wherein the chimeric amplification primer comprises a sequence which is substantially the same as sequence (A) or (B) or a portion of sequence (A) or (B).

[0089] In some embodiments a double stranded nucleic acid product comprising a hybridized first and second primer extension product as depicted in **Figures 1 and or 2**, may be amplified, or a region of the first and second primer extension product may be amplified, by a method known to the art that is not SPIA. The amplification methods include but are not limited to PCR, linear PCR, SPIA, rolling circle amplification, loop-mediated isothermal amplification, ligation mediated rolling circle amplification and the like.

[0090] The formation of a substrate for single primer isothermal linear amplification (SPIA) from the double stranded nucleic acid product of the method depicted in **Figure 1** is depicted in **Figure 2**. In **Figure 2**, step I shows the use of a single stranded DNA specific 3'-exonuclease such as, but not limited to, exonuclease 1 or the 3'-exonuclease activity of a DNA polymerase such as, but not limited to, T4 DNA polymerase. The exonuclease or exonuclease activity may remove the single stranded 3'-nucleotides from the 3'-end of the first primer extension product which are not hybridized to the second primer extension product.

[0091] The method depicted in **Figure 2** further comprises step II whereby a DNA polymerase with reverse transcriptase activity (e.g. Bst polymerase) further extends the first primer extension product to produce a sequence (A') or (B') complimentary to the tail sequence of the second primer (A) or (B). This extension produces a double stranded DNA product with two defined ends, referred to as the first primer end in which the first primer is incorporated and the second primer end in which the second primer is incorporated.

[0092] The method further comprises step III, whereby SPIA is used to amplify the further extended first primer extension product of step II. In some embodiments of the SPIA method, an RNA-DNA heteroduplex degrading enzyme such as, but not limited to, RNase H is used to remove the RNA portion of the second primer in the RNA-DNA heteroduplex. Then a third primer, referred to as the amplification primer, is annealed to the 3' single stranded portion of the first primer extension product. The third primer, the

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amplification primer, comprises a 5'-RNA portion and a 3'-DNA portion. A DNA polymerase with strand displacement activity such as has been described for example in U.S. Pat. Nos. 6,251,639 and 6,692,918 may then extend the primer to create a third primer extension product while displacing the second primer extension product. The enzyme additions to carry out step III may be done sequentially and repeatedly to produce SPIA products, or preferably the RNase H, amplification primer, and strand displacing polymerase are added as a mixture and the reaction allowed to proceed under conditions that allow amplification as described in US Patent Nos. 6,251,639 and 6,692,918. The amplification products of the SPIA method as described in **Figure 2** all have a 3' end sequence (A') that is the compliment of the first primer tail sequence (A), when the all DNA first primer is a tailed primer comprising sequence (A).

Stem loop nucleic acid structures for SPIA amplification

[0093] Alternatively, in some embodiments, the double stranded nucleic acid product of **Figure 1** may be used to generate amplified nucleic acid by way of a different method than depicted in **Figure 2**. In this method, the chimeric DNA-RNA second primer may comprise a tail sequence (A) that is the same or substantially the same as the tail sequence (A) of the all DNA first primer. The primer annealing sequences may be specific, or random sequence. The double stranded nucleic acid product of **Figure 1** in which the tail sequences of the first and second primer are substantially the same comprises a second primer extension product that may be separated from the first primer extension product by heat, chemical or enzymatic means and annealed to form a stem-loop structure.

[0094] The stem loop structure may comprise a DNA-RNA heteroduplex of RNA sequence (A), and complementary DNA sequence (A'). The annealing reaction may then be treated with a single strand DNA specific 3'-exonuclease such as for example exonuclease 1, and a single stranded nucleic acid specific RNase such as for example RNase 1. The exonuclease and RNase treatment may provide a clean-up step in that a substantial portion of extraneous nucleic acids such as the target nucleic acid, and the first and second primers are degraded. The degradation products may then be removed by a number of methods known in the art for removing nucleotide degradation products from polynucleic acids such as size exclusion spin columns, ion-exchange resin, ultrafiltration, dialysis, or any method known to the art. In some cases, removal of degradation products may not be required. In other cases, additional steps may be used to remove other undesirable compounds from the stem loop structure such as pyrophosphate and the like. In still other cases, the exonuclease and RNase may be inactivated by heat or chemical denaturation.

[0095] The resulting stem loop structure, for example, the purified stem loop structure may then be amplified by SPIA by first degrading the RNA portion of the DNA-RNA heteroduplex with for example RNase H, thus yielding a substantially linear nucleic acid comprising a sequence A' at the 3'-end. Amplification further comprises providing a chimeric amplification primer and a DNA polymerase with strand displacement activity. The chimeric amplification primer may comprise a DNA portion and a 5'-RNA portion wherein the chimeric amplification primer comprises a sequence which is substantially the same as sequence (A) or a portion of sequence (A).

[0096] Alternatively, the stem loop structure may be amplified by any other method of nucleic acid amplification known to the art such as but not limited to rolling circle template amplification, polymerase chain reaction (PCR), and linear polymerase chain reaction.

[0097] **Figure 3** depicts an embodiment for producing amplification products by the use of the SPIA method by way of a stem-loop intermediate product. Here, the tailed regions of both the first primer and the second

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primer comprise the sequence (A). In this case the hybridized first and second primer extension products of **Figure 1** are denatured, for example, by heat or chemical denaturation and subsequently are allowed to anneal. The result of having the same tailed sequence for the first and second primers is that the 3'-end and 5'-end of the second primer extension product comprises complimentary or substantially complementary sequences such that they are capable of hybridization to one another to form a stem-loop structure. Hybridization to form the stem –loop structures is carried out under conditions that are readily determined by one skilled in the art.

[0098] The annealing step can be carried out in such manner that the unimolecular nature of the self annealing of the second primer extension product is preferred over renaturation of the double stranded product of the method of **Figure 1.** The self annealed product thus forms a stem loop type structure. In some cases the length of time or rate of the annealing process, the addition of DNA binding proteins or enzymes, the use of chaotropic or cosmotropic solutes, or the concentration of first and primer extension products may be varied in order to promote stem-loop structure formation. Self annealing may be favored for certain sizes of the second primer extension product. In some cases, self annealing is favored by having a second primer extension product size that is smaller than about 100 kilobases or about 10 kilobases. In some cases, self annealing is favored for second primer extension product having sizes that are smaller than about 1 kilobase. In other embodiments, self annealing is favored for second primer extension product sizes are smaller than about 500 bases or between 10 and 500 bases. Where the stem-loop formation does occur for a given size range, this property can be used in order to select for that size range at the exclusion of the others, as the products which do not form the stem-loop structure may be susceptible to degradation by the enzymes described above. In some cases, the process selects for second primer extension products less than about 50, 80, 100, 200, 400, 500, 800, 1,000, 2,000, 4,000, 5,000, 8,000, 10,000, or 20,000 bases.

[0099] Step II shows the process of degradation of extraneous polynucleotides such as the target nucleic acid, the first primer extension product, and unincorporated primers. Degradation of the reaction components described above, is accomplished by using one or more enzymes that degrade single stranded nucleic acids, and to not degrade or do not substantially degrade double-stranded nucleic acids. Extraneous DNA polynucleotides may be removed by digestion with 3'-single stranded DNA specific exonucleases. Since sequences (A) and (A') of the second primer extension product are hybridized to form a double stranded portion, the stem-loop products are not degraded by these enzymes. Extraneous RNA polynucleotides may be removed by digestion with RNase 1. The combination may be used to remove extraneous chimeric primers such as the second primer depicted in **Figure 1.**

[00100] Step III shows a method for removing the RNA from the stem loop structure created in step I. RNase H is used to degrade the RNA portion of the DNA-RNA heteroduplex formed by the stem loop structure. This leads to an essentially linear polynucleic acid with a defined 3'- sequence (A'). The step of degradation of the RNA in the RNA-DNA heteroduplex by RNase H may be carried out in the reaction mixture for carrying SPIA amplification of the primer extension product as described below.

[00101] In step IV, SPIA amplification is performed in a manner as described in US Patent Nos. 6,251,639 and 6,692,918 using a mixture comprising a chimeric amplification primer that hybridizes to the A' sequence of step III, a strand displacing DNA polymerase, and RNase H.

[00102] In an alternative step not depicted, a chimeric oligonucleotide may be employed to allow the generation of amplified DNA with a defined 5' end. The suitable chimeric oligonucleotide may comprise a 3'-DNA sequence (A) capable of hybridizing with the (A') sequence of the linear polynucleic acid product of step

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III. The chimeric oligonucleotide may also comprise a 5'-RNA tail sequence (C). The linear polynucleic acid is extended with an RNA dependent DNA polymerase creating an RNA DNA heteroduplex of DNA sequence (C') and RNA sequence (C) which may be amplified by the SPIA method using a mixture comprising RNase H which cleaves RNA tail sequence C, an amplification primer that hybridizes to DNA tail sequence C', and a suitable polymerase. The amplified DNA products may then comprise a defined sequence (A) at or near its 5'-end.

Capture of target sequence on immobilized first primers

[00103] In some embodiments of the present invention the first primer is immobilized on a surface. The surface may be a chip or a slide such as for example a gene chip or a microarray. In other cases, the surface may comprise a bead such as for example an agarose bead, a polymeric bead, polystyrene beads, or a magnetic bead. In still other cases, the surface may be any surface known to the art capable of being derivatized with oligonucleotides. In some cases, the first primers are immobilized to discrete sites. In some cases, the discrete sites comprise immobilized primers with a specific known annealing sequence or set of sequences such that target nucleic acids of known sequence are immobilized at known discrete sites. The immobilized first primer, or primers, may be useful for capturing a targeted nucleic acid sequence (e.g. RNA or DNA) for subsequent amplification or analysis. The subsequent amplification may comprise SPIA, PCR, rolling circle template amplification or linear PCR. The immobilization may be performed by a number of methods known to the art. For example, the first primer, or primers, may be covalently linked to the surface by a photolabile, heat labile, reduction labile, oxidation labile, acid labile, or base labile linker. Alternatively, the immobilized first primer, or primers, may be hybridized to another nucleic acid or nucleic acid analog such as a locked nucleic acid, pyroanosyl nucleic acid, or peptide nucleic acid that is covalently linked to the surface. The immobilized first primer, or primers, may comprise a linker, as described in the following.

[00104] In some embodiments, because the captured target sequences that are produced are isolated as discrete populations, subsequent clonal amplification products can be generated and analyzed, for example sequenced, in a parallel manner. Therefore, in some embodiments, at least at least 100, 500, 1000, 10000, 50000, 100000, 300000, 500000, or 1000000 populations of clonal amplification products can be analyzed in parallel. The skilled artisan will appreciate that various methods can be suitable for parallel analysis of clonal amplicons. Generally, such methods can produce a discrete detectable signal that can be associated or linked to individual populations of clonal amplicons.

[00105] In some embodiments the target nucleic acid comprises DNA. In some cases, the DNA comprises genomic DNA such as for example the human genomic DNA from a subject or set of subjects. In other cases, the DNA comprises a subset of a genome. In still other cases, the DNA comprises cDNA generated from an RNA source. In some cases, the target nucleic acid comprises RNA. In some cases, the RNA comprises messenger RNA or total RNA. In some cases, the RNA comprises a transcriptome or a subset of a transcriptome.

[00106] In one aspect of the present invention the first primer comprises an immobilized all-DNA first primer. The first primer may comprise an annealing sequence (P), and a tail sequence (A). The annealing sequence may be random such that it is capable of hybridizing to substantially any portion of the target nucleic acid. The random annealing sequences may comprise for example random hexamers or random decamers. The annealing sequences alternatively may comprise specific sequences for hybridizing to a specific region of

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the target nucleic acid. In some cases, the annealing sequence of first primers comprise a set of specific target annealing sequences, wherein each first primer of the method comprises a specific target annealing sequence, such that a specific set of target nucleic acid regions are captured and or subsequently amplified. The sets may comprise a set of related sequences for hybridizing to a set of related genes, introns, exons, splice variants and the like. For example, the annealing sequences may hybridize to nucleic acid encoding serine/threonine kinases or their complement. In some cases, the annealing sequences may be randomized at certain positions and specific at other positions, such that they may hybridize to more than one member of a set of homologous target nucleic acids.

[00107] The immobilized all DNA first primer may be annealed to a target nucleic acid and extended using a polymerase to generate a first primer extension product. In some cases, the polymerase may comprise substantially no strand displacement activity. In other cases, a polymerase may be chosen that possesses a substantial amount of strand displacement activity. In some cases, a substantially thermostable polymerase that is maximally active above about 60°C such as Taq polymerase for example may be used, for example to enable higher processivity or avoid problems with secondary structure within primers and or target nucleic acid. In other cases, a polymerase that is slightly thermostable that is maximally active between 40°C and 60°C may be used. In still other cases, a mesophilic polymerase that is maximally active below 40°C may be used to extend the first and or second primer. In some cases, an RNA-dependant DNA polymerase may be used, while in other cases a DNA dependant DNA polymerase may be used. In some cases, it may be advantageous to use a polymerase that does not exhibit either 3'-5' or 5'-3'-exonuclease activity. In other cases 3'-5' or 5'-3'-exonuclease activity may be advantageous. In some cases, the polymerase used to generate the first primer extension product may be a reverse transcriptase. In some cases, the use of a polymerase with high processivity may be advantageous. In some cases, the target nucleic acid may be removed after generation of the first primer extension product by heat or chemical denaturation and or degradation. In other cases, the target nucleic acid may be removed by enzymatic methods.

[00108] In some embodiments of the present invention, target nucleic acids are amplified using a chimeric DNA-RNA second primer. The chimeric second primer may comprise an RNA segment and a DNA segment. In some cases, the RNA segment is generally at the 5' end and the DNA segment is generally at the 3' end of the chimeric primer. In some cases, a portion of the RNA segment comprises a tail sequence that may comprise the same or substantially the same sequence (A) as the tail sequence of the all DNA first primer. In some cases, the tail sequence comprises a different tail sequence (B) than the tail sequence (A) of the all DNA first primer.

[00109] In some cases, a portion of the DNA segment of the chimeric second primer comprises a target nucleic acid annealing sequence. In some cases, the target nucleic acid annealing sequence is random such that it may hybridize with any, or substantially any, region of the target nucleic acid. In other embodiments, the annealing sequence is specific for a region of the target nucleic acid. In still other embodiments, a set of chimeric DNA-RNA second primers comprising a set of non-random annealing sequences may be utilized to amplify a set of target nucleic acid regions in a multiplex fashion as described previously.

[00110] In some cases the chimeric DNA-RNA second primer may be annealed to a target nucleic acid or to the immobilized first primer extension product and extended using a polymerase to generate an immobilized second primer extension product. The polymerase may be a thermostable or mesophilic DNA dependant

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DNA polymerase as described above. Additionally, the polymerase may or may not exhibit substantial strand displacement or error correcting activity.

[00111] **Figure 4** depicts a method for using DNA primers immobilized on a solid substrate that can capture of target nucleic acid from solution. Step I shows a surface to which all DNA first primers are immobilized. The first primers comprise a DNA tail sequence (A), and a DNA target nucleic acid annealing sequence (P) as described above. While the solid substrate is depicted in **Figure 4** as a spherical particle, it is to be understood that the method depicted can be carried out on a solid substrate of any suitable geometry, including wells, planar surfaces, and other geometries described herein. The surface may comprise, for example a particle or bead, a nanoparticle, a microarray, a glass slide, a chip or cassette, a polynucleic acid chip, a fused fiber optic array, a polymer, or any type of surface known to the art to which nucleic acid, such as an oligonucleotide may be attached.

[00112] The method of attachment may or may not require a linker element between the first primer and the surface. The linker element may be a spacer element such as by way of example ethylene glycol, polyethylene glycol, polyvinyl, polystyrene, polyether, a saturated carbon chain, an unsaturated carbon chain, a nucleic acid sequence, an amino acid sequence or any other spacer element capable of linking a nucleic acid to a surface known to the art. The spacer element may also comprise a cleavable element for removal of immobilized first primers from the surface. The cleavable spacer element may be cleaved through the action of a strong base, a strong acid, heat, light, reduction, oxidation, enzymatic reaction, or any of a variety of other methods known to those skilled in the art.

[00113] Step I also shows the annealing of a target nucleic acid to the immobilized first primer. The target nucleic acid may comprise RNA or DNA. The target nucleic acid may comprise fragments from an RNA or DNA sample. The annealing may occur between the sequence (P) of the first primer and a sequence on the target nucleic acid. The sequence on the primer that anneals to the target nucleic acid may be a random sequence, a target specific sequence, or a set of target specific sequences. In some embodiments a plurality of specific primer sequences are used, wherein, for example, different primer sequences are immobilized on different regions of a microarray or different sequences are immobilized on different beads.

[00114] In step II, the first primer is extended by the action of a polymerase to create a first primer extension product. In the case of a DNA target nucleic acid, it may be advantageous to use a DNA dependent DNA polymerase. In the case of an RNA target nucleic acid, it may be advantageous to use an RNA dependent DNA polymerase or reverse transcriptase. In some embodiments it may be advantageous to utilize a polymerase that does or does not exhibit substantial strand displacement activity. It may also be advantageous to extend the first primer with a thermostable or a thermolabile polymerase. The thermostable polymerase may exhibit higher rates of polymerization, fewer difficulties with secondary structure of target nucleic acid or first primer, or greater fidelity. The thermolabile polymerase may exhibit greater fidelity, greater rates of polymerization, or enable facile removal of polymerase activity by heat denaturation. In some cases, the use of other nucleic acid binding proteins, or reaction additives, during the extension step is anticipated. The proteins or additives include but are not limited to DMSO, DMF, cations, anions, glycerol, single-stranded nucleic acid binding proteins including but not limited to T4 gene 32 protein (gp32), buffers, and salts.

[00115] The target nucleic acid and the first primer extension product are then separated by heat, chemical, or enzymatic treatment, and a second DNA-RNA chimeric primer is then annealed to the first primer extension product in step III. The primer may comprise a 5' tail sequence (A) or (B) and an annealing

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sequence (P) as described previously. The annealed second primer then may be used to extend the primer to create a second primer extension product complementary to the entire, or a portion of the entire, first primer extension product as depicted in step IV. As described for extension of the first primer, it is anticipated that for certain aspects of the present invention, the DNA polymerase used to create the second primer extension product may be thermostable, thermolabile, strand displacing, non strand displacing, high fidelity, highly processive, or any combination thereof.

Generation of amplified DNA from captured target sequences

[00116] In some embodiments of the present invention, the immobilized double stranded nucleic acid product depicted in **Figure 4** may be used to generate amplified DNA. Amplified DNA may be generated by a number of methods known to the art. Preferred amplification methods include but are not limited to PCR, SPIA, rolling circle amplification, loop-mediated isothermal amplification, ligation mediated rolling circle amplification and the like. In the case of SPIA amplification, the method of amplification may further comprise providing a single stranded DNA specific 3'-exonuclease such as exonuclease 1, or a polymerase with 3'-exonuclease activity such as for example T4 DNA polymerase. The exonuclease or exonuclease activity may degrade the 3'-portion of the first primer extension product that is not hybridized to the second primer extension product. The first primer extension product may then be extended with a polymerase such as Bst that exhibits reverse transcriptase activity. The extension may generate a DNA-RNA heteroduplex at one end. The immobilized first and second primer extension products comprising a DNA-RNA heteroduplex at one end may be amplified by SPIA by providing an RNase such as RNaseH to cleave the RNA portion of the heteroduplex, a chimeric amplification primer, and a polymerase with strand displacing activity. The chimeric amplification primer may comprise a DNA portion and a 5'-RNA portion wherein the chimeric amplification primer comprises a sequence which is substantially the same as sequence (A) or (B) or a portion of sequence (A) or (B). The amplified nucleic acid may comprise a sequence tail (A') at the 3'-end.

[00117] A method of generating amplified DNA using SPIA from the immobilized first and second primer extension products depicted in **Figure 4** is shown in **Figure 5**. This method generates amplified product directly from an extension product of the primer immobilized to the solid substrate. Step I of **Figure 5** depicts the use of a single stranded DNA specific 3'-exonuclease such as exonuclease 1 or the 3'-exonuclease activity of a DNA polymerase such as T4 DNA polymerase to remove the portion of the 3'-end of the first primer extension product that is not hybridized to the second primer extension product. In step II, the immobilized first primer extension product or first primer extension products are then extended by a DNA polymerase that exhibits reverse transcriptase activity. A DNA polymerase that exhibits reverse transcriptase activity includes but is not limited to Bst polymerase. Step III depicts the use of RNase H to cleave the RNA portion of the DNA-RNA heteroduplex. This provides a site for annealing an amplification primer to the priming site A' or B', or a portion of site A' or B', as depicted in step IV. Thus amplified DNA products with a defined 3'-end sequence (A') may be generated by SPIA methods using a reaction mixture comprising RNase H, a DNA polymerase with strand displacement activity, and a chimeric amplification primer.

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Generation of amplified DNA from immobilized target sequence using solution phase SPIA

[00118] In one aspect of the present invention, the immobilized double stranded first and second primer extension product depicted in **Figure 4** may be amplified by solution phase SPIA. In some cases, the double stranded nucleic acid product depicted in **Figure 4** may be treated with a single stranded DNA specific 3'-exonuclease to remove the 3'-portion of the first primer extension product that is not hybridized to the second primer extension product. The immobilized first and second primer extension product may then be cleaved from the surface releasing the first and second primer extension product into the solution phase. The cleavage may be performed by application of heat, base, acid, reducing agent, oxidation or any method known to the art. The cleavage may occur at or within the linker between the nucleic acid portion of the all DNA first primer and the surface. Alternatively, the cleavage may occur before exonuclease treatment.

[00119] The first primer extension product may then be extended using a polymerase such as Bst that exhibits substantial reverse transcriptase activity. The extension may produce a double stranded nucleic acid a portion of which comprises a DNA-RNA heteroduplex. The double stranded nucleic acid may be amplified using any method of nucleic acid amplification known to the art such as but not limited to PCR, linear PCR, SPIA, rolling circle amplification, loop-mediated isothermal amplification, ligation mediated rolling circle amplification and the like. Alternatively, the extension of the first primer extension product in the complex with the second primer extension product may be extended with a DNA polymerase comprising an RNA-dependent DNA polymerase activity, such as for example Bst polymerase, prior to the release from the surface, such that the released complex comprises an RNA-DNA heteroduplex at one end.

[00120] **Figure 6** depicts an alternative method for producing amplified DNA using SPIA from the immobilized first and second primer extension products depicted in **Figure 4.** Here, the products of the first steps of the method are cleaved from the solid surface such that the SPIA amplification occurs in solution rather than occurring on the solid surface. While the figure depicts cleavage occurring after the exonuclease step of the method, it is understood that the cleavage can occur at any suitable step before or after this step. Step I depicts a 3'-exonuclease step as described previously for degrading extraneous nucleic acids including but not limited to target nucleic acid and unincorporated primers, as well as trimming the unhybridized 3'-end of the first primer extension product. Step II depicts the use of a cleaving step to release the hybridized first and second primer extension products from the surface or particle to which they are immobilized. The cleavage mechanisms anticipated include chemical, enzymatic, or light as described previously. Following cleavage, the remaining steps are carried out in solution. Step III depicts the use of a DNA polymerase such as but not limited to Bst polymerase to extend the first primer extension product. Step IV depicts the use of RNase H to produce an amplification primer hybridization site. Step V depicts the use of SPIA to generate amplified DNA products having a defined 3'-DNA sequence (A').

Capture of target sequences for formation of stem loop strucutres

[00121] In some embodiments of the present invention an all DNA first primer comprising a tail sequence (A) immobilized to a bead or surface may be annealed to capture target nucleic acid sequences and extended, and a chimeric second primer may be annealed comprising a tail sequence (A) which is the same or substantially the same as the tail sequence of the all DNA first primer and extended. The capture and first and second primer extension steps may be performed by the methods described previously. The second primer extension products may be used further to generate a stem loop nucleic acid structure. The stem loop nucleic acid structure may be amplified by a number of methods known the art as described previously

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such as but not limited to PCR, linear PCR, SPIA, rolling circle amplification, loop-mediated isothermal amplification, ligation mediated rolling circle amplification and the like.

[00122] **Figure 7** depicts an alternative method for amplification of captured of target nucleic acid using immobilized primers. This method utilizes the same sequence, e.g. sequence (A) in the 5'-tail of both the first and the second primers. As described above, this method produces a product which can form a stem-loop structure, which facilitates clean-up of the desired products prior to SPIA amplification. Step I depicts the use of immobilized all DNA first primers with a 5'-tail sequence (A) and a 3'-template annealing sequence (P) for annealing to a target nucleic acid as described previously. Step II depicts the use of an RNA or DNA dependant DNA polymerase to extend the first primer and produce a first primer extension product as described previously. In step III, the target nucleic acid is removed by heat, enzymatic, or chemical means, and a DNA-RNA chimeric second primer comprising a 5'-tail sequence (A) and 3' template annealing sequence (P) as described previously. For the method depicted in this figure, it may be advantageous to ensure that the 5'-tail sequence of the second primer is identical to or substantially the same as the 5'-tail sequence of the first primer. In step IV the annealed second primer is extended with a DNA polymerase as described previously to yield a second primer extension product.

Generation of stem loop amplification substrate from captured target sequences

[00123] In some embodiments of the present invention, the second primer extension product or products of a captured target sequence as described previously, may be utilized to generate amplified DNA. The amplified DNA may be separated from the first primer extension product by heat or chemical denaturation, and annealed to form a stem loop structure. In some cases, primers and other extraneous nucleic acids may be removed from the reaction by degradation before or after separation and annealing of the second primer extension product. For example, unextended first and second primers and target nucleic acid may be degraded with a single stranded DNA specific 3'-exonuclease and or a single and or double stranded RNA specific RNase as described previously. The stem loop structure may be resistant to degradation by the exonuclease and RNase because of a lack of a single stranded 3'–DNA or RNA end. The stem loop structure may then be amplified by any methods known in the art such as but not limited to PCR, linear PCR, SPIA, rolling circle amplification, loop-mediated isothermal amplification, ligation mediated rolling circle amplification and the like.

[00124] **Figure 8** depicts a method of utilizing the products depicted in **Figure 7** for the generation of a substrate suitable for DNA amplification using the SPIA method. Step I is an optional step. In step I, a 3'-exonuclease is used as described previously to remove extraneous nucleic acids, and trim the 3'-end of the first primer extension product. In step II, the second primer extension product is separated from first primer extension product by heat or chemical means. The second primer extension product is then annealed to create a stem loop structure as described previously. Exonucleases and RNase 1 can then be added to remove extraneous polynucleotides and unincorporated primers. The stem loop structure then acts as a suitable substrate for SPIA amplification to generate amplified DNA. Alternatively, a chimeric oligonucleotide may be employed to generate amplified DNA with a defined 5'-end as described in **Figure 3** alternative step.

Amplification of RNA using a chimeric second primer

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[00125] In some embodiments of the present invention, amplified DNA can be produced from an RNA template using an all DNA first primer comprising a tail sequence (A) or (B) and an annealing sequence (P) as described previously for amplification of a specific sequence, multiplex amplification, or whole transcriptome amplification. In some cases, the first primer extension product is formed by providing a RNA dependant DNA polymerase. In some cases, further steps may include the removal of RNA target nucleic acid by heat, chemical, heat and chemical, or enzymatic means. For example, RNA template may be removed by providing RNase H and RNase1 to degrade any single and or double stranded RNA and any RNA portion of an RNA-DNA heteroduplex. In one aspect of the present invention a chimeric DNA-RNA second primer as described previously comprising a tail sequence (B) that is substantially different from the tail sequence (A) in the all DNA first primer may be annealed to the first primer extension product and extended as described previously to create a second primer extension product. In some cases, a single stranded DNA specific 3'-exonuclease may be provided to degrade unincorporated first and second primers as well as any unhybridized 3'-end of the first primer extension product. The degraded 3'-end of the first primer extension product may then be extended using a polymerase such as for example Bst that exhibits reverse transcriptase activity to generate a double stranded nucleic acid product comprising the first and second primer extension product and a DNA-RNA heteroduplex at one end. The first and second primer extension product may then be used to generate amplified DNA using a number of methods known to the art such as but not limited to PCR, linear PCR, SPIA, rolling circle amplification, loop-mediated isothermal amplification, ligation mediated rolling circle amplification and the like. In some cases, SPIA is used to generate amplified products comprising a defined 3'-end sequence (A').

[00126] **Figure 9** depicts a method for producing amplified DNA from an RNA template using a SPIA method using an all DNA first primer of tail sequence (A), a DNA-RNA chimeric second primer of tail sequence (B), and an RNA target nucleic acid. It is also anticipated that in some cases, a second primer tail sequence (A) may be employed. In this method, the first primer is annealed to the target in a target specific or random fashion and is extended with an RNA dependent DNA polymerase or reverse transcriptase to generate a first primer extension product. Target nucleic acid is removed by RNase H and RNase 1 as depicted, or any other RNases or combination thereof known in the art, which could be easily inactivated so as not to interfere with the following steps of the methods of the invention. It is also anticipated that other methods may be employed to remove RNA target nucleic acid selectively such as a thermal or chemical method. Following removal of target nucleic acid, the chimeric second primer comprising RNA containing tail sequence (B) is annealed to the first primer extension product and extended. A single stranded DNA specific 3'-exonuclease is then employed to trim back the unhybridized 3'-end of the first primer extension product, and a third DNA polymerase step is then used to create a double stranded hybridized product with a defined DNA-RNA heteroduplex end of DNA sequence (B') and RNA sequence (B), and a DNA-DNA duplex end of sequence (A) and (A'). This product of the method depicted in **Figure 9** may then be amplified DNA of a defined 3'-sequence (A') is then produced by the SPIA method using a mixture of RNase, amplification primer, and a suitable polymerase.

Generation of amplified DNA having a defined 5' and 3' end

[00127] In some embodiments of the present invention, a double stranded nucleic acid comprising a first and second primer extension product and further comprising a DNA-RNA heteroduplex at one end may be used to generate amplified DNA comprising a defined 5' and 3'-end. In some cases, the RNA portion of the DNA-

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RNA heteroduplex may be cleaved or degraded by an enzyme such as RNase H. The method may further include providing a DNA-RNA chimeric oligonucleotide. The chimeric oligonucleotide may comprise a 5'-segment and a 3'-segment. The 5'-segment may comprise RNA and the 3'-segment may comprise DNA. The 3'-DNA segment may comprise a sequence (B) the same or substantially the same as the RNA portion of the DNA-RNA heteroduplex degraded by RNase H. The 5'-RNA segment may comprise a sequence (C) that is substantially different from previously described sequences (A) and (B). The chimeric oligonucleotide may be annealed to the first primer extension product such that sequence (B) of the chimeric oligonucleotide hybridizes with sequence (B') of the first primer extension product. The first primer extension product may then be extended by a polymerase as described previously. The resulting double stranded nucleic acid comprising a first and second primer extension product and a DNA-RNA heteroduplex may then be amplified by a number of methods known to the art such as but not limited to PCR, linear PCR, SPIA, rolling circle amplification, loop-mediated isothermal amplification, ligation mediated rolling circle amplification and the like. In some cases, SPIA may be used to generate amplified products comprising a defined 3' end sequence (A') and a defined sequence at or near its 5'-end comprising sequence (B).

[00128] **Figure 10** depicts an alternative method of generating amplified DNA using the SPIA method from the product the method depicted in **Figure 9**. In this method, RNase H is used to degrade the RNA portion of the DNA-RNA (B')-(B) heteroduplex and a chimeric oligonucleotide comprising 3'-DNA sequence (B) and a 5'-RNA sequence (C) is annealed to the DNA sequence (B'). The first primer extension product is then extended using a suitable polymerase to create a double stranded nucleic product with a DNA-RNA heteroduplex at one end of DNA sequence (C') and RNA sequence (C) and a DNA-DNA duplex at the other end of sequence (A) and (A'). Following the extension step, SPIA amplification is then employed using a mixture of RNaseH, an RNA-DNA chimeric amplification primer, and a suitable polymerase as described previously. In this case, the products of the SPIA amplification have a defined 3' sequence (A') and a defined 5' sequence (B).

Generating a SPIA substrate with a ligand

[00129] In some embodiments of the present invention a double stranded nucleic acid comprising a first and second primer extension product and further comprising a ligand may be generated. The ligand may be useful for capture of the double stranded nucleic acid, purification, or further downstream manipulation such as sequencing for example. The ligand may be but is not limited to a biotin molecule or other small organic molecule like a fluorophore, a peptide, a nucleic acid, a magnetic particle, a bead, a nanocrystal or quantum dot, a protein, or any other ligand capable of being linked to a nucleic acid covalently or non-covalently. In some cases, the ligand is linked to an all DNA first primer comprising a tail sequence (A) and an annealing sequence (P) as described previously. The all DNA first primer is annealed to a target nucleic acid (e.g. RNA or DNA) and extended using a polymerase as described previously to generate a first primer extension product linked to a ligand. The first primer extension primer may comprise random sequences, a specific sequence, or a set of multiple specific sequences as described previously.

[00130] In some cases, a DNA-RNA chimeric second primer comprising a tail sequence (A) or (B) and an annealing sequence as described previously may be annealed to the first primer extension product and extended using a DNA polymerase to generate a second primer extension product. The first and second primer extension product may further be treated with a single stranded DNA specific 3'-exonuclease to

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generate a double stranded nucleic acid comprising the first and second primer extension products, a ligand, and a DNA-RNA heteroduplex. In some embodiments, the double stranded nucleic acid product may be amplified using a number of methods known to the art such as but not limited to PCR, linear PCR, SPIA, rolling circle amplification, loop-mediated isothermal amplification, ligation mediated rolling circle amplification and the like. In some cases SPIA may be used to generate an amplified DNA product with a 3'-end comprising a defined sequence (A').

[00131] **Figure 11** depicts a method for using a ligand attached to an all DNA first primer, a DNA-RNA chimeric second primer and an RNA template nucleic acid to generate amplified DNA products using SPIA. The method follows the method outlined in **Figure 9** with exception of the ligand attached all DNA first primer of tail sequence (A). Various nucleic acid ligands anticipated by the present invention are known to the art and include but are not limited to biotin, proteins such as antibodies, antibody fragments, avidin, streptavidin, maltose binding protein, S-protein, enzymes, peptides such as hexahistidine, myc tag, flag tag, HA tag, or any affinity tag or member of any pair of binding agents known in the art, fluorophores, particles, nanoparticles, magnetic particles, or other nucleic acid sequences. Methods for attaching ligands to nucleic acid primers are known to the art. The ligand may be used for attachment to a solid substrate, for example for archiving and clonal expansion. The ligand may be useful for functionalizing the first primer extension product for further chemical or enzymatic manipulation, or for purification of the first and or second primer extension products. The SPIA amplified products of the method depicted in this figure have a 3' end comprising the defined sequence (A').

Generating a SPIA substrate with a ligand and amplified product with two defined ends

[00132] In some embodiments of the present invention a double stranded nucleic acid SPIA substrate may be generated having a ligand and a two defined ends comprising a first and second primer extension product. The ligand may be useful for capture of the double stranded nucleic acid, purification, or further downstream manipulation such as sequencing for example. The ligand may be but is not limited to a biotin molecule or other small organic molecule like a fluorophore, a peptide, a nucleic acid, a magnetic particle, a bead, a nanocrystal or quantum dot, a protein, or any other ligand capable of being linked to a nucleic acid covalently or non-covalently. In some cases, the ligand is linked to an all DNA first primer comprising a tail sequence (A) and an annealing sequence (P) as described previously. The all DNA first primer is annealed to a target nucleic acid (e.g. RNA or DNA) and extended using a polymerase as described previously to generate a first primer extension product linked to a ligand. The first primer extension primer may comprise random sequences, a specific sequence, or a set of multiple specific sequences as described previously.

[00133] In some cases, a DNA-RNA chimeric second primer comprising a tail sequence (A) or (B) and an annealing sequence as described previously may be annealed to the first primer extension product and extended using a DNA polymerase to generate a second primer extension product. The first and second primer extension product may further be treated with a single stranded DNA specific 3'-exonuclease to generate a double stranded nucleic acid comprising the first and second primer extension products, a ligand, and a DNA-RNA heteroduplex.

[00134] The double stranded nucleic acid product may be further treated with RNase H to degrade or cleave the RNA portion of the DNA-RNA heteroduplex. In some cases, a DNA-RNA chimeric oligonucleotide comprising a 5'-RNA sequence (C) and a 3'-DNA sequence (B) as described previously may be annealed

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to the first primer extension product. The first primer extension product may be further extended with a polymerase exhibiting substantial reverse transcriptase activity such as for example Bst polymerase. The further extension may generate a double stranded first and second primer extension product comprising a ligand and DNA-RNA heteroduplex. In some embodiments, the double stranded extension product may be amplified using a number of methods known to the art such as but not limited to PCR, linear PCR, SPIA, rolling circle amplification, loop-mediated isothermal amplification, ligation mediated rolling circle amplification and the like. In some cases SPIA may be used to generate an amplified DNA product with a 3'-end comprising a defined sequence (A') and a 5'-end comprising sequence (B).

[00135] **Figure 12** depicts a method as in the previous figure for producing amplified DNA using a ligand attached all DNA first primer. In this method a chimeric oligonucleotide is used to enable the generation of amplified DNA using the SPIA method comprising a defined 3'-end sequence (A') and a defined 5'-end sequence (B).

Amplification of a target nucleic acid using an non-tailed all DNA first primer

[00136] In some embodiments of the present invention, a method for amplification of a target nucleic acid (DNA or RNA) may comprise using an all DNA first primer that comprises an annealing sequence (P) as described previously but the all DNA first primer may not comprise a tail sequence. The all DNA first primer may be annealed to a specific, random or multiple regions of the target nucleic acid and extended with a polymerase as described previously to generate a first primer extension product. In some cases, a set of first primers may be used for amplification of a set of target sequences within the target nucleic acid in a multiplex fashion. A DNA-RNA chimeric second primer comprising a tail sequence (A) or (B) and an annealing sequence (P) as described previously may then be annealed to the first primer extension product and or the target nucleic acid and extended with a polymerase. The first and second primer extension products, primers, and target nucleic acid may then be treated with a single stranded DNA specific 3'-exonuclease such as exonuclease 1 and or a single and or double stranded RNA specific RNase such as RNase 1 to degrade unincorporated primers, unhybridized target nucleic acid, unhybridized 3'-ends of the first primer extension product, and unincorporated primers. The remaining target nucleic acid, and first and second primer extension products may then be extended with a polymerase exhibiting substantial reverse transcriptase activity such as for example Bst polymerase to generate a double stranded nucleic acid with a DNA-RNA heteroduplex. In some cases, the double stranded nucleic acid may be amplified using a number of methods known to the art such as but not limited to PCR, linear PCR, SPIA, rolling circle amplification, loop-mediated isothermal amplification, ligation mediated rolling circle amplification and the like. In some cases SPIA may be used to generate an amplified DNA product with a 3'-end comprising a defined sequence (A') and in some cases a 5'-end comprising sequence (B).

[00137] **Figure 13** depicts a method of producing amplified DNA using the SPIA method with an all DNA first primer where the first primer has no tail sequence, but comprises a template annealing sequence (P). The template annealing sequence may be target specific or randomized. The method further comprises a DNA-RNA second primer of tail sequence (B), an annealing sequence for annealing to the first primer extension product, and a DNA target nucleic acid. The use of randomized annealing sequences may require the use of a non-strand displacing polymerase in order to avoid whole genome amplification. In some cases, a non-strand-displacing polymerase may enable sequence specific amplification when using a sequence-specific annealing sequence with one primer (first or second) and a randomized annealing sequence with another

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primer. In some cases, a non-strand-displacing polymerase may enable sequence specific amplification when using a sequence-specific annealing sequence with an all DNA first primer and a random annealing sequence with an RNA-DNA chimeric second primer. Alternatively, whole genome amplification may be desired in which case it may be advantageous to employ a strand displacing polymerase to generate the first or second primer extension products or both. In some cases, the use of a strand displacing polymerase is advantageous to generate longer primer extension products or to enable the generation of second primer extension products.

[00138] **Figure 14** depicts a method of producing amplified DNA using the SPIA method with a sequence specific non-tailed all DNA first primer comprising an annealing sequence (P). In step I, the first primer is hybridized to a DNA target nucleic acid, and extended using a DNA polymerase. The method further comprises a DNA-RNA chimeric second primer comprising tail sequence (B) and an annealing sequence for annealing to the first primer extension product. The annealing sequence of the chimeric second primer may be a randomized sequence. In step II, the double stranded complex of the DNA target nucleic acid and the first primer extension product is denatured, and the second primer is annealed to the first primer extension product. Figure 14 also shows that some of the second primer may anneal to the other DNA strand, not containing the target sequence, or other portions of the DNA strand comprising the target sequence, but that most of the products from these reactions may result in SPIA amplified products. The second primer is extended in step III with a DNA polymerase. The DNA polymerase may lack strand displacement activity. In step IV a 3'-exonuclease is used to remove the non-hybridized portion of the first primer extension product. In step V, a DNA polymerase is used to extend the first primer extension product. The product of step V can then be used as a substrate for SPIA amplification using a chimeric amplification primer as shown in step VI. and a DNA target nucleic acid.

Additional amplification strategies using all DNA first primers

[00139] In **Figure 15**, a ligand attached all DNA first primer comprising a 5' tail sequence (A), a 3'-annealing sequence (P), and a DNA template nucleic acid. This figure depicts a method for amplifying two stands of the DNA template nucleic acid simultaneously. The ligand attached primer also allows functionalization and or purification of one of the strands to enable amplification of either strand selectively. In the first step, the ligand attached all DNA first primer is annealed to a target DNA and extended with a DNA dependent DNA polymerase to create a first primer extension product. The method further comprises denaturing the hybridization complex between the target nucleic acid and the first primer extension product, and annealing a second primer to the first primer extension product as well as the target DNA. The second primer comprises a 5'-segment a portion of which comprises RNA comprising a tail sequence (B) and a 3'-segment a portion of which comprises DNA comprising an annealing sequence (P) as described previously. The second primer may then be extended by a DNA dependent DNA polymerase to create a second primer extension product. In some cases, the first and second primer extension product form a double stranded nucleic acid product. In other cases, the second primer extension product and the target DNA form a double stranded nucleic acid product. The method further comprises treating the double stranded nucleic acid products with a single stranded DNA specific 3'-exonuclease and a DNA polymerase to create two double stranded nucleic acid products. In one case the double stranded nucleic acid product comprises a ligand at one end and a DNA-RNA heteroduplex comprising sequence (B) and (B') at the other end. In another case, the double stranded nucleic acid product does not comprise a ligand at one end. These double

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stranded nucleic acid products may act as substrates for SPIA amplification by providing a reaction mixture comprising RNase H, a chimeric amplification primer comprising a 5'-RNA segment and a 3'-DNA segment as described previously, and a DNA polymerase with substantial strand displacing activity.

[00140] **Figure 16** depicts a method of producing amplified DNA using the SPIA method without the use of a DNA-RNA chimeric second primer. In this method, an all DNA first primer of 5'-tail sequence (A) and a random or target specific template nucleic acid annealing sequence (P) is used to form the first primer extension product hybridized to a DNA or RNA template nucleic acid as previously described. In step II, the template nucleic acid is separated from the first primer extension product and an all DNA second primer comprising an annealing sequence that is annealed to the first primer extension product. The resulting double stranded first and second primer extension products are optionally treated with a 3'-exonuclease as described, the exonuclease is removed or inactivated and the first and second primer extension products are denatured. A chimeric oligonucleotide comprising a 5'-RNA sequence (C) and a 3'-DNA sequence (A) is then annealed to the second primer extension product, generating a substrate suitable for subsequent SPIA amplification. The SPIA amplifications products comprise a defined 5'-sequence (A).

[00141] **Figure 17** depicts an alternative method for producing amplified DNA using the SPIA method without the use of a DNA-RNA chimeric second primer. This method allows for the production of amplified product with defined sequences at or near both ends of the amplified product. The method comprises an all DNA second primer comprising a 5'-tail sequence (B) and a 3'-template annealing sequence (P). The method generates a substrate suitable for subsequent SPIA amplification. The SPIA amplification products comprise a defined 3'-sequence (B') and a defined 5'-end comprising sequence (A). In step I, an all DNA first primer comprising a 5'-segment comprising tail sequence (A) is annealed to a target nucleic acid and extended with a DNA polymerase to create a first primer extension product. In step II, the first primer extension product is denatured from the target nucleic acid, and an all DNA second primer as described hereinabove is annealed to the first primer extension product. In some cases, the target nucleic acid comprises RNA which is degraded in this step by heat, enzymes, chemical degradation etc. In step III, the second primer is extended with a DNA polymerase to create a double stranded nucleic acid product. Step IV depicts an optional step in which the double stranded nucleic acid product is treated with a single stranded DNA specific 3'-exonuclease. In step V, the double stranded nucleic acid product of step III or IV is denatured by heat or chemical means. In step VI a DNA-RNA chimeric olignucleotide (or "oligo") as described previously is annealed to the second primer extension product. In step VII, the chimeric oligonucleotide is extended to yield a third primer extension product comprising a 3'- portion comprising sequence (B') and a 5'-portion comprising RNA comprising sequence (C). Step VII further comprises extending the second primer extension product such that the 3'- segment comprises sequence (C'). Step VII yields a double stranded nucleic acid comprising sequence (B) and (B') at one end and a DNA-RNA heteroduplex comprising sequence (C) and (C') at the other end.

[00142] **Figure 18** depicts a method of amplifying a target sequence from the double stranded nucleic acid product of **Figure 17** by employing the double stranded nucleic acid product as a substrate for SPIA amplification. In the first step of **Figure 18**, RNase H cleaves the RNA portion of the (C')-(C) DNA-RNA heteroduplex of the double stranded nucleic acid product, and a chimeric amplification primer as described previously is annealed to the DNA sequence (C'). In the next step, SPIA amplification products comprising a 3' end comprising sequence (B') and a 5'-end comprising sequence (A) are generated by providing RNase H, a chimeric amplification primer, and a strand displacing DNA polymerase.

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[00143] .While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

General Techniques

[00144] The practice of the invention may 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).

[00145] The terms "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, carbocyclic sugar analogs, .alpha.-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

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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.

[00146] "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. The oligonucleotide(s) are generally comprised of a sequence of at least 5 nucleotides, generally from about 10 to about 100 nucleotides, about 20 to about 50 nucleotides, and often about 10 to about 30 nucleotides in length. The oligonucleotides of the invention can be DNA, RNA, DNA-RNA, or other polynucleotide. The terms oligonucleotide or sequence may be used interchangeably herein.

[00147] Various techniques can be employed for preparing an oligonucleotide utilized in the present invention. Such oligonucleotide can be obtained by biological synthesis or by chemical synthesis. For short sequences (up to about 100 nucleotides) chemical synthesis may frequently be more economical as compared to the biological synthesis. In addition to economy, chemical synthesis provides a convenient way of incorporating low molecular weight compounds and/or modified bases during the synthesis step. Furthermore, chemical synthesis is very flexible in the choice of length and region of the target polynucleotide binding sequence. The oligonucleotide can be synthesized by standard methods such as those used in commercial automated nucleic acid synthesizers. Chemical synthesis of DNA on a suitably modified glass or resin can result in DNA covalently attached to the surface. This may offer advantages in washing and sample handling. For longer sequences standard replication methods employed in molecular biology can be used such as the use of M13 for single stranded DNA as described by J. Messing (1983) Methods Enzymol, 101, 20-78.

[00148] In the present invention, nucleoside triphosphates are incorporated by a polymerase enzyme in the extension of the primer to produce an extension product. Nucleoside triphosphates are generally nucleosides having a 5'-triphosphate substituent. The nucleosides are pentose sugar derivatives of nitrogenous bases of either purine or pyrimidine derivation, covalently bonded to the 1'-carbon of the pentose sugar, which is usually a deoxyribose or a ribose. The purine bases include adenine (A), guanine (G), inosine (I), and derivatives and analogs thereof. The pyrimidine bases include cytosine (C), thymine (T), uracil (U), and derivatives and analogs thereof. Nucleoside triphosphates include deoxyribonucleoside triphosphates such as the four common triphosphates dATP, dCTP, dGTP and dTTP and ribonucleoside triphosphates such as the four common triphosphates rATP, rCTP, rGTP and rUTP. The term "nucleoside triphosphates" also includes derivatives and analogs thereof, which are exemplified by those derivatives that are recognized in a similar manner to the underivatized nucleoside triphosphates. Examples of such derivatives or analogs, by way of illustration and not limitation, are those which are biotinylated, amine modified, alkylated, and the like and also include phosphorothioate, phosphite, ring atom modified derivatives, and the like.

[00149] As used herein, the term “nucleotide” generally refers to a base-sugar-phosphate combination that is the monomeric unit of nucleic acid polymers, i.e., DNA and RNA. In some aspects of the invention modified nucleotides are used, for example, where a nucleotide is connected to a ligand. A modified nucleotide is generally the unit in a nucleic acid polymer that results from the incorporation of a modified nucleoside triphosphate during an amplification reaction and therefore becomes part of the nucleic acid polymer.

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[00150] As used herein, a "nucleoside" is generally a base-sugar combination or a nucleotide lacking a phosphate moiety.

[00151] The term "double stranded product" is used herein to refer to products that are produced by the extension of a primer. It is understood that the products are at least partially double stranded, for example, in the region comprising the primer extension product and its complement. The double stranded product need not be completely double-stranded, and may have single stranded regions. It is also understood that the double stranded product can have heteroduplex regions in which one strand comprises RNA and the complementary strand comprised DNA in that region.

Reaction Conditions and Detection

[00152] Appropriate reaction media and conditions for carrying out the methods of the invention include those that permit nucleic acid extension, copying, and 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.sup.2+, or Mn.sup.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. 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. In some embodiments, the amplification reactions (particularly, primer extension and transcription; and generally not the step of denaturing) are performed isothermally, which substantially avoids the thermocycling process. The isothermal amplification reaction is carried out at a temperature that permits hybridization of the oligonucleotides (primer) 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.

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Extension of primers

[00153] The methods of the present invention involve the extension of primers. In general, primers hybridize to, and are extended along (chain extended), a sequence within the target polynucleotide and, thus, the target polynucleotide acts as a template. The extended primers are chain "extension products." The sequence over which the primer is extended may lie between two defined sequences but need not be. In general, the primers hybridize with a sequence within the target polynucleotide. The target sequence usually contains from about 10 to 30 to 50 to 100 or up to 5,000 or more nucleotides, often 10 to 1,000 nucleotides, or 10-10,000 nucleotides. The target polynucleotide may be a fraction of a larger molecule or it may be substantially the entire molecule (polynucleotide analyte).

Single Primer Isothermal Amplification Using a Complex Comprising an RNA/DNA Partial Heteroduplex as a Template

[00154] In some aspects of the invention, the amplification method that is used is a single primer isothermal amplification using a complex comprising an RNA/DNA partial heteroduplex as a template. In this method, termed single primer isothermal amplification, a complex comprising an RNA/DNA partial heteroduplex is a substrate for further amplification as follows: an enzyme which cleaves RNA sequence from an RNA/DNA heteroduplex (such as RNase H) cleaves RNA from the partial heteroduplex, leaving a partially double stranded polynucleotide complex comprising a 3' single stranded DNA sequence. The 3' single stranded sequence (formed by cleavage of RNA in the complex comprising an RNA/DNA partial heteroduplex) is generally the complement of the amplification composite primer, and thus forms a specific binding site for a composite primer. Extension of a bound composite primer by a DNA-dependent DNA polymerase with strand displacement activity produces a primer extension product, which displaces the previously bound cleaved primer extension product, whereby polynucleotide (generally, DNA) product accumulates. See, for example, U.S. Pat. Nos. 6,251,639 and 6,692,918.

[00155] Amplification using a complex comprising an RNA/DNA partial heteroduplex as a template for further amplification (also termed single primer isothermal amplification) generally occurs under conditions permitting composite primer hybridization, primer extension by a DNA polymerase with strand displacement activity, cleavage of RNA from an RNA/DNA hybrid and strand displacement. In so far as the composite primer hybridizes to the 3'-single stranded portion (of the partially double stranded polynucleotide which is formed by cleaving RNA in the complex comprising an RNA/DNA partial heteroduplex) comprising, generally, the complement of at least a portion of the composite primer sequence, composite primer hybridization may be under conditions permitting specific hybridization.

[00156] In some embodiments, the methods of the invention result in amplification of a multiplicity, a large multiplicity, or a very large multiplicity of template polynucleotide sequences. In some embodiments, essentially all of the template polynucleotide present in the initial sample (e.g., all of the mRNA or all of the genomic DNA) is amplified. In other embodiments, at least 1, at least 2, at least 3, at least 4, at least 5, at least 10, at least 20. at least 50, at least 100, at least 200, at least 300, at least 500, at least 1,000, at least 5,000, at least 10,000 or more than 10,0000 distinct sequences (such as a gene or other sub-segment of a polynucleotide, transcripts of a nucleic acid target, a marker (such as a SNP or other polymorphism) are amplified, as assessed, e.g., by analysis of marker sequences known to be present in the template sample under analysis, using methods known in the art. Template polynucleotide sequences that are amplified may be present on the same polynucleotide (e.g., a chromosome or portion of a chromosome for genomic DNA

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template or on the same RNA for RNA template) or on different template polynucleotides (e.g., different chromosome or portions of chromosomes for DNA template, or different RNAs for RNA template). In some case, amplification of genomic DNA is exemplified herein, it will be understood by those of skill in the art, however, that the global amplification methods of the invention are suitable for amplification of any pool or subset of polynucleotides.

[00157] In some embodiments, the methods of the invention are used to globally amplify double stranded DNA target. It is understood that in these cases, the amplified product generally is a mixture of sense and antisense copies of the template DNA. In some embodiments, the methods of the invention are used to globally amplify a single stranded DNA or RNA target. In these cases, the amplification product may generally be a copy of either the target polynucleotide (sense copy) or of the complement to the target nucleotide (antisense copy). Whether the sense or antisense copy is produced will depend on the method, as will be understood by one of ordinary skill in the art. In some embodiments, the amplification product of different senses can be annealed to form a double stranded (or partially double stranded) complex. In other embodiments, they can be prevented from annealing (or subsequently denatured) to produce a mixture of single stranded amplification products. The amplified products may be of differing lengths.

[00158] As illustrated in these embodiments, all steps are isothermal (in the sense that thermal cycling is not required), although the temperatures for each of the steps may or may not be the same. It is understood that various other embodiments may be practiced, given the general description provided above. For example, as described and exemplified herein, certain steps may be performed as temperature is changed (e.g., raised, or lowered).

[00159] For simplicity, the isothermal amplification methods of the invention are described as two distinct steps or phases, above. It is understood that the two phases may occur simultaneously in some embodiments (for example, if the enzyme that cleaves RNA from RNA/DNA hybrid is included in the first reaction mixture).

[00160] Although generally only one composite primer is described above, it is further understood that the amplification methods may be performed in the presence of two or more different composite primers that randomly prime template polynucleotide. In addition, the amplification polynucleotide products of two or more separate amplification reactions conducted using two or more different composite primers that randomly prime template polynucleotide can be combined.

Other amplification methods

[00161] Some aspects of the invention comprise the amplification of polynucleotide molecules or sequences within the polynucleotide molecules. Amplification generally refers to a method that results in the formation of one or more copies of a nucleic acid or polynucleotide molecule or in the formation of one or more copies of the complement of a nucleic acid or polynucleotide molecule. Amplifications can be used in the invention, for example, to amplify or analyze a polynucleotide bound to a solid surface. The amplifications can be performed, for example, after archiving the samples in order to analyze the archived polynucleotide.

[00162] In some aspects of the invention, exponential amplification of nucleic acids or polynucleotides is used. These methods often depend on the product catalyzed formation of multiple copies of a nucleic acid or polynucleotide molecule or its complement. The amplification products are sometimes referred to as "amplicons." One such method for the enzymatic amplification of specific double stranded sequences of DNA is known as the polymerase chain reaction (PCR). This in vitro amplification procedure is based on repeated cycles of denaturation, oligonucleotide primer annealing, and primer extension by thermophilic

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template dependent polynucleotide polymerase, resulting in the exponential increase in copies of the desired sequence of the polynucleotide analyte flanked by the primers. The two different PCR primers, which anneal to opposite strands of the DNA, are positioned so that the polymerase catalyzed extension product of one primer can serve as a template strand for the other, leading to the accumulation of a discrete double stranded fragment whose length is defined by the distance between the 5' ends of the oligonucleotide primers.

[00163] Another method for amplification involves amplification of a single stranded polynucleotide using a single oligonucleotide primer. The single stranded polynucleotide that is to be amplified contains two non-contiguous sequences that are complementary to one another and, thus, are capable of hybridizing together to form a stem-loop structure. This single stranded polynucleotide already may be part of a polynucleotide analyte or may be created as the result of the presence of a polynucleotide analyte.

[00164] Another method for achieving the result of an amplification of nucleic acids is known as the ligase chain reaction (LCR). This method uses a ligase enzyme to join pairs of preformed nucleic acid probes. The probes hybridize with each complementary strand of the nucleic acid analyte, if present, and ligase is employed to bind each pair of probes together resulting in two templates that can serve in the next cycle to reiterate the particular nucleic acid sequence.

[00165] Another method for achieving a nucleic acid amplification is the nucleic acid sequence based amplification (NASBA). This method is a promoter-directed, enzymatic process that induces in vitro continuous, homogeneous and isothermal amplification of a specific nucleic acid to provide RNA copies of the nucleic acid. The reagents for conducting NASBA include a first DNA primer with a 5'-tail comprising a promoter, a second DNA primer, reverse transcriptase, RNAse-H, T7 RNA polymerase, NTP's and dNTP's.

[00166] Another method for amplifying a specific group of nucleic acids is the Q-beta-replicase method, which relies on the ability of Q-beta-replicase to amplify its RNA substrate exponentially. The reagents for conducting such an amplification include "midi-variant RNA" (amplifiable hybridization probe), NTP's, and Q-beta-replicase.

[00167] Another method for amplifying nucleic acids is known as 3SR and is similar to NASBA except that the RNAse-H activity is present in the reverse transcriptase. Amplification by 3SR is an RNA specific target method whereby RNA is amplified in an isothermal process combining promoter directed RNA polymerase, reverse transcriptase and RNase H with target RNA. See for example Fahy et al. PCR Methods Appl. 1:25-33 (1991).

[00168] Another method for amplifying nucleic acids is the Transcription Mediated Amplification (TMA) used by Gen-Probe. The method is similar to NASBA in utilizing two enzymes in a self-sustained sequence replication. See U.S. Patent No. 5,299,491 herein incorporated by reference.

[00169] Another method for amplification of nucleic acids is Strand Displacement Amplification (SDA) (Westin et al 2000, Nature Biotechnology, 18, 199-202; Walker et al 1992, Nucleic Acids Research, 20, 7, 1691-1696), which is an isothermal amplification technique based upon the ability of a restriction endonuclease such as HincII or BsoBI to nick the unmodified strand of a hemiphosphorothioate form of its recognition site, and the ability of an exonuclease deficient DNA polymerase such as Klenow exo minus polymerase, or Bst polymerase, to extend the 3'-end at the nick and displace the downstream DNA strand. Exponential amplification results from coupling sense and antisense reactions in which strands displaced from a sense reaction serve as targets for an antisense reaction and vice versa.

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[00170] Another method for amplification of nucleic acids is Rolling Circle Amplification (RCA) (Lizardi et al. 1998, Nature Genetics, 19:225-232). RCA can be used to amplify single stranded molecules in the form of circles of nucleic acids. In its simplest form, RCA involves the hybridization of a single primer to a circular nucleic acid. Extension of the primer by a DNA polymerase with strand displacement activity results in the production of multiple copies of the circular nucleic acid concatenated into a single DNA strand.

[00171] In some embodiments of the invention, RCA is coupled with ligation. For example, a single oligonucleotide can be used both for ligation and as the circular template for RCA. This type of polynucleotide can be referred to as a "padlock probe" or a "RCA probe". For a padlock probe, both termini of the oligonucleotide contain sequences complementary to a domain within a nucleic acid sequence of interest. The first end of the padlock probe is substantially complementary to a first domain on the nucleic acid sequence of interest, and the second end of the padlock probe is substantially complementary to a second domain, adjacent to the first domain near the first domain. Hybridization of the oligonucleotide to the target nucleic acid results in the formation of a hybridization complex. Ligation of the ends of the padlock probe results in the formation of a modified hybridization complex containing a circular polynucleotide. In some cases, prior to ligation, a polymerase can fill in the gap by extending one end of the padlock probe. The circular polynucleotide thus formed can serve as a template for RCA that with the addition of a polymerase results in the formation of an amplified product nucleic acid. The methods of the invention described herein, can produce amplified products with defined sequences on both the 5'-and 3'-ends. Such amplified products can be used as padlock probes.

[00172] Some aspects of the invention utilize the linear amplification of nucleic acids or polynucleotides. Linear amplification generally refers to a method that involve the formation of one or more copies of the complement of only one strand of a nucleic acid or polynucleotide molecule, usually a nucleic acid or polynucleotide analyte. Thus, the primary difference between linear amplification and exponential amplification is that in the latter process, the product serves as substrate for the formation of more product, whereas in the former process the starting sequence is the substrate for the formation of product but the product of the reaction, i.e. the replication of the starting template, is not a substrate for generation of products.. In linear amplification the amount of product formed increases as a linear function of time as opposed to exponential amplification where the amount of product formed is an exponential function of time.

[00173] In some embodiments, amplification methods can be solid-phase amplification, polony amplification, colony amplification, emulsion PCR, bead RCA, surface RCA, surface SDA, etc., as will be recognized by one of skill in the art. In some embodiments, amplification methods that results in amplification of free DNA molecules in solution or tethered to a suitable matrix by only one end of the DNA molecule can be used. Methods that rely on bridge PCR, where both PCR primers are attached to a surface (see, e.g., WO 2000/018957 and Adessi et al., Nucleic Acids Research (2000): 28(20): E87) can be used. In some cases the methods of the invention can create a "polymerase colony technology", or "polony", referring to a multiplex amplification that maintains spatial clustering of identical amplicons (see Harvard Molecular Technology Group and Lipper Center for Computational Genetics website). These include, for example, in situ polonies (Mitra and Church, Nucleic Acid Research 27, e34, Dec. 15, 1999), in situ rolling circle amplification (RCA) (Lizardi et al., Nature Genetics 19, 225, July 1998), bridge PCR (U.S. Pat. No. 5,641,658), picotiter PCR (Leamon et al., Electrophoresis 24, 3769, November 2003), and emulsion PCR

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(Dressman et al., PNAS 100, 8817, Jul. 22, 2003). The methods of the invention provide new methods for generating and using polonies.

Compositions

Primers and Oligonucleotides

[00174] The invention provides compositions, kits, complexes, reaction mixtures and systems comprising various components and their combinations used in the amplification methods described herein. In one aspect, for example, the invention provides compositions comprising an all DNA primer. All DNA or RNA-DNA chimeric primers may comprise 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 DNA, or a primer extension product) and is capable of promoting polymerization of a polynucleotide complementary to the template. An all DNA primer or an RNA-DNA chimeric primer maybe, 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.

[00175] The primers of the invention are usually oligonucleotide primers. A primer is generally an oligonucleotide that is employed in an extension on a polynucleotide template. The oligonucleotide primer is usually a synthetic nucleotide that is single stranded, containing a sequence at its 3'-end that is capable of hybridizing with a sequence of the target polynucleotide. Normally, the 3'-region of the primer that hybridizes with the target nucleic acid has at least 80%, preferably 90%, more preferably 95%, most preferably 100%, complementarity to a sequence or primer binding site. The number of nucleotides in the hybridizable sequence of a specific oligonucleotide primer should be such that stringency conditions used to hybridize the oligonucleotide primer may prevent excessive random non-specific hybridization. Usually, the number of nucleotides in the hybridizing portion of the oligonucleotide primer may be at least as great as the defined sequence of the target polynucleotide, namely, at least five nucleotides, at least about ten nucleotides, at least about 15 nucleotides, generally from about 5 to about 200, and usually about 10 to about 50 nucleotides.

[00176] A "random primer," or "random annealing sequence" 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 random primers used herein are generally tailed random primers comprising a 3'-segment that acts as a random primer to the target polynucleotide, and a 5'-sequence that generally does not hybridize to the target polynucleotide. The sequence of any one primer molecule from a sample of random primers (or their complement) may or may not be naturally-occurring, and may or may not be present in the sequences in a sample of interest. The amplification of a plurality of polynucleotides, e.g. DNA or 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

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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. In some embodiments one portion of a primer is random, and another portion of the primer comprises a defined sequence. For example, in some embodiments, a 3'-portion of the primer may comprise a random sequence, while the 5'-portion of the primer comprises a defined sequence. In other embodiments, for example, a 3'-portion of the primer may comprise a defined sequence, while the 5' portion of the primer comprises a random sequence. In some embodiments a 3'-random portion of the primer may comprise DNA, and a 5'-portion defined portion of the primer may comprise RNA, in other embodiments, both the 3'-and 5'-portions may comprise DNA.

[00177] All DNA primers generally comprise polynucleotide annealing sequences. The length of the annealing sequence can be for example from about 2 to about 100 nucleotides. In some cases, the all DNA primers comprise polynucleotide annealing sequences of length about 3 nucleotides to about 50 nucleotides. In other cases, the all DNA primers comprise short polynucleotide annealing sequences of about 4 nucleotides to about 20 nucleotides in length. In still other embodiments, the all DNA primers comprise polynucleotide annealing sequences of about 4 to about 10 or 15 nucleotides in length. In some embodiments, the all DNA primer annealing sequence is about 8 nucleotides. In other embodiments, the all DNA primer annealing sequence is about 6 nucleotides. In some cases the primer may consist of only the annealing sequence, whereas in other cases the primer may also comprise other portions which are not annealing sequences such as a tail sequence or an element other than the annealing sequence and the tail sequence such as a linker for example. In other cases, the all DNA primers also comprise a ligand. The ligand may or may not be comprised of nucleic acid. In some cases, the ligand is a small organic molecule such as for example biotin or a fluorophore. The primers may be synthesized by a number of common methods known in the art. In some cases, the methods of synthesizing primers of the present invention include solid phase synthesis methods such as provided in US Patent 5,623,068.

[00178] Precise nucleotide lengths and compositions can be determined by one with ordinary skill in the art for designing DNA primers for hybridization to an RNA or DNA template nucleic acid and extension by a polymerase. The nucleotide length and sequence composition may take into account the temperature of the hybridization and polymerization reactions to be performed, the propensity for secondary structure inherent in the all DNA primer or the nucleic acid template, the likelihood of a given all DNA primer to hybridize to non-specific or closely related sequences, and the sequence of the nucleic acid template. Suitable primer lengths include from about 5 to about 200 or more nucleotides including about 5, 6, 7, 8, 9, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70 , 80, 90, or about 100 or more nucleotides in length.

[00179] The nucleotide length and sequence composition may be designed to provide specific hybridization to a desired sequence, specific hybridization to a family of related sequences, or hybridization to any sequence or substantially any sequence of a genome or transcriptome. In the case of designing a primer that anneals to any sequence, randomized sequences may be used such as for example random heptamers, hexamers, septamers, octamers, nonamers, or random decamers. In some cases, non-standard bases which exhibit base pairing interactions with more than one partner such as inosine which can base pair with uracil, adenine, and cytosine may be used. The non-standard bases with greater propensity for multiple base pairing partners are referred to as wobble bases. In other cases, specific annealing sequences may be used in combination with such wobble bases. In some cases, all DNA primers comprise specific annealing sequences in which one or more positions within the annealing sequence are randomized. Such a

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combination of specific sequences with randomized positions may be used for example to amplify a set of related or homologous nucleic acid sequences. In still other cases, all DNA primers comprise specific annealing sequences, or a set of specific sequences. In an exemplary embodiment, the annealing sequence comprises a poly thymine sequence such that messenger RNA molecules are preferentially amplified.

[00180] In some cases, all DNA primers may comprise modification of bases or the introduction of nonstandard bases in addition to or other than the inosine base previously described. Non standard bases may be used to increase the thermal stability of the hybridized primer to its target nucleic acid, to increase specificity, or to increase stability (i.e. resistance to degradation). Non standard bases may include but are not limited to locked nucleic acids, peptide nucleic acids, pyranosyl nucleic acids, methylated nucleic acids, or any other modified or nonstandard nucleic acid known to the art. In some cases, the first primer, second primer, composite primer, or chimeric primer comprises non-standard bases which can be recognized by a polymerase such as for example a DNA-dependent DNA polymerase or an RNA-dependent DNA polymerase and copied in a consistent and efficient manner. In some cases, an amplification primer, composite amplification primer, or chimeric amplification primer may comprise any variety of nonstandard bases as described herein.

[00181] All DNA primers may also comprise a tail sequence. The tail sequence may provide a useful way for further manipulation of amplified products such as purification, functionalization, capture, immobilization or other downstream processes such as sequence determination or further amplification, the tag may also be integral to the amplification process itself such as but not limited to the process described in **Figures 3** and **8**. The tail sequence may comprise a sequence that does not hybridize or does not substantially hybridize to the target nucleic acid under the conditions of the method. In some cases, the tail sequence may be designed such that it does not hybridize or substantially hybridize to other primers, oligonucleotides, or tail sequences employed in the method such as sequence (B) of **Figure 4** or sequence (C) of **Figure 10**. . In other cases, the tail sequence may be designed such that it does hybridize to other primers, oligonucleotides, or tail sequences employed in the method. The tail sequence may comprise a sequence between about 1 or 2 nucleotides in length to about 200 nucleotides in length including about 3, 4, 5, 8, 10, 15, 20, 25, 30, 50, 75, 100, 150, or 200 nucleotides in length.

[00182] In some embodiments, all DNA primers comprising a 3'-template annealing sequence and a 5'-sequence tail (A) are used in a first primer extension reaction. The all DNA first primer may be annealed to the target nucleic acid subsequent to an elevated temperature denaturing step to remove secondary structure and or separate complementary strands. Annealing of the all DNA first primer may be followed by addition of polymerase and nucleotide triphosphates as well as buffers, cations, anions, and solute modifying reagents such as glycerol, polyethylene glycol, bovine serum albumin, dimethyl sulfoxide, or single-stranded binding proteins, and the like to initiate a first primer extension reaction. In some cases, a strand displacing polymerase may be used, while in other cases it may be advantageous to utilize a polymerase that does not exhibit substantial strand displacing activity. The first primer extension reaction may provide a template for further manipulation including but not limited to annealing of a chimeric DNA-RNA second primer.

[00183] In some aspects of the present invention, chimeric primers (also referred to as composite primers) are utilized. Chimeric primers can be for example a second primer in the methods of the invention, chimeric extension primers or chimeric amplification primers. Chimeric primers of the present invention include primers that comprise an RNA segment and a DNA segment. The RNA segment may be generally located at the 5' end of the chimeric primer and the DNA segment may generally be located at the 3' end of the

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chimeric primer. In some cases the RNA and DNA segments are adjacent. In other cases, there is an intervening element between the RNA and the DNA segment. The intervening element may comprise additional nucleic acid sequence. In one embodiment, a portion of the DNA segment comprises a template annealing sequence. The template annealing sequence as described for the all DNA primer may be target specific, a set of target specific sequences, or a random sequence.

[00184] In some embodiments of the present invention, the DNA-RNA chimeric primer annealing sequences may be between about 2 nucleotides in length to about 100 nucleotides in length. In some cases, annealing sequences may be between about 3 nucleotides in length to about 50 nucleotides in length. In still other cases, annealing sequences may be about 4 to about 30 nucleotides in length. For example, but without limitation, DNA-RNA chimeric primer annealing sequences may be about 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, or 25 nucleotides in length. In some cases, random annealing sequences comprise random pentamers, hexamers, heptamers, octomers, decamers, or larger.

[00185] In some cases, for example with the use of random template annealing sequences, it may be advantageous to extend the chimeric primer with a polymerase that does not exhibit substantial strand displacement activity. In other cases, it may be advantageous to extend the chimeric primer with a polymerase that does exhibit substantial strand displacement activity. In some cases, the use of a polymerase that exhibits substantial strand displacement activity may be useful for whole genome and or whole transcriptome amplification.

[00186] In some embodiments of the present invention, a portion of the RNA segment of the chimeric primer comprises a sequence tail. The sequence tail may in some cases comprise the same sequence (A) as the sequence tail used for an all DNA first primer extension reaction. In other cases, the sequence tail may comprise a different sequence (B) as compared to the sequence tail used for an all DNA first primer extension reaction.

[00187] In some embodiments of the present invention, a chimeric primer comprising a 5'-RNA segment comprising a sequence tail and a 3' DNA segment comprising a template annealing sequence is annealed to a first primer extension product, a polymerase and is added to the annealing reaction, and a second primer extension product is created. In some embodiments of the present invention, a 3'-exonuclease is added after the second primer extension reaction is completed. The 3'-exonuclease may remove the 3'-unhybridized portion of the first primer extension product. Removal of the exonuclease by heat, the addition of inhibitors, or nucleic acid purification methods may allow further extension of the first primer extension product. The further extension may provide sequence complementary to the sequence tail of the chimeric primer.

[00188] Amplification primers of the present invention include chimeric or composite amplification primers. Composite amplification primers are RNA/DNA composite primers that can be used to create multiple copies of (amplify) a polynucleotide sequence isothermally using RNA cleavage, and DNA polymerase activity with strand displacement. Amplification with such primers is described, for example in U. S. Patents 6,251,639, 6,692,918, and 6,946,251. The composite amplification primer comprises sequences capable of hybridizing to a portion of a DNA template, and most often comprises sequences hybridizable to a defined 3'-portion of the DNA.

[00189] A composite amplification primer comprises at least one RNA portion that is capable of (a) binding (hybridizing) to a sequence on a DNA template independent of hybridization of the DNA portion(s) to a sequence on the same extension product; and being cleaved with an RNase H when hybridized to the DNA

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template. The composite amplification primers bind to the DNA template 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 DNA template remains intact, thus enabling annealing of another composite primer.

[00190] The composite amplification primers also comprise a 3'-DNA portion that is capable of hybridization to a sequence on the DNA template such that its hybridization to the DNA is favored over that of the nucleic acid strand that is displaced from the DNA template 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 some aspects of the present invention, the 3' DNA portion of a composite amplification primer may be about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 nucleotides in length.

[00191] In some embodiments of the present invention chimeric oligonucleotides are utilized. In some cases, the chimeric oligonucleotides may be used to provide a defined end sequence for the SPIA amplified DNA products. The chimeric oligonucleotides may comprise a 5' segment comprising RNA and a 3' segment comprising DNA. In some embodiments such as for example those depicted in **Figures 10 and 12**, a portion of the 3' segment of the chimeric oligonucleotide is substantially the same as the RNA sequence removed from the DNA-RNA heteroduplex of a SPIA substrate by RNase H. In other cases, such as for example that depicted in **Figure 16**, a portion of the 3' segment of the chimeric oligonucleotide is substantially the same as the complement of the tail sequence of the first primer.

Enzymes

[00192] Polymerases are used in the methods of the invention, for example to extend primers to produce primer extension products. A polymerase, or nucleotide polymerase, is a catalyst, usually an enzyme, for forming an extension of a polynucleotide along a DNA or RNA template where the extension is complementary thereto. The nucleotide polymerase is a template dependent polynucleotide polymerase and utilizes nucleoside triphosphates as building blocks for extending the 3'-end of a polynucleotide to provide a sequence complementary with the polynucleotide template. Usually, the catalysts are enzymes, such as DNA polymerases, for example, prokaryotic DNA polymerase (I, II, or III), T4 DNA polymerase, T7 DNA polymerase, Klenow fragment, Bst DNA polymerase, reverse transcriptase, Vent DNA polymerase, Pfu DNA polymerase, Taq DNA polymerase, and the like, derived from any source such as cells, bacteria, such as E. coli, plants, animals, virus, thermophilic bacteria, and so forth. RNA polymerases include T7 RNA polymerase, AMV polymerase, Q-beta-replicase, and so forth. In some cases, Bst DNA Polymerase Large Fragment can be used. Bst DNA polymerase Large Fragment is the portion of the *Bacillus stearothermophilus* DNA Polymerase protein that contains the 5′ → 3′ polymerase activity, but lacks the 5′ →3′ exonuclease domain. Where the polymerase forms an extension product on a DNA template, it is referred to herein as a DNA dependent polymerase. Where the polymerase forms an extension product on a RNA template, it is referred to herein as a RNA dependent polymerase.

[00193] In some embodiments, the methods of the present invention provide enzyme inhibitors. In some cases, polymerase inhibitors are provided such as for example DNA-dependent DNA polymerase inhibitors. In some cases, DNA-dependent DNA polymerase inhibitors such as actinomycin that do not substantially inhibit RNA-dependent DNA polymerization are provided. Actinomycin may be particularly useful in the methods of the present invention when the target sequence to be amplified comprises RNA that is present in a sample that also comprises DNA. In such cases actinomycin may be used to selectively inhibit the

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production of extension product complementary to the DNA during, for example extension of the first primer.

[00194] Exonucleases are used in the methods of the invention, for example an exonuclease specific for single-stranded DNA, , whereby treatment of the double stranded first and second primer extension products with the single-strand specific exonuclease enable the removal of the single stranded 3' nucleotides from the 3' region of a first primer extension product that are not hybridized to a second primer extension product. In another example an exonuclease may be used to remove unincorporated primers or target nucleic acids that are not hybridized to first or second primer extension products. In yet another example the treatment of the stem loop product of the second primer extension product generated using an all DNA tailed first primer and a chimeric second primer both comprising a sequence A at their 5'-ends, with a single strand specific 3'-exonuclease is used for clean-up of the reaction mixture as described previously, by the selective degradation of the non incorporated primers and other reaction products. Non-limiting examples of an exonuclease include single-strand specific 3'-exonucleases such as exonuclease 1. The exonuclease should remove all of the single-stranded 3' nucleotides which are not hybridized to form a double stranded nucleic acid. In some embodiments, the exonuclease may remove additional 3' nucleotides which are hybridized to a primer extension product. In other embodiments, a polymerase comprising exonuclease activity may be used. Non-limiting examples include a T4 polymerase comprising 3' exonuclease activity. . Exonucleases may be used in the methods of the present invention for clean up of reaction mixtures, such as amplification reaction mixtures, for example single strand specific DNA exonucleases may be used for clean-up of PCR or SPIA amplification products without the need for purification prior to performing sequencing reactions.

[00195] RNases are used in the methods of the present invention, for example in some cases a single and or double stranded RNA specific RNase is used to degrade single and double stranded RNA from template nucleic acid and or unincorporated primers. In some cases, single and double stranded RNA specific RNases includes but is not limited to RNase 1. In another example RNase which is specific for the cleavage of the RNA portion of a DNA-RNA heteroduplex is used in the methods of the present invention to degrade or cleave the RNA portion of the DNA-RNA heteroduplex of a partial heteroduplex generated by the methods of the invention. This may have the effect of making the DNA portion of the DNA-RNA heteroduplex available to hybridize with other oligonucleotides such as but not limited to primers, amplification primers and chimeric oligonucleotides. By way of example and without limitation an example of a RNase specific for cleaving the RNA portion of a DNA-RNA heteroduplex is RNase H.

Nucleic Acid Target

[00196] A nucleic acid target, also referred to as a template, as defined herein includes but is not limited to any length nucleotide, for example, DNA and or RNA. Examples of nucleic acid templates include but are not limited to complementary cDNA, mRNA, total RNA, genomic DNA, and DNA. In some cases, the nucleic acid target may be derived from a natural source such as for example an organism or from the environment. In other cases, the nucleic acid target may be derived from a synthetic source such as a nucleic acid synthesizer. In still other cases, the nucleic acid target may comprise both natural and synthetic nucleic acids in any proportion. In some embodiments of the present invention, target nucleic acid may be purified prior to use by the methods of the present invention. In some cases, target nucleic acid may be pre-fractionated according to size and or sequence by electrophoresis, ion exchange, gel filtration, reverse phase, or hybridization techniques. In some cases, target nucleic acid may be amplified by other methods

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known to the art as described herein prior to application of the methods of the present invention. Sources for target nucleic acid include but are not limited to animals including but not limited to humans, non-human primates, dogs, cats, cows, pigs, sheep, guinea pigs, hamsters, birds, frogs, fish, rats, mice, rodents and any other animal from which nucleic acid may be obtained. Other sources for target nucleic acid include but are not limited to bacteria, viruses, plants, insects, worms, fruit flies, and fungi. Target nucleic acid may be obtained from tissue or bodily fluids such as skin, blood, cells, hair follicles, sebaceous glands, cells obtained from fine needle aspiration or other surgical and non surgical biopsy techniques, saliva, urine, stool, menses and the like, or may be obtained from cultures, environmental samples and the like..

Surface or Solid Substrate

[00197] In various exemplary embodiments, a solid surface may have a wide variety of forms, including membranes, slides, plates, micromachined chips, microparticles, beads and the like. Solid surfaces may comprise a wide variety of compositions including, but not limited to, glass, plastic, silicon, alkanethiolate derivatized gold, cellulose, low cross linked and high cross linked polystyrene, silica gel, polyamide, and the like, and can have various shapes and features (e.g., wells, indentations, channels, etc.). As used herein, the terms "solid surface" and "solid substrate" are used interchangeably. In some cases these may be referred to as the surface or the support. The surface can be hydrophilic or capable of being rendered hydrophilic and may comprise inorganic powders such as silica, magnesium sulfate, and alumina; natural polymeric materials, particularly cellulosic materials and materials derived from cellulose, such as fiber containing papers, e.g., filter paper, chromatographic paper, etc.; synthetic or modified naturally occurring polymers, such as nitrocellulose, cellulose acetate, poly (vinyl chloride), polyacrylamide, cross linked dextran, agarose, polyacrylate, polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), nylon, poly(vinyl butyrate), etc.; either used by themselves or in conjunction with other materials; glass available as Bioglass, ceramics, metals, and the like. Natural or synthetic assemblies such as liposomes, phospholipid vesicles, and cells can also be employed. The surface can have any one of a number of shapes, such as strip, rod, particle, including bead, and the like.

[00198] In some embodiments, the solid surface comprises a bead or plurality of beads. The beads may be of any convenient size and fabricated from any number of known materials. Example of such materials include: inorganics, natural polymers, and synthetic polymers. Specific examples of these materials include: cellulose, cellulose derivatives, acrylic resins, glass, silica gels, polystyrene, gelatin, polyvinyl pyrrolidone, co- polymers of vinyl and acrylamide, polystyrene cross-linked with divinylbenzene or the like (as described, e. g, in Merrifield, Biochemistry 1964,3, 1385-1390), polyacrylamides, latex gels, polystyrene, dextran, rubber, silicon, plastics, nitrocellulose, natural sponges, silica gels, control pore glass, metals, cross-linked dextrans (e. g., Sephadex) agarose gel (SepharoseTM), polystyrene and other solid phase supports known to those of skill in the art. The beads are generally about 2 to about 100 um in diameter, or about 5 to about 80 pm in diameter, in some cases, about 10 to about 40, um in diameter. In some embodiments the beads can be magnetic. Having magnetic beads can be useful for isolation and purification of the beads comprising nucleic acids described herein. Other methods to separate beads can also be used. For example, the capture beads may be labeled with a fluorescent moiety which would make the nucleic acid- bead complex fluorescent. The target capture bead complex may be separated, for example, by flow cytometry or fluorescence cell sorter.

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Attachment of oligonucleotides to solid surfaces

[00199] One aspect of the invention involves attaching oligonucleotides to solid surfaces such that the oligonucleotides can hybridize with polynucleotides produced by the methods of the invention. Many methods of attaching oligonucleotides to surfaces are known. In some embodiments, the oligonucleotide is attached covalently to the solid surface. In some embodiments such attached oligonucleotides act to capture nucleic acids such as amplification products, and in some cases such attached oligonucleotides also act as primers. Methods of attaching oligonucleotides and primers to a surface are known in the art (see, e.g., Beier et al., 1999, Nucleic Acids Res. 27(9):1970-1977; Brison et al., 1982, Molecular and Cellular Biology 2:578 587; Cheung et al., 1999, Nat. Genet 21(1 Suppl):15-19; Chrisey et al., 1996, Nucleic Acids Res. 24(15):3031-3039; Cohen et. al., 1997, Nucleic Acids Res. 1997 Feb. 15; 25(4):911-912; Devivar et al., 1999, Bioorg. Med. Chem. Lett. 9(9):1239-1242; Heme et al., 1997. J. Am. Chem. Soc. 119:8916-8920; Kumar et al., 2000, Nucleic Acis Res. 28(14):e71; Lipshutz et al., 1999, Nat. Genet. 21(1 Suppl):20-24; Milner et al., 1997, Nat. Biotechnol. June;15(6):537-541; Morozov et al., 1999, Anal. Chem. 71(15):3110-3117; Proudnikov et al., 1998, Anal Biochem. 259(1):34-41; Rasmussen et al., 1991, Anal Biochem. 198(1):138-142; Rogers et al., 1999, Anal. Biochem. 266(1):23-30; Salo et al., 1999, Bioconjug Chem. 10(5):815-823; Singh-Gasson et al., 1999, Nat. Biotechnol. 17(10):974-978, and Pierce Chemical Company Catalog 1994, pp. 155-200), incorporated herein by reference). Attached oligonucleotides may also be synthesized directly on the solid surface.

Uses of the present invention

Multiplex Amplification of Desired Nucleic Acid Targets from a Complex Sample

[00200] The methods of the present invention are useful for amplification of multiple different nucleic acid targets from a complex sample. The amplification of multiple different nucleic acid targets from a sample may be referred to as multiplex amplification. In some embodiments, multiplex amplification may be carried out by the methods of the present invention by the use of a set of first primers with different annealing sequences each of which is specific for a different nucleic acid target. In some cases, the template may be removed or degraded after the first primer extension product has been formed by the methods of the present invention. The multiple first primer extension products may then be contacted with a second primer with a random, degenerate, or specific annealing sequence. In other embodiments, the annealing sequence of the first primer may be random or degenerate, while a set of second primers may be used with different annealing sequences each of which is specific for a different nucleic acid target. Multiplex amplified products of the present invention may be detected by sequencing, nucleic acid array hybridization, electrophoresis, capture on solid phase (e.g. beads), or chromatography.

[00201] In a particular embodiment, multiplex amplification of a set of targets in a nucleic acid sample may be carried out to reduce the complexity in that sample. For example, the nucleic acid sample may be a whole genome or whole transcriptome. In some embodiments of the present invention it may be desirable to amplify only a set of the sequences in the whole genome or whole transcriptome such a set may include for example, the exons in a genome, the set of kinases, the set of protein kinases, the set of lipid kinases, proteases, serine proteases, cysteine proteases, oncogenes, transcription factors, polymorphic regions related to a particular disease or condition such as single nucleotide polymorphisms, or any set or subset of target sequences within a sample. In some cases, multiplex amplification may be used to amplify 5 or more sequences from a complex sample, 10 or more sequences, 25 or more, 50 or more, 100 or more sequences,

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200 or more sequences, 300 or more sequences, 500 or more sequences, 1000 or more sequences, 2000 or more sequences, 5000 or more sequences, 10,000 or more sequences, 25,000 or more sequences, 50,000 or more sequences, or 100,000 or more sequences. In some cases, the methods of the present invention provide for improved multiplex amplification of target sequences as compared to other methods known in the art such as for example PCR because the linear amplification methods provided herein do not substantially alter the relative abundance of different sequences in a sample by preferentially amplifying some sequences over others. In some cases, the methods of the present invention are useful for processing nucleic acid samples by reducing their complexity by amplifying sequences of interest and/or removing sequences that are not of interest and preparing a sample for downstream processing such as sequencing, quantifying, and array hybridization.

Capture and Amplification of Single or Multiple Nucleic Acid Target or Targets from a Sample

[00202] The methods of the present invention may be used for capture and amplification of single or multiple nucleic acid targets from a sample. In some embodiments, the first primer of the present invention may be attached to a solid phase such as a bead for example and used to capture a nucleic acid target from a sample such as a complex sample by hybridization. In some cases, un-captured nucleic acid may then be removed by washing way the nucleic acid not bound to the solid phase or by removing the solid phase from the nucleic acid that is not bound to the bead, or by a combination thereof. Methods and compositions for hybridization of specific sequences are provided herein. In some embodiments, the first primer and the target nucleic acid may hybridize in solution and the resulting hybridized product may then be captured. In some cases, the capture step may include the use of a solid phase that hybridizes to or has an affinity for a ligand that is bound to, or covalently bound to, the first primer. For example, the first primer may include a tail sequence that is complementary to a sequence bound to a bead. For another example, the first primer may include a ligand such as biotin that may bind to a solid phase (e.g. a bead) derivatized with streptavidin. In still another example, the first primer may include a streptavidin molecule and the solid phase may comprise biotin. In some embodiments the first primer has a specific annealing sequence for capture and amplification of a specific target nucleic acid. In other cases, the first primer may provide a degenerate annealing sequence for capture and amplification of multiple target nucleic acids. In some cases, the first primer may be provided as a set of first primers each with a different specific annealing sequence for capture and amplification of multiple target nucleic acids.

[00203] The captured target nucleic acid may be amplified by the methods provided herein. In some embodiments, a first primer extension product may be formed from the first primer bound to a captured target nucleic acid using the methods of the present invention by the use of a polymerase. The first primer extension product may be contacted with a second primer and a second primer extension product may be formed using the methods of the present invention. The second primer extension product may then be amplified using an amplification primer of the present invention, a polymerase, and RNAse H as described herein. The resulting amplification product may be useful for simplifying complex nucleic acid samples, (i.e. complexity reduction) by capturing and amplifying a subset of the nucleic acid that is desired for further analysis such as sequencing, array based genome or transcriptome analysis, expression profiling and analysis, genetic analysis, SNP analysis etc.

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Characterization of Nucleic Acids

[00204] The methods of the invention are amenable to quantitative analysis, as in some embodiments, amplification can yield sufficient single stranded polynucleotide (generally, DNA and RNA) products which accurately reflect the representation of the various DNA or RNA sequences (e.g. genomic DNA or 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. Pat. 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.

[00205] In another embodiment, the amplification methods of the invention are utilized to generate multiple copies of single stranded polynucleotide products from RNA or DNA targets that are labeled by the incorporation of labeled nucleotides during DNA 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 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 RNA or 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. Labeling can also be accomplished by random priming of amplification products with the incorporation of labeled dNTPs. Other methods for labeling and or fragmenting of the amplification products of the invention are known in the art, for example the methods provided by U.S. Patent Application Serial Nos. 10/304,035 filed November 22, 2002, and 10/441,663 filed May 19, 2003 which are herby incorporated by reference in their entirety and which are useful for the generation of products suitable for analysis using various designs of microarrays, such as for example high density oligonucleotides microarrays .

[00206] The labeled amplified products are 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) a DNA or RNA sequence of interest by generating labeled polynucleotide (generally, 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. 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 DNA or 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 DNA or

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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 or to detect the presence or absence of defined target DNA or RNA in a sample. The measurement is also useful for measuring the relative amounts of various DNA sequences corresponding, for example, to genetic regions in the sample. 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 of the corresponding target DNA or target RNA species in the sample.

Sequencing of the polynucleotide products of the invention

[00207] As described above, the methods can be used to obtain sequence information about a target RNA or target DNA of interest. The sequencing can be carried out on the primer extension products or amplification products produced by the methods herein. In some embodiments the sequencing is performed on the polynucleotides attached to solid surfaces as described herein. In one embodiment sequencing is performed on polynucleotides that are attached to the beads through oligonucleotides attached to the beads which capture amplified product, and are extended to produce a polynucleotide attached to the surface comprising a defined sequence at its 3' end.

[00208] The methods of the invention are useful, for example, for sequencing of a polynucleotide sequence of interest. The sequencing process may be carried out as described for the methods described herein. In some cases, the methods of the invention are useful for providing amplified products having defined 3' and 5' ends that are useful for further analysis such as for example for sequencing, array hybridization, or quantitative PCR. In some cases, the methods provide for multiplex amplification such as from complex mixtures such as whole genome or whole transcriptome or a portion thereof, or multiple whole genomes or whole transcriptomes or portions thereof.

[00209] Known methods for sequencing include, for example, those described in:, Sanger, F. et al. , Proc. Natl. Acad. Sci. U. S. A. 75,5463-5467 (1977); Maxam, A. M. & Gilbert, W. Proc Natl Acad Sci USA 74,560-564 (1977); Ronaghi, M. et al. , Science 281, 363,365 (1998); Lysov, 1. et al., Dokl Akad Nauk SSSR 303, 1508-1511 (1988); Bains W. & Smith G. C. J. Theor Biol 135,303-307 (1988); Drnanac, R. et al. , Genomics 4,114-128 (1989); Khrapko, K. R. et al. , FEBS Lett 256.118-122 (1989); Pevzner P. A. J Biomol Struct Dyn 7, 63-73 (1989); and Southern, E. M. et al., Genomics 13, 1008-1017 (1992). Pyrophosphate-based sequencing reaction as described, e. g., in US patent 6,274,320; 6,258, 568 and 6,210,891), may also be used.

[00210] In some cases, the methods and compositions of the present invention may be useful for capture and amplification of a plurality of target sequences from a complex sample, such as for example a whole genome or genomes, or a whole transcriptome or transcriptomes. The capture and amplification of target sequences from a complex sample may be desirable for complexity reduction. For example, complexity reduction may be advantageous for further use of the amplified products in highly parallel analytical techniques such as the use of high density microarrays, or high throughput sequencing methods including for example the use of the methods and instrumentation developed by Roche 454 Life Sciences, Illumina, Sequenom, ABI, or any of the sequencing methods described herein such as those described in US Patent Nos. 7,211,390; 6,355,431; 5,750,341; 5,969,119; 6,274,320; 6,258, 568; 6,210, 891; and 6,306,597. In

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some embodiments, this complexity reduction may be used to capture and in some cases amplify a single RNA or DNA sequence of interest from a complex sample.

[00211] The methods of the invention are useful, for example, for sequencing of an RNA sequence of interest. The sequencing process can be carried out by processing and amplifying a target RNA containing the sequence of interest by any of the methods described herein. Addition of nucleotides during primer extension can be analyzed using methods known in the art, for example, incorporation of a terminator nucleotide or sequencing by synthesis (e.g. pyrosequencing).

[00212] In embodiments wherein the end product is in the form of 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. 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. The amount of time can be determined empirically by one skilled in the art.

[00213] Suitable dNTP analogs 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.

[00214] 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, 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

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