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Sir:

Prior to the examination of the above-referenced application on the merits, please enter the amendments below.

IN THE SPECIFICATION

Please insert the following paragraph after the title on page one of the specification:

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a filing under §371 of International Patent Application No. PCT/IB2010/002243, filed August 13, 2010, which claims priority to US Provisional Application No. 61/235,595, filed August 20, 2009 and US Provisional Application No. 61/288,792, filed December 21, 2009; all of which are herein incorporated by reference.

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AMENDMENTS TO THE CLAIMS

Please amend the claims as shown below. A complete listing of the claims in this case, including their current status identifier, is shown below.

1. (Currently Amended) A method of **sample analysis**, ~~moving a domain in a nucleic acid, the method~~ comprising:

a) annealing a reflex sequence in a polynucleotide strand in a sample with a complement of the reflex sequence present in the polynucleotide strand, wherein the polynucleotide strand comprises the following in a 5' to 3' orientation:

a first domain **comprising i. a multiplex identifier (MID) whose identity can be used to differentiate polynucleotides in the sample, and ii. a primer site**;

the reflex sequence;

a polynucleotide of interest **having a desired site that is distal to the first domain**; and

the complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand; ~~and~~

b) extending from the 3' end of the complement of the reflex sequence through the first domain to produce a double stranded region; **comprising** ~~whereby~~ a complement of the first domain ~~is present~~ at the 3' end of the extended polynucleotide strand, thereby moving the first domain ~~in the polynucleotide strand~~ **into proximity with the desired site; and**

c) sequencing the MID and the desired site using a primer that complementary to the primer site in the complement of the first domain.

2-3 (Canceled)

4. (Original) The method of claim 1, wherein the reflex sequence is from 10 to 50 nucleotides in length.

5. (Currently Amended) The method of claim 1, wherein the **first domain** ~~reflex sequence~~ comprises an RNA polymerase site.

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6. (Original) The method of claim 1, the method further comprising removing the first domain and the reflex sequence from the 5' end of the double stranded region.

7. (Original) The method of claim 6, wherein the removing step comprises treating the nucleic acid of step b with T7 exonuclease.

8. (Original) The method of claim 1, wherein the first domain is present in an adapter region attached to the polynucleotide of interest.

9. (Original) The method of claim 8, wherein the reflex sequence is present in the adapter region.

10. (Original) The method of claim 8, wherein the reflex sequence is a sequence present within the 5' region of the polynucleotide of interest.

11. (Original) The method of claim 1, wherein the sample is a multiplexed sample, wherein the first domain in each of the polynucleotide strands in the multiplexed sample comprises a MID corresponding to its parental sample.

12. (Original) The method of claim 1, wherein the sample comprises multiple polynucleotide strands, and wherein each polynucleotide strand in the sample comprises a unique MID in the first domain.

13. (Original) The method of claim 1, wherein the polynucleotide strand is produced by:

annealing a nucleic acid synthesis primer to a parent polynucleotide strand, wherein the parent polynucleotide strand comprises the following in a 5' to 3' orientation:

the first domain;

the reflex sequence; and

a second domain comprising the polynucleotide of interest;

wherein the nucleic acid synthesis primer comprises:

a 3' priming domain that is complementary to a site adjacent to and 3' of the polynucleotide of interest in the second domain; and

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a 5' reflex sequence;

extending the nucleic acid synthesis primer to produce an extension product; and

replicating the resultant extension product with a second nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain, thereby producing the polynucleotide strand.

14. (Original) The method of claim 13, wherein the length of the second domain is from 30 bases to 30 kilobases.

15. (Original) The method of claim 13, wherein the parent polynucleotide strand is enriched prior to the annealing step based on its sequence.

16. (Original) The method of claim 1, further comprising the steps of:

c) replicating the polynucleotide strand of step b with a first nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain to produce a first extension product;

d) annealing a second nucleic acid synthesis primer to the first extension product, wherein the second nucleic acid synthesis primer comprises a 3' priming domain that is complementary to a site within the polynucleotide of interest in the first extension product; and

e) extending the second synthesis primer to produce a second extension product.

17. (Original) The method of claim 16, wherein the second synthesis primer comprises a 5' domain that is not complementary to the polynucleotide of interest in the first extension product.

18. (Original) The method of claim 17, wherein the first domain comprises a first primer site and the 5' domain of the second synthesis primer comprises a second primer site.

19. (Original) The method of claim 18, wherein the length of the polynucleotide of interest in the second extension product is from 50 to 10,000 nucleotides.

20. (Currently Amended) **A kit comprising**:

a polynucleotide strand comprising the following in a 5' to 3' orientation:

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a first domain;

a reflex sequence;

a polynucleotide of interest; and

a complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand**; and**

instructions for performing the method of claim 1.

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REMARKS UNDER 37 CFR § 1.115

Formal Matters

Claims 1 and 3-20 are pending.

Claims 2-3 have been canceled without prejudice. Claims 1, 5 and 20 are amended. Support for the amendments is found at, for example: the section bridging pages 30 and 31; the paragraph bridging pages 30 and 31; page 20, lines 18-19; the sentence bridging pages 17 and 18; page 24 lines 20-23; page 20 line 16-17; the sentence bridging pages 7 and 8; page 21, lines 22-26; page 24, lines 20-24, claims 2 and 3; the paragraph bridging pages 7 and 8; page 21 lines 22-26; page 13 lines 13-15; page 25, lines 5-7 and page 21, lines 22-26. No new matter has been added.

CONCLUSION

Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number CGLC-020.

Respectfully submitted,

Date: January 26, 2012

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(54) EFFICIENT METHOD OF PREPARING DNA INVERTED REPEAT STRUCTURE

(57) Conventional techniques for preparing a chimera gene having an inverted repeat sequence of a target sequence suffer from complications since a target sequence is inserted into 2 sites on a vector in sense and antisense orientations, and restriction enzyme recognition sequences must be independently provided at an insertion site of a vector and both ends of the target sequence.

In this invention, a cassette construct comprising an arbitrary adaptor sequence and an inverted adaptor sequence separated by an arbitrary spacer sequence or a plasmid vector comprising such cassette construct incorporated therein is prepared, a target sequence is ligated to either or both ends of the cassette construct, or a target sequence is inserted into one end of the cassette construct on the plasmid vector, followed by PCR. Thus, a chimera gene having an inverted repeat sequence is prepared.

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Description

Technical Field

[0001] The present invention relates to an RNA interference technique that can be used as a means for analyzing gene functions and that can be used for preparing functionally modified mutant animals or plants.

[0002] The present invention further provides a method for efficiently preparing a chimera gene having an inverted repeat sequence of a DNA fragment of a target gene that is used for RNA interference and a cassette construct therefor.

Background Art

[0003] The antisense method (Non-Patent Document 1) and the sense method (Non-Patent Document 2) have been heretofore known as techniques for post-transcriptional regulation of gene expression. However, both of these techniques are insufficient in terms of efficiency of gene regulation in transformants.

[0004] In recent years, double-stranded RNA (dsRNA) has been found to be more effective in regulation of lowering of post-transcriptional gene expression than antisense or sense RNA in *Caenorhabditis elegans*. This phenomenon is referred to as "RNAi." RNAi starts from degradation of dsRNA into small fragments referred to as small interfering RNAs or siRNAs mediated by a Dicer enzyme, which is a member of the RNase III nuclease family.

[0005] Upon introduction of a very small amount of dsRNA into mammalian cells, interferon is generated, and apoptosis is induced. Accordingly, a method that would not induce apoptosis was studied. It was discovered that siRNA, which is short dsRNA generated upon Dicer-induced degradation of dsRNA, would not induce apoptosis and that it would be capable of post-transcriptional regulation. Further, several *Caenorhabditis elegans* genes were found to encode hairpin RNA and to regulate other genes. Based on such findings, whether or not hairpin-folded small RNA could inhibit functions of a given gene instead of siRNA was examined. As a result, short hairpin RNA (shRNA) was found to be capable of regulating gene expression at the same level as siRNA.

[0006] As a method for further efficiently suppressing post-transcriptional gene expression, a method involving the use of a chimera gene having an inverted repeat sequence of a DNA fragment of a target gene is known (Non-Patent Document 3).

[0007] A method, wherein a chimera gene having an inverted repeat sequence of a DNA fragment of a target gene is stably introduced into an organism, and dsRNA generated upon such introduction in the organism induces degradation of endogenous RNA in a sequence-specific manner (i.e., RNA interference), has been extensively used as a means for effectively analyzing gene functions.

[0008] A technique wherein homologous gene recombination is applied to the insertion of a DNA fragment of a target gene has been reported (Patent Document 1; Non-Patent Document 4).

[0009] Patent Document 1: U.S. Patent Publication No. 20030049835
Non-Patent Document 1: Proc. Natl. Acad. Sci. U.S.A., Vol. 83, 5372-5376 (cited in Non-Patent Document 3)
Non-Patent Document 2: Plant Cell, Vol. 2, p. 291 (cited in Non-Patent Document 3)
Non-Patent Document 3: The Plant Journal, Vol. 33, pp. 793-800
Non-Patent Document 4: Plant J., 27, 581-590, 2001

Disclosure of the Invention

[0010] Conventional techniques for constructing a chimera gene having an inverted repeat sequence of a DNA fragment of a target gene (a target sequence) require separate insertion of the target sequence to 2 sites on an arbitrary vector, and usually on a plasmid vector, in sense and antisense orientations or vice versa.

[0011] In general, such target sequence insertion is carried out via cleavage of DNA with a restriction enzyme and via ligase-mediated binding. In order to accurately insert the target sequence in sense and antisense orientations or vice versa, restriction enzyme recognition sequences must be independently present at 2 target-sequence-insertion sites on the vector and the target sequence must have restriction enzyme recognition sequences corresponding to the target-sequence-insertion sites.

[0012] Construction of a chimera gene having an inverted repeat sequence of a target sequence may be occasionally difficult via standard procedures, depending on the type of nucleotide sequence of the target sequence. Thus, this technique is not suitable for extensive analysis of gene functions.

[0013] Also, a technique wherein homologous gene recombination is applied to target sequence insertion has been reported (Wesley et al., Plant J., 27, 581-590, 2001; U.S. Patent Publication No. 20030049835). In this technique, a pair of recognition sequences for an enzyme used for homologous gene recombination must be provided at both ends of the target sequence, and it is necessary to carry out PCR involving the use of an oligonucleotide primer specific to the nucleotide sequences at both ends of the target sequence or to insert a target sequence between a pair of recognition

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sequences of the enzyme used for homologous gene recombination on a vector. In order to construct a chimera gene having an inverted repeat sequence with the use of a target sequence comprising the aforementioned enzymes used for homologous gene recognition sequence, each target sequence must be independently subjected to homologous gene recombination.

[0014] At the outset, a cassette construct, which is a DNA construct comprising an arbitrary nucleotide sequence (an adaptor sequence) and an inverted repeat nucleotide sequence of the adaptor sequence (an inverted adaptor sequence) separated by an arbitrary spacer sequence or a plasmid vector having such cassette construct incorporated therein is prepared. Subsequently, a DNA fragment of the target gene (the target sequence) is bound to either or both ends of the cassette construct. Alternatively, the target sequence is inserted into a site outside either end of the cassette construct on the plasmid vector. Further, the cassette construct comprising the target sequence bound thereto is used as a template to conduct PCR with the use of a single type of primer corresponding to either end of the target sequence. Alternatively, the plasmid vector having the target sequence inserted therein is used as a template to conduct PCR with the use of a primer (primer A) corresponding to a nucleotide sequence of a plasmid vector located outside of the aforementioned target-sequence-insertion site and a primer (primer B) corresponding to a nucleotide sequence comprising an adaptor sequence or an inverted adaptor sequence located on the side of the cassette construct opposite from the target-sequence-insertion site and part of a spacer sequence. This can yield a double-stranded PCR product, i.e., a chimera gene that contains an inverted repeat sequence of the target sequence via a lariat configuration resulting from temporary annealing between the adaptor sequence and the inverted adaptor sequence in a single single-stranded PCR product generated upon PCR or via a dimmer configuration resulting from temporary annealing between these adaptor sequences of 2 single-stranded PCR products, followed by elongation and PCR (Fig. 1 to Fig. 5).

[0015] In the present invention, a cassette construct, which is a DNA construct comprising an arbitrary nucleotide sequence (an adaptor sequence) and an inverted repeat nucleotide sequence of the adaptor sequence (an inverted adaptor sequence) separated by an arbitrary spacer sequence or a plasmid vector having such cassette construct incorporated therein is first prepared. Thus, a chimera gene having an inverted repeat sequence of a target sequence as shown in Fig. 1 can be efficiently prepared in the same manner, regardless of the type of nucleotide sequence of the target sequence.

[0016] When a large quantity of various genes are knocked out via the RNAi method, it is required that chimera genes having inverted repeat sequences of a target sequence can be easily prepared in the same manner. The present invention sufficiently satisfies such requirement.

[0017] This description includes part or all of the contents as disclosed in the description and/or drawings of Japanese Patent Application No. 2003-330569, which is a priority document of the present application.

Brief Description of the Drawings

[0018]

Fig. 1 schematically shows an inverted repeat sequence of a target sequence prepared in the present invention.
Fig. 2 shows a procedure 1 for preparing a cassette construct.
Fig. 3 shows a procedure 2 for preparing a cassette construct.
Fig. 4 shows a procedure for preparing a plasmid vector for preparing an inverted repeat sequence of a target sequence comprising a cassette construct incorporated therein.
Fig. 5 shows a procedure for preparing a chimera gene having an inverted repeat sequence of a target sequence.
Fig. 6 illustrates a cassette construct 1 for facilitating target sequence binding.
Fig. 7 illustrates a cassette construct 2 for facilitating target sequence binding.
Fig. 8 illustrates a cassette construct 3 for facilitating target sequence binding.
Fig. 9 illustrates a cassette construct 4 for facilitating target sequence binding.
Fig. 10 illustrates a cassette construct 5 for facilitating target sequence binding.
Fig. 11 illustrates a cassette construct 6 for facilitating target sequence binding.
Fig. 12 shows an embodiment of modification of a plasmid vector for preparing an inverted repeat sequence of a target sequence comprising a cassette construct incorporated therein.
Fig. 13 shows a method for preparing pGEMA and pGEMAS.
Fig. 14 shows a method for preparing pRNAi.
Fig. 15 shows a procedure for inserting the target sequence Z8755 into pRNAi.
Fig. 16 shows a procedure for preparing a chimera gene having an inverted repeat sequence of the target sequence Z8755.

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Preferred Embodiments of the Invention

1. Cassette construct

[0019] The present invention includes a DNA construct (a cassette construct) wherein an arbitrary sequence (a spacer or intervening sequence) is located between another arbitary nucleotide sequence (an adaptor sequence) and an inverted repeat nucleotide sequence of the adaptor sequence (an inverted adaptor sequence) (Figs. 2 and 3).

1-1. Spacer sequence (intron)

[0020] A spacer sequence must be a nucleotide sequence that is not related to a target sequence or an adaptor sequence (i.e., a sequence that does not complementarily bind to a target sequence or an adaptor sequence *in vitro* or *in vivo*). For example, an arbitrary sequence that does not complementarily hybridize to a target sequence or an adaptor sequence at the time of asymmetric PCR for chimera gene amplification can be used as a spacer sequence. A spacer sequence is preferably composed of 10 to 10,000 nucleotides, and more preferably 50 to 2,000 nucleotides, so that inverted repeat adaptor sequences can easily complementarily anneal to each other in a single molecule in the state of single-stranded DNA or RNA and they can be easily amplified in *E. coli*. Specific examples of spacer sequences that can be used include a partial fragment of a green fluorescent protein (GFP) from *Aequorea coerulescens* or that of a β-glucuronidase (GUS) structural gene from *E. coli*. More preferable examples include intron sequences, such as an intron sequence from the fatty acid desaturase (FAD2) gene from *Arabidopsis thaliana* or that from the ABC transporter gene of a fruit fly, so that a spacer sequence can be removed via splicing in the body of a plant, fruit fly, or the like.

1-2. Adaptor sequence

[0021] An adaptor sequence and an inverted adaptor sequence are each independently a nucleotide sequence that is not related to the nucleotide sequence of a target sequence, a spacer sequence, or a plasmid vector used (i.e., a sequence that does not complementarily bind to a target sequence, a spacer sequence, or a plasmid vector used *in vitro* or *in vivo*). Use of a sequence that enables annealing of an adaptor sequence to an inverted adaptor sequence under general PCR annealing conditions is preferable. Specifically, such sequence is designed to have 18 to 28 nucleotides, GC content of 50% to 60%, and Tm of 55°C to 80°C, in general. More specifically, a lone linker sequence reported by Ko et al. can be used as an adaptor sequence and a complementary sequence thereof can be used as an inverted adaptor sequence (Ko, M. S. H. et al., Nucleic Acids Res., 18, pp. 4293-4294, 1990).

[0022] An inverted adaptor sequence is not necessarily a completely inverted sequence of an adaptor sequence. However, it is necessary that such inverted adaptor sequence be able to complementarily hybridize to the target adaptor sequence at the time of asymmetric PCR for chimera gene amplification.

2-1. Preparation of cassette construct

[0023] The cassette construct can be prepared via conventional techniques. Preferably, a spacer sequence having blunt ends is first prepared, as shown in Fig. 2. Further, linker sequences each having a noncohesive protruding end and a blunt end, which are referred to as "lone linkers," can be ligated to the both blunt ends of the spacer sequence as an adaptor sequence and as an inverted adaptor sequence. Examples of lone linkers include LL-Sse8387I, such as LL-Sse8387IA (5'-GAGATATTACCTGCAGGTACTC-3') and LL-Sse8387IB (5'-GAGTACCTGCAGGTAATAT-3') and LL-SalI, such as LL-SalIA (5'-ATTGACGTCGACTATCCAGG-3') and LL-SalIB (5'-CCTGGATAGTCGACGTC-3') (Ko, M. S. H. et al., Nucleic Acids Res., 18, pp. 4293-4294, 1990). LL-Sse8387IA,B or LL-SAlIA,B is phosphorylated and annealed. The annealed LL-Sse8387I or LL-SalI is ligated to a spacer sequence having blunt ends. The thus prepared cassette construct can be amplified via PCR using, for example, LL-Sse8387I or LL-SalIA as a primer.

[0024] Alternatively, the cassette construct can be prepared in the following manner. A cassette construct is prepared on a plasmid vector via conventional techniques, and a portion of the cassette construct can be cleaved via restriction enzyme reaction or can be amplified via PCR. A cassette construct can be prepared on a plasmid vector by, for example, inserting an adaptor sequence and an inverted adaptor sequence at both ends of the spacer sequence on the plasmid vector comprising the spacer sequence inserted therein via a conventional technique, i.e., restriction enzyme reaction, followed by ligation, as shown in Fig. 3A. Alternatively, an oligonucleotide having an adaptor sequence and an inverted adaptor sequence adjacent to each other and a recognition sequence for an arbitrary restriction enzyme between these adaptor sequences and an oligonucleotide consisting of a complementary strand of the former oligonucleotide are prepared as shown in Fig. 3B. These oligonucleotides are annealed to each other to prepare an inverted repeat adaptor sequence, the resultant is incorporated into an arbitrary plasmid vector, and a spacer sequence can be then inserted in the middle of the inverted repeat adaptor sequence with the use of the arbitrary restriction enzymes. Restriction enzymes,

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the recognition sites for which do not exist on an adaptor sequence, a plasmid vector, and a spacer sequence can be used as the aforementioned restriction enzymes. Preferably, restriction enzymes that generate cohesive ends with both ends of the spacer sequence to be inserted can be used. A plasmid vector for TA cloning, such as the pGEM-T Easy Vector (Promega), can be preferably used as the plasmid vector with the addition of a single nucleotide, i.e., adenine (A), to the 3' end of the oligonucleotide.

2-2. Preparation of a plasmid vector for preparing an inverted repeat sequence of a target sequence comprising a cassette construct incorporated therein

[0025] A plasmid vector for preparing an inverted repeat sequence of a target sequence comprising a cassette construct incorporated therein can be prepared via conventional techniques. For example, a restriction enzyme recognition site at which a target sequence can be inserted into the plasmid vector located adjacent to the cassette construct comprised in the plasmid vector 3A and the plasmid vector 3B as shown in Fig. 3 may be provided, and the resultant can be used as a plasmid vector for preparing an inverted repeat sequence of the target sequence having the cassette construct incorporated therein. Alternatively, a restriction enzyme recognition site that generates blunt ends on a plasmid vector adjacent to the cassette construct upon incorporation of the cassette construct prepared in 2-1 into an arbitrary plasmid vector may be provided as shown in Fig. 4. Thus, a plasmid vector for preparing an inverted repeat sequence of a target sequence that can easily insert a target sequence can be designed.

[0026] The plasmid vector having the cassette construct incorporated therein can be used for preparing the pool of chimera genes having inverted repeat sequences of a variety of gene DNA fragments based on cDNA of arbitrary animals or plants, and preferably based on homogenized cDNA.

2-3. Binding of a target sequence to a cassette construct and insertion of a target sequence into a plasmid vector for preparing an inverted repeat sequence comprising a cassette construct incorporated therein

[0027] A target sequence can be selected in accordance with the Patent Document 1. For example, a DNA fragment of the gene, the phenotype expression of which is to be suppressed, can be used as a target sequence. A target sequence is composed of 10 to a number of nucleotides required for sufficiently suppressing the phenotype expression of the target sequence, and such number is preferably at least 19 nucleotides, at least 21 nucleotides, at least 25 nucleotides, at least about 50 nucleotides, at least about 100 nucleotides, at least about 150 nucleotides, at least about 200 nucleotides, or at least about 500 nucleotides. A target sequence may be composed of about 1,000 nucleotides, if possible.

[0028] A target sequence is bound to either or both ends of a cassette construct. In such a case, use of a target sequence having unphosphorylated blunt ends is preferable. Such target sequence can be prepared via PCR with the use of a set of primers having the dephosphorylated 5' end and thermostable DNA polymerase that does not add (dA) to the 3' end or via PCR with the use of common thermostable polymerase, followed by removal of a (dA) protrusion at the 3' end via conventional techniques and dephosphorylation of PCR products. Alternatively, PCR is carried out with the use of a set of primers having the phosphorylated 5' end and thermostable DNA polymerase that does not add (dA) to the 3' end, followed by dephosphorylation of the 5' end of PCR products. Thus, a target sequence of interest can be prepared.

[0029] A target sequence is inserted into a site outside of an adaptor sequence or an inverted adaptor sequence on a plasmid vector for preparing an inverted repeat sequence of the target sequence, which comprises the cassette construct incorporated therein. Preferably, a DNA fragment of the target sequence is inserted into a site outside one end of the cassette construct with the use of a restriction enzyme, and the recognition sequence for which is present at a site outside one end of the cassette construct on the plasmid vector only. More preferably, a target gene is inserted into a site outside either end of the cassette construct, where it is proximal to the cassette construct. When a recognition sequence of the blunt-end-generating restriction enzyme is located outside the adaptor sequence, for example, the plasmid vector is cleaved with such restriction enzyme, and the cleavage product can be ligated to a blunt-ended target sequence with the aid of a ligase. When another restriction enzyme recognition sequence is present outside the adaptor sequence, a plasmid vector and the target sequence are treated with the same restriction enzyme, and they are allowed to bind to each other at their cohesive ends.

2-4. Modification of a cassette construct (pretreatment for binding a target sequence to either or both ends of a cassette construct)

[0030] In order to facilitate the binding of the target sequence to the cassette construct and the preparation of an inverted repeat sequence of the target sequence via PCR using the cassette construct having the target sequence bound thereto as a template, either or both ends of the cassette construct can be modified as a pretreatment.

[0031] Cassette constructs for facilitating target sequence binding are as described below.

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[0032] The cassette construct 1 for facilitating target sequence binding shown in Fig. 6 (hereafter abbreviated to "CC1") is designed to facilitate the binding of the target sequence, both ends of which are dephosphorylated, via phosphorylation of both 5' ends. CC1 can be easily prepared with the use of thermostable DNA polymerase that does not add (dA) to the 3' end, such as KOD-Plus- (Toyobo). The cassette construct 2 for facilitating target sequence binding shown in Fig. 7 (CC2) is designed to facilitate the binding of the target sequence, both ends of which are dephosphorylated, to a single end of the cassette construct via phosphorylation of a single end.

[0033] The cassette construct 3 for facilitating target sequence binding shown in Fig. 8 (CC3) is designed to facilitate the binding of the target sequence, which is amplified with the use of common thermostable DNA polymerase that adds (dA) to the 3' end and both ends of which are dephosphorylated, to the 3' end of the cassette construct via addition of (dT) to both 3' ends of CC1.

[0034] The cassette construct 4 for facilitating target sequence binding shown in Fig. 9 (CC4) is designed to facilitate the binding of the target sequence, which is amplified with the use of common thermostable DNA polymerase that adds (dA) to the 3' end and both ends of which are dephosphorylated, to a single 3' end of the cassette construct via addition of (dT) and phosphorylation of such single 3' end.

[0035] The cassette construct 5 for facilitating target sequence binding shown in Fig. 10 (CC5) is designed to facilitate the binding of the target sequence to a single end of the cassette construct via addition of topoisomerase I (Invitrogen) and phosphorylation of a single 3' end.

[0036] The cassette construct 6 for facilitating target sequence binding shown in Fig. 11 (CC6) is designed to facilitate the binding of the target sequence, which is amplified with the use of common thermostable DNA polymerase that adds (dA) to the 3' end and both ends of which are dephosphorylated, to a single end of the cassette construct via addition of (dT) and topoisomerase I to a single 3' end and phosphorylation of such single 3' end.

2-5. Modification of a plasmid vector for preparing an inverted repeat sequence of a target sequence comprising a cassette construct incorporated therein

[0037] In order to facilitate the insertion of the target sequence into a plasmid vector for preparing an inverted repeat sequence of the target sequence comprising the cassette construct incorporated therein, the plasmid vector for preparing an inverted repeat sequence is cleaved with a restriction enzyme or the like, and the ends thereof can be processed so as to facilitate the insertion of the target sequence. More specifically, the cleaved ends are blunt-ended as with the case of pRN Ai/blunt shown in Fig. 12, (dT) is added to the cleaved 3' end as with the case of pRNAiT shown in Fig. 12, topoisomerase I is added to the cleaved 3' end as with the case of pRNAiTP shown in Fig. 12, or (dT) and topoisomerase I are added to the cleaved 3' end as with the case of pRNAiTTP shown in Fig. 12.

3. Amplification of chimera gene

[0038] A DNA product in which the target sequence has been bound to either or both ends of the cassette construct as shown in any of Fig. 6 to Fig. 11 is used as a template to conduct arbitrary gene amplification techniques, and preferably, PCR, with the use of a primer specific to one end of the target sequence (primer I or II). Thus, a chimera gene comprising a target sequence and an inverted repeat sequence thereof separated by the cassette construct can be prepared. Specifically, generation of a single-stranded DNA construct via thermal denaturation, PCR with the use of primer I or II may be carried out. Fig. 6 to Fig. 11 show the examples of PCR with the sole use of a primer specific to the target sequence, and different linkers (or adaptors) can be ligated to both ends of a cDNA or genomic DNA fragment via conventional techniques. In such a case, a primer set of the linker (or adaptor) portion can be used instead of a primer specific to the target sequence.

[0039] A plasmid vector comprising the target sequence inserted into a site located outside either end of the cassette construct is used as a template to conduct arbitrary gene amplification techniques, and preferably asymmetric PCR (in which the amount of a primer used is excessively large compared with another primer; asymmetric amplification, 4-1-2-6, "Amplification and detection of nucleic acid," Standard Techniques, (the Japanese Patent Office)). Thus, a chimera gene comprising the target sequence and an inverted repeat sequence separated by the cassette construct can be prepared. A set of the following primers is used: (1) a part of a plasmid vector located on the opposite side of the cassette construct from the inserted target sequence; and (2) an oligonucleotide designed to comprise an adaptor sequence or an inverted adaptor sequence located at a position farther from the target sequence and part of a spacer sequence adjacent thereto. When asymmetric PCR is carried out, it is preferable to use the primer (1) in amounts that are 5, 10, or approximately 100 times or more greater than that of the primer (2).

[0040] An arbitrary sequence composed of several nucleotides may be incorporated into the 5' side of the primer to facilitate the incorporation of the amplified chimera genes into various types of vectors.

[0041] The aforementioned asymmetric PCR results in amplification of excessive amounts of single-stranded PCR products composed of a target sequence, an adaptor sequence, a spacer sequence, and an inverted adaptor sequence

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ligated in that order. The adaptor sequence and part of the inverted adaptor sequence in the resulting single-stranded PCR product are temporarily annealed to each other in a single molecule to form a lariat configuration, or these sequences are annealed to each other between 2 molecules, followed by elongation and PCR amplification with the sole use of the primer (1). Thus, a chimera gene having an inverted repeat sequence can be obtained as a double-stranded PCR product.

[0042] Also, a chimera gene having a target sequence and an inverted repeat sequence thereof can be obtained as a double-stranded PCR product without asymmetric PCR. For example, general PCR (symmetric PCR) is carried out, a single-stranded PCR product elongated from the aforementioned primer (1), i.e., a single-stranded PCR product composed of a target sequence, an adaptor sequence, a spacer sequence, and an inverted adaptor sequence ligated in that order, is recovered, and the resultant is subjected to PCR with the sole use of the aforementioned primer (1). The adaptor sequence and part of the inverted adaptor sequence in the resulting single-stranded PCR product are temporarily annealed to each other in a single molecule to form a lariat configuration, or these sequences are annealed to each other between 2 molecules, followed by elongation and PCR amplification with the sole use of the primer (1). Thus, a chimera gene having an inverted repeat sequence can be obtained as a double-stranded PCR product.

4. Incorporation into expression vector and preparation of transformant

[0043] An amplification product comprising the inverted repeat sequence of the target sequence can be incorporated into an adequate expression vector.

[0044] The resulting expression vector can be introduced into a host cell, such as a plant cell.

[0045] Hereafter, the present invention is described in greater detail with reference to the examples, although the technical scope of the present invention is not limited thereto.

Examples

[Example 1]

[0046] A chimera gene shown in Fig. 1 was prepared in accordance with the following steps (1) to (3) as an example of preparation of a chimera gene having an inverted repeat sequence of a DNA fragment of a target gene (a target sequence) according to the present invention.

(1) Preparation of pRNAi

[0047] An oligonucleotide having an arbitrary adaptor sequence and an inverted arbitrary sequence between which a spacer sequence can be inserted in the center is synthesized. An oligonucleotide (5'-GAGATATTACCTGCAGGTACT-CACCCGGGTGAGTACCTGCAGGTAATATCTC A-3') having a recognition sequence CCCGGG for the restriction enzyme *Sma*I in the center thereof and having A at its 3' end was synthesized herein, and this oligonucleotide was annealed (Fig. 13B), followed by cloning into a plasmid vector. Since protrusions (A) were generated at both 3' ends via annealing, the resultant was cloned into a commercially available TA cloning vector (the pGEM-T Easy Vector, Promega) (Fig. 13A) to prepare pGEMA (Fig. 13C). Further, a spacer sequence was inserted into the center of the cloned inverted repeat sequence (Fig. 13D to 13F). The center portion of the inverted repeat sequence of pGEMA was cleaved with the restriction enzyme *Sma*I (Fig. 13D), as a spacer sequence, a DNA fragment containing the intron sequence of the FAD2 genes from *Arabidopsis thaliana* was amplified via PCR (Fig. 13E, SEQ ID NO: 4), and the amplified fragment was inserted with the aid of T4 DNA ligase to prepare pGENAS (Fig. 13F). Subsequently, a region containing a cassette construct was cleaved with the restriction enzyme *Eco*RI, the recognition sequences of which are located at both sides of the cloning site of an oligonucleotide on the pGEM-T Easy Vector, and the cleavage product was inserted and ligated to the *Eco*RI cleavage site of the pBluescript II KS plasmid vector (Stratagene) with the use of T4 DNA ligase to prepare pRNAi comprising the recognition sequence for the restriction enzyme EcoRV at a position located outside of the adaptor sequence of the cassette construct (Fig. 14). Subsequently, the nucleotide sequence of pRNAi was examined via conventional techniques.

(2) Insertion of DNA fragment of target gene (target sequence) into pRNAi

[0048] A target sequence was inserted into a site outside one end of the cassette construct on pRNAi. Part of cDNA of a receptor protein kinase gene of zinnia (Z8755, DDBJ Accession No. AU293996, T. Demura et al., PNAS, 99, 15794-15799), which had been cloned into the pGEM-T Easy Vector (Invitrogen), was inserted into a site outside of the adaptor sequence. At the outset, a set of primers corresponding to the sequence located outside of the cloning site on the vector; i.e., a forward primer (TGTAAAACGACGGCCAGT) and a reverse primer (CAGGAAACAGCTATGACC), were used to amplify via PCR a DNA fragment containing cDNA of Z8755, and a partial DNA fragment having blunt

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ends was prepared using the restriction enzyme *Afa*I (SEQ ID NO: 5). The resultant was inserted and ligated to the restriction enzyme *Eco*RV cleavage site of pRNAi with the aid of T4 DNA ligase (Fig. 15).

(3) Preparation of a chimera gene having an inverted repeat sequence of a target sequence

[0049] A plasmid comprising a target sequence inserted therein was used as a template to conduct PCR with the use of a primer (primer A) designed based on the nucleotide sequence of a vector located immediately outside of the target-sequence-insertion site and a primer (primer B) designed based on part of a spacer sequence and a nucleotide sequence containing an inverted repeat sequence located on the side of the cassette construct opposite from the DNA insertion site. In such a case, the amount of primer A used was excessively large compared with that of primer B. Thus, a double-stranded PCR product comprising a target sequence and an inverted repeat sequence thereof separated by a cassette construct was prepared. This is considered to be via a lariat configuration resulting from temporary annealing between the adaptor sequence and the inverted adaptor sequence in a single single-stranded PCR product generated upon PCR or via a dimmer configuration resulting from temporary annealing between these adaptor sequences of 2 single-stranded PCR products, followed by elongation and PCR. As shown in Fig. 16, oligonucleotide RNAiF (CACCCCTCGAGGTCGACGGTATCGATAAGCTTGAT) comprising a nucleotide sequence (CCCCTCGAGGTCGACGGTATCGATAAGCTTGAT) located immediately outside the recognition site for the restriction enzyme EcoRV of pBluescript II KS and having at its 5' end a sequence for cloning into pENTR/D Topo Vectpr (Invitrogen) was used at a final concentration of 0.6 μmol/l as primer A. Also, oligonucleotide RNAiR (GATTGAGATATTACCTGCAGGTACTCACCCGGGTG) synthesized from a complementary strand of the 3' end (CACCC) of the spacer sequence and the nucleotide sequence (GGGTGAGTACCTGCAGGTAATATCT) of an inverted adaptor sequence was used at a final concentration of 0.6 μmol/l as primer B. The reaction was carried out in 50 μl of a reaction solution comprising about 2 ng of a plasmid (pRNAi-Z8755) comprising target sequence (a partial sequence of Z8755), 1 μl of thermostable DNA polymerase (KOD-Plus-, TOYOBO), 5 μl of 10x buffer (TOYOBO), 2 μl of 25 mM $MgSO_4$ (TOYOBO), 5 μl of 2 mM dNTP (TOYOBO), 3 μl of 10 μM RNAiF, and 3 μl of 1 μM RNAiR, and thermal denaturation was carried out at 94°C for 2 minutes, followed by the reaction at 94°C for 15 seconds and at 68°C for 3 minutes and 30 seconds. Such PCR cycle was repeated 50 times.

(4) Subcloning of a chimera gene having an inverted repeat sequence of a target sequence

[0050] The PCR product amplified via PCR in (3) above was subcloned into a plasmid vector via conventional techniques, and the nucleotide sequence thereof was determined to examine whether or not the target sequence had an inverted repeat sequence. The pENTR/D TOPO vector (Invitrogen) was used as a plasmid vector, and the nucleotide sequence was determined via cycle sequencing.

Industrial Applicability

[0051] The present invention can be applied to technical fields where transformants and knock-out animals or plants are produced via an RNAi technique.

[0052] All publications, patents, and patent applications cited herein are incorporated herein by reference in their entirety.

Sequence Listing Free Text

[0053] SEQ ID NO: 1 represents a ligation product of an adaptor sequence and an inverted sequence thereof. SEQ ID NOs: 2 and 3 represent primers. SEQ ID NO: 4 represents a DNA sequence containing an intron sequence of the FAD2 gene from *Arabidopsis thaliana*. SEQ ID NO: 5 represents a partial DNA sequence of zinnia cDNA (Z8755).

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SEQUENCE LISTING

<110> RIKEN

<120> Method of preparing inverted repeat sequence

<130> PH-2258-PCT

<150> JP 2003/330569

<151> 2003-09-22

<160> 5

<170> PatentIn version 3.1

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<211> 53

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<213> artificial

<220>

<223> adaptor sequence

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<210> 2

<211> 33

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<223> Partial DNA sequence of the Zinnia elegans cDNA, Z8755

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aggagcccgc aacagcgcta atactagcag 90

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Claims

1. A cassette construct for preparing an inverted repeat sequence of a target sequence consisting of an adaptor sequence, a spacer sequence, and an inverted sequence of the adaptor sequence.

2. The cassette construct according to claim 1, wherein the spacer sequence is an intron sequence.

3. The cassette construct according to claim 1 or 2, wherein either or both ends of the cassette construct are pretreated for target sequence binding.

4. The cassette construct according to any one of claims 1 to 3, which comprises a target sequence bound thereto at its either or both ends.

5. A method for preparing an amplification product comprising an inverted repeat sequence of a target sequence via PCR with the use of the cassette construct according to claim 4 as a template and a single primer derived from a sequence at either end of the target sequence.

6. A method for preparing an inverted repeat sequence of a target sequence via PCR with the use of the cassette construct according to claim 4 as a template.

7. A plasmid comprising the cassette construct according to claim 4 incorporated therein.

8. A method for preparing an inverted repeat sequence of a target sequence via PCR with the use of the plasmid according to claim 7 as a template.

9. The method for preparing an inverted repeat sequence of a target sequence according to claim 8, wherein PCR is asymmetric PCR.

10. The method according to claim 8 or 9, wherein a 3' end of a primer used in PCR contains a spacer sequence.

11. An expression vector comprising the inverted repeat sequence of the target sequence prepared by the method according to any one of claims 5, 6, 8, 9, and 10.

12. A host cell transformed with the expression vector according to claim 11.

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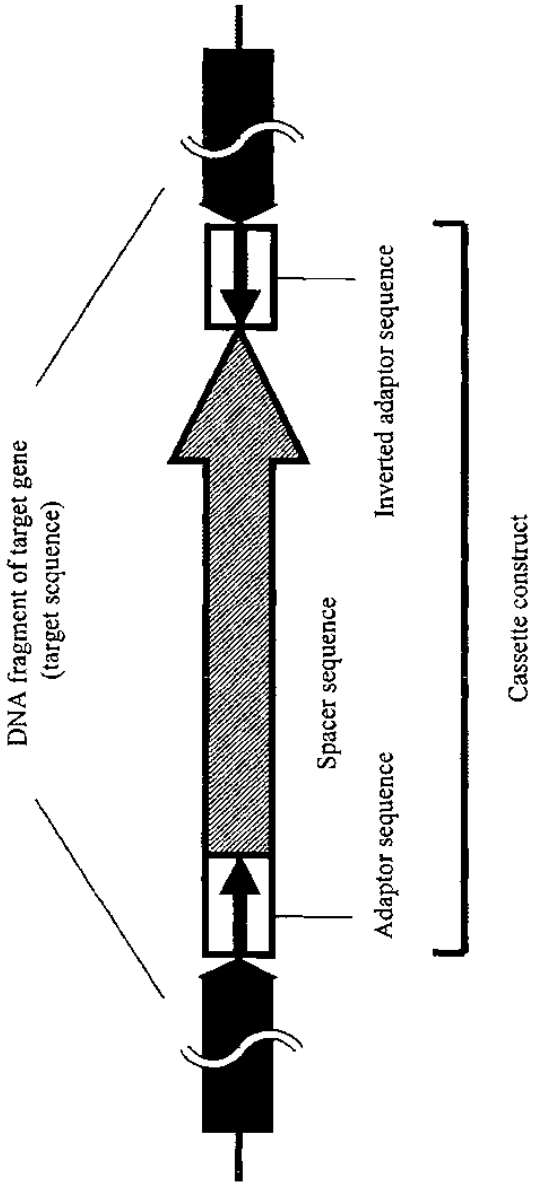


Fig. 1

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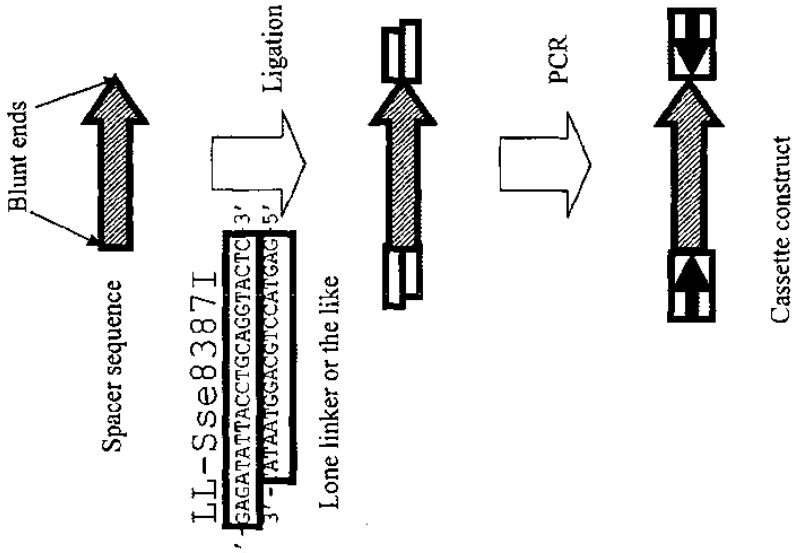
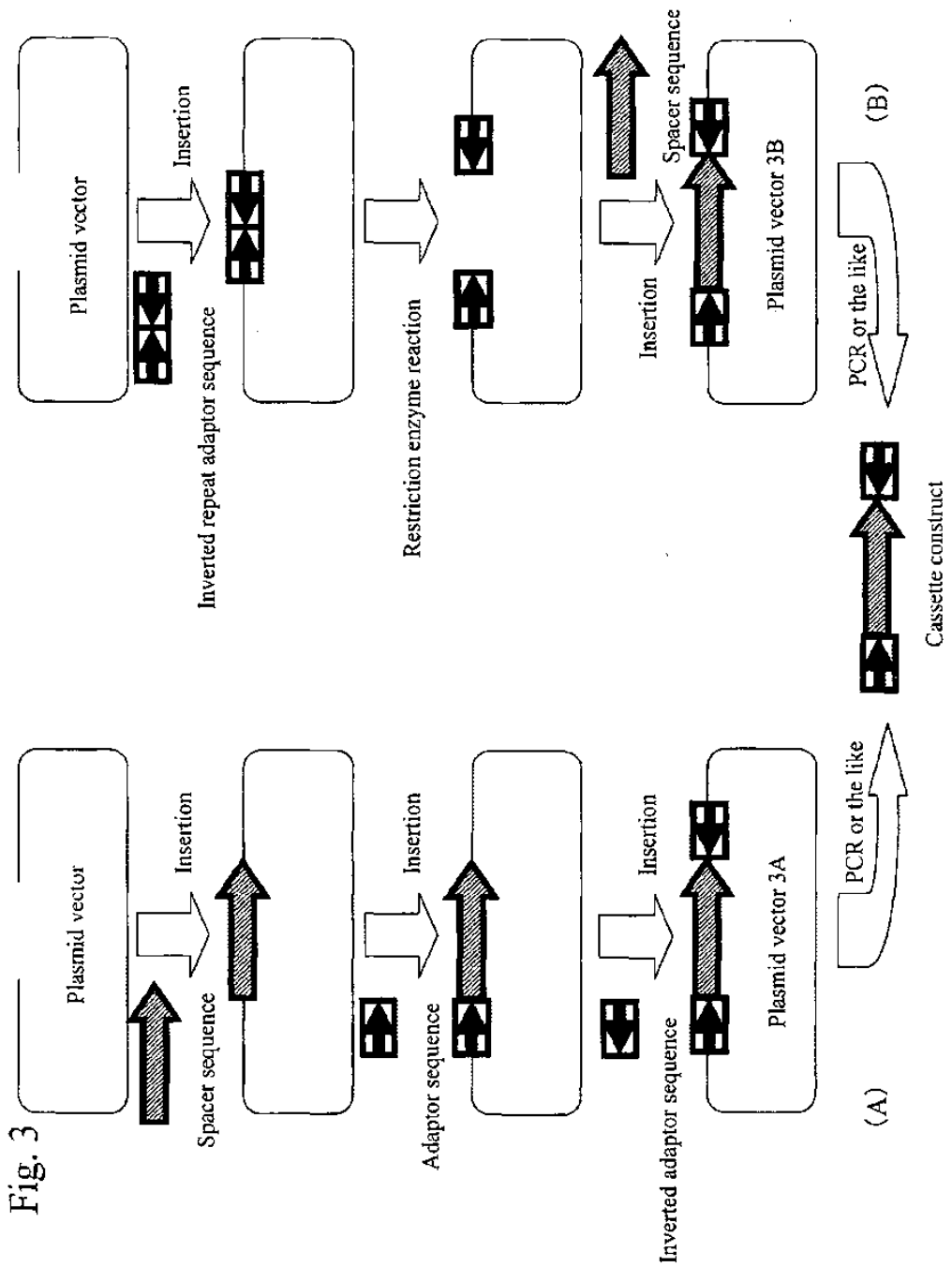


Fig. 2

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Fig. 3



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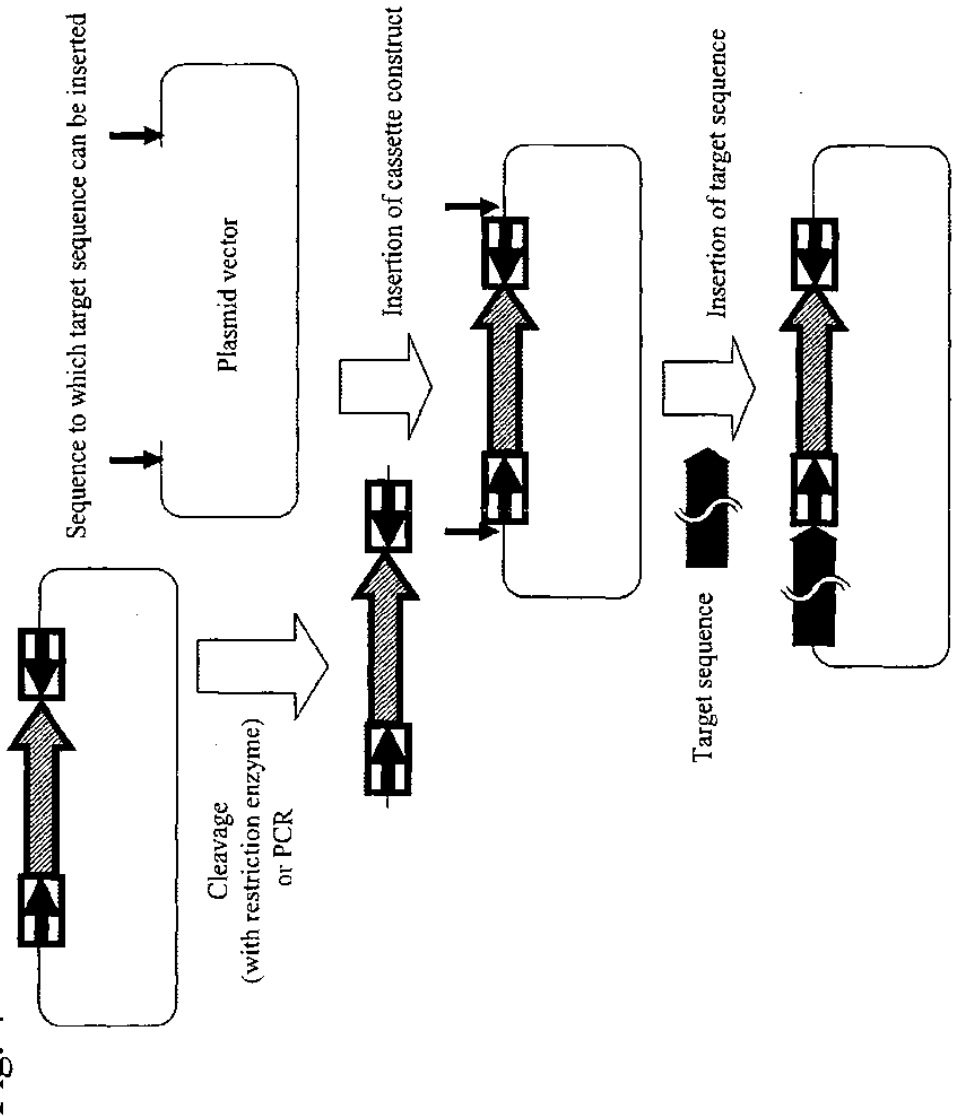
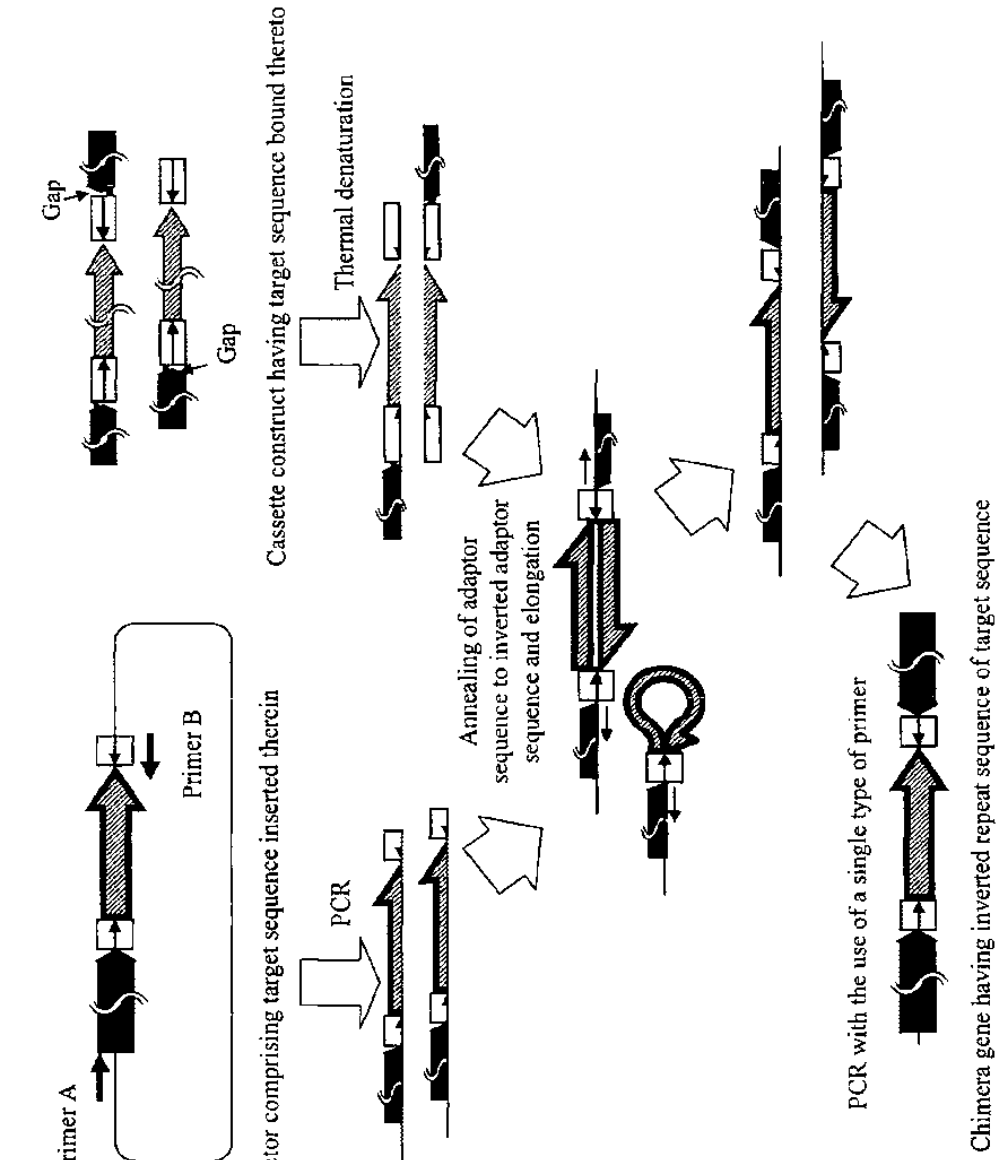


Fig. 4

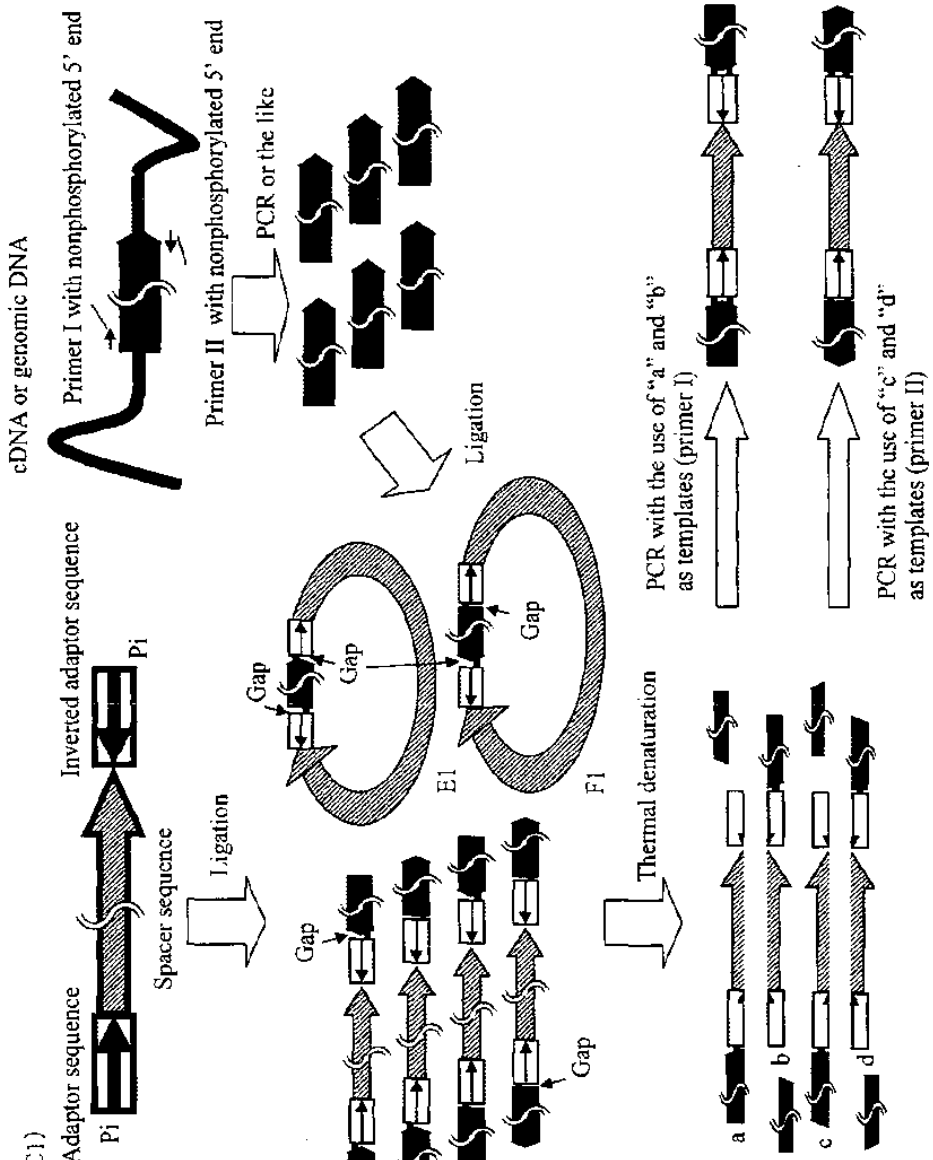
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Fig. 5



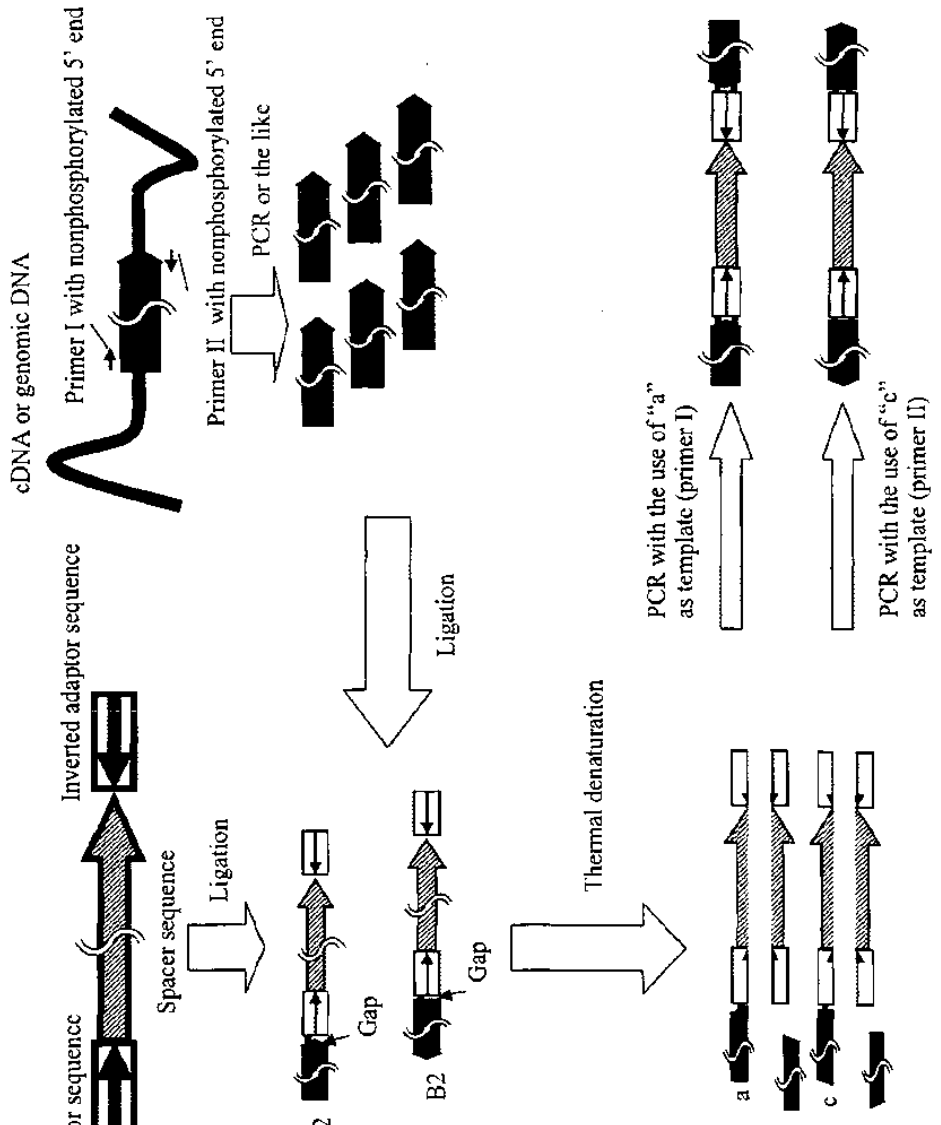
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Fig. 6



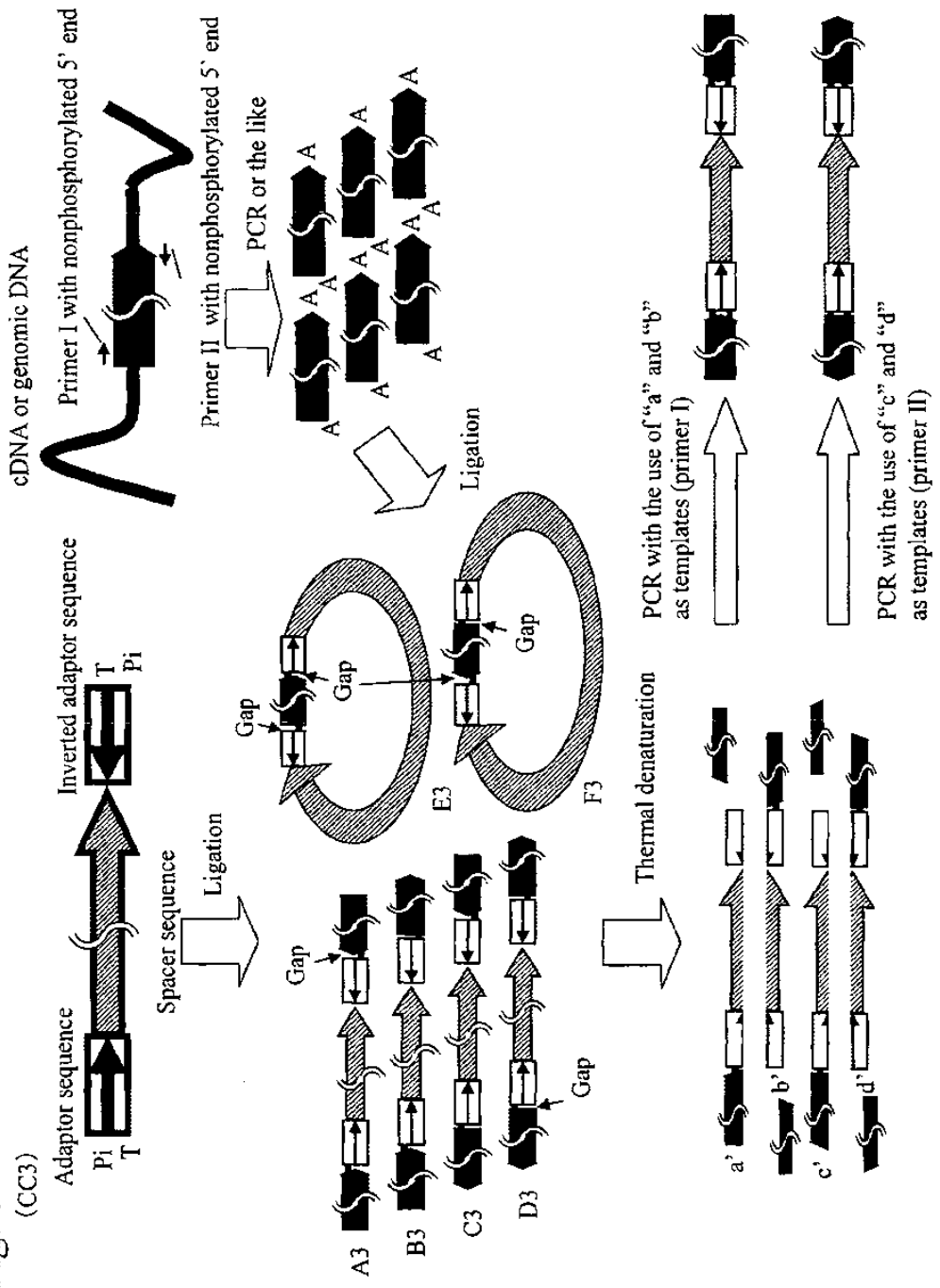
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Fig. 7



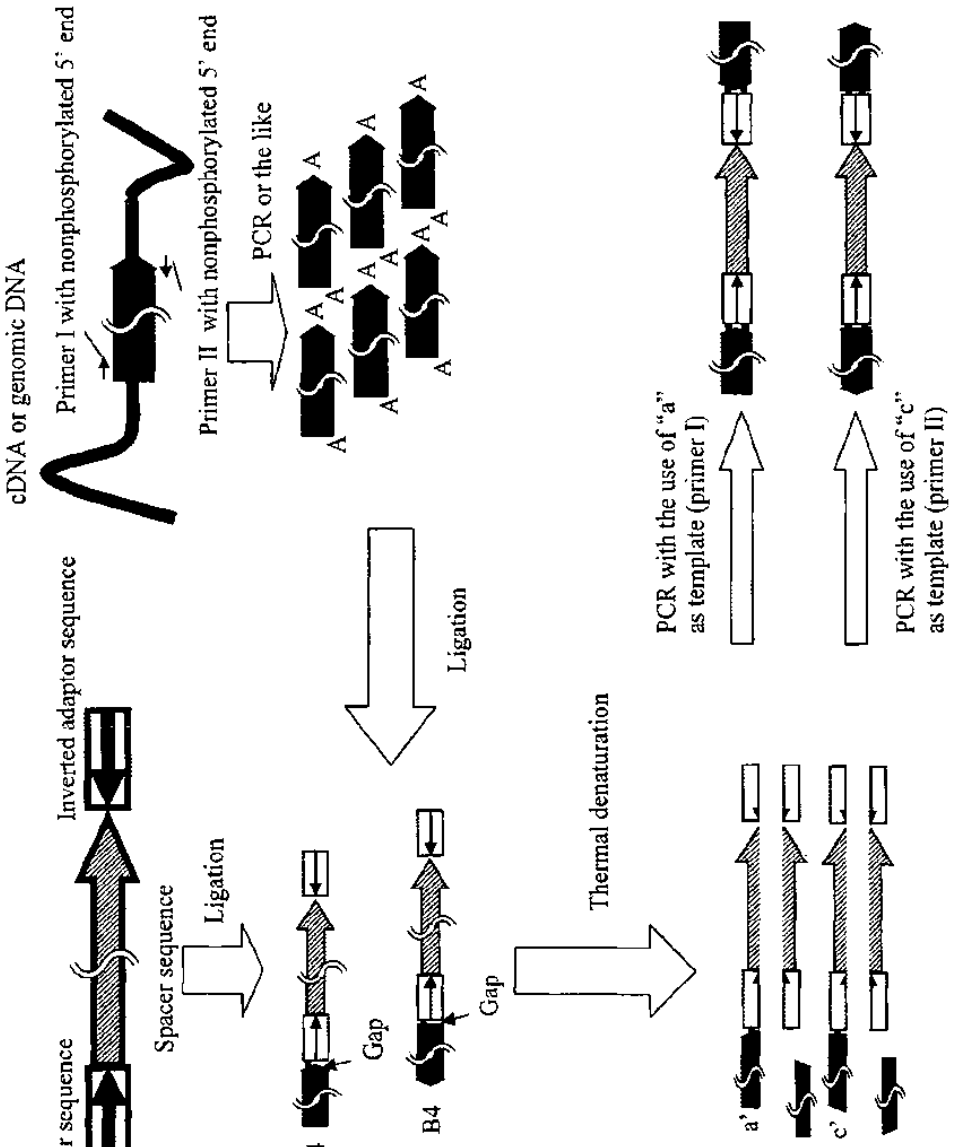
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Fig. 8



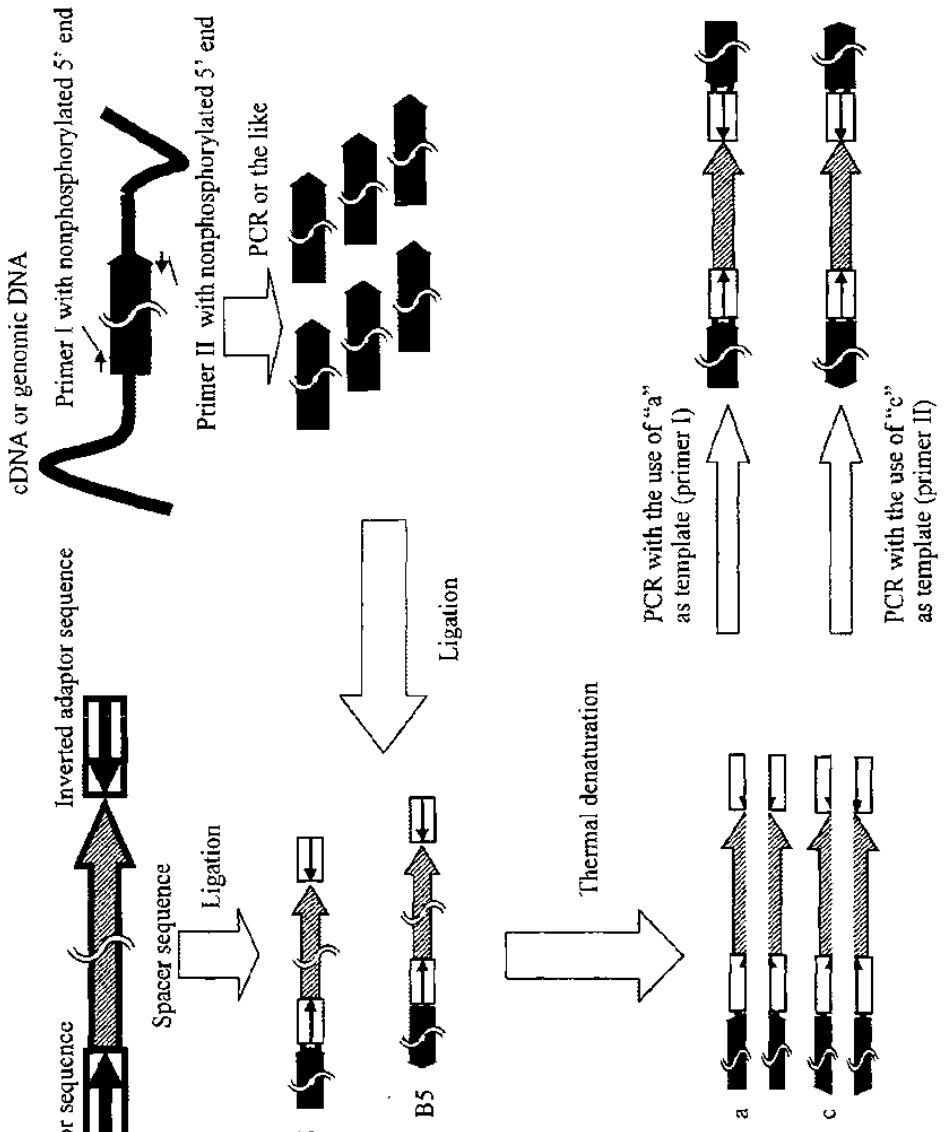
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Fig. 9



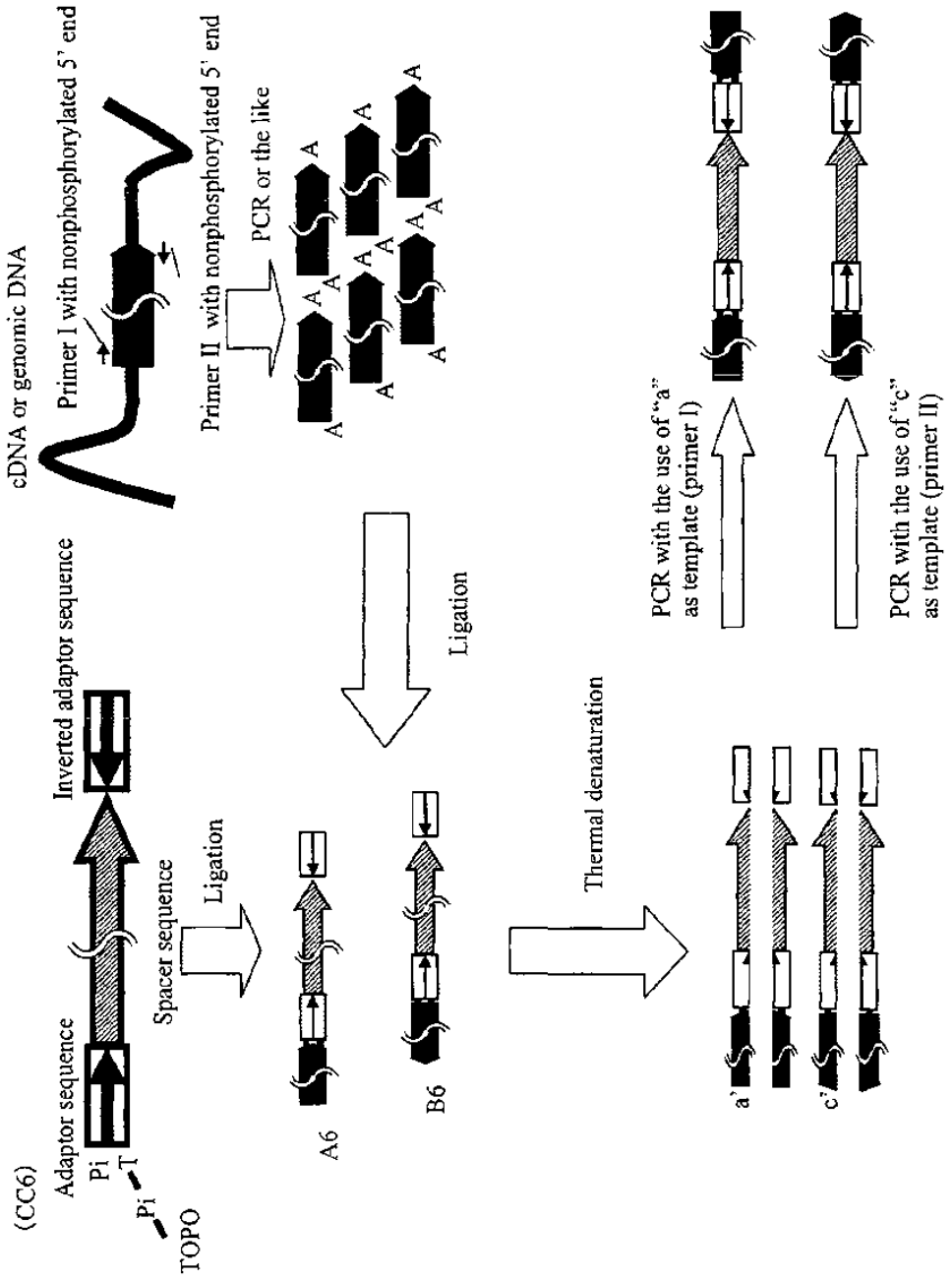
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Fig. 10



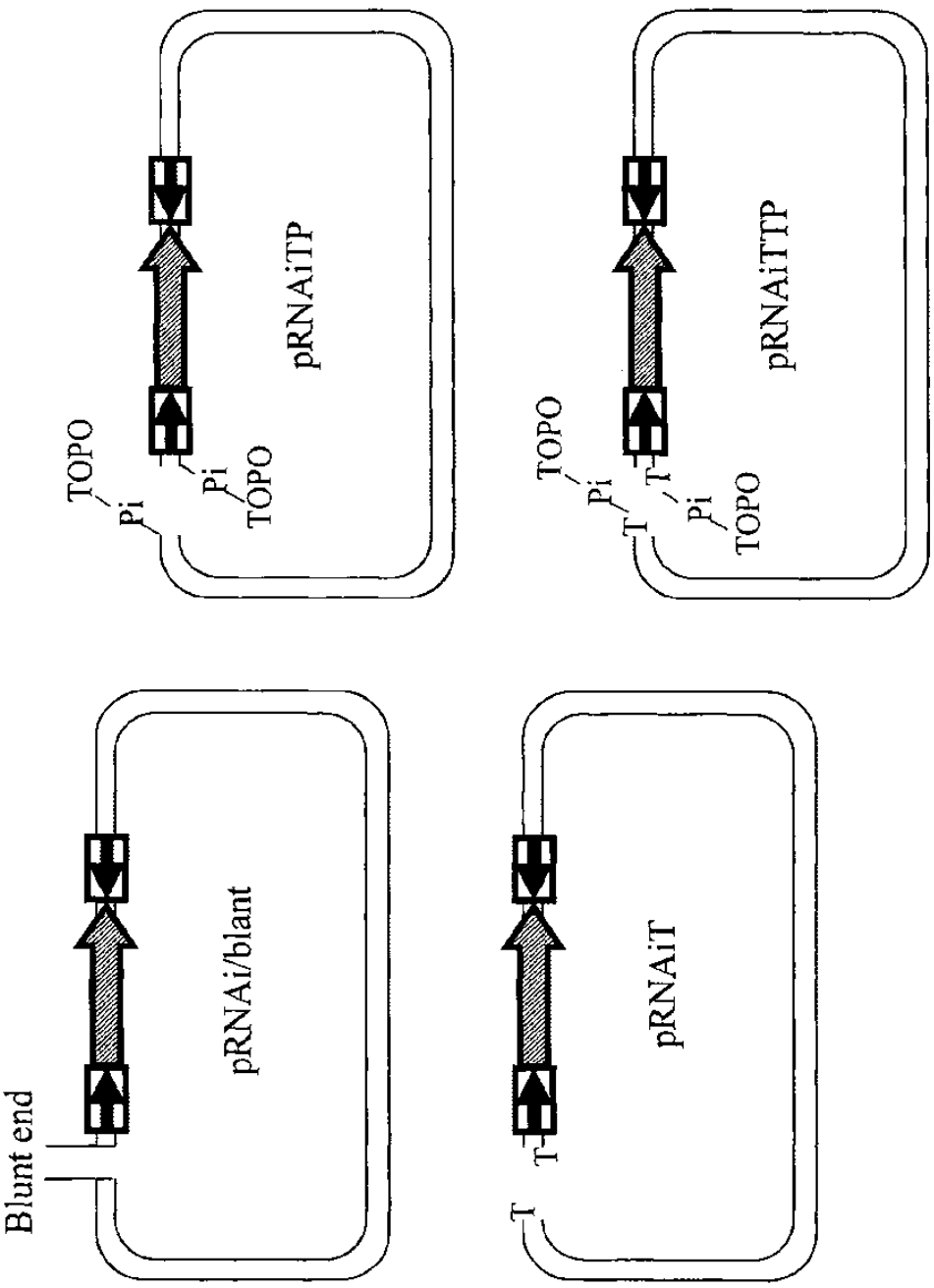
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Fig. 11



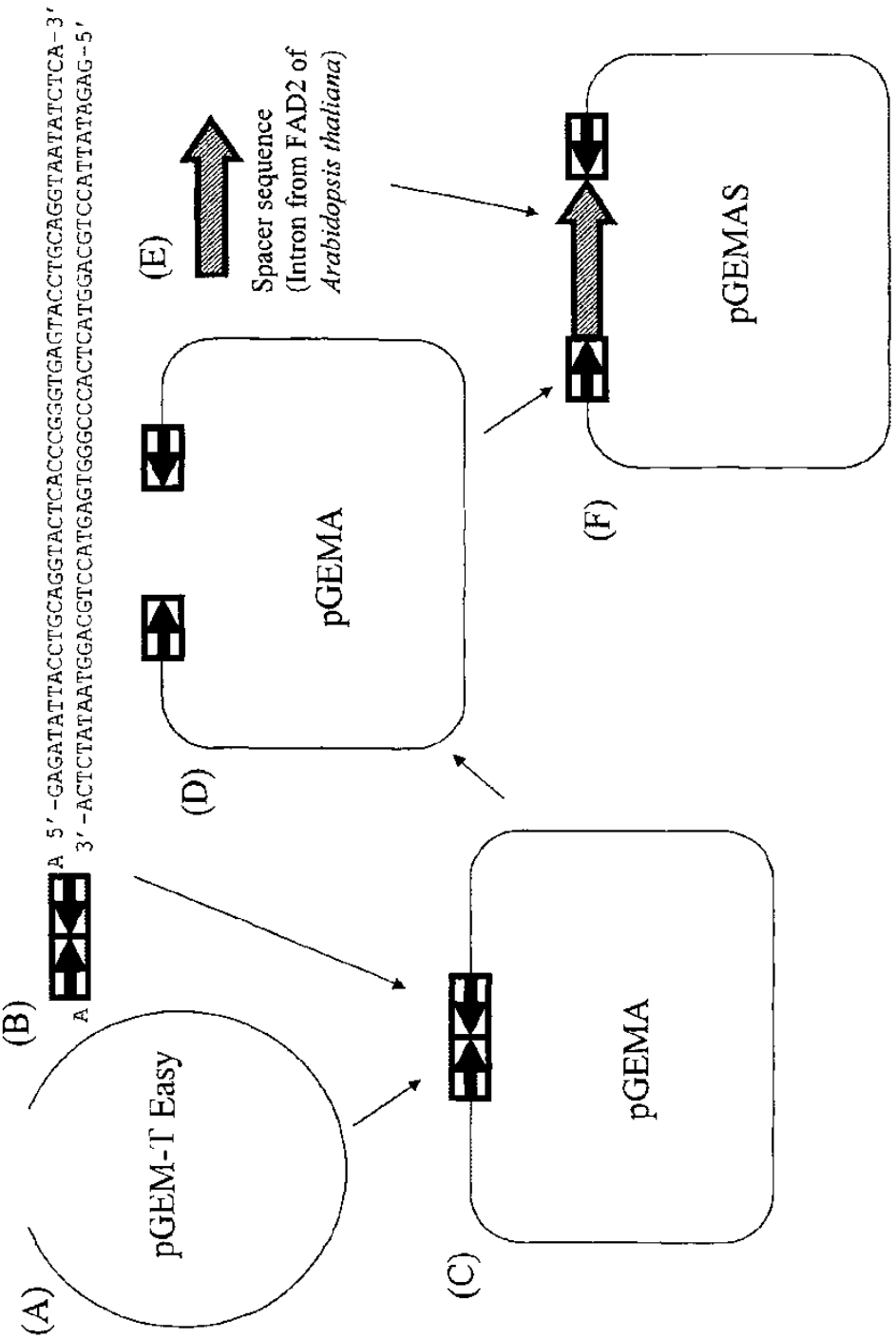
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Fig. 12



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Fig. 13



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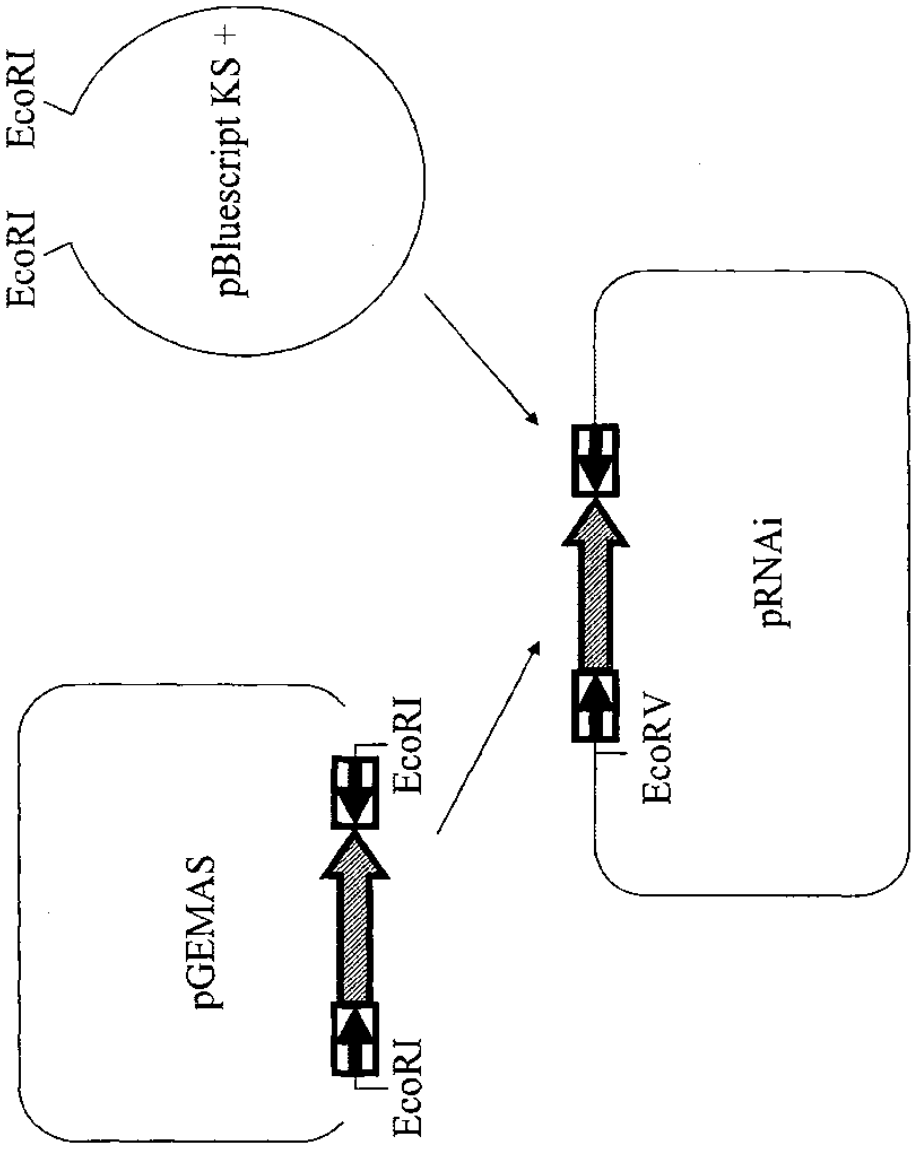


Fig. 14

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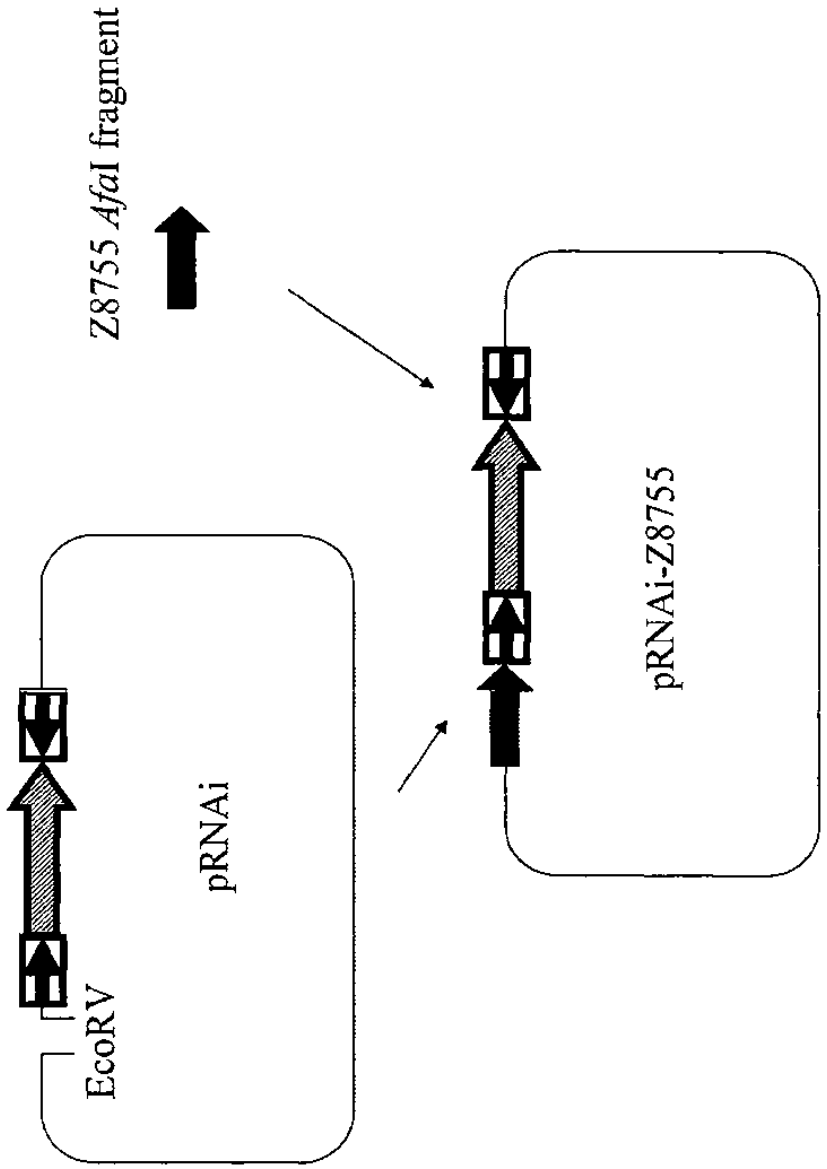


Fig. 15

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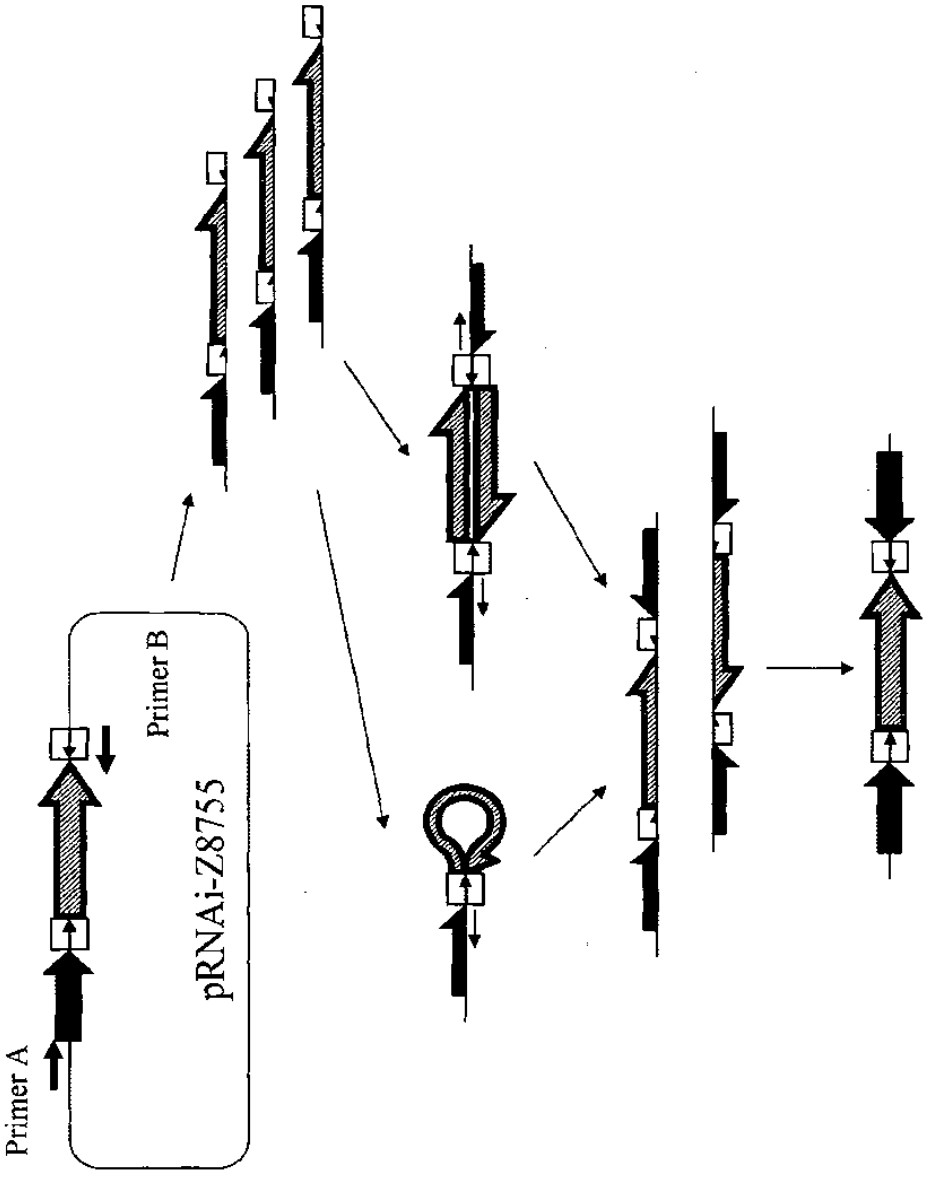


Fig. 16

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2004/014308

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] C12N15/09, C12N1/15, C12N1/19, C12N1/21, C12N5/10

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)
Int.Cl[7] C12N15/09, C12N1/15, C12N1/19, C12N1/21, C12N5/10

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 practicable, search terms used)
BIOSIS/WPI(DIALOG), MEDLINE(STN), JSTPlus/JST7580(JOIS)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Lee, Y.S. et al., Making a better RNAi vector for Drosophila: use of intron spacers, Methods, 2003 August, Vol.30, No.4, pages 322 to 329	1-12
A	WO 2002/066638 A1 (GENCOM CORP.), 29 August, 2002 (29.08.02), & EP 1371727 A1 & US 2004/0010130 A1	1-12
A	WO 2002/059294 A1 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANIZATION), 01 August, 2002 (01.08.02), & US 2003/0049835 A1 & EP 1354035 A1 & AU 2002224568 A1 & JP 2004-516852 A	1-12

☐ Further documents are listed in the continuation of Box C. ☐ 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
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"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
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(54) Title: RNA INTERFERENCE

(57) Abstract: The present invention relates to RNA interference and methods for selecting interfering RNAs. The present invention also relates to modified interfering RNAs. The present invention also relates to methods of reducing the level of a specific mRNA in a cell, methods for reducing the level of a specific protein in a cell, and methods of regulating gene expression. The present invention also relates to methods of screening libraries for an interfering RNA of interest and methods of screening libraries based on a gene function.

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RNA INTERFERENCE

PRIORITY INFORMATION

[001] This application claims priority benefit of U.S. Patent Application No. 60/436,849, filed December 27, 2002. The entire contents of U.S. Patent Application No. 60/436,849 is specifically incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[002] The present invention relates to RNA interference. The present invention also relates to methods of selecting interfering RNAs. The present invention also relates to methods of using interfering RNAs. The present invention also relates to RNA interference based assays to identify target genes.

BACKGROUND OF THE INVENTION

[003] RNA interference is a process by which specific mRNAs are degraded into short RNAs. RNA interference has been observed in organisms as diverse as nematodes, insects, trypanosomes, planaria, hydra, zebrafish, and mice. To mediate RNA interference, a double-stranded RNA with substantial sequence identity to the target mRNA is introduced into a cell. The target mRNA is then degraded in the cell, resulting in decreased levels of that mRNA and the protein it encodes.

[004] Many different mRNAs have been targeted in this manner. Various labs have demonstrated that RNA interference is functional *in vitro*, e.g., in *Drosophila* extracts, and *in vivo,* e.g., in *C. elegans.*

SUMMARY OF THE INVENTION

[005] In certain embodiments, a method of decreasing the level of a target mRNA in a host cell is provided. In certain embodiments, a host cell is contacted with a double-stranded RNA molecule, wherein the double-stranded RNA molecule comprises a sequence complementary to at least a portion of the target mRNA. In certain embodiments, the double-stranded RNA molecule further comprises at least one chemical modification. In

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certain embodiments, the at least one chemical modification is selected from 2'-F, 2'-OMe, and 2'-deoxy. In certain embodiments, the host cell is incubated under conditions whereby RNA interference occurs, thereby decreasing the level of the target mRNA.

[006] In certain embodiments, a method of decreasing the level of a target mRNA in a host cell is provided. In certain embodiments, a vector is delivered to the host cell. In certain embodiments, the vector comprises a first nucleic acid sequence and a second nucleic acid sequence. In certain embodiments, the first nucleic acid sequence encodes a first RNA molecule comprising a first RNA sequence that is complementary to at least a portion of the target mRNA. In certain embodiments, the second nucleic acid sequence encodes a second RNA molecule comprising a second RNA sequence that is substantially identical to at least a portion of the target mRNA. In certain embodiments, the host cell is incubated under conditions that allow transcription of the first nucleic acid sequence and the second nucleic acid sequence. In certain embodiments, the host cell is incubated under conditions that allow RNA interference to occur, thereby decreasing the level of the target mRNA.

[007] In certain embodiments, the first RNA sequence and the second RNA sequence are each longer than about 70 nucleotides. In certain embodiments, the vector further comprises at least one promoter selected from a phage promoter, a viral promoter, a pol II promoter, and a pol III promoter.

[008] In certain embodiments, a method of selecting a double-stranded RNA molecule is provided. In certain embodiments, a target mRNA sequence is inputted into Oligo 5.0™ Primer Analysis software. In certain embodiments, the primer length is selected as 19. In certain embodiments, a primer is identified in the stability window, wherein the primer has a bell-shaped internal energy profile. In certain embodiments, a primer is identified in the stability window, wherein the primer has a substantially flat internal energy profile. In certain embodiments, a primer is identified in the stability

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window, wherein the primer has a maximum internal energy of less than -10 kcal/mol. In certain embodiments, a primer is identified in the stability window, wherein the primer has an internal energy of between -6 and -9 kcal/mol. In certain embodiments, a primer is identified in the stability window, wherein the primer has a melting temperature below 65° C. In certain embodiments, a primer is identified in the stability window, wherein the primer has a melting temperature below 50° C.

[009] In certain embodiments, a BLAST search is performed on the primer against an EST database. In certain embodiments, a double-stranded RNA is synthesized, wherein the double-stranded RNA comprises a first RNA strand comprising a first RNA sequence that is identical to the nucleotide sequence of the primer and a second RNA strand comprising a second RNA sequence that is complementary to the nucleotide sequence of the primer.

[010] In certain embodiments, a method of decreasing the level of a target mRNA in a mammalian host cell is provided. In certain embodiments, a mammalian host cell is contacted with an RNA hairpin molecule. In certain embodiments, the RNA hairpin molecule comprises a first region, a second region, and a third region. In certain embodiments, the first region comprises a sequence that is substantially identical to at least a portion of the target mRNA. In certain embodiments, the third region comprises a sequence that is substantially complementary to the first region. In certain embodiments, the first region and the third region hybridize, thereby forming an RNA hairpin molecule. In certain embodiments, the mammalian host cell is incubated under conditions whereby RNA interference occurs, thereby decreasing the level of the target mRNA in the host cell.

[011] In certain embodiments, a method of decreasing the level of a target mRNA in a host cell is provided. In certain embodiments, a vector is delivered to the host cell. In certain embodiments, the vector comprises a nucleic acid sequence, wherein the nucleic acid sequence encodes an RNA hairpin molecule. In certain embodiments, the RNA hairpin molecule comprises a first region, a second region, and a third region. In certain

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embodiments, the first region comprises a sequence that is substantially identical to at least a portion of the target mRNA. In certain embodiments, the third region comprises a sequence that is substantially complementary to the first region. In certain embodiments, the first region and the third region hybridize, thereby forming an RNA hairpin molecule. In certain embodiments, the host cell is incubated under conditions that allow transcription of the nucleic acid sequence. In certain embodiments, the host cell is incubated under conditions that allow RNA interference to occur, thereby decreasing the level of the target mRNA in the host cell.

[012] In certain embodiments, a method of constructing a library of RNA hairpin molecules is provided. In certain embodiments, a plurality of single-stranded DNA hairpin templates is synthesized. In certain embodiments, each single-stranded DNA hairpin template comprises a first region, a second region, and a third region. In certain embodiments, the first region comprises an RNA polymerase promoter sequence. In certain embodiments, the second region comprises a random nucleotide sequence having between 5 and 500 nucleotides. In certain embodiments, the third region comprises a first nucleotide sequence, a second nucleotide sequence, and a third nucleotide sequence, wherein the first nucleotide sequence hybridizes to the third nucleotide sequence, thereby forming a single-stranded DNA hairpin template. In certain embodiments, the 3' end of the third nucleotide sequence of each of the plurality of single-stranded DNA hairpin templates is extended to form a plurality of double-stranded DNA hairpin templates. In certain embodiments, the plurality of double-stranded DNA hairpin templates is amplified to form a plurality of double-stranded DNA templates. In certain embodiments, the plurality of double-stranded DNA templates is transcribed to form a library of RNA hairpin molecules.

[013] In certain embodiments, a method of identifying a target gene is provided. In certain embodiments, an array comprising a plurality of positions is formed. In certain embodiments, each position comprises at least one mammalian cell. In certain embodiments, the at least one mammalian cell at

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each position is contacted with at least one RNA hairpin molecule. In certain embodiments, the at least one mammalian cell is incubated under conditions that allow RNA interference to occur. In certain embodiments, an at least one mammalian cell exhibiting at least one biological endpoint is selected. In certain embodiments, the at least one RNA hairpin molecule associated with the plurality of cells exhibiting at least one biological endpoint is identified. A BLAST search on the nucleic acid sequence of the at least one RNA hairpin molecule is performed, thereby identifying the target gene.

[014] In certain embodiments, a library comprising a plurality of RNA hairpin molecules is provided. In certain embodiments, each RNA hairpin molecule comprises a first region, a second region, and a third region, wherein the first region comprises a random nucleotide sequence having between 5 and 500 nucleotides and the third region comprises a nucleotide sequence that is substantially complementary to the first region.

BRIEF DESCRIPTION OF THE DRAWINGS

[015] Figure 1 shows a schematic representation of certain proposed mechanisms of RNA interference. Double-stranded RNA (dsRNA) is cleaved into 21-25 nucleotide fragments by an endonuclease. The sense strands of the dsRNA hybridize to the target mRNA, and the hybridized target mRNA is cleaved into 21-25 nucleotide fragments by an endonuclease. Those fragments may then hybridize to another copy of the target mRNA, leading to its cleavage by endonuclease.

[016] Figure 2 shows a schematic representation of certain assays for identifying modified dsRNAs that are active in RNA interference. (A) A chemically-modified dsRNA (A) or a hairpin or circular dsRNA (B) having a sequence identical to a portion of a luciferase gene is incubated with a luciferase reporter gene expression construct in a Drosophila extract. Luciferase expression in the extract is then detected. A decrease in luciferase expression indicates a functional modified dsRNA.

[017] Figure 3 shows a schematic representation of the *in vivo* expression of dsRNA for RNA interference according to certain embodiments.

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(A) A DNA expression construct having a sequence encoding the dsRNA sense strand and a sequence encoding the dsRNA antisense strand is transformed or transfected into a host cell. The DNA expression construct expresses the sense and antisense strands, which associate in the cell to form a dsRNA. The dsRNA is cleaved into 21-25 dsRNA fragments by endonuclease. The fragments hybridize to the target mRNA, causing it to be cleaved into short fragments as well. (B) A DNA expression construct having several sequences that encode a hairpin RNA is transformed or transfected into a host cell. The DNA expression construct expresses the hairpin RNA, which is then cleaved by endonuclease to form dsRNA fragments. The fragments hybridize to the target mRNA, causing it to be cleaved into short fragments as well.

[018] Figure 4 shows the ds-SiRNA and antisense oligonucleotides used in a study (SEQ ID NOS: 1 to 15). Antisense nucleic acid sequences are 25-nucleotide-long ss-DNAs in which the middle seven nucleotides carry a 2'-Omethyl modification and the remaining nucleotides on either side have a phosphorothioate backbone modification. These sequences target three independent sites (#4, #8 and #15) on the PKC-θ mRNA that were picked using a computer program designed to identify the best sites within an mRNA sequence for antisense targeting. The ds-SiRNA molecules are based on the three antisense sequences, except that they have either 19- or 21 nucleotide target homology instead of 25 nucleotide homology as in antisense DNA. The ds-SiRNA molecules have 3' extensions with two deoxythymidines (dTs) that do not base pair with the target mRNA. For each target site, two ds-RNAs, one with a 21 nucleotide (SiRNA-A) and the other with a 19 nucleotide (SiRNA-B) target homology, were chemically synthesized. In a SiRNA molecule the top strand indicates the antisense strand, whereas the bottom strand is the sense strand.

[019] Figure 5 shows sequences and the predicted secondary structures of ss-SiRNA triggers used in a study (SEQ ID NOS. 16 to 21). For each target site, two ss-SiRNA triggers were designed with the propensity to

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form short hairpins. Out of the two molecules, one (SiRNA-HP) with six dT nucleotides in the loop and two dT residues in the 3'-extension was chemically synthesized. The other (SiRNA-HP-T) ss-SiRNA molecule containing six uridines in the loop and two uridines at the 3' recessed end was obtained by in vitro transcription using corresponding synthetic DNA templates. SiRNA-#8-HP-T that contains a single nucleotide deletion within the region of homology was used as a control.

[020] Figure 6 shows reduction of target mRNA by defined short hairpin RNAi triggers in cultured mammalian cells. This work targeted the PKC-θ gene, a gene that is expressed endogenously in NIH 293 cells (a human kidney cell line) by a range of nucleic acid triggers. These triggers include ds-SiRNA, ss-SiRNA with the propensity to form short hairpins, and antisense DNA oligonucleotides. Sequences and secondary structures of triggers are illustrated in Figures 4 and 5.

[021] Various nucleic acid triggers were delivered into NIH 293 cells grown in 96-well plates by transfection with Lipofectamin-2000. Twenty-four hours after the transfection, levels of two specific messages, PKC-θ and Cyclophilin, were quantified by branched-DNA (bDNA) capture detection method. Cyclophilin, a housekeeping gene, was used to detect any nonspecific gene inhibition induced by various nucleic acid triggers. What is shown is the ratio of the mRNA levels of the two genes as a function of various triggers. Arrows indicate the reduction of the ratio triggered by synthetic and transcribed hairpin RNA molecules. The two triangles indicate the lack of gene knockdown by the ss-SiRNA hairpin containing a single nucleotide deletion, indicating that the RNA interference process is selective and uses hairpin triggers containing specific sequences for effective gene silencing.

[022] Figure 7 shows the architecture and synthesis of SiRS RNA hairpin libraries according to certain embodiments. The random sequence hairpin RNA library is obtained by in vitro transcription of a chemically synthesized DNA template whose general structure is illustrated in the inset.

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In this design of the random sequence DNA template, the 5' end carries a biotin moiety for solid phase capture onto a streptavidin-coated microtiter plate. A contiguous randomized region having 15-20 nucleotides is flanked by the top strand of T7 RNA polymerase promoter and a defined nucleotide stretch that forms the closing stem to make the single-stranded DNA hairpin template. As illustrated, the 3' end of the single-stranded DNA hairpin template is extended to complete the hairpin, creating a double-stranded DNA hairpin template, using a high fidelity DNA polymerase. Upon streptavidin capture, the double-stranded DNA hairpin template is briefly amplified by 2 cycles of PCR to obtain a double-stranded DNA template for in vitro transcription. Alternatively, the double-stranded DNA hairpin template is directly used for transcription without brief amplification to obtain a SiRS hairpin library.

[023] Figure 8 shows the making of "Master Plates" and "Lead Plates" using a SiRS RNA hairpin library according to certain embodiments. An initial DNA library having approximately 10^6 individual sequences is distributed into a number of 1,536-well "Master Plates". Although the number of "Master Plates" can vary, one may use 5 such plates to obtain approximately 400 unique template molecules/well. After 2-cycles of PCR amplification with a biotinylated primer, the resulting DNA in a single "Master Plate" is distributed into four 384-well "Lead Plates" in which each well has approximately 400 unique template molecules/well.

[024] Figure 9 is an illustration of a "Primary Screen" with a SiRS RNA hairpin library according to certain embodiments. A SiRS RNA hairpin library is generated within "Lead Plates" by in vitro transcription. Twenty such plates are used to cover the sequence space of the initial random library having approximately 10^6 individual molecules. After in vitro transcription, RNA from each "Lead Plate" is transferred to a new plate ("RNA-Lead Plate"), from which SiRS RNA hairpins are delivered to cells for a number of functional assays that are carried out in parallel.

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[025] Figure 10 shows the identification of lead SiRS hairpin libraries from a primary biological screen according to certain embodiments. Microtiter wells that produce biological end-points are identified in each assay. The SiRS RNA hairpin lead sub-libraries that produce biological endpoints in each functional assay are traced back to the "Lead Plates" to identify DNA templates from which the SiRS RNA hairpin lead sub-libraries are derived. These DNA templates that produce the functional SiRS RNA hairpin triggers (lead sub-libraries) are amplified by 2 cycles of PCR using a biotinylated primer. Resulting PCR products are distributed into several (about three) 384-well microtiter plates ("Daughter Plates") coated with SA.

[026] Figure 11 shows the "Secondary Screen" with lead SiRS RNA hairpin libraries according to certain embodiments. SiRS RNA hairpin libraries produced within "Daughter Plates" are used for secondary screening. Secondary screening gives rise to candidate SiRS hairpin molecules. Finally, wells that contained DNA templates that produce candidate SiRS hairpin molecules are identified, amplified by PCR, cloned and sequenced. A consensus sequence within these candidate sequences is identified.

[027] Figure 12 shows the use of the consensus SiRNA hairpin triggers in identifying candidate gene(s) according to certain embodiments. The consensus sequences that are identified within the candidate SiRNA hairpin molecules are used to perform BLAST searches within the Human Genome Data Base to track the gene responsible for the function tested in the assay.

[028] Figure 13 shows the construction of a SiRS hairpin library for generating a retroviral library according to certain embodiments. The design of the synthetic ssDNA containing the random region with 15-20 positions is shown in the inset. Two fixed regions that provide binding sites for PCR primers flank the random region. A unique restriction site, preferably a six-base pair cutter, is included near the end of each primer to facilitate the construction of inverted repeats. Upon PCR amplification, the resulting duplex DNA is digested with one restriction site to obtain sticky ends for self-

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ligation. The ligated product containing inverted repeats of the random region is digested with the second restriction enzyme and is inserted into the retroviral vector.

[029] Figure 14 shows production of a retroviral vector harboring a SiRS hairpin library according to certain embodiments. About 10-20 µg of synthetic insert containing inverted repeats of a random region is ligated downstream of a human tRNA promoter in the viral vector. The resulting plasmids are transformed into a compatible strain of bacteria and the transformants are isolated by appropriate selection methods. Transformants are pooled and expanded, and the recombinant plasmids are purified. Finally, a retroviral library is produced in a suitable cell line using a triple transfection approach.

[030] Figure 15 shows a method for a biological screen with a retroviral vector expressing a SiRS hairpin library according to certain embodiments. Cells that provide a biological endpoint of interest are transduced with a retroviral vector expressing a SiRS hairpin library at a low MOI in microtiter plates. Subsequently, wells that produce a desirable phenotypic response are identified and the viruses harboring the ss-SiRNA triggers that produce the phenotypic response are rescued. After viral rescue, plasmids carrying the selected SiRS library are prepared and used for the production of a new batch of retrovirus, which are used for the next round of biological screening followed by selection. After several rounds of selection and amplification, specific hairpin sequences that produce the desired phenotype are identified by sequencing. A consensus sequence within the enriched hairpin sequences is used to perform a blast search within the human genome database to identify the candidate gene.

[031] Figure 16 shows the calculated average internal stability profiles and average melting temperatures (T_m) of certain miRNA duplexes identified in *C. elegans*, *D. melanogaster* and *H. sapiens* (Largos-Quintana et al., 2001, Lau et al., 2001; Lee and Ambros, 2001). Both of those parameters were calculated using Oligo 5.0™ Primer Analysis Software available from National

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Biosciences, Inc., Plymouth, MN. **(A)** Calculated average internal stability profiles for antisense strands of miRNAs in their duplex form. For each category, a collection of individual miRNAs were used; 15 for *C. elegans* in Category I (Lee and Ambros, 2001), 17 for *D. melanogaster*, 31 for *H. sapiens* (Largos-Quintana et al., 2001) and 50 for *C. elegans* in Category IV (Lau et al., 2001). For each collection of 19-basepair miRNA duplexes, average internal stability of internal 17 nucleotide positions was calculated by averaging values of the same position in individual miRNAs. **(B)** Comparison between calculated average internal stability profiles of two miRNAs (*C. elegans*-Category I, closed squares and D. melanogaster, closed circles) and the profiles of three collections of random 19-nucleotide duplex RNA molecules. Random collections of 19-nucleotide duplex RNAs were obtained by hybridizing segments of 19-nucleotide antisense oligonucleotides to miRNA sequences of two human genes [Protein kinase C-theta (PKC-θ, open squares) and Insulin-like growth factor receptor 1 (IGF1-R, Open diamonds] and a reporter gene, secreted alkaline phosphatase (SEAP, open circles). Sample sizes of the three collections of random 19-nucleotide duplexes were 189 for IGF1R, 100 for PKC-θ and 70 for SEAP. **(C)** Calculated average T_m values for the four categories of miRNAs. Average T_m was obtained by averaging the calculated T_m values of individual miRNA duplexes in each category. Error bars indicate the average deviation.

[032] Figure 17 shows the calculated average internal stability profiles and calculated T_m values for certain experimentally validated SiRNA molecules targeted to six human genes, one mouse gene, and a single reporter gene, SEAP. **(A)** Calculated average internal stability profiles of functional (closed squares; sample size of 16) and nonfunctional (open squares; sample size of 21) SiRNA molecules. **(B)** Calculated T_m values of individual SiRNA molecules used for experimental validation of target mRNA reduction. Filled bars indicate T_m values of functional SiRNA molecules, whereas the open bars show those of nonfunctional SiRNAs. For each SiRNA molecule, the first part of the name indicates the targeted gene and

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the number reflects the first nucleotide of the target site within the mRNA sequence. **(C)** Calculated average internal stability profiles of functional (closed squares; sample size of 12) and nonfunctional (open squares; sample size of 8) SiRNA molecules with calculated T_m values between 50° - 70° C.

[033] Figure 18 shows the calculated individual internal stability profiles for representative examples of functional (closed squares) and nonfunctional (open squares) SiRNA molecules that do not satisfy both criteria for a functional SiRNA. **(A)** SiRNA (PKB-α-1436; open squares) has high internal stability and a high value for the calculated T_m (85° C), and is nonfunctional. SiRNA (PKC-θ-1303; closed squares) is a functional trigger that barely satisfies the internal stability profile, but has a low value for the calculated T_m (66° C). **(B)** Neither of the two SiRNAs has a preferred internal stability profile. SiRNA (TERT-708; open squares) has a relatively high value for the calculated T_m (79° C) and is non functional. SiRNA (DJ-1-615; closed circles) has a low value for the calculated T_m and is functional. **(C)** Internal stability profiles of three SiRNA molecules targeted to overlapping sites (SEQ ID NOS.22 to 24 and 151) (illustrated at the top of the graph) within the mRNA of PKC-θ. SiRNA-1 (PKC-θ-69) and SiRNA-2 (PKC-θ-70) are both functional (filled squares) and have a preferred bell-shaped internal stability profile. In contrast, SiRNA-2 (PKC-θ-75) that targets only five nucleotides downstream has an undesirable internal stability profile (open circles) and is nonfunctional. This is in spite of its calculated T_m value of 55° C, which is well within the T_m range of the other two functional SiRNA molecules.

[034] Figure 19 shows the characteristics of certain SiRNA triggers (rationally designed and arbitrarily picked) for SEAP mRNA. Panel **A** shows internal stability profiles and sequences of rationally designed SiRNA triggers based on criteria outlined here. **A1** shows SiRNA triggers (SEQ ID NOS. 25 to 32) that were designed to be functional, whereas **A2** indicates those (SEQ ID NOS. 33 to 36) designed not to be functional. Panel **B** shows internal stability profiles and sequences of arbitrarily (randomly) picked SiRNA triggers. **B1** shows the SiRNA triggers (SEQ ID NOS. 37 to 40) picked

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arbitrarily that turned out to be functional (SP-155, which reduces the SEAP level by approximately 50%, is considered marginally functional), whereas **B2** illustrates all the nonfunctional SiRNA molecules (SEQ ID NOS. 41 to 48). For each SiRNA, a calculated internal stability profile of the antisense strand (top) and the T_m of the duplex is shown. Panel **C** shows the endpoint measurements of RNA interference elicited by different SiRNA triggers that were either rationally designed or arbitrarily picked. The enzyme activity of cell medium after transient expression of the SEAP gene using prAAV6-SEAP plasmid in the presence of different SiRNA molecules (at 100 nM) was measured using a chemiluminescent substrate. The black bars (left) indicate the SEAP activity with rationally designed SiRNA molecules. Bars depicted on the right side of the figure labeled "Random Picks" indicate the enzyme activity with arbitrarily designed SiRNA molecules. The open bar shows the SEAP activity of the control transfection in which no SiRNA was used. Each result is an average of three independent transfections, and the error bar indicates the standard deviation of the three readings.

[035] Figure 20 shows the concentration dependence of certain SiRNA triggers in silencing SEAP expression. Transfections were carried out as described in Fig. 19, but a range of concentrations for each SiRNA trigger were used. Twenty-four hours after transfection, alkaline phosphatase activity was quantified using a chemiluminescent assay. **(A)** For each trigger, the SEAP activity is plotted against the concentration of the SiRNA trigger used. Black and gray bars show the SEAP activity in the presence of varying concentrations of SEAP-1035 and SEAP-1070, respectively. The white bars indicate the effect of different concentrations of the most effective SiRNA trigger, SEAP-2217. The two bars to the right show the control in which no SiRNA trigger was included during transfection. **(B)** A different representation of the data in Panel A. Squares and triangles indicate the concentration effect of SEAP-1070 and SEAP-1035, respectively. Circles show the concentration effect of SEAP-2217.

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[036] Figure 21 shows screen snapshots depicting windows of the Oligo 5.0™ Primer Analysis Software program used for identifying effective SiRNA sequences. The "current oligo length" is set to 19. **(A)** The melting temperature window shows the calculated T_m profiles for 19 nucleotide long duplexes annealed to the mRNA sequence (SEQ ID NOS. 49 and 50). **(B)** The internal stability window shows the internal stability profile with a target site having a desired bell-shaped curve indicated in open circles for a "good pick", **(C)** A second representative internal stability window highlights, in open circles, the undesirable internal stability profile reflecting a poor choice for a SiRNA pick.

[037] Figure 22 shows the effect of the length of the helical region in certain SiRNA triggers. Sequences of SiRNA triggers with varying length of the RNA helical region (17-, 19-, 21-, 23-, 25-base pair RNA helical region) are shown on the right (SEQ ID NOS. 31, 32, and 51 to 60). Each trigger, except for SP-19-AR, has two deoxy thymidine residues at the 3' end on each strand (shown in bold). SP-19 is the same as 2217. In SP-19-AR the two nucleotides at the 3' end in the antisense strand are complimentary to the targeted SEAP mRNA. The effect of these triggers in silencing the SEAP expression is shown on the left. Each measurement is an average of triplicate readings derived from independent transfections of SiRNA triggers at a 100 nM concentration. The control bar reflects the amount of SEAP activity measured in cells in the absence of any SiRNA trigger.

[038] Figure 23 shows the silencing of SEAP expression by certain SiRNA triggers with different end structures. Sequences of SiRNA triggers used in the experiment are shown to the right (SEQ ID NOS. 31, 32, 59 to 66). Deoxy-nucleotides at the 3' ends of SP-19 and SP-19-Blunt are underlined and marked in bold. The effect of the silencing of the SEAP gene by these triggers is shown to the left. Each measurement is an average of triplicate readings derived from independent transfections of SiRNA triggers at a 100 nM concentration. The control bar reflects the amount of SEAP activity measured in cells in the absence of any SiRNA trigger.

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[039] Figure 24 shows the primary and secondary structures of certain hairpin triggers carrying four and eight nucleotides in the loops (SEQ ID NOS. 67 to 70). Antisense and sense strands have reverse orientations in SP-HP uucg AS-S and SP-HP uucg S-AS hairpin triggers carrying tetra loops. SP-HP loop 8 AS-S is identical to SP-HP uucg AS-S except that it has an eight-nucleotide loop. SP-HP uucg AS-S + 5' ext has an internal bulge at the base of the stem. In each trigger, the sequence region within the box depicts the antisense and sense strands annealed to form a 19-base pair helical region. Antisense and sense strands are indicated by AS and S.

[040] Figure 25 shows the effect of the silencing of the SEAP gene by certain triggers. Each measurement is an average of triplicate readings derived from independent transfections of SiRNA triggers at a 100 nM concentration. The control bar reflects the amount of SEAP activity measured in cells in the absence of any SiRNA trigger. 2217 shows the reduction of SEAP level with a double-stranded SiRNA trigger carrying a 19-base pair helical region with two dT residues at the 3' extensions as another control.

[041] Figure 26 (A, B, and C) shows eight series of SiRNA triggers in which the variation of antisense strand was studied by keeping the sense strand constant in each series. In each series, SiRNA triggers that are nonfunctional are indicated within a box. In Figure 26A, the left column contains SEQ ID NOS. 31, 51, 52, 58, 60, 62, 71, 72, and 73. In Figure 26A, the middle column contains SEQ ID NOS. 31, 32, 52, 58, 60,. 62, and 71 to 73. In Figure 26A, the right column contains SEQ ID NOS. 31, 52, 58, 60, and 71 to 74. In Figure 26B, the left column contains SEQ ID NOS. 31, 52, 58, 60, 62, 71 to 73, and 75. In Figure 26B, the right column contains SEQ ID NOS. 31, 52, 57, 58, 60, and 72 to 73. In Figure 26C, the left column contains SEQ ID NOS. 31, 52, 58 to 60, 62, and 71 to 73. In Figure 26C, the middle column contains SEQ ID NOS. 31, 52, 58, 60, 62, 71 to 73, and 76. In Figure 26C, the right column contains SEQ ID NOS. 31, 52, 58, 60, 62, 71 to 73, and 77.

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[042] Figure 27 shows the effect of the silencing of the SEAP gene by certain triggers whose structures are shown in Figures 26A-C. Results are provided for different double stranded RNA molecules with varying lengths and end structures. The sense strands of each molecule are identified in the box below the bar graph. Each sense strand is matched with a series of antisense strands identified in the box to the right of the bar graph as indicated by the shading of the bars. Each measurement is an average of triplicate readings derived from independent transfections of SiRNA triggers at a 100 nM concentration.

[043] Figure 28 (A, B, and C) shows eight series of SiRNA triggers in which the variation of sense strand was studied by keeping the antisense strand constant in each series. In each series, SiRNA triggers that are nonfunctional are indicated within a box. In Figure 28A, the left column contains SEQ ID NOS. 32, 51, 52, 57, 59, and 74 to 77. In Figure 28A, the middle column contains SEQ ID NOS. 31, 32, 51, 57, 59, and 74 to 77. In Figure 28A, the right column contains SEQ ID NOS. 32, 51, 57, 59, 71, and 74 to 77. In Figure 28B, the left column contains SEQ ID NOS. 32, 51, 57, 59, 72, and 74 to 77. In Figure 28B, middle column contains SEQ ID NOS. 32, 51, 57 to 59, and 74 to 77. In Figure 28B, the right column contains SEQ ID NOS. 32, 51, 57, 59, 60, and 74 to 77. In Figure 28C, the left column contains SEQ ID NOS. 32, 51, 57, 59, and 73 to 77. In Figure 28C, the right column contains SEQ ID NOS. 32, 51, 57, 59. 62, and 74 to 77.

[044] Figure 29 shows the effect of the silencing of the SEAP gene by triggers whose structures are shown in Figures 28A-C. Results are provided for different double stranded RNA molecules with varying lengths and end structures. The antisense strands of each molecule are identified in the box below the bar graph. Each antisense strand is matched with a series of sense strands identified in the box to the right of the bar graph as indicated by the shading of the bars. Each measurement is an average of triplicate readings derived from independent transfections of SiRNA triggers at a 100 nM concentration.

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[045] Figure 30 depicts several dsRNAs having C-U substitutions, a positive control dsRNA (SP-1260-s + SP-1260-as), and a negative control dsRNA (SP-19) and their activity in an RNA interference assay (SEQ ID NOS. 29 to 32, 35, 36, and 78 to 81).

[046] Figure 31 shows certain antisense oligonucleotides (ASOs) and ds-SiRNA triggers used to target PKC-θ mRNA at three different sites, site #4, #8 and #15, identified by a computer program designed for ASO picking (SEQ ID NOS. 2 to 5, 7 to 15, 82, and 83). In each case, the 25 nucleotide long ASO sequence is underlined. The middle seven nucleotides in the ASOs have a 2'-OMe modification (boxed), whereas the nucleotides that flank the 2'-OMe stretch (nine nucleotides to either side) have a phosphorothioate backbone. Below each ASO are two ds-SiRNAs. Each contains two dT residues in its 3' extension. The ds-SiRNA designated by A has a 21 nucleotide target homology, whereas the other ds-SiRNA designated by B carries a 19 nucleotide target homology.

[047] Figure 32 depicts the nucleotide sequences and the predicted fold-back stem-loop structures of certain ss-SiRNA (SEQ ID NOS. 16 to 21) designed to target the same three sites, site #4, #8 and #15, as indicated in Figure 31. For each site, two ss-SiRNA triggers were designed. The one designated by HP contains six dT residues in the loop and two dT nucleotides in the 3' extension of the predicted folded stem-loop structure, and was obtained by chemical synthesis. The other, denoted by HP-T, contains six U residues in the loop and a 5' extension was produced by in vitro transcription. In these molecules, underlined nucleotides do not share homology to the target site.

[048] Figure 33 shows the quantification of the mRNA level of PKC-θ in 293 cells 24 hours after transfection with certain nucleic acid triggers. The amount of chemiluminescence (RLU) is directly proportional to the amount of PKC-θ mRNA in a single well of a microtiter plate. Each measurement is an average of triplicate measurements derived from independent transfections with error bars representing the standard deviation of the three

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measurements. Bars: 1, 2, 9, 10, 17, 18, 25, and 26 indicate the PKC-θ levels after transfection with ASO; 3, 4, 11, 12, 19, 20, 27, and 28 indicate the PKC-θ levels after transfection with ds-SiRNA; 5, 13, and 21 indicate the PKC-θ levels after transfection with chemically synthesized ss-SiRNA; 14, 15, 22, and 23 indicate the PKC-θ levels after transfection with in vitro transcribed ss-SiRNA; 6 and 7 indicate the PKC-θ levels after transfection with ss-SiRNA with a single nucleotide deletion; 31 indicates the PKC-θ levels after transfection with the scrambled ASO.

[049] Figure 34 shows the quantification of the Cyclophilin mRNA level in 293 cells 24 hours after transfection with certain nucleic acid triggers. The amount of chemiluminescence (RLU) is directly proportional to the amount of Cyclophilin mRNA in a single well of a microtiter plate. Each measurement is an average of triplicate measurements derived from independent transfections with an error bar representing the standard deviation of the three readings. Bars: 1, 2, 9, 10, 17, 18, 25, and 26 indicate the Cyclophilin levels after transfection with ASO; 3, 4, 11, 12, 19, 20, 27, and 28 indicate the Cyclophilin levels after transfection with ds-SiRNA; 5, 13, and 21 indicate the Cyclophilin levels after transfection with chemically synthesized ss-SiRNA; 14, 15, 22, and 23 indicate the Cyclophilin levels after transfection with in vitro transcribed ss-SiRNA; 6 and 7 indicate the Cyclophilin levels after transfection with ss-SiRNA with a single nucleotide deletion; 8, 16 and 24 indicate the Cyclophilin levels after transfection with RNA antisense strand alone; 29 and 30 indicate Cyclophilin levels after no transfection; 31 indicates the Cyclophilin levels after transfection with the scrambled ASO.

[050] Figure 35 shows the selective reduction of PKC-θ message in 293 cells 24 hours after transfection with certain nucleic acid triggers. The level of PKC-θ mRNA (in Figure 33) was normalized by dividing the level of PKC-θ by the level of Cyclophilin mRNA (in Figure 34). Bars: 1, 2, 9, 10, 17,18, 25 and 26 indicate the PKC-θ levels after transfection with ASO; 3, 4, 11, 12, 19, 20, 27 and 28 indicate the PKC-θ levels after transfection with

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ds-SiRNA; 5, 13, and 21 indicate the PKC-θ levels after transfection with chemically synthesized ss-SiRNA; 14, 15, 22 and 23 indicate the PKC-θ levels after transfection with in vitro transcribed ss-SiRNA; 6 and 7 indicate the PKC-θ levels after transfection with ss-SiRNA; 6 and 7 indicate the PKC-θ levels after transfection with a single nucleotide deletion; 8, 16 and 24 indicate the PKC-θ levels after transfection with RNA antisense strand alone; 29 and 30 indicate the PKC-θ levels after no transfection; 31 indicates the PKC-θ levels after transfection with the scrambled ASO.

[051] Figure 36 shows the cytotoxity of 293 cells 24 hours after transfection with certain nucleic acid triggers. The cytotoxicity was measured by the MTT assay. Each measurement is an average of triplicate measurements derived from independent transfections with an error bar representing the standard deviation of the three readings. Bars: 1, 2, 9, 10, 17, 18, 25 and 26 show the toxicity levels after the transfection with ASO; 3, 4, 11, 12, 19, 20, 27 and 28 show the toxicity levels after transfection with ds-SiRNA; 5,13, and 21 show the toxicity levels after transfection with chemically synthesized ss-SiRNA; 14, 15, 22 and 23 show the toxicity levels after transfection with in vitro transcribed ss-SiRNA; 6 and 7 show the toxicity levels after transfection with ss-SiRNA with a single nucleotide deletion; 8, 16 and 24 show the toxicity levels after transfection with RNA antisense strand alone; 29 and 30 show toxicity levels with no transfection; 31 and 32 indicate the toxicity levels after the transfection with the scrambled ASO.

[052] Figure 37 shows the cytotoxity of 293 cells 24 hours after transfection with certain nucleic acid triggers. The toxicity was measured by the AlamarBlue assay. Each measurement is an average of triplicate measurements derived from independent transfections with an error bar representing the standard deviation of the three readings. Bars: 1, 2, 9, 10, 17, 18, 25 and 26 indicate the toxicity levels after transfection with ASO; 3, 4, 11, 12, 19, 20, 27 and 28 indicate the toxicity levels after transfection with ds-SiRNA; 5, 13, and 21 indicate the toxicity levels after transfection with chemically synthesized ss-SiRNA; 14, 15, 22 and 23 indicate the toxicity

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levels after transfection with in vitro transcribed; ss-SiRNA; 6 and 7 indicate the toxicity levels after transfection with ss-SiRNA with a single nucleotide deletion; 8, 16 and 24 indicate the toxicity levels after transfection with RNA antisense strand alone; 29 and 30 indicate the toxicity levels after no transfection; 31 and 32 indicate the toxicity levels after transfection with the scrambled ASO.

[053] Figure 38 shows the reduction of PKC-θ mRNA using certain ds-SiRNA triggers picked by random choice (SEQ ID NOS. 84 to 97). Random pick # 4 is a blunt end ds-RNA sequence. The N19-AA-Rm sequence was picked to have homology throughout the molecule, including the two dT residues at the 3' end of the antisense strand. This molecule has one blunt end, whereas the other end is likely to be unpaired. Each measurement is an average of triplicate measurements derived from independent transfections with an error bar representing the standard deviation of the three readings. Gray bars indicate the level of mRNA when transfected with ds-SiRNA BSS, a ds-SiRNA trigger that should not have homology to any human gene. Black bars indicate the level of mRNA in cells that were not transfected.

[054] Figure 39 shows the reduction of the DJ-1 mRNA level in MDA-MB453 cells upon transfection with certain ds-SiRNAs and ASOs (SEQ ID NOS. 96 to 107). Each measurement is an average of triplicate measurements derived from independent transfections with an error bar representing the standard deviation of the three readings. Grey bars indicate the level of mRNA when transfected with ds-SiRNA BSS, a ds-SiRNA trigger that should not have homology to any human gene. Black bars indicate the level of mRNA in cells that were not transfected.

[055] Figure 40 shows the reduction of KD312 mRNA level in DLD-1 cells upon transfection with ds-SiRNAs and ASOs (SEQ ID NOS. 108 to 113). Ds-SiRNAs are based on the two antisense sequences. Each measurement is an average of triplicate measurements derived from independent transfections with an error bar representing the standard deviation of the three

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readings. The bar at the far right indicates the level of mRNA in cells that were not transfected.

[056] Figure 41 depicts plasmid pAAV6-seap used in transient transfection studies.

[057] Figure 42 shows the silencing of the transient expression of SEAP in 293 cells using certain ds-SiRNAs (SEQ ID NOS. 114 to 119). The activity of SEAP produced after the transfection of pSEAP-AAV into 293 cells either in the presence of: 1. ds-SiRNA-Seap-1 (open), 2. ds-SiRNA-Seap-2 (gray), 3. ds-SiRNA-Seap-3 (black) or absence of any SiRNA trigger (indicated as "None" on the far right of the graph) was quantified using a chemiluminescent substrate. Each data point is an average of three independent transfections with an error bar representing the standard deviation of the three readings.

[058] Figures 43A, B and C show the duration of certain RNA interference in the transient expression of a SEAP reporter gene in 293 cells. SEAP activities are shown after co-transfection of different amounts of pAAV6-seap plasmid (squares-25 pmoles, circles-5 pmoles, diamonds-0.5 pmoles, triangles-0.05 pmoles) with three different concentrations of ds-SiRNA-Seap-1 (Panel A-1 00 nM, Panel B-10 nM and Panel C-5 nM). After the indicated time, 15 µL of the medium was used to assay for the activity of SEAP. Each data point is an average of three independent transfections.

[059] Figure 44 shows the duration of certain RNA interference and antisense effects in reducing PKC-θ mRNA level in 293 calls. Cells were transfected with either ASO AS-8 shown in Figure 31 (filled bars) or ds-SiRNA -8-A, shown in Figure 31 (open bars). The message level of PKC-θ was quantified after 12 hours. (Panel A), 24 hours.(Panel B) and 72 hours. (Panel C). In each panel, the gray bars indicate the control experiment in which nonspecific ASO was used.

[060] Figure 45 shows the certain ds-SiRNAs used for studying the nature and the specificity of SiRNA triggers (SEQ ID NOS. 4, 5, and 120 to

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141). These triggers are designed to interact with the PKC-θ message and contain two dT residues at their 3' ends. Mut-1 and Mut-2 triggers carry one and two base-pair mutations as indicated by bold and underlined nucleotides. Ds-SiRNA triggers 1-3 have 12, 15, and 17 base pairs that are homologous to the target mRNA. Trigger 1 is 14 nucleotides long. Trigger 2 is 17 nucleotides long. Trigger 3 is 19 nucleotides long. Triggers 4-6 have different lengths of target homology, but the physical length of all three triggers is kept at 21 nucleotides. In triggers 4-6, those nucleotides not homologous to the target mRNA are shown in lower case letters. Trigger 7 is the same as ds-SiRNA-BA, shown in Figure 31. Triggers 8- 13 have point mutations in the sense strand while the antisense strand is completely homologous except for two dTs at the 3' end. In each case the point mutation(s) in the sense strand is underlined and indicated by bold.

[061] Figure 46 depicts the reduction of the PKC-θ message level by certain ds-SiRNA triggers with different characteristics. Sequences of these triggers are illustrated in Figure 45. The effect of the physical length of the SiRNA trigger is shown by A (1 -12 base-pairs, 2-15 base-pairs, and 3-17 base pairs). B indicates the length of the homology requirement of ds-SiRNA triggers. Triggers 4, 5 and 6 carry 21-base pairs, out of which the length of target homology varies from 17 (4), 15 (5) and 12 (6). The effect of triggers carrying single (Mut-1) and double (Mut-2) base pair changes on the reduction of the PKC-θ message is indicated by X and Y, respectively. C indicates the effect of mutations located only in the sense strand. Ds-SiRNA trigger 7 is the positive control in which there is no mutation in the sense strand, whereas triggers 8-13 carry 1, 2, 3, 4, 5 and 6 point mutations in the sense strand, respectively. The black bar indicated by 14 shows the PKC-θ message level in the control experiment where no SiRNA was used.

[062] Figure 47 shows the reduction of the PKC-θ message level by a ds-SiRNA (ds-SiRNA-8-A) trigger carrying different chemical modifications. As indicated, ds-SiRNA was modified at the 2'-position in the sugar with Fluorine (F), Methoxy (OMe) and H (DNA). In the case of 2'-F modification,

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only the pyrimidines were modified, whereas the modifications were introduced throughout the strand with the other two modifications. All three combinations, modifications in the antisense sense strand (1), sense strand (2), and both strands (3) were used. Combinations represent a 2'-F modification in the antisense and H- and OMe- modifications in the sense strand. In addition to the modification in the sugar, the 3' ends were also modified with two different caps; inverted dT and inverted abasic modifications.

[063] Figure 48 depicts certain unimolecular (ss-SiRNAs) and bimolecular (ds-SiRNAs) triggers designed for the SEAP mRNA. All pyrimidines in Sphp-1 F are modified with a 2'-F group (SEQ ID NOS. 142 to 150).

[064] Figure 49 shows the silencing of the transient expression of SEAP in 293 cells using certain bimolecular and unimolecular SiRNAs. The activity of SEAP produced after the transfection of pSEAP-AAV into 293 cells in the presence of different RNAi triggers was quantified using a chemiluminescent substrate. Each data point is an average of three independent transfections with an error bar representing the standard deviation of the three readings.

[065] Figure 50 shows genes targeted by certain arbitrarily designed SiRNA molecules. The column on the far right labeled "% Success" shows the number of arbitrarily picked SiRNAs that successfully mediated RNA interference of the target gene as a percentage of the total number of SiRNAs that were arbitrarily picked for that target gene. The G/C content is calculated as the G/C content of the SiRNA.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS OF THE INVENTION

[066] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed. In this application, the use of the singular includes the plural unless specifically stated otherwise. In this

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application, the use of "or" means "and/or" unless stated otherwise. Furthermore, the use of the term "including", as well as other forms, such as "includes" and "included", is not limiting. Also, terms such as "element" or "component" encompass both elements and components comprising one unit and elements and components that comprise more than one subunit unless specifically stated otherwise.

[067] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited in this application, including but not limited to patents, patent applications, articles, books, and treatises, are hereby expressly incorporated by reference in their entirety for any purpose.

[068] Standard techniques may be used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (e.g., electroporation, lipofection, etc.). Enzymatic reactions and purification techniques may be performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures may be generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. See e.g., Sambrook et al. Molecular Cloning: A Laboratory Manual (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)), which is incorporated herein by reference for any purpose. Unless specific definitions are provided, the nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques may be used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

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[069] The following examples, including the experiments conducted and results achieved are provided for illustrative purposes only and are not to be construed as limiting the present invention in any way.

I. Gene inactivation through RNA interference (RNAi)

Introduction

[070] Double-stranded RNA (dsRNA)-mediated specific gene inactivation or gene silencing has been observed in many different organisms. This phenomenon, referred to as RNA interference (or RNAi), is expected to play a significant role in understanding gene function, signal transduction pathways and identifying therapeutic agents in the future. To date, the mechanism by which a dsRNA molecule inactivates the expression of a gene is not completely clear. Certain experimental evidence indicates that the process of RNA interference may be post-transcriptional and involves degradation of specific mRNA. The mRNA that is degraded is dictated by the sequence of the dsRNA that is introduced into the cell. As a result, the RNA interference process has been shown to be extremely sequence-specific. It has been observed that the dsRNA that mediates the target mRNA degradation is processed (or cleaved) into a collection of fragments of 21-25 nucleotides. According to a proposed model (Zamore, P. D. et al., Cell, 101, 25-33, 2000; Bass, B. L., Cell, 101, 235-238, 2000), the sense strands of RNA fragments derived from the dsRNA are hybridized to the target mRNA. This process is believed to be facilitated by a helicase and a protein catalyzing an ATP-dependent strand exchange activity. The target mRNA is subsequently cleaved, resulting in the inactivation of the message before being translated (Figure 1).

[071] In certain embodiments RNA interference may be used in the following areas.

[072] 1) To use as a tool for target validation in functional genomics.

[073] 2) To generate therapeutic molecules that interfere in specific gene expression.

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[074] 3) To generate transgenic animals and plants by introducing gene constructs that express dsRNA targeted to a given gene.

[075] 4) To use as a technique for gene therapy applications in controlling the expression of targeted genes.

[076] 5) To use in oligonucleotide therapeutics using synthetic nucleic acid molecules.

1. For functional genomics and therapeutics

[077] An RNAi technique is envisioned to play a role in functional genomics to validate gene targets in both tissue culture and animal models. At the tissue culture level, inactivation of a target gene has been demonstrated by introducing into cells exogenous dsRNA that has been produced by *in vitro* transcription. These natural dsRNAs are relatively long molecules consisting of 100-800 base pairs. It may be beneficial to identify RNA species that are more nuclease resistant than natural RNA. In certain embodiments, one may screen a variety of chemically- modified RNAs to identify candidates that are both nuclease resistant and active in the RNAi mechanism (Figure 2). In certain instances, one may identify chemically-modified, thereby nuclease resistant, RNA species that are both active in RNA interference and amenable for economical chemical synthesis. Certain relatively small chemically-modified RNA species with appropriate secondary structures that will make them active in RNA interference are discussed. One may find suitable modifications in RNA in light of the recapitulation of the essential features of RNAi *in vitro* using a cell-free system derived from *Drosophila* embryos (Tuschl, T. et al., Genes and Development, 13, 3191-3197, 1999). The cell-free system would enable identification of chemically modified RNA species that retain the RNAi activity. Furthermore, the same system could be used to design a dsRNA molecule that is short, economical and effective in RNA interference. For example, these RNA molecules may have short stem-loop structures.

[078] In certain instances, the dsRNA molecules that mediate the degradation of a specific mRNA may be expressed *in vivo* (Figure 3). They

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may be expressed as fairly long RNA transcripts that could form stem-loop structures. One strand that forms the stem will have the sequence complementary (antisense) to the target mRNA. Alternatively, sense and antisense strands of the dsRNA may be expressed separately under two promoters. In these applications, it is envisioned to use transcriptionally active but translationally inactive cassettes to express RNA *in vivo*. The present disclosure describes the use of RNA expression cassettes *in vivo*. These will include, but are not limited to, the use of promoters for RNA polymerase II and III as well as any viral promoters.

[079] In certain embodiments, a host cell may be contacted with two or more interfering RNAs that may target one or more target mRNAs.

[080] Although an antisense approach may be used in target validation using tissue culture models, the technology sometimes may be challenging in validating the same targets in animal models. This may be due to poor delivery and targeting of short synthetic oligonucleotides to desired tissues in animals. Expression of dsRNA molecules in animals using gene delivery vectors (Examples: Retroviral, Adenovirus and Adeno Virus-Associated vectors) may obviate this challenge and is a subject of this disclosure. Hence, an RNA interference approach could represent a method of choice for validating target genes in animal models.

2. Generating Transgenic animals and plants

[081] Transgenic species that do not express certain genes could be produced by specific introduction of DNA cassettes that transcribe dsRNA specific to messages of genes of interest. In certain embodiments, viral resistant transgenic species may be produced by introducing dsRNA specific to certain viruses known to be pathogenic in that species.

3. Gene Therapy applications

[082] The RNA interference approach could be useful in inhibiting a gene product in patients using a gene therapy approach. For example, genes that are responsible for a multi drug resistance phenotype may be a useful

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target for an RNA interference approach. This has a direct impact in cancer patients undergoing chemotherapy.

Advantages of an RNAi approach:

[083] 1. Accumulating experimental evidence suggests that the process of RNA interference may be a catalytic process mediated by interplay of several proteins. As a result, only a few copies of dsRNA per cell may be required to degrade a large pool of target RNA.

[084] 2. Double-stranded RNA molecules that carry out gene inactivation could be either introduced exogenously or expressed *in vivo*, facilitating the mode of delivery for different applications.

[085] 3. Double-stranded RNA molecules are readily taken up by cells. Unlike certain single-stranded oligonucleotides currently used in antisense research.

[086] Technologies that allow specific inhibition of gene expression are becoming important for drug discovery efforts to identify better human therapeutics. Such techniques may play an important role in the following specific areas.

1. Identifying novel genes with implications in disease states.
2. Identifying specific biological roles of a gene of interest.
3. Characterizing complex biological pathways in details.
4. Establishing experimental animal models to move drug candidates to clinics.

[087] Furthermore, in addition to their role in facilitating drug discovery, molecules that mediate specific gene inhibition could become therapeutics as well. One widely used technique in target validation in cultured mammalian cells has been the antisense approach (reviewed in (Murray, 1992)) in which the specific destruction of target mRNA is triggered by the delivery of a ssDNA molecule that is complimentary to a region within the target. Upon hybridization of the antisense (AS) oligonucleotides to its

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target mRNA, it is believed that the enzyme RNase H is recruited to the site to cleave the message.

[088] To compare the characteristics of RNA interference with those of antisense-mediated gene knockdown, PKC-θ, an endogenous gene expressed in the human kidney cell line HEK 293 (293 cells), was used in the following experiment as the primary target. After the delivery of either SiRNA triggers or antisense oligonucleotides, the level of PKC-θ message was measured by a branched DNA (bDNA) assay, an assay designed to capture and detect a specific nucleic acid sequence such as a unique mRNA. In parallel, cellular toxicity and nonspecific gene inactivation was studied by monitoring the level of a housekeeping gene, cyclophilin. In addition to these studies, experiments were designed to gain further understanding of the nature of SiRNA triggers with respect to their specificity, homology, length requirements, and their tolerance to a range of chemical modifications.

Materials and Methods

SiRNA

[089] SiRNAs were prepared using several different methods. Certain chemically synthesized SiRNAs were synthesized using RNA phosphoramidites containing a 2'-O-TriisopropylsilylOxyMethyl (TOM) protection group from Glen Research (Sterling, VA). Other SiRNAs were obtained from Dharmacon (Longmont, CO) employing 5'-Silyl-2'-bis(2-acetoethoxy)methyl (ACE) Orthoester chemistry. Synthesized SiRNAs using TOM phosphoramidites were HPLC purified, whereas those obtained from Dharmacon were used without further purification, due to their high purity resulting from extremely high coupling efficiency (Scaringe, 2001). Ss-SiRNAs produced by in vitro transcription were obtained using corresponding synthetic DNA templates containing the promoter for the T7 RNA polymerase (Milligan et al., 1987). After transcription, full-length RNAs were purified by polyacrylamide gel electrophoresis run under denaturing conditions. SiRNAs were annealed in an annealing buffer consisting of 100 mM KCl, 30 mM HEPES (pH 7.5), and 2 mM $MgCl_2$ by heating to 75° C for 2 minutes followed

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by slow cooling to ambient temperature. HPLC-purified antisense oligonucleotides (ASO) were obtained by solid phase synthesis using cyanoethyl phosphoramidites. The ASOs contained two chemical modifications: the middle seven nucleotides carried a 2'-OMe modification in the sugar and the remaining flanking regions contained phosphorothioate linkages.

Plasmid expressing Secreted Alkaline Phosphatase (SEAP)

[090] The SEAP gene (from pAP-1 SEAP vector from Clontech) was cloned into an adeno-associated vector (AAV # 6).

Cultured mammalian cells

[091] This work used the following cell lines cultured in DMEM with 10% FBS at 5% CO_2 at 37° C.

Cell line	ATCC number
HEK 293	CRL1573
DLD-1	CCL221
MDA-MB 453	HTB131
TM3	CRL1714
Calu 6	HTB-56

Delivery of nucleic acid triggers

[092] Cells seeded in 96-well plates at approximately 25,000/well in the previous day were transfected with different nucleic acid triggers using Lipofectamin 2000 and Opti-MEM I (from Invitrogen). Briefly, SiRNA was diluted in Opti-MEM-I in 50 µL of volume. This was mixed with an equal volume of Lipofectamin 2000 diluted 12.5-fold in OPTI-MEM I. After incubating the mixture at ambient temperature for 20 minutes, 270 µL of the regular cell medium was added and 95 µL of the solution was immediately transferred onto the cells in the plate with no media. Plates were transferred to a 37° C incubator with 5% CO_2 for either 24 or 48 hours. To monitor the fate of SEAP transiently expressed in 293 cells, prAAV6-seap plasmid was included in the transfection mixture with and without SiRNAs.

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Quantification of mRNA levels

[093] Specific mRNA levels of cells transfected with different nucleic acid triggers were quantified using QuantiGene High Volume Kit (from Bayer) that employs a branched-DNA (b-DNA) method for nucleic acid detection according to the manufacturer's instructions. Specific detection of a given mRNA is based on its selective capture on to the microtiter plate, which is dictated by the capture probes. Probe sets that are unique to each target mRNA were designed using the ProbeDSesigner software (Bayer) according to the manufacturer's instructions. Before using the probe sets in experiments, the probes were tested using the cells expressing each message to make sure that they worked.

Cytotoxicity Assays

[094] After 24 or 48 hour incubation, 25 µL of AlamaBlue reagent (Trek Diagnostic Systems, Inc.) was added to each well and incubated at 37° C with 5% CO_2 for 2 hours. The absorbance at 570 nm was read in a SpectraMax UV/VIS 96-well plate format spectrophotometer. The MTT assay was carried out according to the manufacturer's instructions.

Secreted Alkaline Phosphatase (SEAP) assay

[095] Twenty-four hours after the transfection, 15 µL of medium from each well was transferred to a white opaque 96-well flat bottom microtiter plate, and the amount of SEAP was detected using chemiluminescent SEAP assay (Great EscAPe SEAP assay kit form Clontech) according to the manufacturer's instructions.

Results and Discussion

Design of SiRNA Triggers

SiRNA triggers based on antisense target sites

[096] The initial set of SiRNA triggers were based on three antisense DNA sequences that target three independent sites in the PKC-θ message. A computer program designed to identify the best target sites for antisense oligonucleotides had previously picked these sites. When targeted by antisense DNA molecules, sites 8 and 15 were more effective than site 4 in

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reducing the level of PKC-θ message. Figure 31 shows the sequences of antisense DNA sequences as well as ds-SiRNA triggers used in the study. All antisense DNA sequences used are 25 nucleotides in length and contain 2'-OMe substitutions in seven nucleotides in the middle. This region is flanked by nine phosphorothioate nucleotides to either side. Similar to the design reported by Elbashir, 2001a, the Ds-SiRNA molecules also contained 3'-overhangs having two deoxythymidine (dT) residues. In the previous design by Elbashir, 2001a, the two dT residues in the 3'-overhang of the antisense strand provided enhanced nuclease stability while maintaining target recognition. (Elbashir, 2001a) In contrast, the dT residues in the 3'-overhang of the antisense strand of the ds-SiRNA triggers here do not base pair with the target mRNA, hence the target homology is shortened by two nucleotides. For each target site, two sets of ds-SiRNA triggers with 21 and 19 nucleotide (nts) homology to the target sequence were designed.

[097] Silencing of specific genes has been observed upon the introduction of RNA with the capacity to form long hairpin molecules in which one half of the folded sequence is homologous to the mRNA of the targeted gene (Piccin, 2001; Tavernarakis, 2000; Wang et al., 2000). The experiment here sought to determine whether, short chemically synthesized single-stranded RNA (ssRNA) with the propensity to fold-back and form short hairpin molecules may be used as triggers to induce RNAi in vivo. To investigate whether ss-RNA that forms short hairpin structures could trigger RNAi in mammalian cells, two such molecules for each target site were designed (Figure 32). This class of molecules was named **S**ingle-**S**tranded **S**hort interfering **RNA** Hairpin (ss-SiRNA-HP) triggers to distinguish them from the bimolecular ds-SiRNA triggers having two separate strands complimentary to each other. Out of the two unimolecular ss-SiRNA-HP triggers designed for each site, one trigger contained six dT residues in the loop and two dT residues in the 3' overhang and was produced by chemical synthesis. The other trigger containing six uridines in the loop and two uridines at the 3' recessed end was obtained by in vitro transcription.

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Impact of different nucleic acid triggers upon transfection into 293 cells

[098] We carried out parallel analysis of several end points within the cells transfected by a series of nucleic acids triggers such as single-stranded DNA and RNA antisense molecules, ds-SiRNA, and ss-SiRNA-HP. After delivery of each nucleic acid trigger into 293 cells, the following were measured: (1) reduction of specific mRNA, (2) reduction of nonspecific mRNA and (3) cellular toxicity.

Fate of target-specific and nonspecific mRNA levels in 293 cells

[099] The reduction of the specific message PKC-θ was evaluated by measuring the level of PKC-θ mRNA by a bDNA capture-detection method (Figure 33). This work also measured the message level of the cyclophilin gene, a housekeeping gene, as a control to see any nonspecific effects mediated by different nucleic acid triggers (Figure 34). The level of target mRNA was normalized to the level of the nonspecific mRNA to show the selective reduction of the target mRNA by each nucleic acid trigger (Figure 35). As indicated in Figures 33 and 35, all three antisense sequences reduced the PKC-θ message level in a dose dependent manner (bars, 1, 2, 9, 10, 17 and 18). A combination of all three antisense DNA molecules did not show synergistic effect at 100 nM total DNA concentration (bars 25 and 26). Ds-SiRNA triggers targeted to all three sites were also effective in reducing the message level (bars, 3, 4, 11, 12, 19 and 20). Very similar to antisense-mediated gene knockdown, the efficiency of gene knockdown by SiRNA triggers is also target site dependent, suggesting that the target accessibility presumably plays a role in the RNA interference process. A combination of all three ds-SiRNA triggers did not exert synergy in reducing the mRNA level (bars 27 and 28). Ss-SiRNA-HP triggers also reduced the level of PKC-θ message (bars, 5, 13-15, and 21-23), indicating that ss-SiRNA-HP triggers with the propensity to fold into stem-loop structures serve as triggers for RNA interference in mammalian cells. Both types of ss-SiRNA-HP triggers, ones derived from chemical synthesis that contain dT residues in the loop, and the others, derived from transcription that contain

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uridines in these positions, were effective in reducing specific mRNA levels. This indicates that the nature of nucleotides occupying the loop in this work did not affect the RNA interference process. Interestingly, the ss-SiRNA-HP trigger containing a single nucleotide deletion was inefficient in knocking down the level of PKC-θ mRNA (bars, 6 and 7). This result shows here that a specific RNAi trigger initiated an efficient interference process. It is likely that a single point deletion in a stem-loop sequence could lead to the adoption of several alternate conformers with partial base pairing, effectively decreasing the structure illustrated in Figure 32 for ss-SiRNA-8-HP-T.

[0100] A nonspecific effect of nucleic acid triggers on 293 cells was probed by measuring the message level of a housekeeping gene, cyclophilin (Figure 34). A nonspecific gene knockdown by single-stranded antisense nucleic acid triggers (Figure 34; bars, 1, 8, 9, 10, 16, 17, 24, and 25) was observed. None of the ds-RNAi molecules targeted at three different sites within the PKC-θ message showed significant effect on the cyclophilin message level, indicating the specificity of RNA interference. On the other hand, antisense DNA directed to all three sites in the PKC-θ message significantly decreased the level of the cyclophilin mRNA. Similar to the single-stranded antisense DNA molecules, single-stranded antisense RNA molecules also showed nonspecific reduction of the housekeeping message level. However, in contrast to the single-stranded antisense RNA, none of the ss-SiRNA-HP triggers showed any effect on the housekeeping message level. Although this current observation is limited to a single gene provided as a nonspecific control, these results suggest a nonspecific effect. Namely, single-stranded antisense molecules, whether they were DNA or RNA, have some degree of nonspecific gene inactivation compared to highly selective gene expression interference mediated by dsRNA. The reason for the specific gene inhibition mediated by RNAi may lie within the heart of its mechanism (Hammond. 2001). On the other hand, the antisense effect is expected to occur by simple complementary strand hybridization without facilitation from a protein, and hence can be prone to nonspecific effects.

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Alternatively, nonspecific mRNA reduction could be due to the toxicity induced by single stranded nucleic acid triggers upon transfection (see below), sending cells into a state of shock.

[0101] No nonspecific gene knock down by ss-SiRNA-HP triggers that could fold back to generate short hairpin structures was observed. This result along with the reduction of the specific message by ss-SiRNA suggests that ss-SiRNA-HP molecules, like ds-SiRNA molecules, function through the RNA interference mechanism to specifically reduce target mRNA levels.

Cellular Toxicity

[0102] This work included two commonly used cytotoxicity assays to evaluate cellular toxicity upon delivery of various nucleic acid triggers into cells. Twenty-four hours after the transfection, an AlamarBlue assay was carried out and completed within an hour (Figure 36). The MTT assay that requires a longer incubation time (overnight) was also initiated after 24 hours of transfection (Figure 37),

[0103] The AlamarBlue assay revealed the toxicity associated with cells transfected with single-stranded antisense molecules, both DNA and RNA (Figure 36; bars, 1,2, 8-10, 16, 17, 24, 25 and 32). The observed toxicity of ss-DNA antisense molecules was dose dependent; more pronounced at 100 nM than 10 nM. An unrelated sequence used as a control also exerted cellular toxicity in the AlamarBlue assay (bar, 32), indicating that the observed toxicity is not a function of antisense sequences directed to PKC-θ mRNA, but rather a general phenomenon. The level of toxicity was also dependent on the number of cells used for the transfection. In general, when greater than 40,000 cells per well were used, the level of toxicity observed with single stranded nucleic acids was significantly reduced (data not shown). This result suggests that the undesirable effect of cellular toxicity presumably caused by the lipid-AS nucleic acid complex gets diluted with increased cell number.

Randomly picked ds-SiRNA

[0104] The above ds-SiRNA triggers were based on target sites picked by a computer program designed to identify the best sites for antisense

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targeting. They all worked as expected in reducing the target mRNA level by triggering the RNAi mechanism. This work also tested ds-SiRNA targeted to sites picked by random choice, without applying any pre-selected criteria. Several different ds-SiRNA triggers. Three of these had overlapping target sites within the PKC-θ mRNA (Figure 38A; Random picks # 1-3). Two ds-SiRNA molecules out of these three sharing overlapping target sites were effective in triggering RNAi (Figure 38b). This result demonstrates that effective ds-SiRNA triggers can be picked randomly. However, not all sites were equally effective in eliciting RNA interference. In this experiment, moving the target site by only five nucleotides made the ds-SiRNA trigger ineffective, suggesting the existence of sites that facilitate RNA interference as well as sites that are inert to the mechanism. Sites that are immune to RNAi could be difficult to access by the RNA induced silencing complex (RISC) due to either the folding of RNA, or the masking of RNA with RNA binding proteins, or some combination of these possibilities.

[0105] The two dT residues at the 3'-end of the ds-SiRNAs studied so far do not recognize the target mRNA. These residues were changed to two dC residues and this trigger, containing dC residues in place of dT residues, worked effectively (Figure 38B). This suggests that the nature of nucleotides in the 3' extension does not interfere with the process. The ds-SiRNAs are typically synthesized to contain 3'-extensions, mainly to mimic the digested products of exonuclease III Dicer enzyme. However, it is possible that 3' extensions within ds-SiRNAs are not required to become the substrate for RISC formation and to initiate the RNAi process. This work showed that the 3'- extensions were not necessary to mount an effective RNAi, since triggers with blunt ends worked (Figure 38; Ds-SiRNA random pick #4). In the case of N19-AA-Rm, one end is blunt and the other is not. These results suggest that the end structure was not important to initiate an effective RNAi process.

[0106] By targeting endogenous genes present in several other cell lines (Figures 39-40), this work showed that RNAi can work in several mammalian cells. In the case of silencing the DJ-1 gene in MDA MS453 cells

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(Figure 39), the ds-SiRNAs based on the optimal antisense sequences were not as effective as those identified by random choice, suggesting that the optimal target site for antisense-mediated gene knockdown does not necessarily become the optimal site for SiRNA.

RNAi in a transiently expressed reporter gene

[0107] The above results were obtained with ds-SiRNA triggers directed against endogenous genes in cultured mammalian cells. This study was extended to a gene expressed transiently upon transfection into mammalian cells. A plasmid was designed to express a reporter gene. SEcreted Alkaline Phosphatase (SEAP), under the CMV promoter (prAAV6-seap) was transfected into 293 cells in the presence and absence of a ds-SiRNA trigger designed to target the SEAP mRNA. In this experiment, three different ds-SiRNA triggers picked randomly to target non-overlapping sites within the mRNA of SEAP were used. Out of the three ds-SiRNA tested, one trigger effectively silenced the transient expression of SEAP (Figure 41). The RNAi effect induced by ds-SiRNA trigger 1 was very potent and effective at 10 nM. The previous experiments demonstrated the reduction of the mRNA levels of the specific gene that was targeted by SiRNA. The reduction of the target gene, SEAP, correlated with the presence of a ds-SiRNA trigger that targeted the SEAP gene.

Duration of RNA interference

[0108] Next, the duration of RNA interference triggered by a ds-SiRNA in 293 cells was examined. Figure 43 indicates the period in which RNA interference was effective in silencing a transiently expressed SEAP reporter gene as a function of amounts of prAAV6-seap plasmid and dsSiRNA used. When the amount of plasmid used was low, the persistence of RNAi even up to 170 hours (7 days) after transfection with 100 nM ds-SiRNA was observed(Figure 43 A). With certain increased plasmid concentration, however, the level of expression crept up after 100 hours, and remained at 50 % at 170 hours. A similar trend was observed with ds-SiRNA concentrations as low as 5 nM (Figure 43 B &C). Thus, when the target mRNA concentration

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in cells was relatively low, gene silencing mediated by RNAi persisted longer than with certain higher target mRNA concentrations.

[0109] Next, the duration of RNAi in silencing an endogenous gene, PKC-θ (Figure 44) was investigated. Ds-SiRNA (Ds-SiRNA-8113) was used in the study along with an antisense molecule (AS-8) to measure the duration of RNAi and compare it to that of the antisense effect. After the delivery of nucleic acid triggers, mRNA levels of PKC-θ were measured at 24 hours, 48 hours, and 72 hours, and normalized to the message level of the housekeeping gene at identical time points (Figure 44 A, B and C). Out of the three time points, the strongest effect was observed at 48 hours after transfection. Furthermore, the effect persisted even at 72 hours. The antisense effect, on the other hand, gradually decreased with time. An RNA interference effect peaking at 48 hours may not be a general phenomenon, but could be gene and cell-type specific. Previous studies in mouse zygotes suggested that RNA interference triggered by relatively long ds-RNA (714 bp) lasted for 6.5 days (Wianny, 2000). The efficacy of RNA interference triggered by Ds-SiRNA-813 alone may be generally higher than that induced by Ds-SiRNA-BB mixed with a nonspecific control (data not shown). This result is consistent with previous studies (Parrish, 2000; Tuschl et al., 1999). This suggests that the presence of nonspecific ds-RNA could lead to the formation of unproductive RiSC complexes upon binding to protein components of RiSC. In essence, the addition of nonspecific dsRNA could compete for the limiting amounts of protein components in the cell, thereby decreasing the concentration of the productive RiSC complexes formed with the specific trigger.

Certain SiRNA triggers

[0110] Certain ds-SiRNA triggers may have 21 nucleotides in each strand, out of which 19 nucleotides form base pairs in the duplex leaving two nucleotides in the 3' extension (Caplen, 2001; Elbashir, 2001a). In certain designs, all 21 nucleotides in the antisense strand are homologous to the target mRNA. Tests were conducted to test the effectiveness of different

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lengths of certain ds-SiRNAs, as well as to test the effectiveness of ds-SiRNAs having different homologies. The homology tests involved testing the number of mutations in a ds-SiRNA molecule that may be tolerated certain RNAi processes. These issues were addressed by using a series of ds-SiRNA triggers (Figure 45).

Homology of the SiRNA trigger

[0111] Certain mutations were introduced in both strands of the ds-SiRNA-8A trigger to obtain two triggers (Figure 45); Mut-1 has a single base pair change from G-C to A-U, whereas in Mut-2 two G-C base pairs were changed to two A-U base pairs. These mutations were localized in the middle of the trigger. As shown in Figure 46 C, the RNA interference process worked with a single-base pair mutation, although the efficiency may be somewhat reduced. However, mutation of two base pairs was detrimental to the interference process in this work. This result is in agreement with the previous observations made by Elbashir et al., 2001, who observed a lack of RNA interference with ds-SiRNA triggers containing three base pair changes within a 21 nucleotide homologous region. A significant effect caused by a single point deletion in the sense strand of a ss-SiRNA hairpin trigger was observed (see above), indicating that certain ss-SiRNA hairpin triggers could be very sensitive to mutations.

[0112] Next, the tolerance of the RNA interference mechanism to the mutations localized only in the sense strand of a SiRNA trigger was investigated. Six ds-SiRNA triggers in which the sense strand carried 1-6 point mutations were constructed (Figure 45 and 46, C). Out of these six triggers, only two triggers containing either 1 and 2 point mutations in the sense strand were effective in triggering the RNA interference (Figure 46, C). This result suggested that the sense strand in this work played an active role in the RNAi mechanism and is not only there to protect the antisense strand by base pairing. It may be that mutations in the sense strand affect the initiation step of the RNAi process, rather than the propagation step. Ds-SiRNA triggers with imperfect base pairing may not be an effective

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substrate for assembling into the RiSC complex. At least in *C. elegans*, there is an asymmetry in the two strands with respect to certain chemical modifications (Parrish, 2000); for example the RNAi process accepts substitution of a 2' -NH_2 uracil for regular uracil residues in the sense strand but not in the antisense strand. Thus, in certain instances, these results suggest that both strands of an SiRNA trigger actively participate in the mechanism of the interference process

Short ds-SiRNA triggers

[0113] In certain work, ds-SiRNA triggers of 21-25 nucleotides have been used to elicit RNA interference in mammalian cells (Caplen, 2001; Elbashir, 2001a). A rationale for choosing these lengths was based on the observation that long double-stranded RNAs are cleaved into 21-22 nucleotide fragments that serve as intermediates for the interference process (Elbashir, 2001b). The effect of RNAi triggered by ds-SiRNA triggers consisting of 14, 17, and 19 nucleotides in each strand was investigated (Figure 45 and 46 A and B). Again, these triggers were designed to have 3' extensions of two dT residues that do not base pair with the target mRNA (in the case of the antisense strand). Hence, target homologies of these triggers were 12, 15, and 17 nucleotides, respectively. As shown in Figure 46 (A, bars, 1-3), a ds-SiRNA with 19 nucleotides in each strand was effective in triggering the RNAi response, indicating that ds-SiRNA triggers with a 17 nucleotide homology to the target strand may work effectively. However, as shown in Figure 46 (A and B), certain ds-SiRNA with 17 nucleotides in each strand were not effective in triggering RNAi. There may be several reasons for the inability of ds-SiRNA with 17 nucleotides in each strand to trigger RNAi in this work. One potential reason might be that the length of the duplex is too short to assemble into RiSC upon binding to a number of proteins. Another potential reason could be based on insufficient homology for the RNA interference process to work here.

[0114] To identify which factor might be important for RNA interference, three more ds-SiRNA triggers were designed to differentiate

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between the homology and the length of the trigger (Figure 46, B). The length of the triggers was left at 21 nucleotides, but the length of the homology was 17, 15, and 12 nucleotides, respectively (Figure 45, sequences 4, 5 and 6). The ds-SiRNA triggers of 21 nucleotide length, that also carried either a 15 or 17 nucleotide stretch homologous to the targeted mRNA were effective in RNA interference. The trigger with only a 12 nucleotide homology was not effective(Figure 46, B, Bar 6). These results suggest that both the length of the ds-SiRNA trigger and the number of nucleotides in the homologous region may play a role in certain RNAi processes.

Nuclease resistant SiRNA triggers

[0115] Both ss- and ds-SiRNA triggers (unimolecular and bimolecular) that are stable to nucleases, and hence may be effective in initiating and maintaining an RNAi response in cells, were studied. Nuclease stable SiRNA triggers may survive longer in certain biological fluids both during and after their delivery into the cells. Once inside the cell, mere resistance to degradation may make nuclease stable triggers last longer, thereby establishing long lasting RNA interference. Moreover, certain modifications may facilitate cell penetration. The following modifications in either antisense or sense strand were made to explore the possibilities for creating chemically modified SiRNA triggers (Figure 47).

1. Inverted abasic residue at the 3' end after two dT residues.
2. Inverted dT residue at the 3' end after two dT residues.
3. 2'-F pyrimidines.
4. 2'-OMe in all nucleotides.
5. DNA.

[0116] The inverted abasic residue and the inverted dT residue may function like 3' caps that further protect an oligonucleotide from 3'-5' exonucleases, in addition to the protection provided by two dT residues. Replacement of the 2'-OH group in the sugar by various groups such as NH_2, F and OMe may make RNA more nuclease resistant (Lin et al., 1994; Pieken et al,, 1991). As shown in Figure 47, both modifications at the 3' end do not

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appear to interfere with the RNAi mechanism, suggesting that SiRNA triggers that are protected from 3'-5' exonuclease may be designed. Substitution of all pyrimidines by 2'-F pyrimidines in both strands may also be tolerated by the RNAi mechanism. It has been shown previously that the 2'-F pyrimidine substitution alone may be sufficient to make RNA more nuclease resistant and the half-life of such modified RNA molecules may be increased significantly (Green et al., 1995; Lin et al., 1994; Pagratis et al., 1997). Based on these previous studies, SiRNA triggers carrying all pyrimidines with the 2'-F modifications in combination with 3'-and 5'-cap may be significantly nuclease resistant and may be effective in triggering RNAi. Additionally, the 5'-caps could have any modification that will substantially prevent or reduce exonuclease attack. Certain examples of modifications that may be used are dT, or modifications with amino, cholesterol, thiol groups, or modifications with a dye molecule such as fluoroscein, or modifications with polyethylene glycol, a lipid, or a carrier peptide.

[0117] Ds-SiRNA triggers with one strand with a 2'-OMe substitution inhibited the RNAi process. However, it may be possible to have SiRNA triggers modified with 2'-OMe either in pyrimidines, or in purines, or at specific sites. SiRNA triggers with a single DNA strand may be analogous to SiRNA triggers with a 2'-OMe modification. RNA-DNA hybrids were not efficient in eliciting the RNAi process in this work. The lower level of RNAi in mammalian cells by SiRNA with DNA strands, as demonstrated here, is in agreement with a previous observation made in *C. elegans* with long RNA-DNA hybrid molecules. Previously, using relatively long dsRNA molecules Parrish, et al. observed the tolerance of 2'-F uracil in RNA interference in *C. elegans* (Parrish, 2000).

[0118] Next, a unimolecular trigger based on the fold-back hairpin structure was generated with 2'-F pyrimidines throughout the molecule and with a single inverted dT at the 3' end (Figure 48). As shown in Figure 49, the chemically modified unimolecular ss-SiRNA trigger may be effective in silencing the transient expression of SEAP. This observation supports the

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notion that unimolecular ss-SiRNA triggers may be optimal for certain RNAi. As presented in Figure 49, the use of a unimolecular trigger may be more effective than a bimolecular (ds-SiRNA) trigger to elicit RNAi by targeting an identical site within the target mRNA (Figure 49, compare Sphp-2 and Seap 2).

[0119] The successful use of SiRNA triggers including both pyrimidine nucleotides carrying a 2'-F modification either on both strands (in the case of bimolecular ds-SiRNAs) or throughout the molecule (in the case of unimolecular ss-SiRNAs) was observed. Taken together, these results suggest that the design of SiRNA triggers carrying specific chemical modifications may be a viable approach to initiate RNA interference in mammalian cells. Certain additional modifications to SiRNA triggers include, but are not limited to, the addition of polyethylene glycol, the addition of lipids, and the addition of dyes, such as flourescein. It may be that these triggers will pave the way for creating effective SiRNA triggers for clinical applications.

Conclusions

RNA Interference Compared to Antisense

[0120] 1. Relatively low or almost no toxicity to the cells transfected with SiRNA triggers was observed, whether they were ds-SiRNA or ss-SiRNA that form short hairpins, compared to the single stranded antisense molecules. Transfection with SiRNA triggers in this work did not lead to the reduction of nonspecific mRNAs. In certain instances, this may make RNAi technology more versatile than antisense techniques in general, allowing researchers to transfect cells at low cell density. This also suggests that RNA interference may be a useful tool to study cellular pathways that are sensitive to external insults.

[0121] 2. RNAi in general may be more potent than antisense; about 10-fold lower concentration of SiRNA compared to the concentration of antisense oligonucleotides gave approximately the same level of mRNA reduction in this work. Effective RNA interference with SiRNA triggers transfected at 5 nM was observed.

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[0122] 3. Similar to antisense oligonucleotides, in certain instances, the efficiency of SiRNA triggers may be target site dependent, suggesting that targeting certain sites in a target mRNA may not be effective in eliciting gene silencing through RNAi.

[0123] 4. SiRNA triggers may be picked randomly without any help from a computer algorithm. Since targeting to certain sites on mRNA may not work, a handful of triggers (between 3 and 5) may be tested. This is in contrast to the antisense approach that often involves screening 30-40 oligonucleotides. Since the conversion of a ds-SiRNA trigger that was not effective into a unimolecular trigger that produced effective interference was observed, it may be that the number of unimolecular triggers that are screened may be even lower.

Certain Novel SiRNA triggers

[0124] 1. Effective unimolecular SiRNA triggers that are single-stranded with the propensity to form short hairpins were observed. In those ss-SiRNA HP triggers, the nature of the nucleotides in the loop may not affect the efficiency of RNA interference.

[0125] 2. Certain ss-SiRNA HP triggers appear to be sensitive to nucleotide deletions.

[0126] 3. In certain embodiments, unimolecular siRNA triggers may contain synthetic loops. In certain embodiments, unimolecular siRNA triggers may contain polyethylene glycol loops.

Nuclease stable SiRNA triggers

[0127] 1. The incorporation of a nuclease resistant cap, such as inverted dT and inverted abasic residues at the 3' end of both strands of a ds-SiRNA trigger, may be effective in RNA interference. This also may work in ss-SiRNA triggers. Furthermore, the same strategy may work at the 5' end as well.

[0128] 2. In certain instances, pyrimidines in both strands of ds-SiRNA trigger may be replaced by 2'-F modified pyrimidines, making such triggers resistant to nucleases.

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[0129] 3. In the case of certain unimolecular ss-SiRNA triggers, the 2'-F modification may be introduced throughout the sequence in all pyrimidines without substantially compromising the efficacy of RNA interference.

Features of Certain SiRNA triggers

[0130] 1. Ds-SiRNA triggers may be designed to carry 3' extensions made up of two dT residues that do not recognize the target. In other words, target sites on an mRNA may be any sequence, not restricted to the nature of N_{19}-AA. This opens up a broad range of target sites within a target gene.

[0131] 2. In certain instances, the length of each strand within a ds-SiRNA may be as short as 19 nucleotides out of which 17 nucleotides form a contiguous stretch of target homology with two nonhomologous dT residues at the 3' extensions.

[0132] 3. In certain instances, the region of homology may be as short as 15 nucleotides when the length of the trigger is 21 nucleotides. A 19 nucleotide long ds-SiRNA harboring 15 nucleotides of target homology may be effective in RNA interference.

[0133] 4. In certain instances, ds-SiRNA triggers may tolerate a single base pair mutation.

[0134] 5. In certain instances, mutations in the sense strand of the ds-SiRNA trigger also affect the efficiency of RNA interference. In certain instances, ds-SiRNA triggers with more than two mutations in the sense strand may decrease the RNAi effect. In certain instances, the effectiveness of RNA interference may vary depending on the mutation site in the sense strand, such that an effect may be observed when the mutation is located in the middle, as opposed to near the end of the RNA.

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II. Gene identification and functional analysis using *S*hort *i*nterfering *R*andom *S*equence (SiRS) RNA hairpin libraries

[0135] Identification and characterization of novel genes combined with the functional analysis of identified genes may enable the discovery of novel drug targets. With the completion of the human genome database, companies are racing to identify novel genes. Among these may be genes that could be linked to various diseases.

[0136] Some technologies exist to potentially identify a function of a gene based on sequence information. Antisense oligonucleotides upon pairing-up with a known mRNA sequence mediate the destruction of a message in a cell, leading to the inactivation of gene expression. This technique may be aided by prior knowledge of the mRNA sequence of a gene and may be used to validate gene function. On the other hand, there are many genes in the human genome database whose sequences are not known to date. Furthermore, it may be that alternate splicing will lead to the generation of many splice variants to generate different protein products. This may further complicate the gene identification process.

[0137] A reverse genetic strategy to identify genes exclusively based on their function may be possible, allowing discovery of new genes from the human genome as well as the analysis of the functions of known genes. Furthermore, the proposed strategy may also help identify novel pathways of complex biological processes. This strategy uses epigenetic interference of gene expression using double-stranded RNA (dsRNA) molecules. In this work, certain double stranded RNA (dsRNA) corresponding to a sense and antisense sequence of an mRNA was introduced into a cell, the corresponding mRNA was degraded and the gene was silenced. This post-transcriptional gene silencing phenomenon, mediated by double stranded RNA sequences, is commonly referred to as RNA interference or RNAi (Fire, 1998). RNAi has been observed in a wide range of organisms, including nematodes, insects, trypanosomes, planaria, hydra, zebrafish, and the mouse (Reviewed in Bosher, 2000; Hammond, 2001). RNAi may be

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functionally related to posttranscriptional gene silencing observed in plants and quelling observed in *Neurospora crassa.* Since double stranded RNA mediates all three processes, they can be collectively called "RNA silencing" (Voinnet, 2001; Waterhouse, 2001).

[0138] Biochemical studies carried out with a *Drosophila* in vitro system led to the discovery of short RNA duplexes (21-22-nucleotides) that could effectively trigger gene silencing through RNAi (Elbashir, 2001b; Yang, 2000; Zamore, 2000). Recently, this epigenetic interference of gene silencing by short interfering RNAs (SiRNAs) has been demonstrated in cultured cells of mammals (Caplen, 2001; Elbashir, 2001a), opening the door for using RNAi technology in characterizing the human genome. Although complete details of the cellular mechanism that initiates and propagates gene silencing through RNA interference is unknown, it may be an extremely specific and very potent mechanism to inhibit gene expression by degrading specific mRNA. Only a few molecules of dsRNAi triggers per cell may be needed to inhibit mRNA present at high concentrations, suggesting an inherent amplification step present in the RNAi process. In certain instances, its specificity, efficacy and generality offer an attractive approach for studying gene function.

Short Interfering Random Sequence (SIRS) RNA hairpin libraries

[0139] In certain embodiments, proposed strategies may be based on the use of random sequence RNA libraries to elicit an RNAi response in mammalian cells. In certain embodiments, a library of random sequence short RNA hairpins may be used to elicit RNAi-mediated gene silencing in mammalian cells. Long RNA hairpins have been used to elicit an RNAi response in several species including *C. elegans* (Parrish, 2000; Tavernarakis, 2000) *Trypanosome* (Ngo, 1998), *Drosophila* (Piccin, 2001) and in plants (Chuang, 2000). The use of short hairpin molecules to trigger RNAi in cultured mammalian cells was investigated. This proposition was based on preliminary data that supports successful and specific gene inactivation by short hairpin molecules with defined sequences (Figure 6). The existing data

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suggests that the sequences in the loop of a short hairpin in this work do not have any effect on the efficacy and the selectivity of the RNAi process in mammalian cells. The random region may be placed within the stem of the hairpin in SIRS libraries. Different phenotypic responses (or functions) established in cells that have been exposed to short interfering random sequence (SIRS) RNA hairpin libraries may be looked for using SIRS RNA hairpin libraries. The complexity of the library may be narrowed down by serial dilution to identify small collections of hairpins that trigger the chosen cellular phenotype. Molecules within this sub-library may be split into another array to further reduce the collection of sequences of short RNA hairpins that trigger the specific phenotype. Nucleotide sequences within these hairpins may be identified by cloning and sequencing. The consensus sequence that emerges within this collection of hairpins may be subjected to a blast search to identify the potential candidate gene for the biological function.

[0140] Certain methods may be proposed to screen SIRS hairpin libraries to identify ss-SiRNA hairpins that elicit specific phenotypic responses in cells. Certain methods use a split screening approach; as described above, to cull the original library, whereas certain other methods utilize a biological screening approach using a retrovirus that expresses random sequence short hairpins.

Method 1:

[0141] Method 1 may be based on the use of in vitro synthesized SiRS RNA hairpin libraries from which RNAi triggers may be identified through repetitive screening of sub-libraries. These sub-libraries may be subjected to splitting and amplification to identify the sequence of interest.

A. Library Construction

A. 1. General Design of synthetic DNA templates

Short interfering random sequence (SiRS) RNA hairpin libraries may be generated from synthetic DNA templates. The design of the synthetic DNA template is illustrated in Figure 7 (boxed). These synthetic DNA templates may be single stranded and may contain the top strand of the T7 promoter

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sequence at the 5' end followed by a contiguous stretch of a random region. The promoter for RNA polymerase may be for any RNA polymerase known in the art. Certain examples of polymerases are the T7, T3, and SP6 RNA polymerases. Next to the random region may be a defined nucleotide sequence followed by a loop sequence and a sequence stretch complimentary to the defined nucleotides. In this design, the 3' end may fold back to form an incomplete stem-loop. The 5' end of the DNA template may be biotinylated to facilitate its immobilization on a microtiter plate well surface to facilitate subsequent steps in the screening process. Biotinylation of the DNA template and the use of streptavidin-coated microtiter plates are not required for the screening process.

A. 2. Sequence Complexity

[0142] The number of nucleotides in the random region dictates the sequence complexity of a library. A library with 15 randomized nucleotides has 1.0×10^9 theoretically possible individual molecules, whereas a library with 20 such nucleotides will have 1.0×10^{12} individual molecules. One would expect a single 15-nucleotide-long sequence within a library of approximately 10^9 individual molecules to occur only one time in the human genome. Based on this calculation, such a library may be expected to provide RNAi triggers for all possible genes in the human genome. However, a SiRS RNA hairpin library having one million unique RNAi triggers may be used. This library may be large enough to capture most genes.

A. 3. Synthesis of RNA Libraries

[0143] A general scheme for the production of an SiRS RNA hairpin library from the corresponding synthetic DNA template library using in vitro transcription is outlined in Figure 4. Synthetic DNA templates may be heated to 95° C and allowed to slowly cool to room temperature to facilitate the formation of partial stem-loop structures. The 3' ends of partial DNA stem-loop templates may be extended by using a high-fidelity DNA polymerase such as the Klenow fragment of an E. coli DNA polymerase 1. This reaction allows for the formation of all theoretically possible combinations of perfect

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stems. Once the double stranded DNA template is made, it may be distributed into streptavidin-coated microliter plates (1536-well Master Plates) (Figure 8).

[0144] Since the concentration of each template may not be sufficient to generate enough RNA hairpins of one type, copies of immobilized templates may be amplified by polymerase chain reaction (PCR). PCR-amplified DNA templates may be used for in vitro transcription using T7 RNA polymerase (when T7 RNA polymerase is included in the DNA template). The resulting single stranded RNA molecules may fold into intra-molecular hairpin structures. They also may undergo intermolecular hybridization and generate double-stranded RNA molecules. Both RNA hairpins and dsRNAs may generate an RNAi response. Thus, the formation of intra- or inter-molecular structures should not pose a problem in this strategy. Once RNA molecules are made, they may be ready for use in cell-based screening assays.

B. Functional Screening

[0145] Certain screening exercises may start with a dsDNA template library having approximately $1X10^6$ molecules that has been distributed into five 1536-well microtiter "Master Plates" resulting in approximately 130 unique templates in a single well. Once biotin-conjugated DNA templates are immobilized in the streptavidin (SA) coated 'master plates", excess SA in the well may be blocked with free biotin, and the wells may be thoroughly washed. Subsequently, each template molecule immobilized on the "master plate" may be amplified by PCR using specific primers one of which may be biotinylated at the 5'- end. Upon PCR amplification, amplified, biotinylated, template molecules may be transferred to four 384-well "lead plates" from a single "master plate" (Figure 9). Since the "lead plates'" may also be SA-coated, amplified template molecules may be captured onto the well surface. Next, SiRS RNA hairpin sub-libraries may be generated within the wells of "lead plates" using the standard in vitro transcription protocol. These SiRS RNA hairpin sub-libraries may be delivered by a standard cell transfection protocol into cells grown in 384-well plates in a geometrically addressable

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manner. Several different functional assays in parallel may be performed using SiRS RNA hairpin sub-libraries from "lead plates". Hence, it may be possible to use a relatively low number of cells per well (approximately 5000) for transfecting SiRS RNA hairpin libraries. Functional screening may be based on several biological end-points. Some nonlimiting examples are listed below.

[0146] 1. Cell proliferation.

[0147] 2. Cell death (with and without apoptosis).

[0148] 3. Cell migration.

[0149] 4. Cell senescence.

[0150] 5. Specific reporter assays.

[0151] 6. Triggering the production (or inhibition) of secretary molecules.

[0152] Certain high-throughput cell-based assays may be used to screen an SiRS hairpin library. Each functional screening assay may give rise to the identification of one or more wells with biological endpoints of interest. Corresponding wells in the lead plate contain "lead SiRS libraries". Since functional screening for various biological end-points using different assay formats and cell types may be carried out in parallel, several "lead SiRS libraries" may be identified simultaneously from a single lead plate. These lead libraries may be split into sub-libraries and the resulting sub-libraries may be used for further screening as described below.

[0153] Once a lead library for a particular functional assay is identified, the specific RNAi trigger hairpin may be delineated by further fractionation of the members within the library. This may be done by subjecting the corresponding DNA library in a 'lead plate" to low-level PCR amplification, followed by distributing the amplified products into several 96-well "daughter plates" (Figure 10). If one uses three 96-well plates, it may be that a very low number of unique template molecules per well (approximately

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1-5 unique template/well) may be possible. A low ratio of unique templates per well may also enhance the probability of identifying the unique RNAi trigger for the specific biological outcome. After distribution, template molecules in each well of 96-well plates may be PCR amplified to enrich the number of copies of unique template molecules. RNA may be made within each well of "daughter plates" and used for secondary screening using the same functional assay as used to obtain the lead. Wells from which RNAi triggers were derived to elicit the biological end-point may be identified. If necessary, additional rounds of screening may be carried out to further reduce the complexity of sub-libraries derived from lead libraries. DNA templates from the corresponding wells in "daughter plates" may be cloned and sequenced. Sequences derived from each well may be compared to identify the consensus sequence of the RNAi trigger responsible for the observed biological end-point (Figure 11).

[0154] The biological end-point in the particular functional screen may be confirmed by synthesizing the specific RNA hairpin molecule derived from the consensus sequence. Once confirmed, nucleotide sequences in both arms of the specific hairpin may be used to perform a BLAST search against the human genome database to identify the candidate gene (Figure 12).

[0155] The success of the screening process may depend on the efficiency of library transfection and the amount of RNAi trigger molecules that silence a gene within a single cell. Approaches that make SiRS RNA hairpin libraries more nuclease resistant may help improve the survival of individual molecules during transfection, which may be important during the primary screening step in which the libraries are more diverse. Previous studies in *C. elegans* using long dsRNA triggers suggested that the substitution of 2'-F uracils in place of 2'-OH uracil in either sense or antisense strand did not interfere with the RNAi process (Parrish, 2000). These authors also reported successful RNA interference with long dsRNA triggers synthesized with a single type of α-thio NTP. Substitution of α-thio UTP may produce a somewhat reduced level of RNA interference. A successful reduction of

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specific mRNA in mammalian cells was observed using SiRNA triggers of 21 nucleotides in which all pyrimidines in both strands are modified with 2'-F sugars. The incorporation of 2'-F-modified pyrimidines and α-thio purines into hairpins may make them nuclease stable. SiRS RNA hairpin libraries with chemically-modified pyrimidines on the sugar and phosphorothioate backbone modifications at all purines may be obtained by in vitro transcription employing the appropriately modified NTPs.

[0156] Another approach to enrich the population of each hairpin molecule within target cells employs the transfection of ds-DNA templates (preferably linear or circular in the form of plasmids) carrying the T7 promoter fused to the template for hairpin synthesis. In this case, the target cells may carry the T7 RNA polymerase gene integrated into their genome. Upon transfection, T7 RNA polymerase will transcribe many copies from each template within the cell.

Method 2:

[0157] Method 2 uses a biological screening method using a retroviral vector carrying SiRS RNA hairpin libraries. Retroviruses that mediate a specific biological function in infected cells may be identified, amplified and used for the next cycle of selection.

[0158] A retroviral screening system employing random sequence combinatorial libraries containing the target recognition site for a hairpin ribozyme has been used for target validation. (Kruger, 2000; Li, 2000)

A. Library Construction

[0159] DNA constructs that express SiRS hairpin RNA libraries in vivo may be designed as illustrated in Figure 13. A synthetic single-stranded DNA molecule in which two defined regions flank a randomized region may be obtained by chemical synthesis. The ss-DNA template may be converted to ds-DNA by PCR amplification using a primer pair that anneals to the two defined regions. The two PCR primers may carry two unique restriction sites near their 5' ends. As shown in Figure 13, the dsDNA may be digested with one restriction enzyme and the products may be gel-purified. The resulting

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cohesive ends may be ligated to obtain dsDNA carrying the inverted repeat. These inverted repeats may be cloned into a retroviral vector after digestion with the second restriction enzyme using standard cloning techniques known in the art. For efficient synthesis of SiRS RNA hairpins, the dsDNA library may be cloned downstream of the human pol III tRNA (e.g., tRNA val) promoter which is known to provide a high level of transcription in vivo (Good, 1997). Antibiotic markers such as neomycin and puromycin may be inserted into the retroviral vector to facilitate the selection of vector containing cells after transduction. Several picomoles of the dsDNA library, representing approximately 10^{11} individual molecules, may be ligated into the retroviral vector. The entire ligated mixture may be transformed into a bacterial strain and plated on approximately 10 plates. Transformants may be identified, pooled, and stored at -80° C.

[0160] Plasmid libraries may be obtained by growing the transformant pool followed by carrying out minipreps. The plasmid DNA library may be used to generate the retroviral vector library by triple transfection methods known in the art. This may include the cotransfection of the plasmid DNA library along with two vectors, one expressing Gag-Pol (Landau, 1992) and the other expressing VSG-G (Burns, 1993) into a packaging cell line. The supernatant containing the retroviral library may be harvested, filtered, and used for biological screening.

[0161] Biological screening, as in Method 1, may be performed in parallel. In each case, cells may be transduced with SiRS RNA hairpins carrying retroviral vector at a very low MOI (multiplicity of infection), in certain instances, as low as 1. Cells may be maintained in the presence of the antibiotic to which the retrovirus is resistant and screened for a desired biological response. Individual wells exhibiting a desired biological outcome may be identified and viruses may be rescued from them.

[0162] Viral rescue from selected wells may be achieved by co-transfecting DNAs of two helper vectors expressing Gag-Pol and VSV-G. After co-transfection, the vector supernatant may be selected, pooled, filtered,

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and used for the next round of selection with a fresh plate of cells. Alternatively, PCR rescue may be performed to rescue the sub-library of inserts that provided the desired biological outcome. A single PCR primer carrying the second restriction site in the original DNA construct may be used for PCR rescue. For this, high molecular weight DNA may be extracted from identified wells and PCR amplified with the primer. Resulting PCR products may be digested with the second restriction enzyme, gel purified, and cloned into the retroviral vector as described above. The resulting retroviral sub-library may be used for the next selection round.

[0163] After 2-3 cycles of selection-enrichment of retroviral libraries, inserts may be cloned and sequenced to identify a consensus sequence motif. Based on the consensus sequence motif that emerges, a hairpin RNAi trigger may be synthesized and used to confirm the biological effect of the trigger. Once confirmed, nucleotide sequences in both stems of the RNAi trigger may be used to perform a blast search against the human genome database to identify the candidate gene.

III. Certain methods for designing functional SiRNA triggers for effective gene silencing by RNA interference

[0164] RNA interference (RNAi) is an effective technique for gene silencing in many organisms, including mammals. Short RNA duplexes of 21-22 nucleotides with 2-3 nucleotide 3' extensions, generally referred to as SiRNA molecules, have been effective in specific gene silencing in mammalian cells by triggering the RNAi process. However, not all SiRNA molecules bearing homology to a region within an mRNA sequence work effectively in silencing the cognate gene. A simple, effective, and universal approach is described here for designing productive SiRNA triggers using at least one of the following two criteria: (1) a relatively low calculated melting temperature in the range of 55° - 70° C; and (2) a calculated low energy (-6 to -9 kcal/mol) internal stability profile with either a flat profile or a bell-shaped distribution- suggesting a high internal stability concentrated in the middle of the duplex. Analysis of more than 35 SiRNA triggers targeted to several

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human genes revealed that triggers that satisfy these two criteria were generally effective in silencing the targeted gene. The proposed approach has been experimentally validated by designing functional SiRNA triggers according to the criteria outlined in the method. Hence, choosing SiRNA triggers guided by the proposed approach may be helpful in avoiding the synthesis of a large number of triggers for a given target. Therefore, this technique may represent a more economical and efficient way to analyze gene function.

A. Introduction

[0165] Specific gene silencing has a wide range of applications in biology. Some of these applications include the understanding of the function of a gene, elucidating the individual role of a gene in a complex biological pathway, and the identification of novel therapeutic targets. Validation of therapeutically relevant gene targets may be valuable for the pharmaceutical industry in general, and may also have a broader societal impact in improving the quality of human life. In this regard, techniques that allow for specific gene silencing may play a role in achieving these goals. Some have used the antisense approach at the tissue culture level for target validation. An approach called RNA interference (RNAi) has emerged as an effective way to achieve specific gene silencing. In certain instances, RNAi is a post-transcriptional phenomenon mediated by double-stranded RNA (ds-RNA) with one strand bearing homology to the mRNA of the gene to be silenced. RNAi has been described in the nematode *Caenorhabditis elegans* (*C. elegans*) using long dsRNA molecules (Fire, 1998; Guo, 1995). RNAi also has been demonstrated in a wide range of species (reviewed in Bosher, 2000; Hammond, 2001; Sharp, 2001; Zamore, 2001). Although the exact mechanism of RNAi is not completely understood, an in vitro system derived from *Drosophila* embryonic cells that recapitulates the silencing event has provided some mechanistic insights into the process (Hammond, 2000; Tuschl et al., 1999). These studies led to the demonstration that the long dsRNA molecules that activate the RNAi process are cleaved into fragments

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of approximately two helical turns (approximately 21-22 nucleotides) (Zamore, 2000) by a multidomain ribonuclease III protein called Dicer (Bernstein, 2001). These short dsRNA may then become a platform on to which an ensemble of proteins assembles to form an RNA induced silencing complex (RISC). While the antisense strand of RISC may guide substrate recognition, an endonuclease may perform the cleavage of the target RNA (Elbashir, 2001). The short dsRNA molecules, derived from a long ds-RNA sequence, that become a part of the RISC are called short interfering RNA molecules (SiRNAs) (Elbashir, 2001).

[0166] While certain long dsRNA molecules homologous to a target mRNA may work effectively in lower organisms, in certain instances, they pose a challenge in mammalian cells. In response to the exposure to long dsRNA, mammalian cells are known to activate cellular pathways that lead to the global shut down of gene expression. This nonspecific inhibition of gene expression may be a result of an antiviral response mediated by interferon gamma and RNA-dependent protein kinase pathways (Geiss, 2001; Stark, 1998). Consequently, in certain instances, the use of long dsRNA in mammalian cells for RNAi-mediated gene silencing has been unproductive. However, recent experiments in the field suggest the use of SiRNA molecules as an effective trigger for silencing genes in mammalian cells. In contrast to long dsRNA molecules, the short length of SiRNA may bypass the antiviral response. This discovery opened up the use of an RNAi approach for analyzing mammalian genes, including those of humans. SiRNAs have been designed to carry 3' extensions with two nucleotides that mimic the products of Dicer cleavage. To confer protection from potential 3'-5' exonucleases, the two nucleotides in the 3' extensions may be substituted with dT, instead of natural RNA bases.

[0167] A procedure to design SiRNA molecules with dT 3' extensions has been suggested by Tuschl and colleagues ((http://www.mpibpc.gwdg.de/abteilungen/100/105 SiRNA.html) and (http://www.dharmacon.com/tech/tech003.html). This suggested approach for

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choosing SiRNAs involves the following criteria: (1) locate the first AA dimer 75 nucleotides downstream of the start codon within the mRNA of the gene; (2) record the next 19 nucleotides following the AA dimmer; (3) calculate the GC (guanosine and cytidine) content of the 21 nucleotide sequence to see if it is within 30 - 70 %; (4) perform a BLAST search against the EST database with the 21 nucleotide sequence to make sure only the gene of interest is targeted.

[0168] In order to silence a gene of interest using RNAi, it is customary to synthesize a handful of SiRNA triggers that target 19-22 nucleotide regions in the target mRNA. Out of this collection of molecules, one SiRNA molecule may be effective. This has been the case for some genes, but not for every gene. Additionally, for some targets, there are instances where none of these molecules in the first round of screening will work in triggering the RNAi process. If this occurs, the researcher must screen another set of SiRNAs with the hope of identifying one that will work. This can become an expensive exercise, especially given that the cost of RNA synthesis can be ten times as high as that for DNA. This scenario still occurs whether one arbitrarily screens SiRNA molecules or designs them using the approach described by Tuschl and colleagues (http://www.mpibpc.gwdg.de/abteilungen/100/105/SiRNA.html). Hence, criteria to intelligently guide researchers through the process of designing effective SiRNA molecules may minimize the overall cost of target validation by effectively reducing the number of SiRNA molecules required for screening per target, as well as reducing the associated burden of running additional functional assays.

[0169] While SiRNA molecules may regulate specific gene expression through targeted mRNA degradation, small temporal RNAs (StRNA) may regulate developmental timing by causing sequence-specific repression of mRNA translation. Similar to SiRNA, StRNA appear to be excised from long RNA molecules by the Dicer ribonuclease, and hence may be of similar size, 21-23 nts. Certain StRNAs that have been identified by genetic analysis were

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lin-4 and *let*-7 in *C. elegans* (Hutvagner et al., 2001; Rasquinelli, 2000). During the search for SiRNAs in *Drosophila* embryonic cell extract, Tuschl and colleagues identified several other small RNAs of the same size (Largos-Quintana et al., 2001). This novel class of RNA, now referred to as microRNA (miRNA), is evolutionary conserved among invertebrates and vertebrates. Initial experiments suggest these miRNAs may serve as gene regulators during development. MiRNAs have been identified in *C. elegans*, *Drosophila melanogaster* embryonic extracts and cultured human cells (Largos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001).

[0170] These miRNAs served as a rich source of sequence information to look for cues to design effective SiRNA molecules. Certain criteria present in miRNA molecules were identified. A collection of arbitrarily designed and functionally validated SiRNA molecules were also analyzed. This analysis revealed that, in general, these criteria were met in certain SiRNA molecules that function in gene silencing, but were not found within certain nonfunctional SiRNA molecules. This result suggests that these characteristics may be important for functional SiRNAs. Certain functional SiRNA triggers were designed according to the guidelines that were identified. Analysis of these designed SiRNA triggers suggested that they are functional in RNA interference in mammalian cells. Therefore, rationally designed SiRNA triggers may not only be effective in eliciting RNAi response, but may also work at very low concentrations. These features may be helpful in therapeutic applications of SiRNA triggers by lowering the effective dose and improving the efficacy of the procedure.

B. Results and Discussion

[0171] The observed failure to silence a gene by certain SiRNA molecules bearing homology to an arbitrary chosen site within the cognate mRNA invokes certain non-exclusive possibilities. First, the possible inability of the SiRNA within the RISC to access the target site within the substrate mRNA may be responsible for this observation. Either the folding of mRNA to conceal the target site or the occupancy of the site by RNA binding proteins

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may dictate the target site accessibility. Unlike the RNAi process that is facilitated by a number of proteins, the antisense process relies on the passive hybridization of antisense oligonucleotide strands to the target mRNA. For several mRNAs, certain SiRNA molecules were designed to target the same sites that antisense molecules have successfully targeted, and no correlation between antisense effect and RNAi was observed (unpublished observation). These observations suggested that target site accessibility alone is not necessarily enough for SiRNA molecules to initiate RNAi. Furthermore, addressing the issue of target site accessibility may be complicated and rather complex, primarily due to the fact that the RNA folding pattern will vary from target to target. As a result, it may be optimal for interfering RNAs to be tailor made to target individual mRNAs.

[0172] Another possibility for the inefficiency of certain SiRNA molecules to trigger RNAi could lie in the innate characteristics of the targeting SiRNA. Consequently, identification and understanding of the characteristics of SiRNA molecules that favor the RNAi process may be useful.

[0173] Analysis of a naturally occurring population of RNA molecules that may be processed by a mechanism similar to RNAi may yield some insight into the RNAi process. To that end, miRNA sequences were chosen that were identified recently in three organisms, *C. elegans* (Lau et al., 2001; Lee and Ambros, 2001), *D. melanogaster* and *H. sapiens* (Largos-Quintana et al., 2001). These miRNAs, embedded within long precursor RNA molecules as stem-loop structures, are processed by Dicer. The stems within which miRNAs exist before processing contain bulges and G/U wobble pairs. Hence, miRNAs depart structurally from certain SiRNAs that typically contain perfect helices. Furthermore, there may be differences between miRNAs and SiRNAs on the basis of Dicer processing. In the case of miRNAs, RNA from only one arm of the stem-loop precursor accumulates in the cell (Hutvagner et al., 2001), whereas both antisense and sense strands exist in SiRNA molecules. The nature of the two nucleotides in the 3'-extension (usually

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dTdT) may not affect the efficacy of RNAi (unpublished observation). So, SiRNA molecules may be treated as duplexes of 19 base pairs. To be consistent with this, each miRNA may also be calculated as 19 nucleotides in length. One may also use sense strands with perfect base pairing for miRNA duplexes, which may be different from their natural form.

[0174] The average internal stability of the miRNA duplexes of the three organisms was investigated (Fig. 16, A). Since an internal stability of a base pair within a duplex may be calculated using the contribution of its nearest neighbor, the two terminal base pairs were omitted to avoid the end effects. In all cases, the average internal stability of duplex miRNAs may be in the range of approximately -6.5 to -8.0 Kcal/mol. The distribution of the average internal stability of the internal 17 nucleotides forms a curve somewhat resembling a bell shape- with high internal stability concentrated in the middle nucleotides compared to the two ends. This implies that the middle part of the miRNA duplex may be relatively stable while the two ends may fray easily. This profile was very distinct in *C. elegans* sequences in category I (Lee and Ambros, 2001) and *D. melanogaster* sequences in category II (Largos-Quintana et al., 2001) compared to those in the other two categories. MiRNA sequences in categories III and IV have relatively stable nucleotide pairs near the 5' end of sequences. To investigate whether a collection of any 19 nucleotide RNA duplexes, chosen randomly, would also have a bell-shaped average internal stability profile, mRNA sequences of three different genes were chosen, and the average internal stabilities of duplexes resulting from 19 nucleotide antisense strands annealed from one end to the other (Fig. 16, B) was calculated. The calculated average internal stabilities of all three random collections of 19 nucleotide RNA duplexes, derived from three genes, were higher than those of the four miRNA duplex populations. Furthermore, their average internal stability profiles do not have the bell-shaped appearance. This may suggest that the calculated average internal stability profile, characteristic to miRNA duplexes, may be a special

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feature to RNA duplexes processed by Dicer and utilized by cellular mechanisms analogous to RNA interference.

[0175] The average internal stability of a nucleic acid duplex also reflects its propensity for melting. The calculated average melting temperatures (T_m values) of miRNA duplexes are relatively low, and hence agree with their low average internal stabilities. The average T_m values within the four groups of miRNA duplexes vary from 52° - 58° C (Fig. 16, C). In contrast, the average T_m values of the collections of 19 nucleotide duplexes, scanning the entire mRNAs of the three genes, are higher (60° - 66° C) than those of miRNA duplexes. In fact, the calculated T_m values and internal energy of miRNA duplexes may be higher than in natural forms containing unpaired nucleotides and G-U wobble base pairs. In reality, these values may be even lower than the values presented here. It is noted, however, that the average T_m of 19 nucleotide RNA duplexes may vary depending on the G/C content of the genes that are chosen. Since T_m values of nucleic acid duplexes of the same length may vary as a function of their G/C content, the G/C content of miRNAs was also analyzed. The G/C content of antisense strands of miRNAs was below 50% (40-44%), whereas those of the random collections of 19 nucleotide RNA duplexes were between 48 - 60 %.

[0176] MiRNA duplexes may be distinguished from the random RNA duplexes based on certain parameters; a bell-shaped calculated average internal stability profile and a low calculated T_m value. A collection of SiRNA molecules were analyzed. This collection included a series of SiRNA molecules that were experimentally validated against six different human genes, one mouse gene, and a single reporter gene SEAP (secreted alkaline phosphatase). In this collection, a total of 37 SiRNA molecules were tested, out of which 16 were effective in reducing the level of a targeted mRNA by greater than 70%. These molecules were mostly designed arbitrarily, although some were picked by using the suggestions made by Tuschl and colleagues (http://www.mpibpc.gwdg.de/abteilungen/f100/105/SiRNA.html). The average internal stability profiles of the two classes within the collection of

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experimentally validated SiRNA molecules were analyzed (Fig. 17A). The average internal stability profile of the functional SiRNAs (closed squares, Fig. 17A) exhibits a bell-shaped curve with an overall low internal energy (-6 to -8 kcal/mol), a profile reminiscent of the profiles of the miRNA duplexes. On the other hand, the collection of nonfunctional SiRNAs exhibits a markedly different average internal stability profile that may be characterized by overall high internal stability (-8 to -15 kcal/mol) and a lack of (or the mirror image of) a bell-shaped curve. Thus the analysis of the internal stability profiles for the two classes of SiRNA molecules suggests that a bell-shaped internal stability profile may be associated with productive SiRNA triggers that effectively promote RNA interference.

[0177] The calculated melting temperatures of the two classes of SiRNAs (Fig. 17B) provides another parameter that may be important for defining the effectiveness of an SiRNA molecule. SiRNAs in both classes have a broad range of calculated T_m values. Certain functional SiRNAs have T_m values from 48° - 70° C, with an average T_m of 58.2° C, which is close to the average T_m of the miRNA duplexes (54.5° C). In contrast, the T_m values of certain nonfunctional SiRNAs ranged from 55° - 83° C with an average T_m of 70.9° C. In general, the latter class has a higher T_m value than the former, reflecting their overall high average internal stability. This analysis of T_m data suggests that, in general, SiRNAs with relatively low T_m values (close to 55° C) may be attractive as triggers that promote the RNAi process. The average G/C content of certain functional SiRNAs is 52%, whereas that of certain nonfunctional SiRNAs is 72%, again suggesting that more stable SiRNA duplexes, in certain instances, may be generally nonfunctional.

[0178] Interestingly, there may be SiRNAs in both classes, functional and nonfunctional, with calculated T_m values between 55° and 70° C. The subpopulation of SiRNAs with calculated T_m values between 55° and 70° C from both classes were analyzed and their average internal stability profiles were calculated (Fig. 17C). The two classes of SiRNAs have internal stability profiles that were almost mirror images. Certain functional SiRNAs exhibit an

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average internal stability profile with a bell-shaped distribution, the characteristic signature for productive SiRNA triggers in certain instances. Whereas, certain nonfunctional SiRNA molecules, with their melting temperatures within the same range, have an average internal stability profile reflecting the mirror image of a bell-shaped curve. This analysis suggests that, in certain instances, when one picks two SiRNAs with roughly the same T_m values, e.g., in the 50° - 70° C range, the one with a bell-shaped internal stability profile may be more likely to function as a productive trigger.

[0179] It may be that one will find SiRNA molecules that are either functional, but may not follow the criteria, or have the criteria fulfilled but fail to function. In fact, such outliers were searched for in the rather small collection of experimentally validated SiRNAs, and molecules in either category that failed both criteria were not found. However, two sets of examples in which SiRNAs fulfilled one criterion but not the other were found, (Fig. 18 A, B and C). In the first case (Panel A), the two SiRNAs targeted to two different genes, PKC-θ and PKB-α, have internal stability profiles close to the profile preferred for a productive RNAi trigger. However, the one with a calculated T_m of 66° C, indicated by closed squares, was functional in silencing the cognate gene. The other SiRNA, which was nonfunctional, had a calculated T_m of 85° C. However, the nonfunctional SiRNA also had a bell-shaped internal stability profile. In the second case (Panel B), neither of the two SiRNAs targeted to two different genes had the preferred internal stability profile. However, the SiRNA molecule with a relatively flat internal stability profile (ΔG approximately -6 kcal/mol) and low calculated T_m (53° C; closed squares) was effective in gene silencing. The one that had a somewhat bell-shaped internal stability profile was nonfunctional, perhaps due to its high calculated T_m value (79° C).

[0180] An example in which three SiRNA molecules are targeted to a single gene is depicted in Panel C of Fig. 18. All three SiRNAs had overlapping sites within the targeted mRNA of the PKC-θ gene. Two SiRNAs, 1 and 2, (closed squares) had almost identical target sites, displaced by only

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a single nucleotide. Both these SiRNAs were functional and had the preferred internal stability profile and calculated low T_m values (55° and 57° C). The third SiRNA still targeted approximately the same site but was shifted by five nucleotides. This displacement by five nucleotides resulted in a different internal stability profile, which was the mirror image of the preferred form. Interestingly, this SiRNA (open squares) may be nonfunctional, although it also has the calculated T_m of 55° C. This example suggests the importance in certain instances of the internal stability profile for an SiRNA molecule to be productive as an RNAi trigger when its calculated T_m is in the preferred range. This example suggests that picking SiRNA triggers based on the calculation of T_m alone, in certain instances, may not be helpful if the internal stability profile is not preferred. However, SiRNA triggers characterized by T_m and a low internal stability profile that is flat across the duplex may also be functional (Fig. 18B, closed squares and see below).

[0181] So far an analysis of the experimentally validated SiRNA collection with respect to certain criteria suggests a good correlation between the efficacy of SiRNA molecules and meeting the criteria. To put the proposed method of choosing functional SiRNA molecules using certain criteria to the test, six SiRNA molecules were designed to target mRNA of SEAP (Fig. 19). Out of six SiRNAs, four were chosen to be functional (SEAP-309, -1035, -1795 and -2217); three SiRNA molecules met both criteria (SEAP-309, -1035 and -1795), whereas one molecule (SEAP-2217) possessed a calculated low T_m value (46.4° C) and had a relatively low and flat internal energy profile (Fig. 19A-1). This trigger was specially chosen to test whether in this instance a low internal stability energy profile with a more or less even distribution across the duplex is acceptable as a functional SiRNA molecule. Two SiRNA molecules were designed (SEAP-1070 and -1260) that did not meet the two criteria and were predicted to be nonfunctional (Fig. 19A-2). In addition to these rational picks, four other SiRNA molecules were picked arbitrarily (SEAP- 147, - 500, -1113, 1271). Along with SEAP-68 and -155, six randomly picked SiRNAs were used (Fig. 19B-1&2) to compare

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with the results of the rationally picked triggers. As shown in Fig. 19C, all four SiRNA triggers that were rationally picked to be functional using the criteria were functional in reducing the expression of the target gene SEAP. Out of those functional SiRNA triggers, the most effective trigger, SEAP 2217, has a flat internal energy profile. This result, in combination with that presented in Fig. 18B, suggests that a flat internal energy profile may also be acceptable for a functional SiRNA trigger. One trigger, out of the two triggers that were rationally designed not to be functional, was nonfunctional, whereas the other reduced the SEAP expression by approximately 50%. The efficacy of arbitrarily picked SiRNA triggers in RNAi was evaluated and only one trigger (SEAP 68) out of six molecules was functional. SEAP-155, which came from the same selection, may be moderately functional; reducing the SEAP level by approximately 50%. These results suggest that the rational design of SiRNA triggers using these criteria may be reliable in indentifying functional SiRNA triggers.

[0182] Next, the minimal effective concentration of three SiRNA triggers designed for SEAP was investigated. At a fixed 100 nM concentration, the degree of effectiveness of these three triggers in silencing SEAP expression was different (Fig. 19). As shown in Fig. 20, the minimal concentration required by three triggers to silence the gene varied 0.5, 2.5 and greater than 100 nM for SEAP-2217, -1035, and -1070, respectively. With SEAP-2217 and -1035, the magnitude of gene silencing remained constant above the minimum effective concentration. These results suggest that certain SiRNA triggers that are effective at high concentration (100 nM) may not be functional at low concentrations. Hence, the identification of optimally effective SiRNA triggers, facilitated by the guidelines outlined here, may be valuable in developing SiRNA based therapeutics. Such effective SiRNA triggers may cut down the effective dose requirement.

[0183] It appears that in certain instances SiRNA triggers with low calculated T_m values may be functional triggers, provided the internal energy criteria are met. Target sites with low melting temperatures may be common

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within mRNAs that are low in G/C content. Consequently, the probability of success in identifying functional SiRNA molecules by random picking may be high. However, as indicated in Figure 50, the success of identifying arbitrarily designed SiRNA molecules in this work does not correlate with the G/C content of mRNA.

[0184] Certain criteria of SiRNA molecules, T_m in the range of 55° C, and a bell-shaped or flat internal stability profile, that promote RNAi emerged from the initial analysis of a set of naturally occurring miRNA molecules. Subsequently, the existence of these criteria in functional SiRNA molecules that were designed arbitrarily and validated experimentally was confirmed. The success of designing functional SiRNA molecules using the two criteria was demonstrated.

[0185] A possible, but nonlimiting, rationale for the benefits of the criteria follows. It is reasonable to assume that within the RISC the antisense strand of the SiRNA may come off from the duplex and subsequently anneal to the substrate mRNA. These events may be facilitated by either individual proteins or multi-domain proteins within the RISC. Under such a scenario, the two strands within an SiRNA would be expected to melt easily. In light of this view on the mechanism, the interplay of molecular forces between the two strands of an SiRNA to keep them in a dynamic environment may be important to an effective SiRNA trigger. Hence, SiRNA molecules with a relatively low T_m may be advantageous. SiRNA duplexes with a relatively high internal stability in the middle may keep the two strands together, while the two ends with low internal stability provide easy entry to a protein like helicase to facilitate strand separation when needed. In contrast, SiRNA duplexes that are highly stable, as characterized by a high T_m and high internal stability may resist the strand separation step in the RNAi mechanism and hence fail to trigger RNA interference. Recently, the participation of RNA-dependent RNA polymerase (RdRP) has been demonstrated in the RNA interference process in *C. elegans* (Sijen et al., 2001) and Drosophila embryo extracts (Lipardi et al., 2001). An antisense strand of SiRNA once annealed

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to the target mRNA may serve as a primer for an RdRP to convert mRNA into dsRNA that is degraded to generate more SiRNA molecules *in situ*. Even for this activity to take place, strand separation of an SiRNA molecule may be an important prerequisite.

C. Conclusion

[0186] As demonstrated experimentally, rational design of functional SiRNA triggers based on the criteria set forth herein may help facilitate target validation and other applications of RNA interference. However, there may be instances that SiRNA molecules may not necessarily adhere to these guidelines. This may be because the predictions are based on the pattern analysis of sequences alone and do not factor in the cellular environment in which the target mRNAs exist. In reality, RNA is not a linear target as treated in this analytical approach, but is folded and interacts with a host of RNA-binding proteins. Hence, these criteria are considered guidelines for a high probability for success, but may not always provide effective SiRNA molecules.

[0187] The following is a stepwise procedure for designing functional SiRNA molecules for a mRNA target sequence according to certain embodiments. Windows depicted in Fig. 21 will be helpful in understanding the process of picking effective SiRNA triggers.

1. Download the target mRNA sequence from the NCBI database (http://www.ncbi.nlm.nih.gov).
2. Open the sequence file in Oligo 5.0™ Primer Analysis Software.
3. Select the length of the primer as 19 using the dropdown menu under "Change".
4. Search in the (lower) internal stability window for a bell-shaped region with the internal energy preferably below -10 (kcal/mol) highlighted for the 19-mer.
5. If the calculated T_m for that site is below 65° C in the (upper) melting temperature window, pick the antisense strand.

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6. Alternatively, pick a site with a flat internal stability profile with energy of approximately -6 to -9 kcal/mol and T_m of <50° C.

7. Perform a BLAST search against the EST database with the 19 nucleotide antisense to ensure only the gene of interest is targeted.

8. If these criteria are met, synthesize the SiRNA molecule with a 19 nucleotide base pair duplex consisting of two dT residues at the 3' ends.

D. Materials and Methods

SiRNA

[0188] SiRNAs were prepared using several different methods. Certain chemically synthesized SiRNAs were synthesized using RNA phosphoramidites containing a 2'-O-TriisopropylsilylOxyMethyl (TOM) protection group from Glen Research (Sterling, VA). Other SiRNAs were obtained from Dharmacon (Longmont, CO) employing 5'-Silyl-2'-bis(2-acetoethoxy)methyl (ACE) Orthoester chemistry. Synthesized SiRNAs using TOM phosphoramidites were HPLC purified, whereas those obtained from Dharmacon were used without further purification due to their high purity resulting from extremely high coupling efficiency (Scaringe, 2001). SiRNAs were annealed in an annealing buffer including 100 mM KCl, 30 mM HEPES (pH 7.5), and 2 mM $MgCl_2$ by heating to 75° C for 2 minutes followed by slow cooling to ambient temperature.

Experimental evaluation of SiRNAs in tissue culture

[0189] The efficiency of certain SiRNA molecules designed for each human gene target was evaluated upon transfection into cultured human cells expressing the target. The specific mRNA level of the target gene was measured following transfection. In parallel, the mRNA level of a housekeeping gene, cyclophilin, as a nonspecific target was also quantified. Changes in the cyclophilin mRNA levels with SiRNA triggers were not observed. The efficiency of SiRNA to specifically reduce the targeted mRNA was calculated as a ratio of the target mRNA to cyclophilin mRNA. SiRNA

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molecules that gave greater than 70% reduction of the target mRNA level were taken as functional SiRNAs.

Delivery of nucleic acid triggers

[0190] Cells seeded in 96-well plates at approximately 25,000/well the previous day were transfected with different nucleic acid triggers using Lipofectamin 2000 and Opti-MEM I (from Invitrogen). Briefly, SiRNA was diluted in Opti-MEM-I in a 50 µL volume. This was mixed with an equal volume of Lipofectamin 2000 diluted 12.5-fold in OPTI-MEM I. After incubating the mixture at ambient temperature for 20 minutes, 270 µL of regular cell medium was added, and 95 µL of the solution was immediately transferred onto the cells in the plate with no media. Plates were transferred to a 37° C incubator with 5% CO_2 for either 24 or 48 hours. To monitor the fate of SEAP transiently expressed in 293 cells, prAAV6-seap plasmid was included in the transfection mixture with and without SiRNAs.

Quantification of mRNA levels

[0191] Specific mRNA levels of cells transfected with different nucleic acid triggers were quantified using QuantiGene High Volume Kit (from Bayer) that employs a branched-DNA (b-DNA) method for nucleic acid detection according to the manufacturer's instructions. Specific detection of a given mRNA is based on its selective capture on to the microtiter plate, which is dictated by the capture probes. Probe sets that are unique to each target mRNA were designed using the ProbeDSesigner software (Bayer) according to the manufacturer's instructions. For each case, probe sets were validated using the cells expressing each message before being used for experiments.

Secreted Alkaline Phosphatase (SEAP) assay

[0192] The SEAP gene (from pAP-1 SEAP vector from Clontech) was cloned into an adeno-associated vector (AAV # 6) upstream of the EF1-α 3' UTR. Twenty-four hours after the transfection, 15 µL of medium from each well was transferred to a white opaque 96-well flat bottom microtiter plate, and the amount of SEAP was detected using a chemiluminescent SEAP assay (Great EscAPe SEAP assay kit form Clontech) according to the

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manufacturer's instructions. RLU values obtained in the presence of an SiRNA trigger were normalized to that obtained in the absence of a trigger. SiRNA molecules that gave greater than 70% reduction of the RLU level compared to the control (no SiRNA added) were taken as functional SiRNAs.

Calculation of Internal stability profiles and Melting Temperatures (T_m values)

[0193] Internal stability profiles were calculated using the software program Oligo 5.0™ Primer Analysis Software (National Biosciences, Inc., Plymouth, MN), a program that is generally used for designing oligonucleotides for PCR and various nucleic acid hybridization applications. The ΔG value for each position reflects the average of all overlapping pentamer sequences of the 19 base-pair duplex. The program calculates the average ΔG value by adding ΔG values of the 4 nucleotide pairs within the pentamer. The terminal base pairs were excluded to avoid end effects and only the internal 17 nucleotide sequence was considered. Furthermore, the internal stability profiles of antisense strands were calculated using nearest neighbor calculations of DNA, not of RNA. Although the absolute values for RNA may vary, the general trends may still be valid.

[0194] T_m values were also calculated using the same software program according to the nearest neighbor thermodynamic values. Again, this calculation is also based on DNA and not on RNA, but the general trend for T_m also holds true.

IV. Certain functional SiRNA triggers in mammalian cells

[0195] It has been reported that SiRNA triggers with a 19-base pair helical region with two nucleotide 3' extensions may be optimal triggers for gene silencing through RNA interference. These conclusions were born out based on two observations: (1) empirical design of RNA triggers to mimic cleavage products of Dicer, the enzyme involved in processing long double-stranded RNA into short interfering RNA or SiRNA; (2) Results obtained by using an in vitro system derived from *Drosophila* embryo extract. Certain 21

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nucleotide triggers with a 19 nucleotide helicial region in fact work in silencing gene expression in mammalian cells.

[0196] The work described herein suggests that certain SiRNA triggers of variable functional anatomies work effectively in mammalian cells, a result that is different from certain results obtained using an in vitro system. Salient features of the current discovery are summarized below.

[0197] Certain SiRNA triggers with a 17-base pair RNA helical region with an antisense strand of 17 RNA nucleotides are nonfunctional in triggering RNA interference in mammalian cells. On the other hand, certain SiRNA triggers with a 17-base pair RNA helical region with an antisense strand of at least 19 RNA nucleotides are effective triggers.

[0198] Recently, it has been shown that instead of ssDNA antisense oligonucleotides, injection of dsRNA into the nematode *C. elegans* resulted in the loss of function of a gene to which the injected dsRNA had homology (Fire, 1998; Guo, 1995). RNAi is one manifestation of dsRNA-induced gene silencing. Other forms include post-transcriptional gene silencing (PTGS) and co-suppression observed in plants (Ketting, 2000), as well as quelling in the fungus *Neurospora crassa* (Reviewed in (Fire, 1999; Matzke, 2001; Waterhouse, 1999)). Nature may use the RNA silencing phenomenon to protect the cell from viral infections and from mobilization of transposons. A growing body of evidence suggests that it may also be used to regulate the expression of endogenous genes.

[0199] RNAi has been demonstrated in a host of species, including invertebrates such as hydra, planaria, trypanosomes, nematodes and insects as well as vertebrates (mouse and zebra fish) (reviewed in Bosher, 2000; Hammond, 2001; Sharp, 2001; Zamore, 2001). These studies revealed certain important aspects of RNA interference. In certain instances, it has been shown that only the gene to which the dsRNA shares homology becomes silenced (Fire, 1998; Kennerdell and Carthew, 1998). In certain instances, the dsRNA should be homologous to the exons of a gene to observe effective gene silencing, indicating that the silencing mechanism may

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not interfere with mRNA processing, but may occur post transcriptionally after splicing (Fire, 1998). In certain instances, the dsRNA molecule may be substantially complimentary to the coding or noncoding regions of the target mRNA. In certain instances, the dsRNA molecule may be substantially complimentary to the untranslated regions of the target mRNA, including but not limited to the 5' and 3' untranslated regions. In certain instances, only a few copies of the dsRNA trigger may be required to degrade mRNA present in large excess (Fire, 1998; Kennerdell and Carthew, 1998), suggesting that there may be a possible amplification step included within the molecular mechanism of RNA interference. In both worms and plants, RNA interference may spread across cell boundaries (Fire, 1998; Hamilton, 1999).

[0200] Mutants that are defective in RNA interference have been isolated in *C. elegans* (Grishok, 2000), *Neurospora* (Cogoni, 1999), *Arabidopsis* (Dalmay, 2000) and *Chlamydomonas* (Wu-Scharf, 2000). Biochemical analysis of the RNAi process was facilitated by the in vitro system derived from *Drosophila* embryonic cells (Hammond, 2000; Tuschl et al., 1999). Studies on the in vitro system that recapitulates the RNAi process provided some insight into the fate of the dsRNA. It was suggested that the dsRNA may be cleaved into discrete 21-23 nucleotide fragments by an ATP-dependent process which does not require the presence of target mRNA (Zamore, 2000). This suggests that the small 21-23 nucleotide dsRNA fragments may not be by products of the process, but may be intermediates in the RNAi process. These results obtained in vitro were in agreement with the previous observation of the existence of small dsRNA of 25 nucleotides in plants undergoing PTGS either by viruses or trans genes (Hamilton, 1999). These small dsRNAs found in plants undergoing PTGS included both sense and antisense strands corresponding to the silenced gene. The conversion of dsRNA into small dsRNAs was suggested in vivo in *C. elegans* and *Drosophila* as well (Parrish, 2000; Yang, 2000). The multidomain RNAse III protein Dicer may be the enzyme that processes dsRNA into 21-23 nucleotide short dsRNA (Bernstein, 2001) that are called SiRNAs (Short Interfering

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RNAs)(Elbashir, 2001b). According to one of the proposed models for RNAi (Hammond, 2001; Zamore, 2001), the SiRNA molecules generated upon the cleavage of the long dsRNA become a part of a ribonucleo-protein complex called RiSC (RNA-induced silencing complex). The RISC may then find and bind to the target mRNA through a homology searching mechanism facilitated by a protein(s) within the complex and the antisense strand of the SiRNA. Once the site of homology is identified, the target mRNA may be cleaved by an endonuclease, which may be a member of RISC as well. SiRNA guided cleavage of the target RNA has been suggested in vitro (Elbashir, 2001b). It has been suggested that the cleavage within the target RNA takes place near the center of the homology to the SiRNA.

[0201] Earlier attempts in applying relatively long (approximately 800 nts) dsRNA to initiate RNAi in mammalian cells failed (Calpan et al., 2000). However, RNAi has been observed in mouse oocytes and early embryos (Svoboda, 2000; Wianny, 2000), suggesting the possible existence of RNAi machinery in mammalian cells. The failed attempts in demonstrating RNAi in mammalian cells have been attributed to the nonspecific effects induced by long dsRNAs in mammalian cells. Certain dsRNAs are known to induce nonspecific effects in mammalian cells by activating several pathways through the rapid induction of IFNγ (Geiss, 2001; Stark, 1998). DsRNA is known to activate dsRNA-dependent protein kinase, PKR, which in turn phosphorylates and inactivates the translation factor eIF2α. The overall result is the global shut down of protein synthesis in the cell and subsequent cell death. In addition, dsRNAs may also induce the production of 2'-5'-polyadenylic acid which in turn activates the nonspecific nuclease RNase L that nonspecifically degrades RNA. The induction of nonspecific effects by dsRNA in mammalian cells may be related to the length of the dsRNA; for example, the activation of PKR may require dsRNA that is longer than 30 base pairs. The efficiency of the activation of PKR increases with the length of RNA and 85 base pairs may provide optimal activation in certain instances (Manche, 1992).

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[0202] The use of short dsRNA molecules, such as SiRNA, has the potential to keep the dsRNA-induced nonspecific pathways from activating. In other words, being intermediates of the RNA interference process, SiRNA may trigger RNAi in mammalian cells. Recent results with the delivery of SiRNA into mammalian cells suggest this theory may be accurate (Caplen, 2001; Elbashir, 2001a). These authors used SiRNA of 21-25 nucleotides to silence genes expressed either transiently or endogenously in cultured mammalian cells. The SiRNAs used in these studies were designed to have 2 nucleotide 3'-overhangs mimicking a digested RNA fragment resulting from cleavage of the ribonuclease III enzyme, Dicer. A phosphate group is present at the 5' end of the Dicer cleavage products, yet it may not be required for a SiRNA molecule to trigger efficient RNA interference (Caplen, 2001; Elbashir, 2001a). Furthermore, the two RNA nucleotides in the 3'-overhang contain two dT residues to presumably protect the functional SiRNA triggers from possible 3'-5' exonuclease activities (Elbashir, 2001a). Certain characteristics of certain SiRNA triggers have been delineated empirically. An attempt was made to understand the functional anatomy of SiRNA triggers using an in vitro system derived from *Drosophila melanogaster* embryo lysate (Elbashir et al., 2001). In this system, SiRNA triggers with 21 nucleotides in each strand and a 19-base pair helical region with 2 nucleotide 3' extensions were the most efficient triggers for mediating RNA interference.

[0203] The results herein suggest that the functional anatomy of SiRNA molecules may be quite flexible in silencing genes in mammalian cells, since triggers with three possible end structures (3'-extension, 5'-extension and blunt) mediate effective gene silencing. Also, the single-stranded RNA molecules that fold into hairpin structures may be equally in triggering RNA interference in mammalian cells. These results may suggest either the mechanism by which RNA interference is mediated in mammalian cells has differences compared to the one found in lower organisms or there are some limiting factors in the in vitro system based on Drosophila embryo extract.

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Materials and Methods

SiRNA

[0204] All SiRNA triggers and hairpin molecules used in this work were chemically synthesized in house using RNA phosphoramidites based on 5'-Silyl-2'-bis(2-acetoethoxy)methyl (ACE) Orthoester chemistry purchased from Dharmacon (Longmont, CO). After deprotection, short RNA molecules (all SiRNA triggers and short RNA hairpins) were used without further purification, due to their high purity resulting from extremely high coupling efficiency (Scaringe, 2001). The long RNA hairpin, SP-HP uucg AS-S, was purified by reverse phase high pressure liquid chromatography. Two strands of SiRNA molecules were annealed in an annealing buffer including 100 mM KCl, 30 mM HEPES (pH 7.5), and 2 mM $MgCl_2$ by heating to 75° C for 2 minutes followed by slow cooling to ambient temperature. Hairpin molecules were also heated and slowly cooled down to ambient temperature in the same annealing buffer.

Delivery of nucleic acid triggers

[0205] Cells seeded in 96-well plates at approximately 95% confluence were transfected with different nucleic acid triggers using Lipofectamin 2000 and Opti-MEM I (from Invitrogen). Briefly, SiRNA was diluted in Opti-MEM-I in 100 µL volume. This was mixed with an equal volume of Lipofectamin 2000 diluted 25-fold in OPTI-MEM I with SuperRNAsin at 1.4 U/µL (from Ambion) and prAAV6-seap plasmid (1 ng/µL). After incubating the mixture at ambient temperature for 5-20 minutes., 550 µL of regular cell medium was added and 100 µL of the solution was immediately transferred onto cells in the plate with no media. Plates were transferred to a 37° C incubator with 5% CO_2 for either 24 or 48 hours. In each case, the final concentration of SiRNA triggers was 100 nM.

Secreted Alkaline Phosphatase (SEAP) assay

[0206] Twenty-four hours after the transfection, 15 µL of medium from each well was transferred to a white opaque 96-well flat bottom microtiter plate, and the amount of SEAP was detected using a chemiluminescent

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SEAP assay (Great EscAPe SEAP assay kit form Clontech) according to the manufacturer's instructions.

Results and Discussion

[0207] RNA interference may be used to study gene function in mammalian cells. Two to three nucleotide 3'-extensions within SiRNA triggers for effective silencing of cognate genes in lower organisms has been reported (Elbashir et al., 2001). Effective gene silencing by hairpin SiRNA triggers in mammalian cells has been observed. In order to investigate the effect of the end structure in effective gene silencing in mammals, a reporter gene, **SE**creted **A**lkaline **P**hosphatase (SEAP) was expressed under a strong CMV promoter and was used as a target gene. A plasmid expressing SEAP mixed with SiRNA triggers was transfected into HEK 293 cells. Silencing of the SEAP gene was monitored 24 hours after transfection using a chemiluminescence assay directed to detect the activity of alkaline phosphatase.

[0208] A site within the SEAP mRNA that was previously characterized to provide effective reduction of gene expression by SiRNA was chosen as the target site (Figure 22; SiRNA trigger 2217). SiRNA triggers targeting this site were chemically synthesized with different lengths and end structures.

Variation of SiRNA trigger length

[0209] It has been demonstrated that SiRNA triggers with a 19 base pair helical region and two nucleotides at the 3' overhang may be optimal for effective gene silencing in *Drosophila* extract (Elbashir et al., 2001). These triggers may be effective in mammalian cells as well (Caplen, 2001; Elbashir, 2001a). The effect of the change in the helical length of certain SiRNA triggers was investigated using triggers with 17, 19, 21, 23, and 25 RNA base pairs. Although these triggers carry helical regions of different lengths they have the same end structure; two dT residues as 3' overhangs. As shown in Fig. 22, the trigger with a 17-bp helical region may be nonfunctional, whereas all other triggers appeared to be effective in silencing SEAP expression.

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These include triggers with 19, 21, 23 and 25 base pair helical regions. There are some minor variations in the degree of silencing; SiRNA with a 25-bp helical region had a comparable silencing effect to that of a 19-bp helical region, whereas triggers with 23 and 25 bp helical regions did not work at the same level. This result is in contrast with the length results observed in certain *Drosophila* extract work in which 24 and 25 nucleotide SiRNA triggers were ineffective, whereas 22 and 23 nucleotide SiRNA triggers produced 60% silencing of the targeted gene (Elbashir et al., 2001). The SiRNA trigger made up of all RNA nucleotides (SP-19-AR) with no deoxy residues at the 3' end extensions may be as effective as the one containing two dT residues (SP-19) in silencing the targeted gene. In the case of SP-19-AR, the entire antisense strand is complimentary to the targeted mRNA, extending the complimentary region from 19 to 21 nucleotides. The effective gene silencing by all RNA SiRNA triggers is consistent with the previous observations made by Caplen et al. in mammalian cells using 21-, 22- and 23 nucleotide SiRNA triggers (Caplen, 2001).

SiRNA triggers with different end structures

[0210] Figure 23 summarizes the results of gene silencing mediated by SiRNA triggers with three possible end structures: 3'-extention, 5'-extension and blunt. The SiRNA trigger with 5'-extensions (SP-19-5'-ext and SP-19) as well as that with a blunt end (SP-19-Blunt) work equally well as that with 3' extensions (SP-19), indicating that in certain instances the end structure of SiRNA triggers may not be important in mediating RNA interference in mammalian cells. This is a different result compared to certain work in *Drosophila* embryo extract (Elbashir et al., 2001). In a *Drosophila* based in vitro system, SiRNA triggers with 5'-extensions were nonfunctional, whereas the triggers with blunt ends produced ambiguous results, some blunt-end triggers may be functional whereas others may not be functional, in spite of all of them targeting to a common site in an mRNA. The blunt end trigger that did not mediate RNA interference (SP-19-Blunt) has only a 17 base paired RNA helical region, which may be too short to be functional as

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observed with SP-17 (Figure 22). Unimolecular hairpin triggers may also be effective in triggering RNA interference in mammalian cells. In the current study, four different synthetic hairpin molecules were used (Figure 24). Hairpin triggers with either four or eight nucleotides in the loop were synthesized. Three hairpin triggers with tetra loops were designed. The first one contains the 3' end of the antisense strand ending at the loop (SP-HP uucg AS-S). The second has the sense strand terminating at the loop (SP-HP uucg S-AS). In the third trigger, the helical region is flanked by a loop and an internal bulge (SP-HP uucg AS-S + 5'ext) providing a 5'-extension. It may be that Dicer would process these hairpin molecules into SiRNA triggers of correct lengths. Hairpin SiRNA triggers with both tetra- and octa-loops may be effective in silencing the target gene (Figure 25), suggesting that the size of the loop may not be important for mediating RNA interference. Hairpin triggers in which the antisense strand is placed in either orientation with respect to the loop may also be used (Figure 25; SP-HP uucg S-AS and SP-HP uucg AS-S), suggesting that in certain instances Dicer may have no preferred symmetry in processing a hairpin molecule into an SiRNA trigger. Furthermore, the hairpin molecule in which the helical region is flanked by a loop and an internal bulge (SP-HP uucg AS-S + 5'ext) may be used to silence the SEAP expression, suggesting that in certain instances both ends may be processed by Dicer. This result suggests that in certain instances the end structure of a double helical RNA fragment may be insignificant to the efficacy of RNA interference in mammalian cells.

[0211] The effect of gene silencing mediated by SiRNA triggers possessing asymmetric lengths in the two strands was explored. Several series of SiRNA triggers were designed in which the length of the sense strand was kept constant and the length of the antisense strand, as well as the nature of its end structure, was changed. Sequences of SiRNA triggers belonging to eight series are listed in Figure 26A, B and C. The results of the efficiency of silencing the SEAP expression by each of these triggers are shown in Figure 27. In each series, SiRNA triggers carrying an antisense

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strand with 17 RNA nucleotides (both SP-17-as and SP-19 blunt-as) may be ineffective in silencing the targeted gene. The triggers that appear to be ineffective in silencing the SEAP gene are indicated in boxes in Figures 26A, B and C. In these cases, the helical length of the contiguous RNA region is 17 base pairs and that length may be too short to mediate effective RNA interference. Except for these SiRNA triggers, triggers with all other combinations of different end structures may be effective in silencing the targeted gene, which may suggest in certain instances a lack of preference for end structures in mammalian cells.

[0212] A similar series of triggers, keeping the antisense strand constant and varying the length of the sense strand, was constructed. Sequences of SiRNA triggers of eight series are illustrated in Figure 28A, B and C, and the results of targeted gene silencing mediated by each trigger are shown in Figure 29. Analogous to the previous results in Figures 23 and 27, triggers with an antisense strand with 17 RNA nucleotides may lack the ability to silence the targeted gene. However, SiRNA triggers in which the sense strand including 17 RNA nucleotides is hybridized to antisense strands that are 19 nucleotides or longer may silence the SEAP gene. These results indicate that in certain instances a short sense strand with 17 nucleotides may be acceptable for the silencing process, but a short antisense strand of the same length may not be acceptable. Thus, in certain instances minimal length, 19 RNA nucleotides, and an antisense strand of 19 nucleotides is used as an SiRNA trigger.

[0213] Certain end structures that effectively work in silencing the SEAP gene in mammalian cells are shown below.

END STRUCTURE	EXAMPLES
Asymmetric 3'-extensions	1. SP-17-S + SP-19-AS 2. SP-17-S + SP-19-RNA-AS 4. SP-19-S + SP-21-AS

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	3. SP-21-S + SP-19- AS 4. SP-21-S + SP-23-AS 5. SP-23-S + SP-21-AS 6. SP-23-S + SP-25-AS 7. SP-25-S + SP-23-AS 8. SP-19-RNA-S + SP-21-AS 9. SP-21-S + SP-19-RNA-AS
Symmetric 3'-extensions	1. SP-19-S + SP-19-AS 2. SP-19-S + SP-19-RNA-AS 3. SP-21-S + SP-21-AS 4. SP-23-S + SP-23-AS 5. SP-19-RNA-S + SP-19-AS 6. SP-19-RNA-S + SP-19-RNA-AS
Blunt ends	1. SP-19-S + SP-19-5'-AS 2. SP-19-RNA-S + SP-19-5'-AS 3. SP-19-5'-S + SP-19-AS 4. SP-19-5'-S + SP-19-RNA-AS

END STRUCTURE	EXAMPLES
Blunt end and 5' extension	1. SP-17-S + SP-21-AS 2. SP-19-S + SP-23-AS 3. SP-21-S + SP-25-AS 4. SP-19-RNA-S + SP-23-AS 5. SP-19-Blunt-S + SP-19-AS

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	6. SP-19-Blunt-S + SP-19-RNA-AS
	7. SP-19-Blunt-S + SP-19-5'-AS
	8. SP-25-S + SP-21-AS
Blunt end and 3' extension	1. SP-23-S + SP-19-AS
	2. SP-23-S + SP-19-RNA-AS
	3. SP-25-S + SP-21-AS
	4. SP-17-S + SP-21-AS
	5. SP-19-S + SP-23-AS
	6. SP-21-S + SP-25-AS
	7. SP-19-RNA-S + SP-23-AS
	8. SP-19-Blunt-S + SP-19-RNA-AS
Symmetric 5' extension	1. SP-19-5'-S + SP-19-5'-AS
	2. SP-19-RNA-S + SP-19-AS
	3. SP-25-S + SP-25-AS
Asymmetric 5' extension	1. SP-23-S + SP-25-AS

END STRUCTURE	EXAMPLES
5' and 3' extensions on one strand	1. SP-17-S + SP-23-AS
	2. SP-17-S + SP-19-5'-AS
	3. SP-19-S + SP-25-AS

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4. SP-21-S + SP-19-5'-AS
5. SP-23-S + SP-19-5'-AS
6. SP-25-S + SP-19-AS
7. SP-25-S + SP-19-RNA-AS
8. SP-25-S + SP-19-5'-AS
9. SP-19-RNA-AS + SP-25-AS
10. SP-19-5'-S + SP-21-AS
11. SP-19-5'-AS + SP-23-AS
12. SP-19-5'-AS + SP-25-AS
13. SP-19-Blunt-S + SP-21-AS
14. SP-19-Blunt-S + SP-23-AS
15. SP-19-Blunt-S + SP-25-AS
16. SP-17-S + SP-25-AS

[0214] In the current work, certain anatomical structures of SiRNA triggers that efficiently work well in certain mammalian cells were different than certain triggers that work in certain in vitro systems. Caplen et al. have shown gene silencing in primary mouse embryonic fibroblasts, 293, and HeLa cells with SiRNA triggers longer than a 19 base pair helical region with 2-3 nucleotides at the 3' extensions (Caplen, 2001). Taken together these results suggest that it may be possible that certain factors that are involved in effective utilization of double-stranded RNA with a helical region longer than 19 base pair in mammalian cells may be missing in an in vitro system. These factors may include nucleases for trimming the long double stranded RNA into effective short triggers or other proteins that help activate and/or facilitate this process.

Substitution of G-U base pairs

[0215] During an attempt to rationally design functional SiRNA triggers, SiRNA triggers were observed with relatively low internal stability that tend to be functional in silencing target genes in mammalian cells. In certain instances, SiRNAs containing contiguous G-C base pairs may not be optimal

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in eliciting RNA interference. In certain instances, one may desire SiRNA triggers that do not have higher than 4 contiguous G-C base pairs in an SiRNA trigger. An approach to make G-C base pairs less stable by substituting uridines in place of cytosines to generate G-U base pairs with a higher propensity for melting. In fact, G-U base pairs are present in certain microRNA molecules that are also processed by Dicer, the same enzyme that processes SiRNA molecules. Hence, the inclusion of G-U base pairs in an SiRNA molecule may not affect the RNA interference process.

[0216] Two SiRNA triggers, one functional (SP-1795) and the other non functional (SP-1260), were used to explore the effect of substituting G-U base pairs for G-C base pairs (C-U substitution). In each SiRNA trigger, all cytosines in either antisense or sense strand were replaced with uridines. These strands containing uridines were combined with complimentary strands with cytosines to generate SiRNA triggers with uridines in either antisense or sense strands.

[0217] The effect of gene silencing by these SiRNA triggers is shown in Figure 27. C-U substitution in either strand had no effect on the nonfunctional SiRNA trigger. In other words, introduction of G-U base pairs in place of G-C base pairs did not convert the particular nonfunctional SiRNA into a functional SiRNA trigger. However, the results may be different when the G-C base pairs are replaced by G-U base pairs in a functional SiRNA trigger. When cytosines in the antisense strand in certain instances were replaced with uridines, the SiRNA trigger appeared to become less functional (Figure 27; 5th bar), suggesting that in certain instances targeting through G-U wobble base pairing is undesirable to mediate RNA interference. On the other hand, substitution of cytosines with uridines in the sense strand may not affect the ability of a functional SiRNA to mediate RNA interference. Based on this observation of the tolerance of C-U substitution in the sense strand, it may be possible to improve the performance of nonfunctional SiRNA triggers. The failure of C-U substitution in the SiRNA trigger SP-1260 may be due to the extremely high number (14 out of 19 total) of contiguous G-C base pairs.

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If one uses a nonfunctional SiRNA with a modest number of contiguous G-C base pairs and carries out a C-U substitution in the sense strand the resulting trigger may be functional.

Conclusions

[0218] SiRNA triggers with a 17-base pair RNA helical region with an antisense strand of greater than or equal to 19 RNA nucleotides may be effective triggers.

[0219] SiRNA triggers carrying an RNA helical region(s) greater than or equal to19 base-pairs with different end structures may be functional in eliciting RNA interference in mammalian cells.

[0220] SiRNA triggers having a 17-base pair RNA helical region with an antisense strand of greater than or equal to 19 RNA nucleotides with different end structures may be effective triggers.

[0221] The 3'-ends may be either ribo- or two deoxy-nucleotides in certain functional SiRNA triggers.

[0222] SiRNA triggers in which a sense strand having 17 nucleotides is annealed to an antisense strand that is greater than or equal to 19 nucleotides may be functional in mammalian cells.

[0223] Unimolecular RNA molecules with the propensity to fold into hairpin structures may serve as functional SiRNA triggers in silencing gene expression in mammalian cells.

[0224] C-U substitutions in the sense strand but not in the antisense strand may be tolerated in functional SiRNA triggers.

[0225] These results suggest that SiRNA triggers with a 19-base pair helical region with two nucleotide 3' extensions may not be the only structure with the ability to mediate highly efficient RNA interference in mammalian cells.

V. RNA interference using DNA delivery

[0226] Gene silencing using RNA interference may be an attractive approach as a tool for understanding gene function and as a therapeutic approach to inhibit undesirable gene expression implicated in disease. A

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double stranded RNA molecule having homology to the target mRNA mediates the silencing process.

[0227] It has been demonstrated that the introduction of certain synthetic SiRNA molecules into cultured cells elicits silencing of the target gene, suggesting that the initial cleavage by the Dicer enzyme may be bypassed. Not all SiRNA triggers identified against a target gene are equally efficacious in silencing that gene, and hence screening of several triggers may be carried out. On the other hand, a relatively long dsRNA molecule cleaved by the Dicer enzyme generates several different such triggers inside the cell. The latter approach may have a higher probability of getting an effective trigger for the silencing process. Transfection of dsRNA longer than 70 base pairs has been shown to elicit cytotoxicity, and, therefore, may not be used as a functional trigger in certain instances. However, the intracellular expression of longer dsRNA may not induce cellular toxicity. Furthermore, linear dsDNA fragments having a U6 promoter upstream of either antisense or sense strands of a targeted reporter gene were constructed using PCR. These PCR fragments were intended to produce either antisense or sense strands of RNA approximately of 22 nucleotide homology to the target gene. The transfection of both types of PCR fragments (antisense and sense), along with a plasmid expressing the target reporter gene, into HEK 293 cells was able to silence the reporter gene expression.

[0228] 1. One may use DNA constructs with the capacity to express RNA having significant homology to a target gene of interest in RNAi. In certain embodiments, these constructs may encode dsRNAs of 70 to 150 nucleotides.

[0229] a. RNA may be single stranded with either antisense or sense polarity to the target mRNA. DNA constructs expressing both polarities may be used in certain embodiments. However, due to the possibility of aberrant RNA generation with an orientation opposite to the promoter, even one of the constructs expressing either sense or antisense RNA may be used.

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[0230] b. RNA may have a double-stranded nature due to the presence of self-complimentary regions. An example of this type of RNA is a fold-back stem loop. When RNA molecules having a double-stranded nature are expressed, a single type of DNA construct may be used.

[0231] 2. In DNA constructs described in 1, the promoters that drive RNA synthesis may be of phage derived, virus-derived, pol II, or pol III type.

[0232] 3. DNA constructs described in 1, may or may not contain extra nucleotides that serve additional functions such as termination of transcription or a poly A signal.

[0233] 4. DNA constructs described in 1, may be either linear or circular. In the case of circular DNA, it may be a plasmid with additional genes conferring different functions such as resistance to one or more antibiotics.

[0234] 5. DNA constructs described in 1, may be synthetic, derived from PCR, or derived from growing inside a host. Alternatively, DNA constructs may be derived from one or more methods described above.

[0235] 6. For application in tissue culture, DNA constructs described in 1, may be introduced into cells by transfection, electroporation, or microinjection.

[0236] 7. For in vivo applications in animals and humans, DNA constructs described in 1, may be delivered by:

a. Simple injection into tissues or blood or any other body fluid;
b. Under pressure;
c. Electroporation;
d. Using micro pumps;
e. Using DNA guns;
f. Orally.

[0237] 8. Adjuvants or formulations that may either stabilize DNA constructs or facilitate a delivery method may be used in the delivery methods outlined in 7.

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We claim:

1. A method of decreasing the level of a target mRNA in a host cell, comprising

a) contacting the host cell with a double-stranded RNA molecule, wherein the double-stranded RNA molecule comprises a sequence substantially complementary to at least a portion of the target mRNA, and wherein the double-stranded RNA molecule further comprises at least one chemical modification;

b) incubating the host cell under conditions whereby RNA interference occurs;

c) thereby decreasing the level of the target mRNA.

2. The method of claim 1, wherein the at least one chemical modification is selected from 2'-F, 2'-OMe, and 2'-deoxy.

3. A method of decreasing the level of a target mRNA in a host cell, comprising

a) delivering to a host cell a vector comprising a first nucleic acid sequence and a second nucleic acid sequence, wherein

i) the first nucleic acid sequence encodes a first RNA molecule comprising a first RNA sequence that is substantially complementary to at least a portion of the target mRNA; and

ii) the second nucleic acid sequence encodes a second RNA molecule comprising a second RNA sequence that is substantially identical to at least a portion of the target mRNA;

b) incubating the host cell under conditions

i) that allow transcription of the first nucleic acid sequence and the second nucleic acid sequence; and

ii) that allow RNA interference to occur;

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c) thereby decreasing the level of the target mRNA.

4. The method of claim 3, wherein the first RNA sequence and the second RNA sequence are each longer than about 70 nucleotides.

5. The method of claim 3, wherein the vector further comprises at least one promoter selected from a phage promoter, a viral promoter, a pol II promoter, and a pol III promoter.

6. A method of selecting a double-stranded RNA molecule, comprising

a) inputting a target mRNA sequence into Oligo 5.0™ Primer Analysis software;

b) selecting 19 for the primer length;

c) identifying a primer in the internal stability window, wherein the primer has a bell-shaped internal energy profile, a maximum internal energy of less than -10 kcal/mol, and a melting temperature below 65°C;

d) performing a BLAST search on the primer against an EST database; and

e) synthesizing a double-stranded RNA molecule comprising a first RNA strand comprising a first RNA sequence that is substantially identical to the nucleotide sequence of the primer and a second RNA strand comprising a second RNA sequence that is substantially complementary to the nucleotide sequence of the primer.

7. A method of selecting a double-stranded RNA molecule, comprising

a) inputting a target mRNA sequence into Oligo 5.0™ Primer Analysis software;

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b) selecting 19 for the primer length;

c) identifying a primer in the internal stability window, wherein the primer has a substantially flat internal energy profile, an internal energy of between -6 and -9 kcal/mol, and a melting temperature below 50°C;

d) performing a BLAST search on the primer against an EST database; and

e) synthesizing a double-stranded RNA molecule comprising a first RNA strand comprising a first RNA sequence that is substantially identical to the nucleotide sequence of the primer and a second RNA strand comprising a second RNA sequence that is substantially complementary to the nucleotide sequence of the primer.

8. A method of decreasing the level of a target mRNA in a mammalian host cell, comprising

a) contacting the mammalian host cell with an RNA hairpin molecule, wherein the RNA hairpin molecule comprises a first region, a second region, and a third region, wherein

i) the first region comprises a sequence that is substantially identical to at least a portion of the target mRNA;

ii) the third region comprises a sequence that is substantially complementary to at least a portion of the first region; and

iii) wherein the first region and the third region hybridize, thereby forming an RNA hairpin molecule; and

b) incubating the mammalian host cell under conditions whereby RNA interference occurs;

thereby decreasing the level of the target mRNA in the mammalian host cell.

9. A method of decreasing the level of a target mRNA in a host cell, comprising

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a) delivering to a host cell a vector comprising a nucleic acid sequence, wherein the nucleic acid sequence encodes an RNA hairpin molecule, and wherein the RNA hairpin molecule comprises a first region, a second region, and a third region, wherein
   i) the first region comprises a sequence that is substantially identical to at least a portion of the target mRNA;
   ii) the third region comprises a sequence that is substantially complementary to at least a portion of the first region; and
   iii) wherein the first region and the third region hybridize, thereby forming an RNA hairpin molecule; and
b) incubating the host cell under conditions that
   i) allow transcription of the nucleic acid sequence; and
   ii) allow RNA interference to occur;
c) thereby decreasing the level of the target mRNA.

10. A method of constructing a library of RNA hairpin molecules comprising
a) synthesizing a plurality of single-stranded DNA hairpin templates, wherein each single-stranded DNA hairpin template comprises a first region, a second region, and a third region, wherein
   i) the first region comprises an RNA polymerase promoter sequence;
   ii) the second region comprises a random nucleotide sequence having between 5 and 500 nucleotides; and
   iii) the third region comprises a first nucleotide sequence, a second nucleotide sequence, and a third nucleotide sequence, wherein the first nucleotide sequence hybridizes to the third nucleotide sequence, thereby forming a single-stranded DNA hairpin template;

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b) extending the 3' end of the third nucleotide sequence of each of the plurality of single-stranded DNA hairpin templates to form a plurality of double-stranded DNA hairpin templates;
c) amplifying the plurality of double-stranded DNA hairpin templates to form a plurality of double-stranded DNA templates;
d) transcribing the plurality of double-stranded DNA templates to form a library of RNA hairpin molecules.

11. A method of identifying a target gene comprising
a) forming an array comprising a plurality of positions, wherein each position comprises at least one mammalian cell;
b) contacting the at least one mammalian cell at each position with at least one RNA hairpin molecule;
c) incubating the at least one mammalian cell under conditions that allow RNA interference to occur;
c) selecting an at least one mammalian cell exhibiting at least one biological endpoint;
d) identifying the at least one RNA hairpin molecule associated with the plurality of cells exhibiting at least one biological endpoint; and
e) performing a BLAST search on the nucleic acid sequence of the at least one RNA hairpin molecule, thereby identifying the target gene.

12. A library comprising a plurality of RNA hairpin molecules, wherein each RNA hairpin molecule comprises a first region, a second region, and a third region, wherein the first region comprises a random nucleotide sequence having between 5 and 500 nucleotides and the third region comprises a nucleotide sequence that is substantially complementary to at least a portion of the first region.

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Proposed Mechanism for RNA Interference

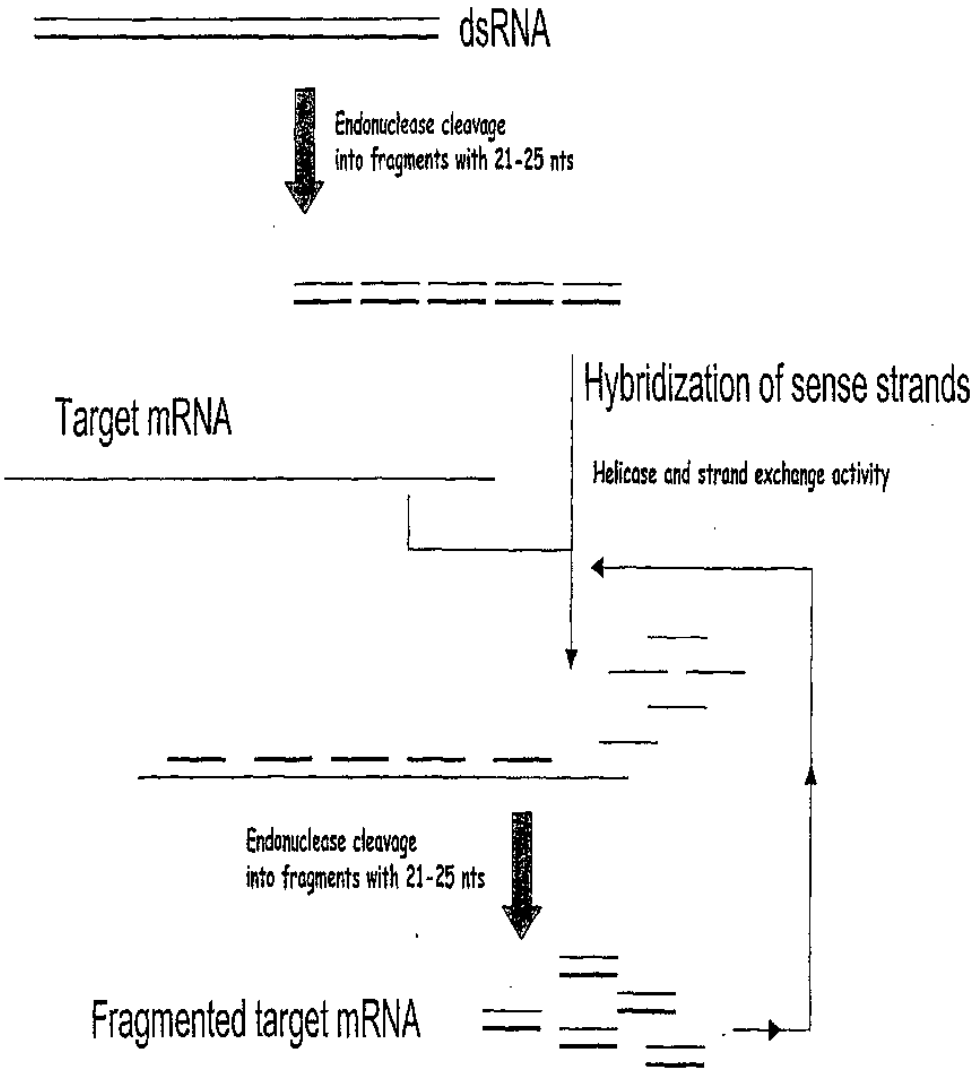


Figure 1

WO 2004/061083
1/56
PCT/US2003/041377

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Identification of chemically-modified dsRNA for RNAi

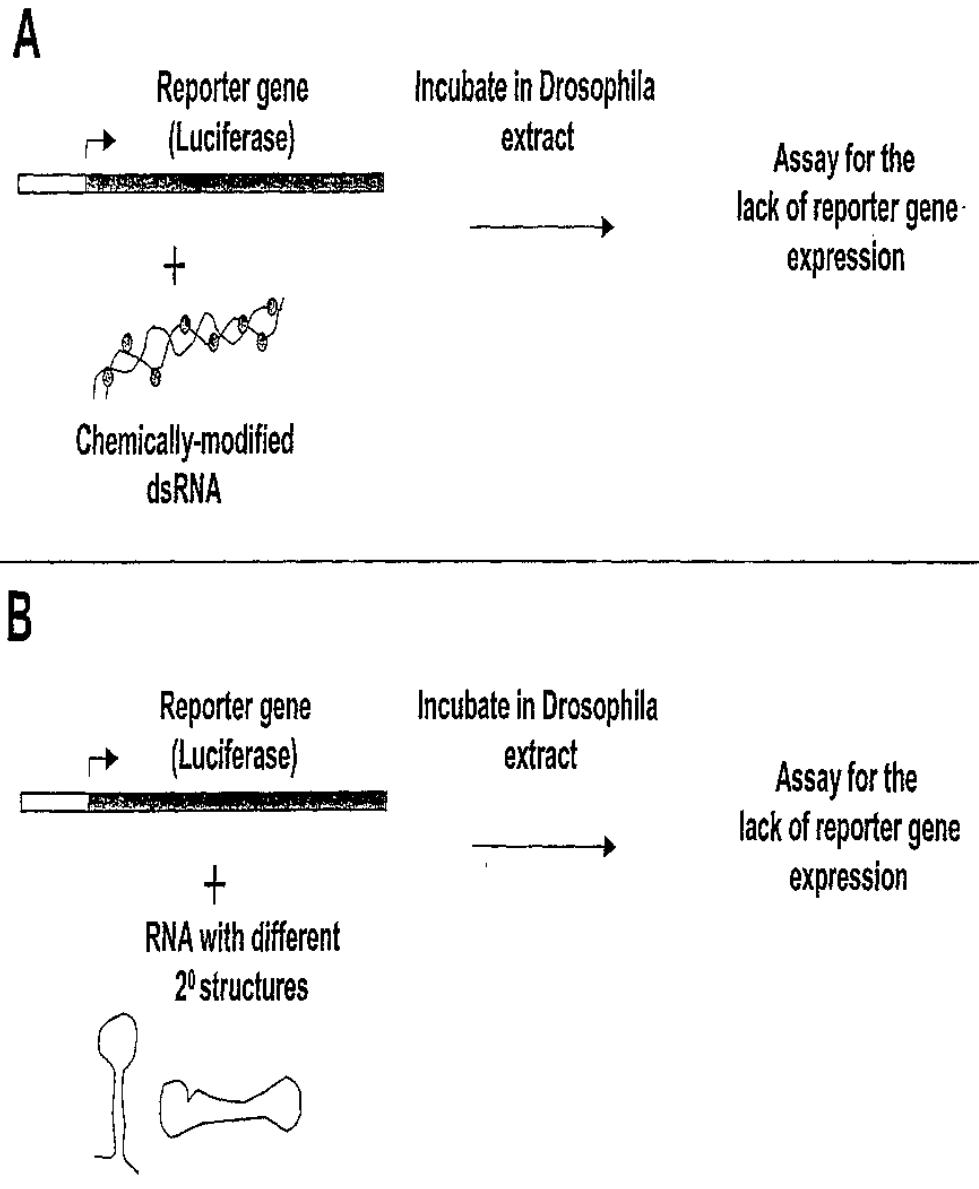


Figure 2

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In vivo expression of RNAs for RNA interference

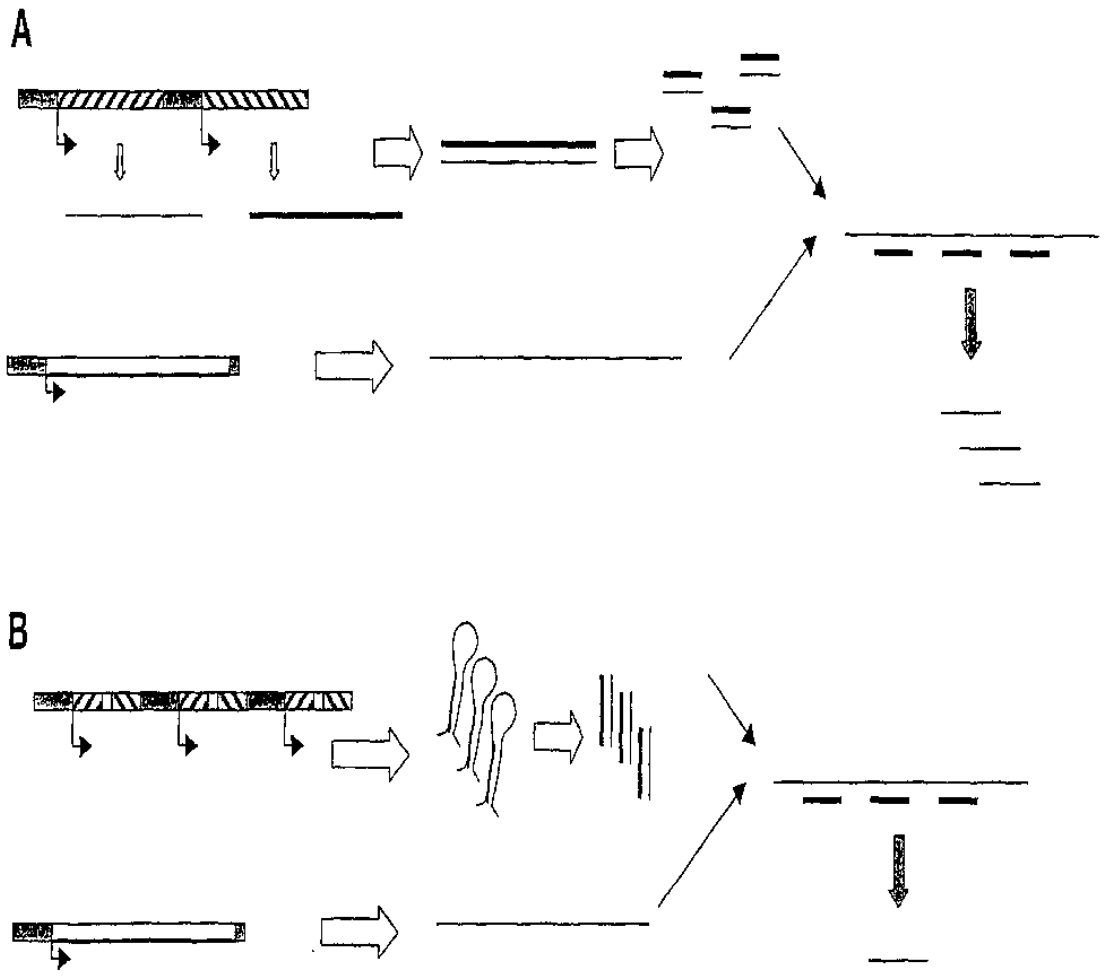


Figure 3

Ds-SiRNA Triggers used in the study

			Target homology (nts)
	AS-8	5'-CAG AAA CGG AUG CUC CCA GGC CAA G-3'	25
SiRNA # 8 A	AS-1	5'-CAG AAA CGG AUG CUC CCA GGC dTdT-3'	21
	S-1	3'-dTdT GUC UUU GCC UAC GAG GGU CCG –5'	
SiRNA # 8 B	AS-2	5'-CAG AAA CGG AUG CUC CCA GdTdT-3'	19
	S-2	3'-dTdT GUC UUU GCC UAC GAG GGU C –5'	
	AS-15	5'-CAG UGU CUC UUG AAC CAG TTC CCA G-3'	25
SiRNA # 815 A	AS-1	5'-CAG UGU CUC UUG AAC CAG UUC dTdT-3'	21
	S-1	3'-dTdT GUC ACA GAG AAC UUG GUC AAG –5'	
SiRNA # 15 B	AS-2	5'-CAG UGU CUC UUG AAC CAG U dTdT-3'	19
	S-2	3'-dTdT GUC ACA GAG AAC UUG GUC A –5'	
	AS-4	5'-GTG GGA AGA AGG TGG CAG TGA ACT C-3'	25
SiRNA # 4 A	AS-1	5'-GUG GGA AGA AGG UGG CAG UGA dTdT-3'	21
	S-1	3'-dTdT CAC CCU UCU UCC ACC GUC ACU –5'	
SiRNA # 4 B	AS-2	5'-GUG GGA AGA AGG UGG CAG U dTdT-3'	19
	S-2	3'-dTdT CAC CCU UCU UCC ACC GUC A –5'	

Figure 4

ss-SiRNA Hairpin Triggers used in the study

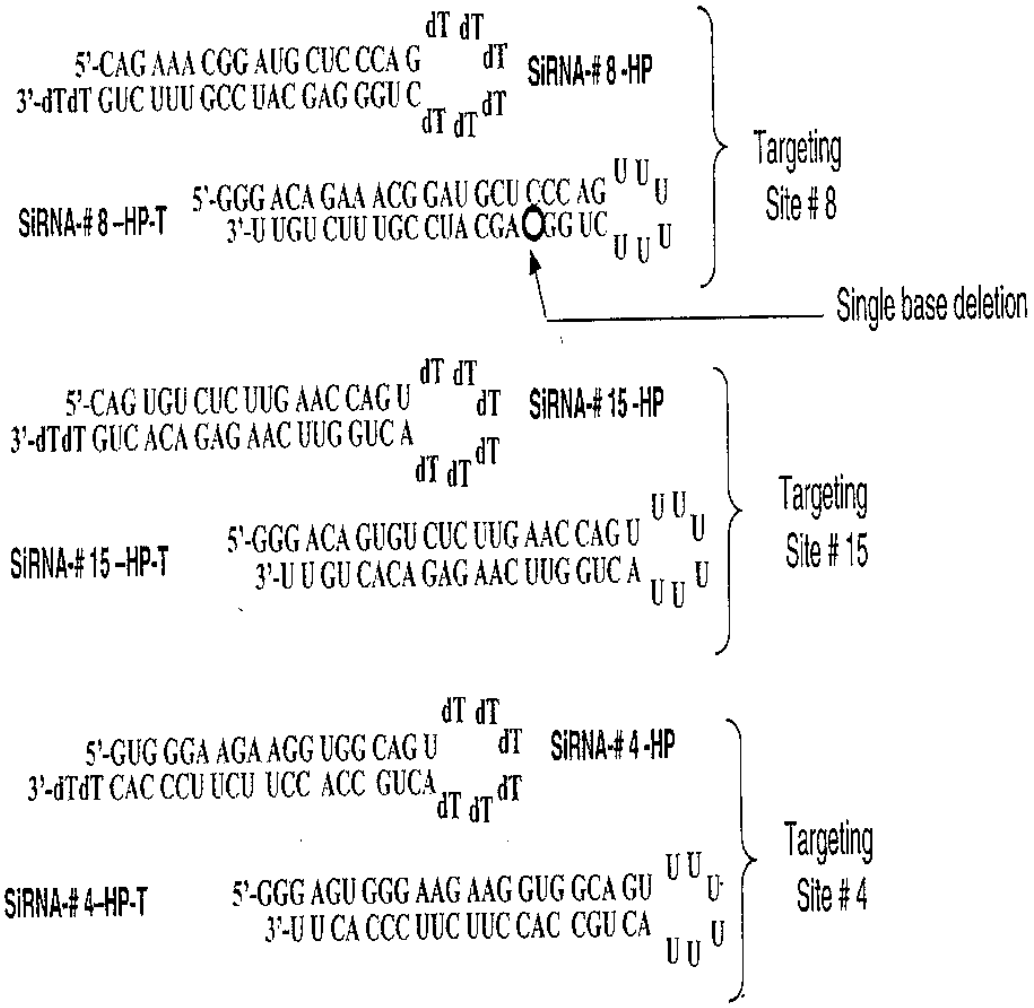


Figure 5

Reduction of targeted mRNA by different nucleic acid triggers

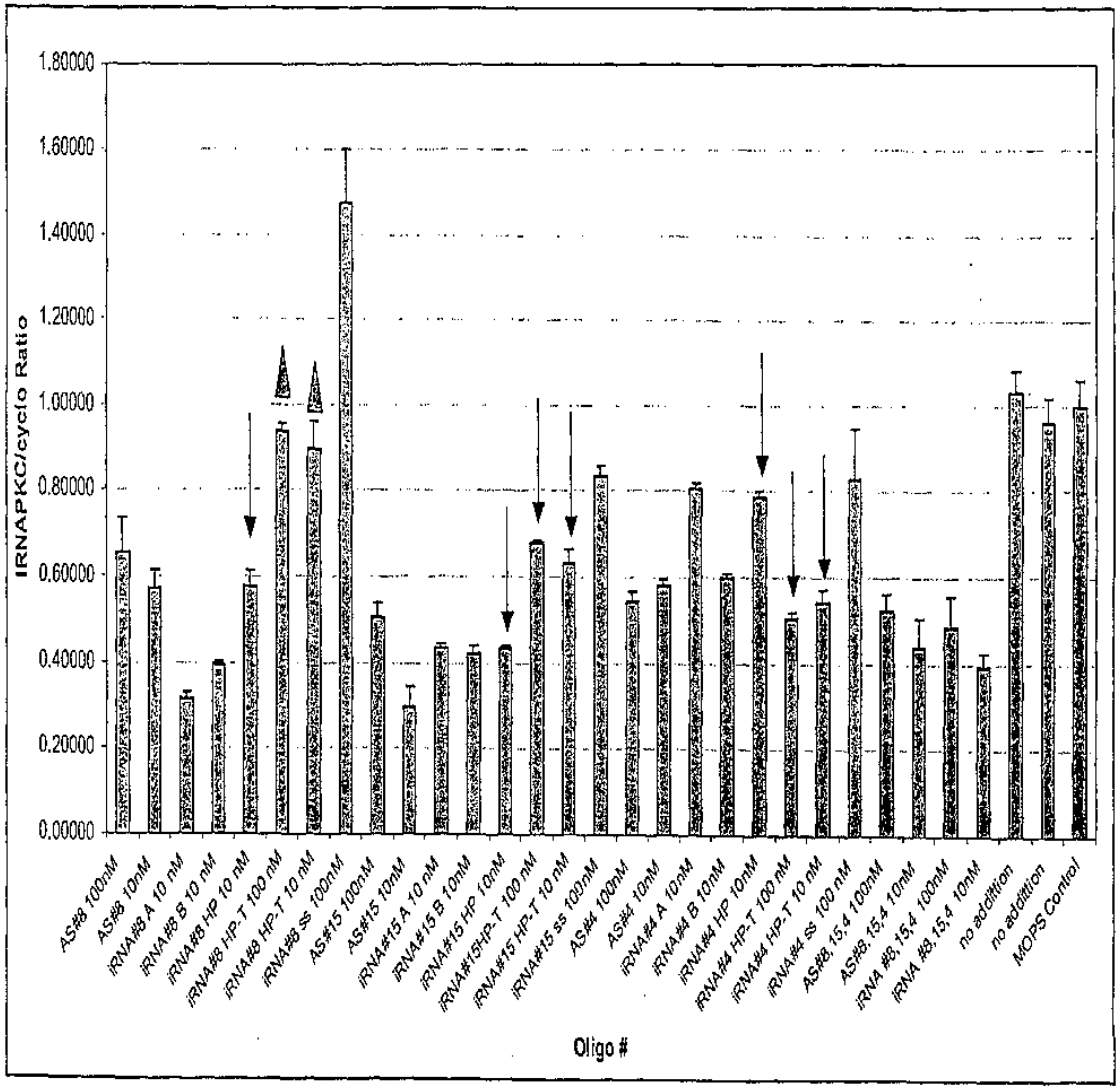


Figure 6

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Synthesis of SiRS RNA hairpin libraries

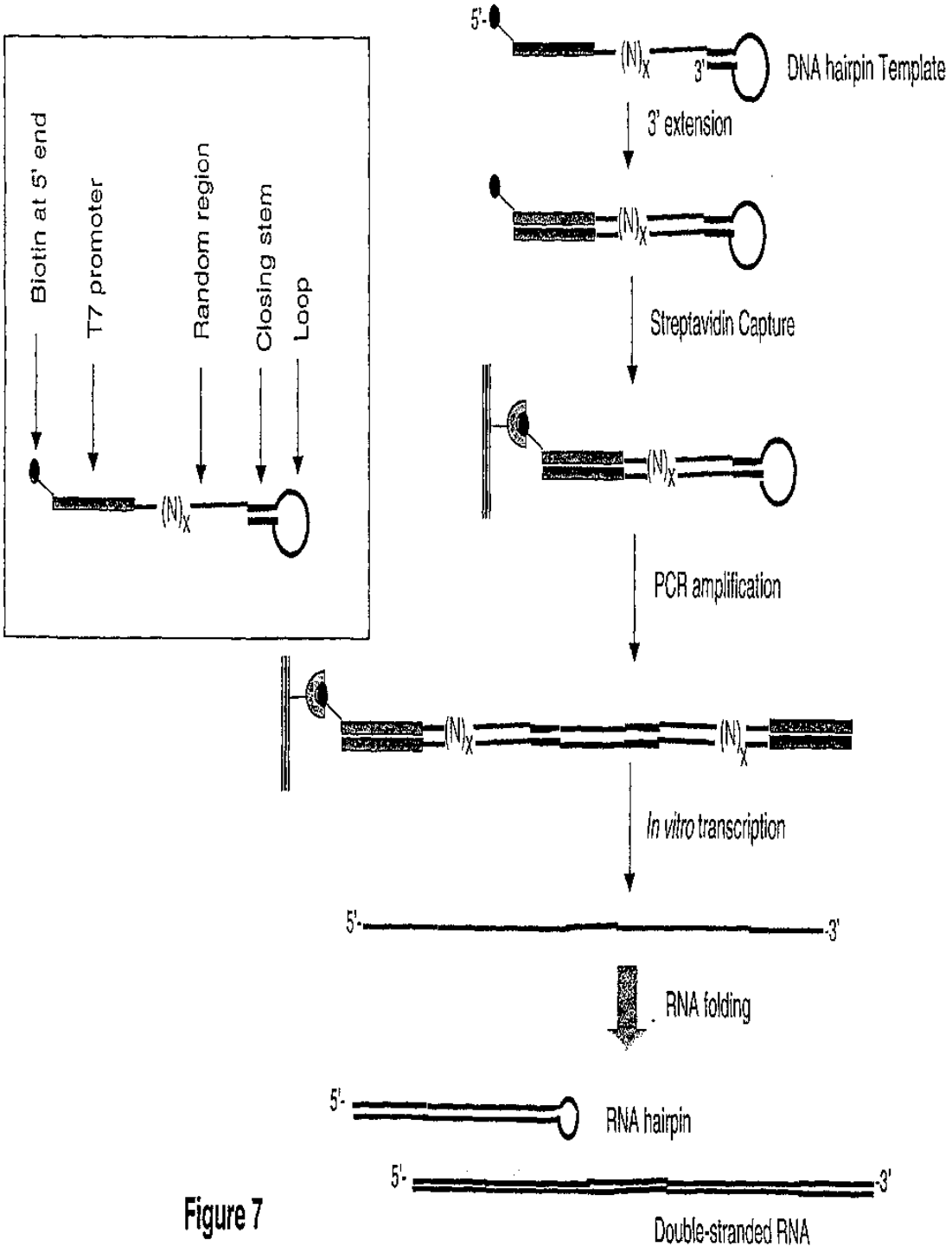


Figure 7

Constructing Short interfering Random Sequence (SiRS)RNA Hairpin Libraries

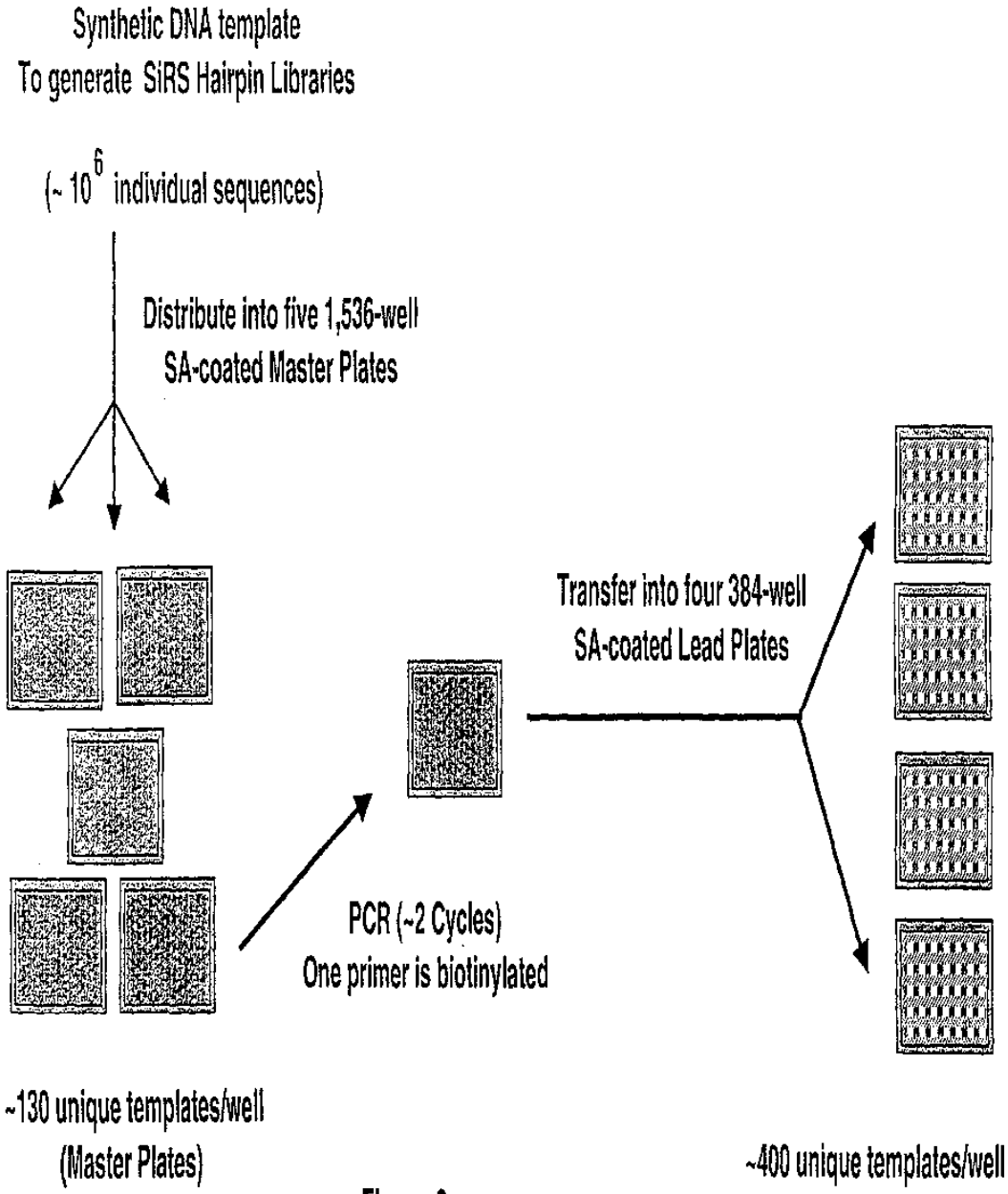


Figure 8

Primary Screening with SIRS RNA Hairpin Library

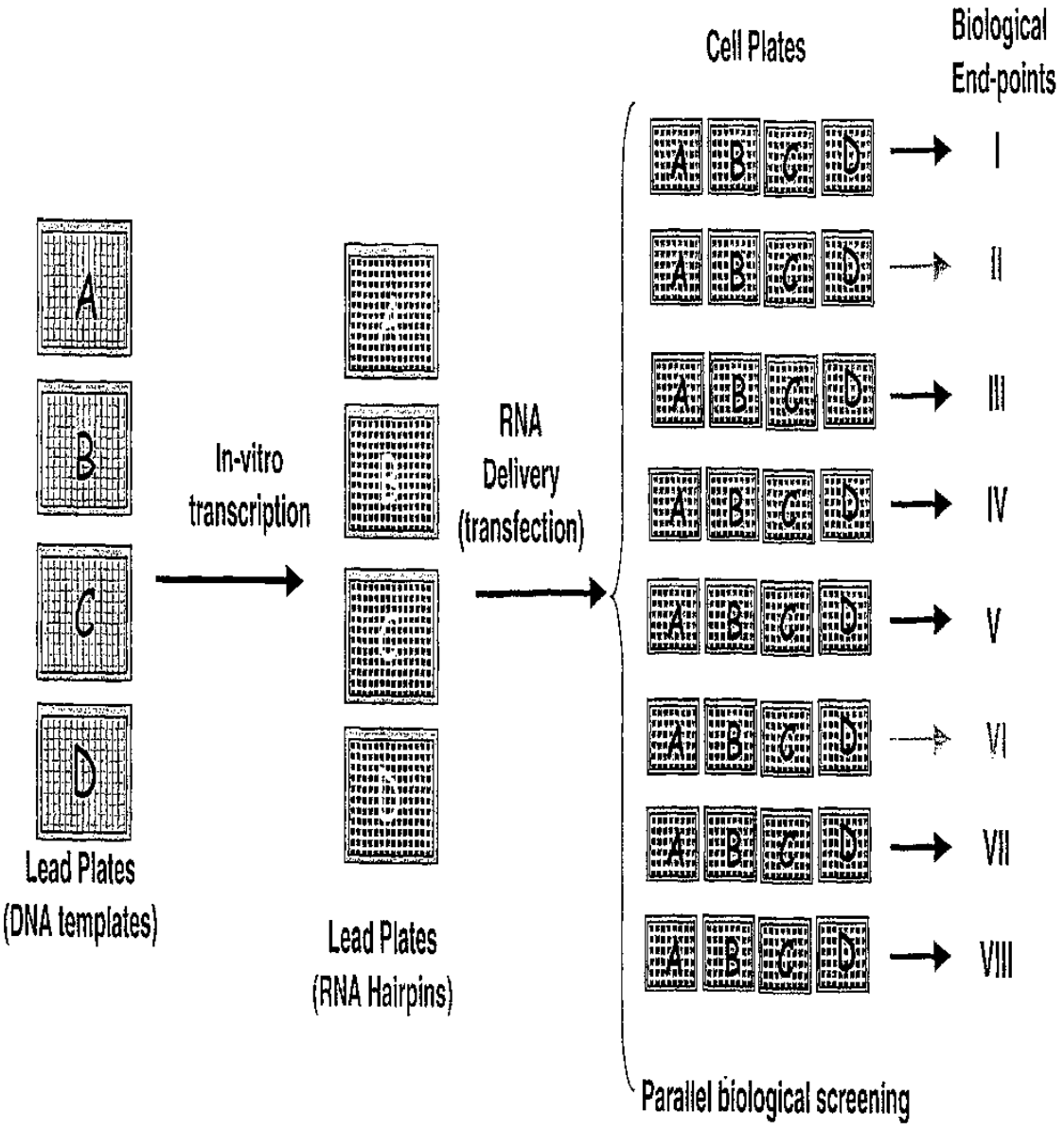


Figure 9

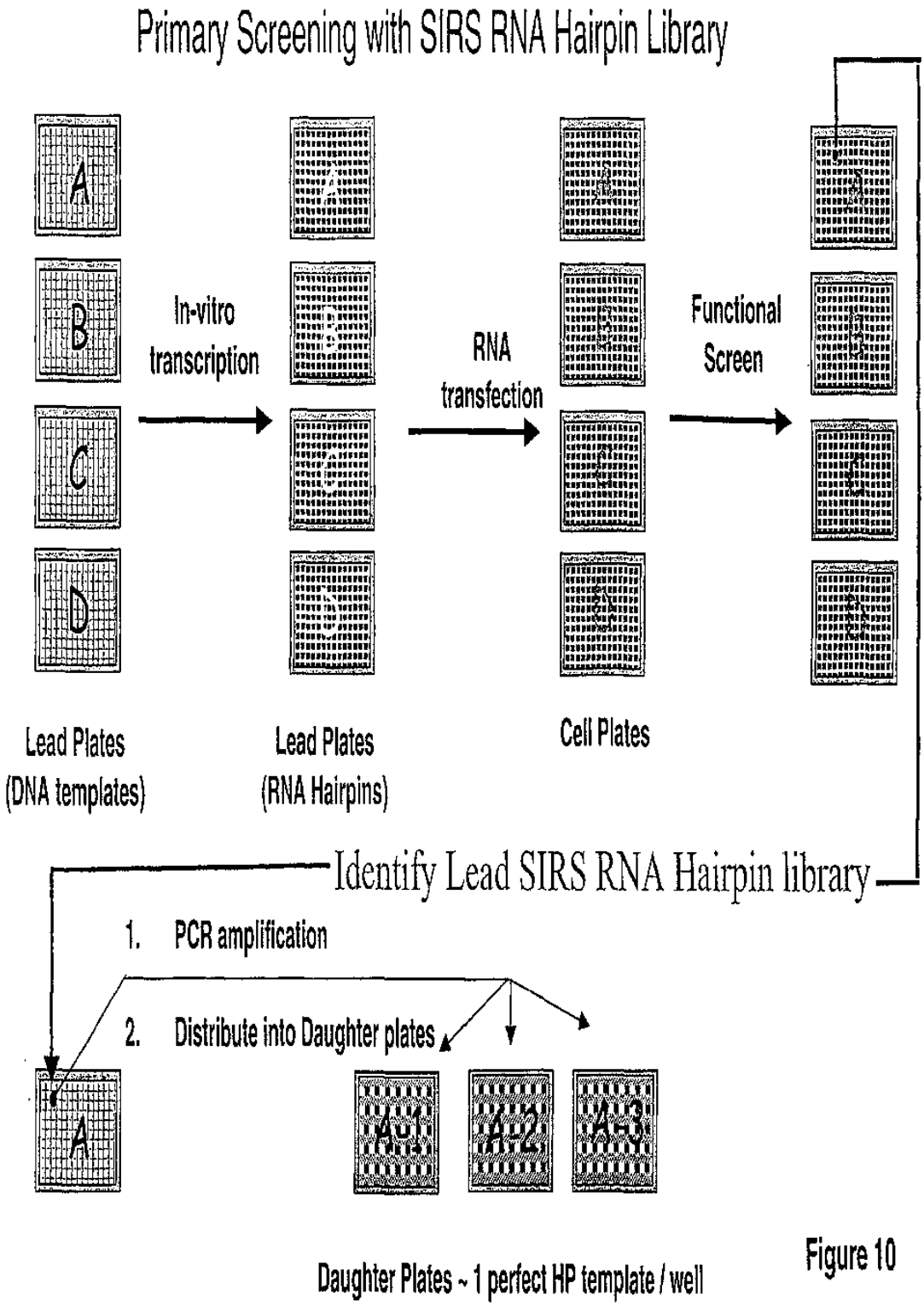

Primary Screening with SIRS RNA Hairpin Library
A
B
C
D
In-vitro transcription
RNA transfection
Functional Screen
Lead Plates (DNA templates)
Lead Plates (RNA Hairpins)
Cell Plates
Identify Lead SIRS RNA Hairpin library
1. PCR amplification
2. Distribute into Daughter plates
A
Daughter Plates ~ 1 perfect HP template / well

Figure 10

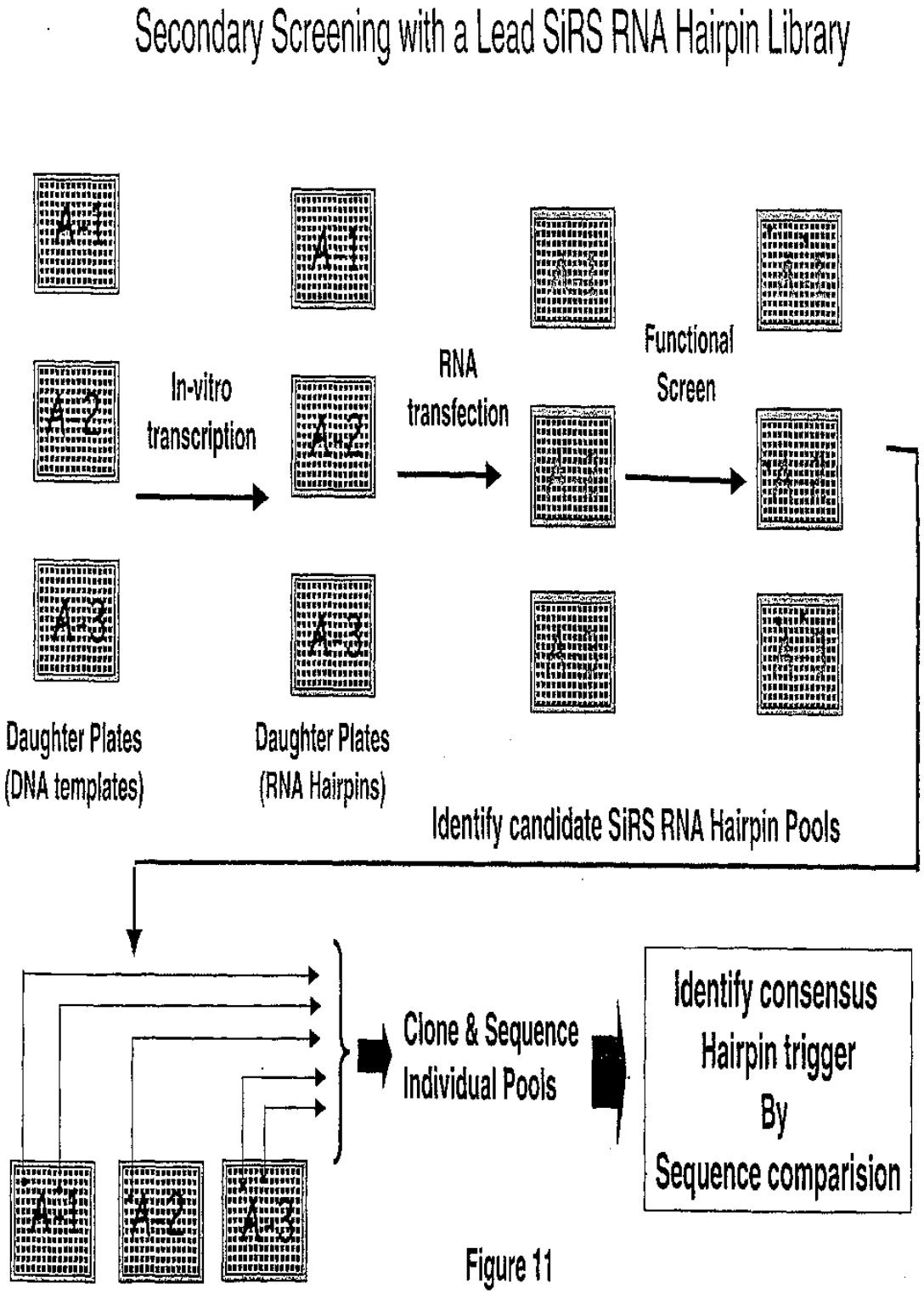
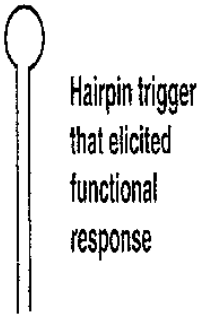

Secondary Screening with a Lead SiRS RNA Hairpin Library
In-vitro transcription
RNA transfection
Functional Screen
Daughter Plates (DNA templates)
Daughter Plates (RNA Hairpins)
Identify candidate SiRS RNA Hairpin Pools
Clone & Sequence Individual Pools
Identify consensus Hairpin trigger By Sequence comparision

Figure 11

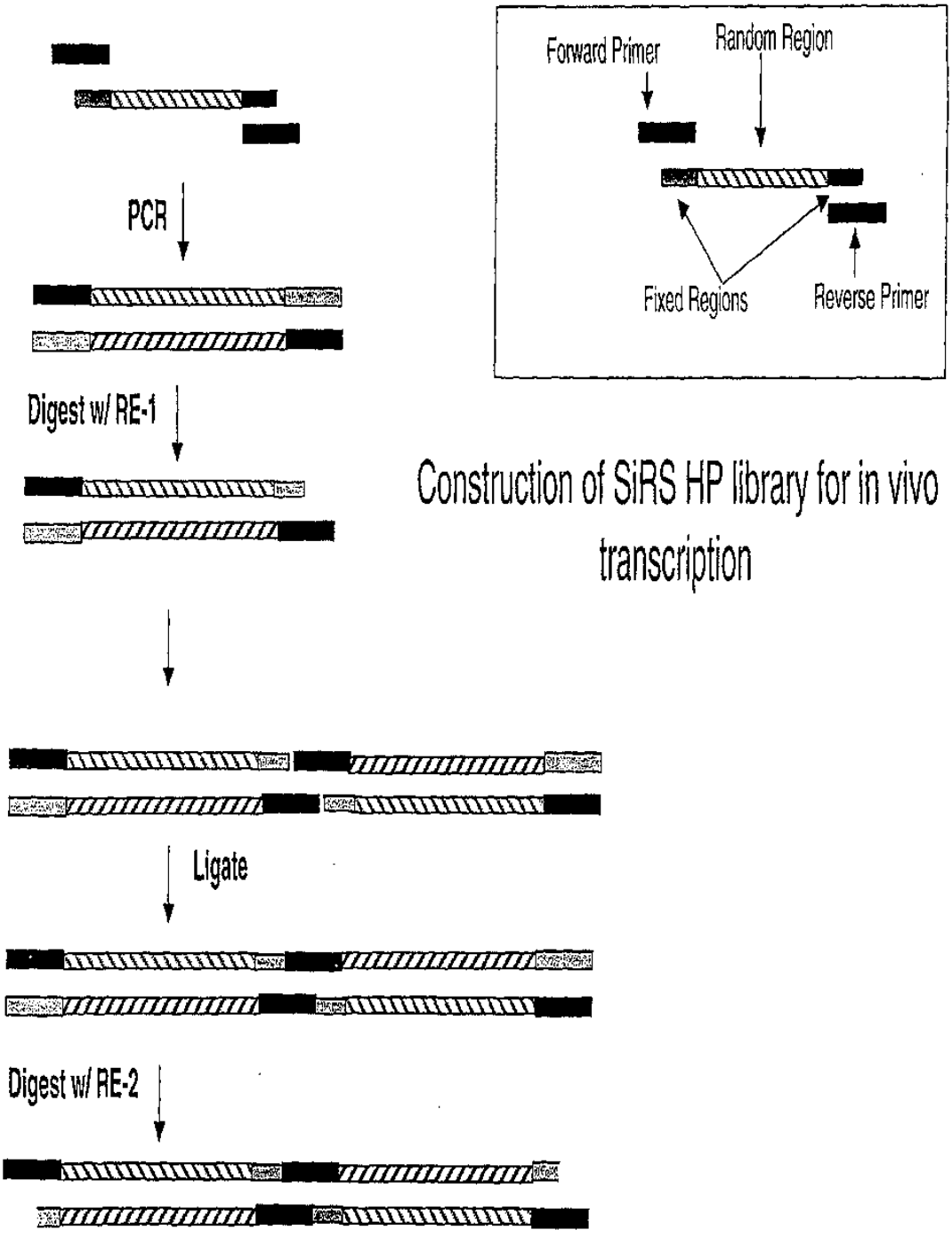


Blast both sequences in the stem against Human Genome Data Base

Identify the Candidate Gene

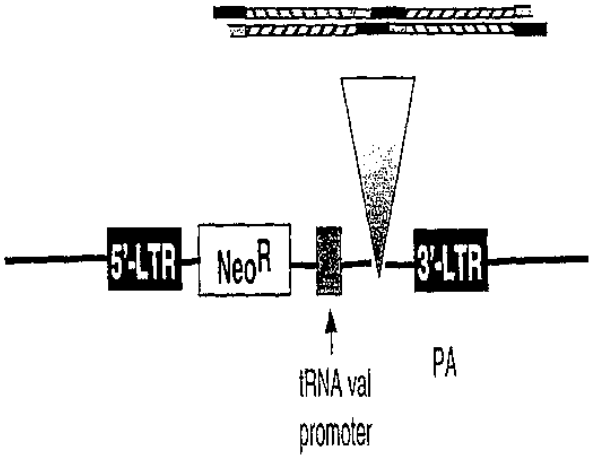
Figure 12

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Construction of SiRS HP library for in vivo transcription

Figure 13



Transform into
Bacteria

Pool and expand transformants

Purify recombinant plasmids

Triple transfection

Produce viral vector
Harboring SiRS HP library

Figure 14

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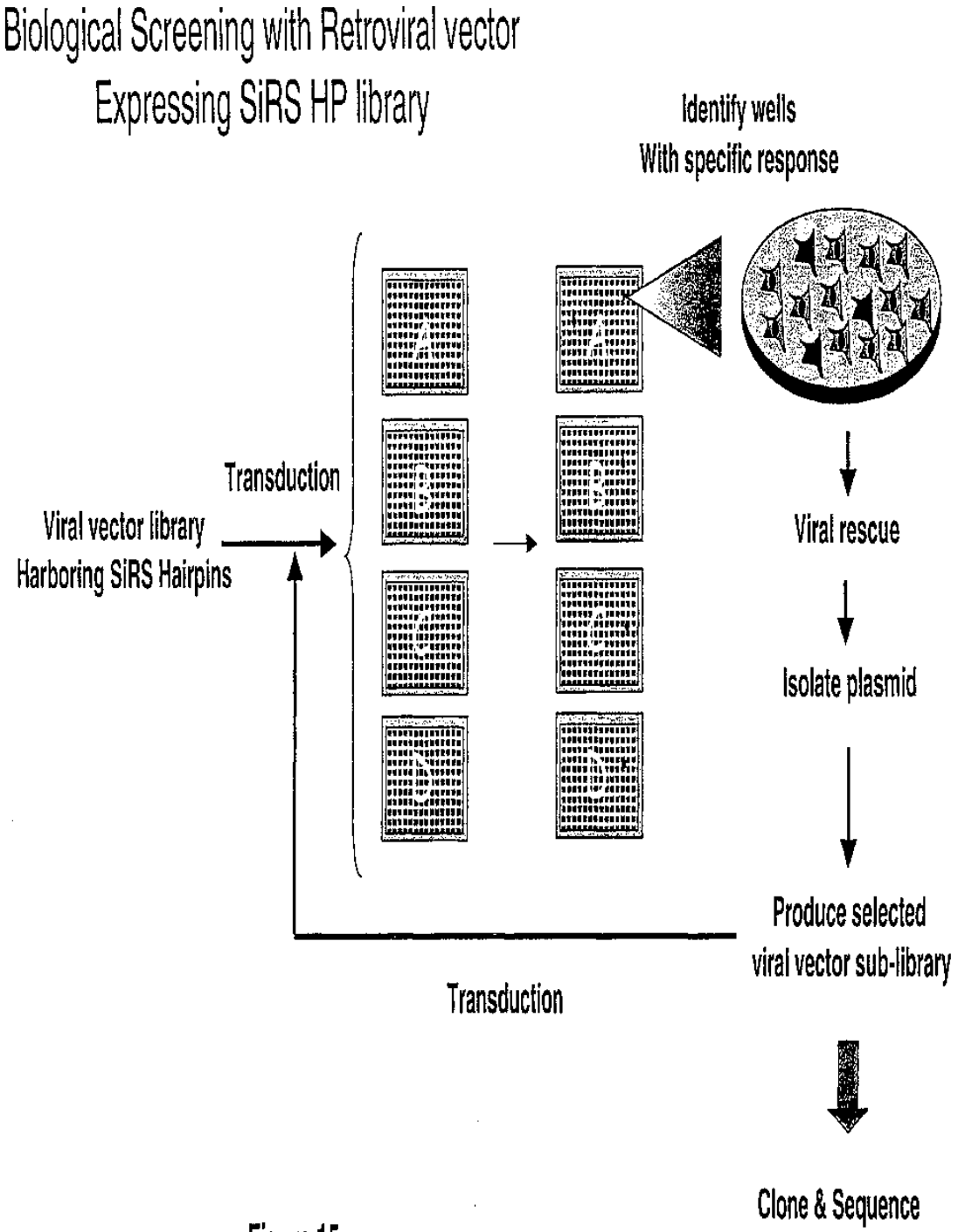

Biological Screening with Retroviral vector
Expressing SiRS HP library
Identify wells
With specific response
Transduction
Viral vector library
Harboring SiRS Hairpins
Viral rescue
Isolate plasmid
Produce selected
viral vector sub-library
Transduction
Clone & Sequence

Figure 15

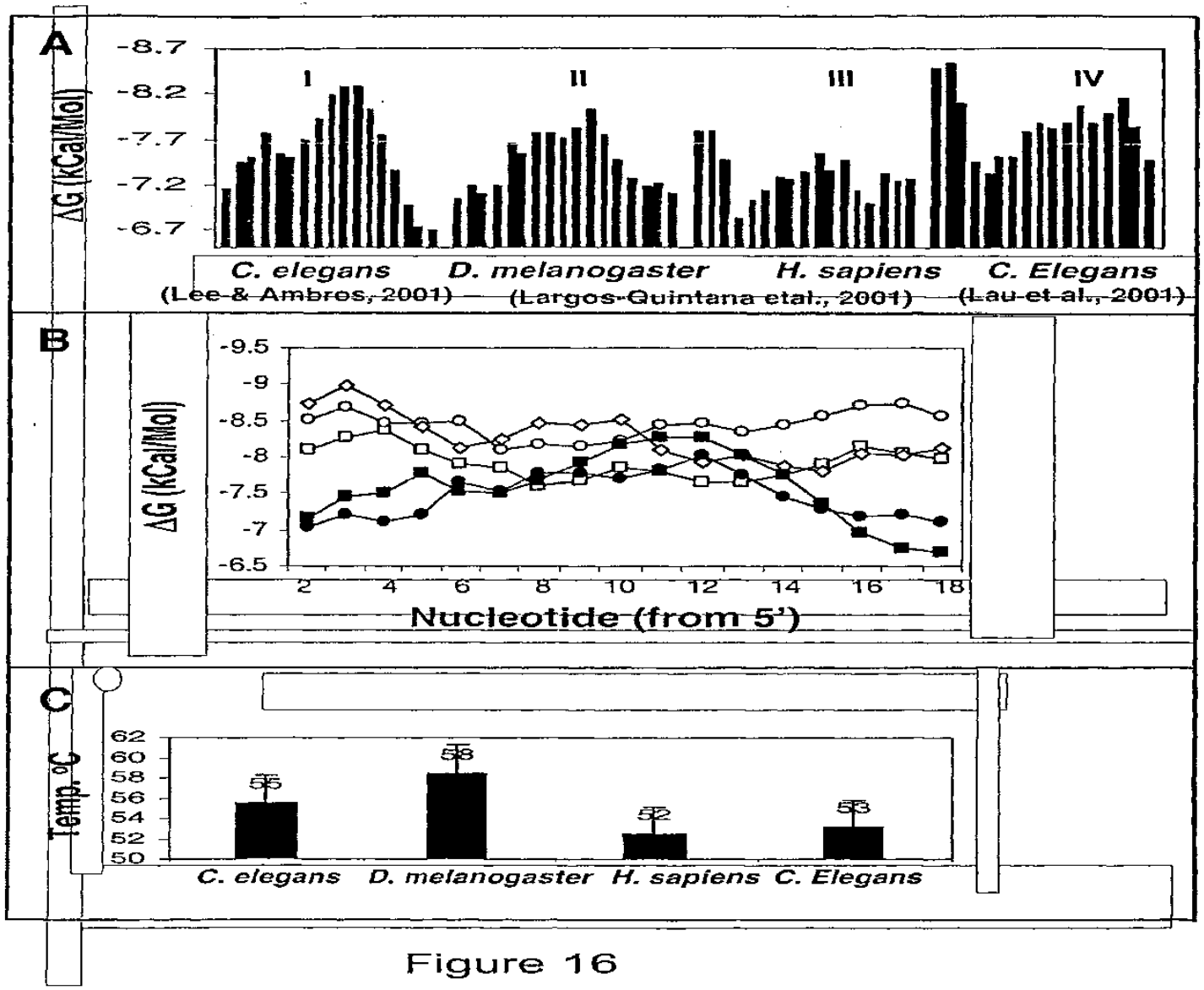


Figure 16

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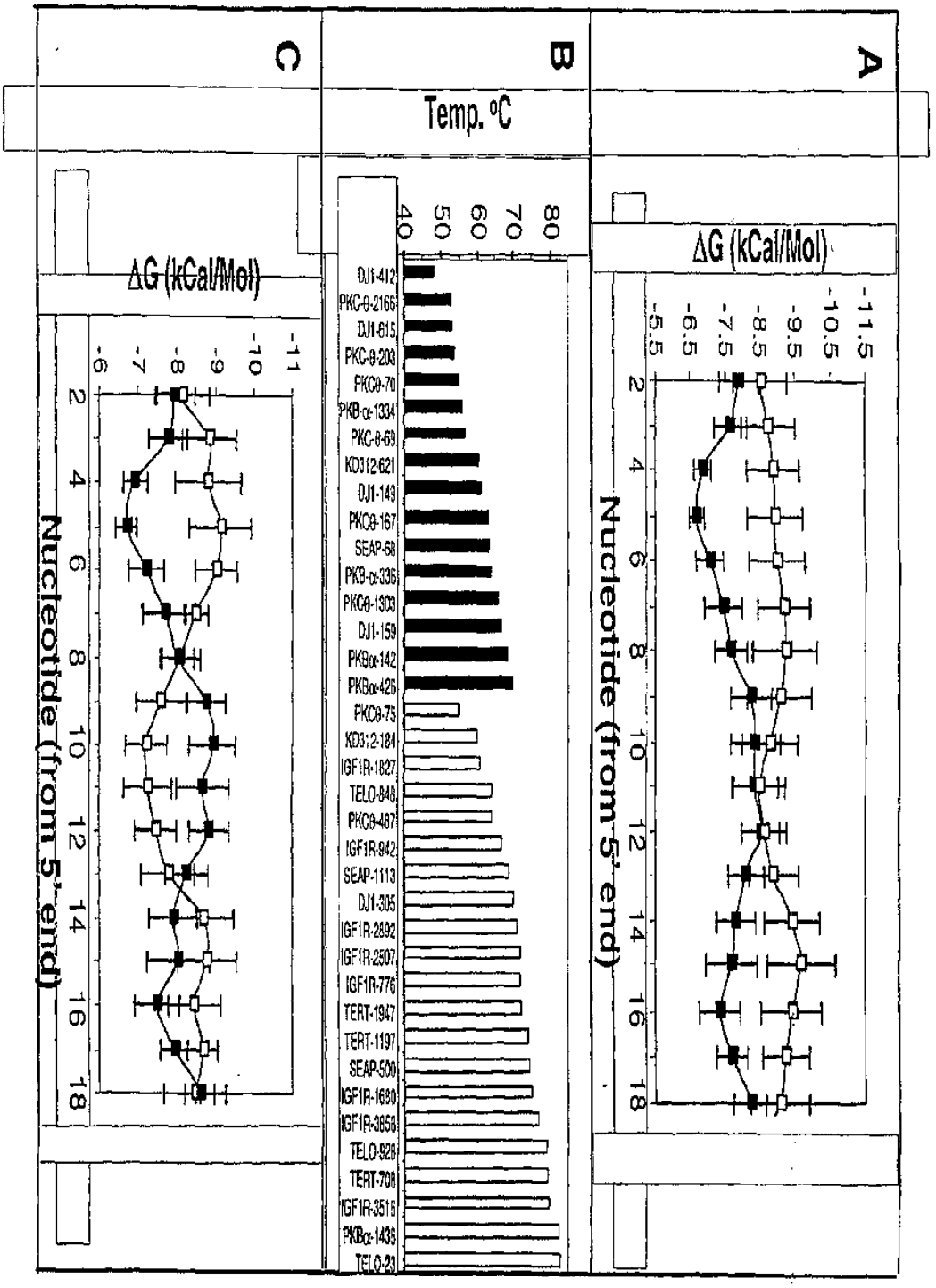

A
ΔG (kCal/Mol)
-11.5
-10.5
-9.5
-8.5
-7.5
-6.5
-5.5
2
4
6
8
10
12
14
16
18
Nucleotide (from 5' end)
B
Temp. °C
40
50
60
70
80
DJ1-412
PKC-θ-2166
DJ1-615
PKC-θ-208
PKCθ-70
PKB-α-1334
PKC-θ-69
KD3I2-621
DJ1-149
PKCθ-167
SEAP-68
PKB-α-335
PKCθ-1303
DJ1-159
PKBα-142
PKBα-425
PKCθ-75
KD3:2-184
IGF1R-1827
TELO-846
PKCθ-487
IGF1R-942
SEAP-1113
DJ1-305
IGF1R-2892
IGF1R-2507
IGF1R-776
TERT-1947
TERT-1197
SEAP-500
IGF1R-1630
IGF1R-3858
TELO-928
TERT-706
IGF1R-3515
PKBα-1435
TELO-23
C
ΔG (kCal/Mol)
-6
-7
-8
-9
-10
-11
2
4
6
8
10
12
14
16
18
Nucleotide (from 5' end)

Figure 17

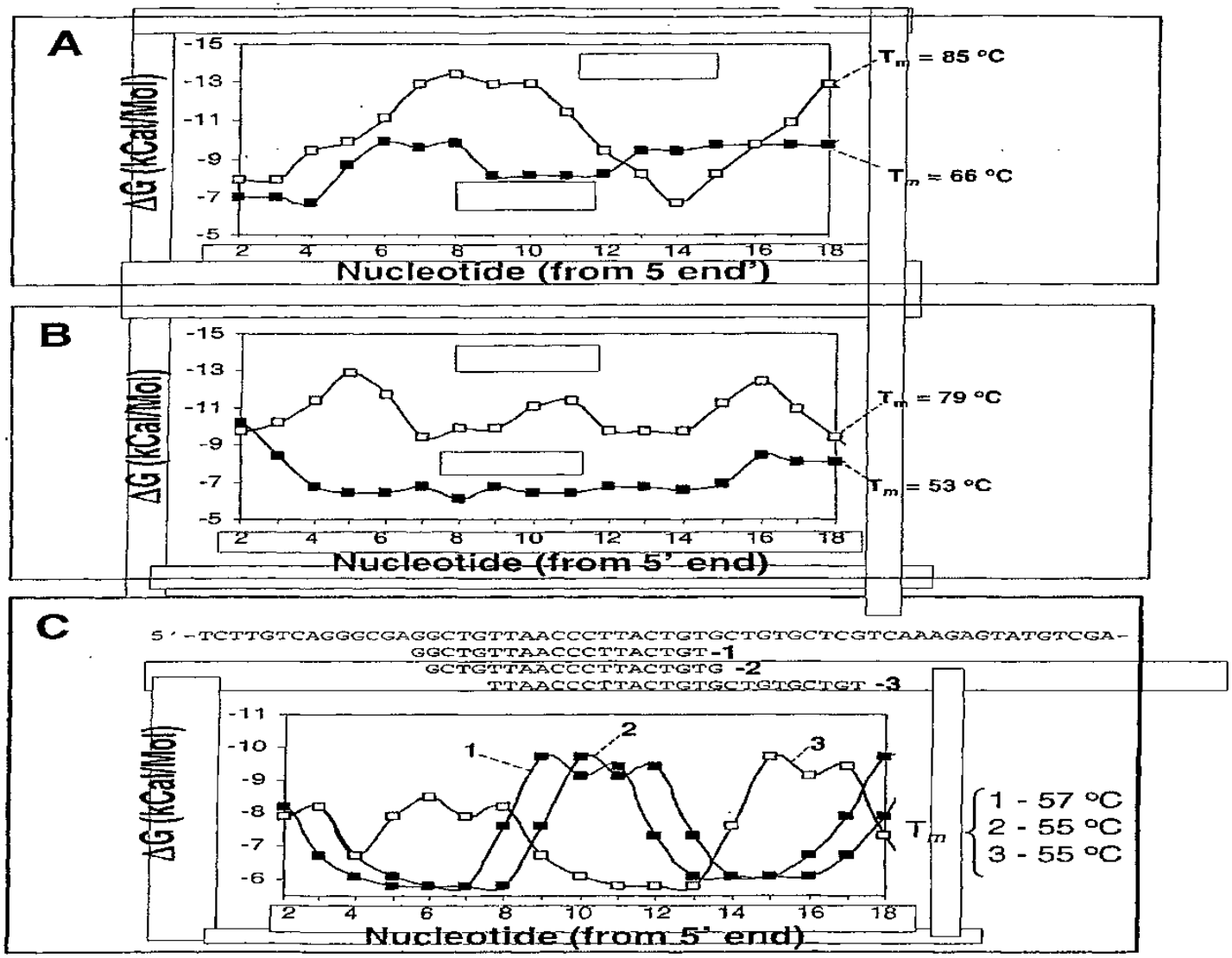
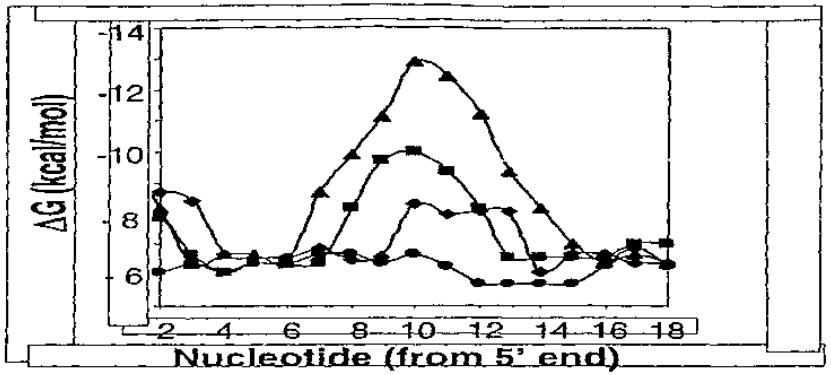


Figure 18

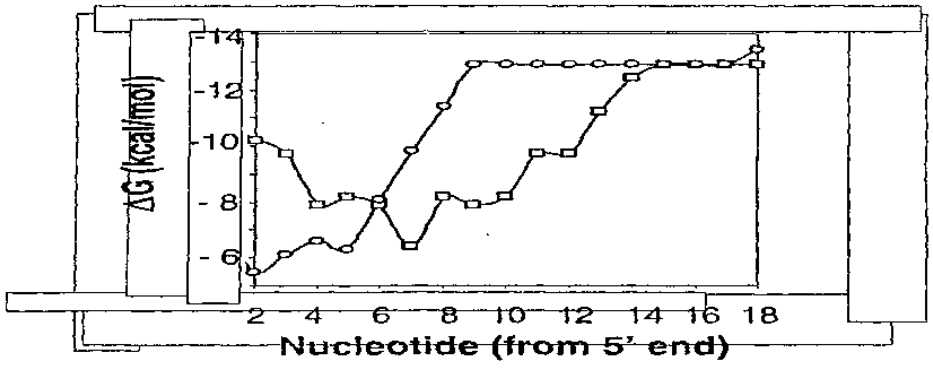
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A-1



		T_m
SP-1035 (◆)	GAACAUGAUCGUCUCAGUCdTdT dTdTCUUGUACUAGCAGAGUCAG	53.3
SP-309 (■)	ACUGUCUGGCACAUGUUUGdTdT dTdTUGACAGACCGUGUACAAAC	58.2
SP-1795 (▲)	UGACAACGGGCCACAACUCdTdT dTdTACUGUUGCCCGGUGUUGAG	66.6
SP-2217 (●)	ACUCUCUGACAUACAUCACdTdT dTdTUGAGAGACUGUAUGUAGUG	46.4

A-2

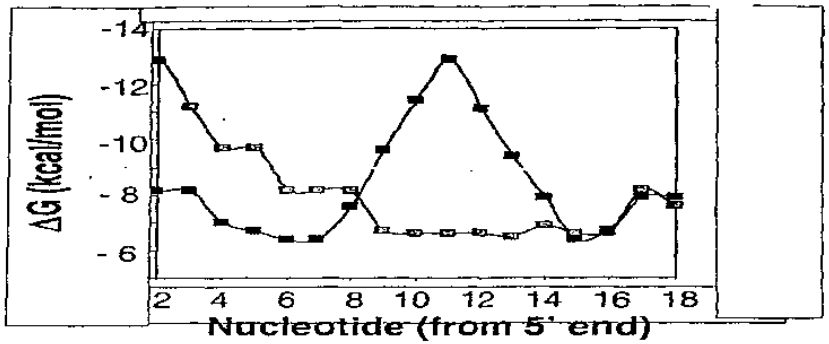


		T_m
SP-1070 (□)	CUGGUGAGCUGGCCCGCCCdTdT TdTdGACCACUCGACCGGGCGGG	77.4
SP-1260 (○)	AACAUCCGGCCGGGCGCCGdTdT TdTdUUGUAGGCCGGCCCGCGGC	83.6

Figure 19

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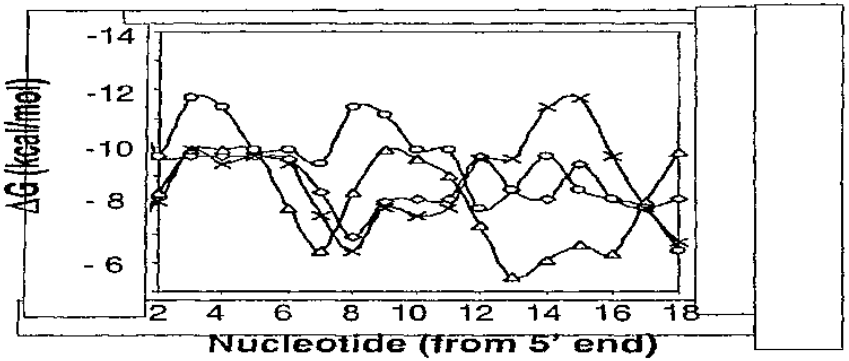
B-1



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TdTdGUCUUCAGGCCCAAGAGGA

SP-155 (■) ACCUCAUCAUCUUCCUGGGdTdT
TdTdUGGAGUAGUAGAAGGACCC

B-2



SP-500 (○) GGCGAGGCGUGCUGCACUCdTdT
TdTdCCGCUCCGCACGACGUGAG

SP-1113 (x) GUGGGAGUGGUCGGCAGUGdTdT
TdTdCACCCUCACCAGCCGUCAC

SP-147 (◇) UCUGGUGCAGGAAUGGCUGdTdT
TdTdAGACCACGUCCUUACCGAC

SP-1271 (△) CGGAUGUUACCGAGAGCGAdTdT
TdTdGCCUACAAUGGCUCUCGCU

Figure 19

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C

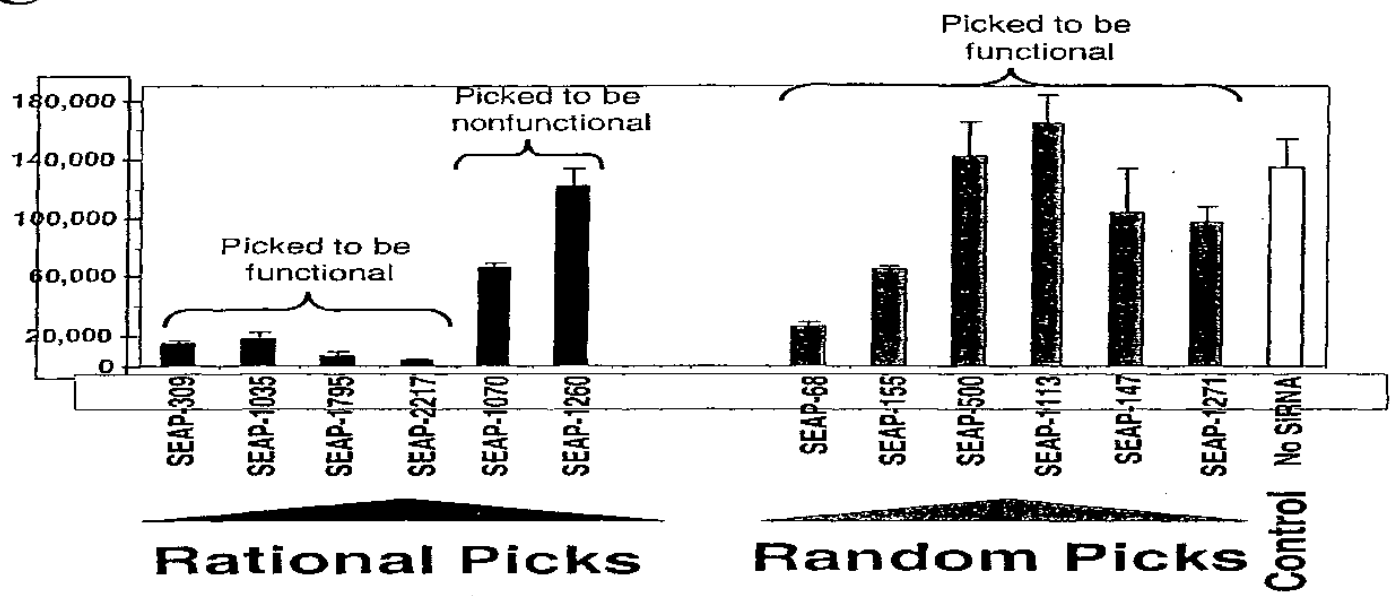


Figure 19

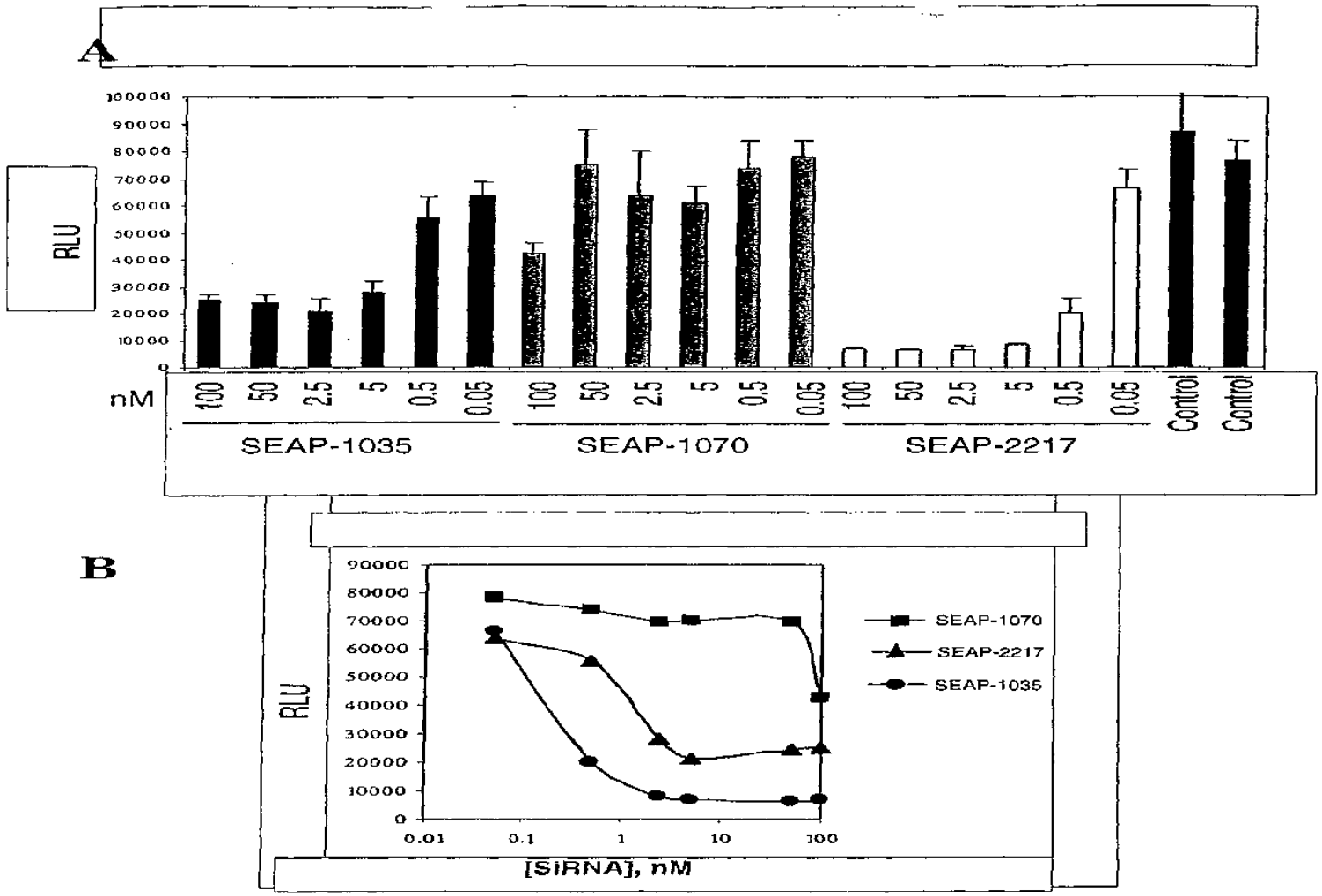


Figure 20

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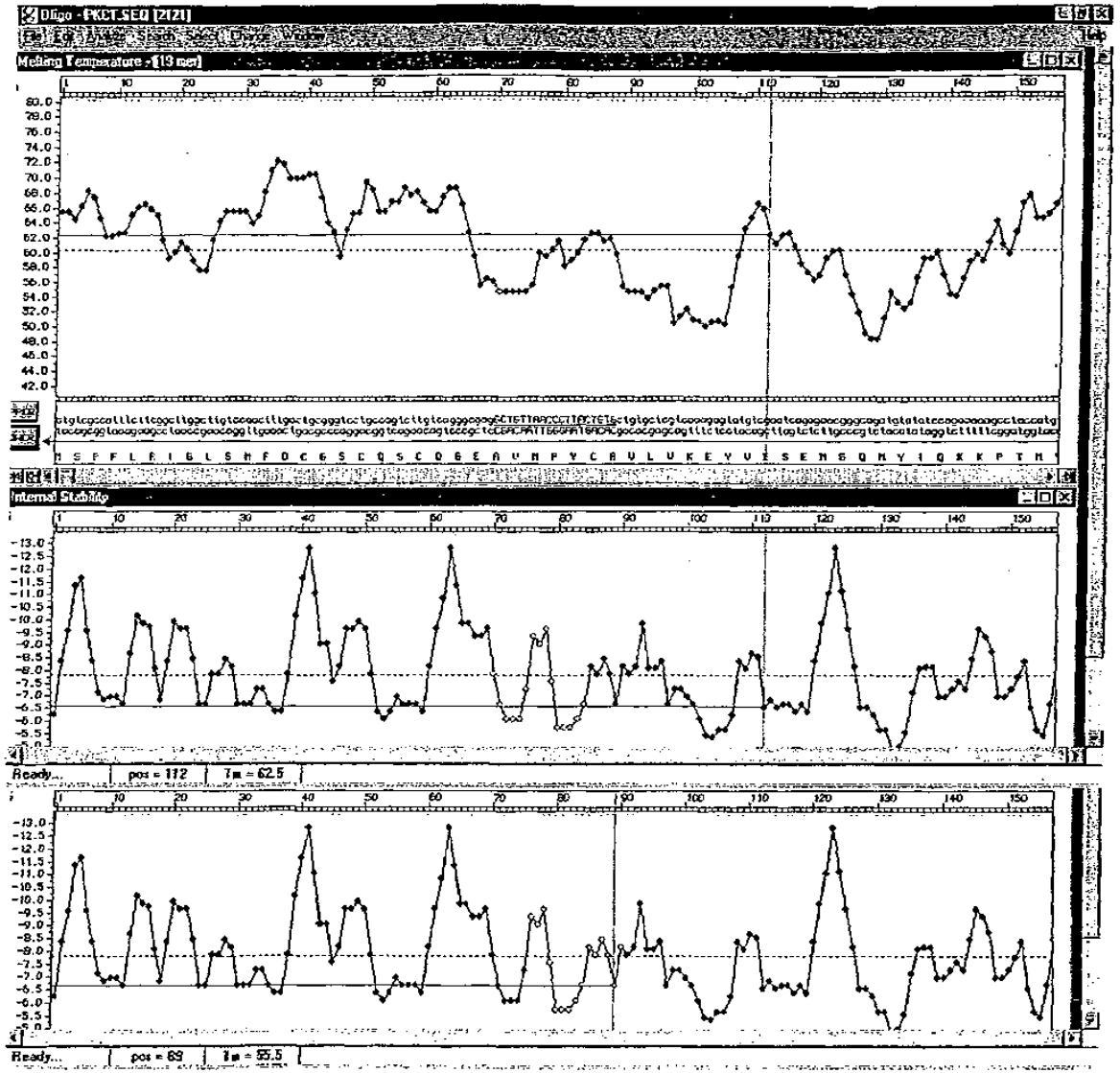
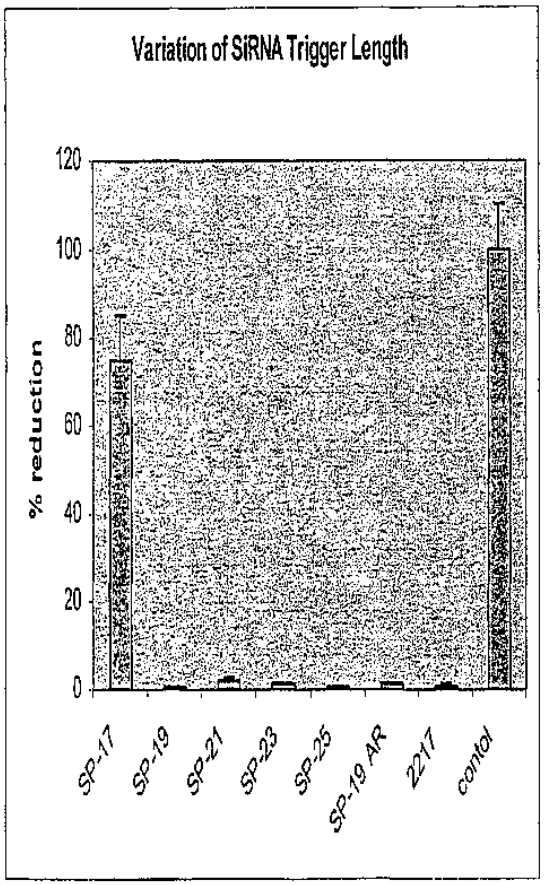


Figure 21

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SP-17 UGAUGUAUGUCAGAGAG**dTdT**
TdTdACUACAUACAGUCUCUC

SP-19 (2217) GUGAUGUAUGUCAGAGAGU**dTdT**
TdTdCACUACAUACAGUCUCUCA

SP-21 UGUGAUGUAUGUCAGAGAG**dTdT**
TdTdACUACAUACAGUCUCUCAC

SP-23 CUGUGAUGUAUGUCAGAGAGU**dTdT**
TdTdGACUACAUACAGUCUCUCACA

SP-25 GCUGUGAUGUAUGUCAGAGAGUGUU**dTdT**
TdTdCGACACUACAUACAGUCUCUCACAA

SP-19-AR GUGAUGUAUGUCAGAGAGUGU
GACACUACAUACAGUCUCUCA

Figure 22

SiRNA Triggers with Different End Structures
% reduction
120
100
80
60
40
20
0
SP-19
SP-19-AR
SP-19-Blunt
SP-19-AR-Blunt
SP-19-AR-5'-ext
SP-21-Blunt
2217
Control
3'-ext
Blunt
Blunt 5'-ext
3'-ext
SP-19
(2217)
GUGAUGUAUGUCAGAGAGUTT
TTCACUACAUACAGUCUCUCA
SP-19-AR
GUGAUGUAUGUCAGAGAGUGU
GACACUACAUACAGUCUCUCA
SP-19-Blunt
GUGAUGUAUGUCAGAGAGU
CACUACAUACAGUCUCUCA
SP-19-AR-Blunt
GUGAUGUAUGUCAGAGAGU
CACUACAUACAGUCUCUCA
SP-19-AR-5'-ext
CUGUGAUGUAUGUCAGAGAGU
CACUACAUACAGUCUCUCACA
SP-21-Blunt
CUGUGAUGUAUGUCAGAGAGUGU
GACACUACAUACAGUCUCUCACA

Figure 23

Chemically synthesized hairpins

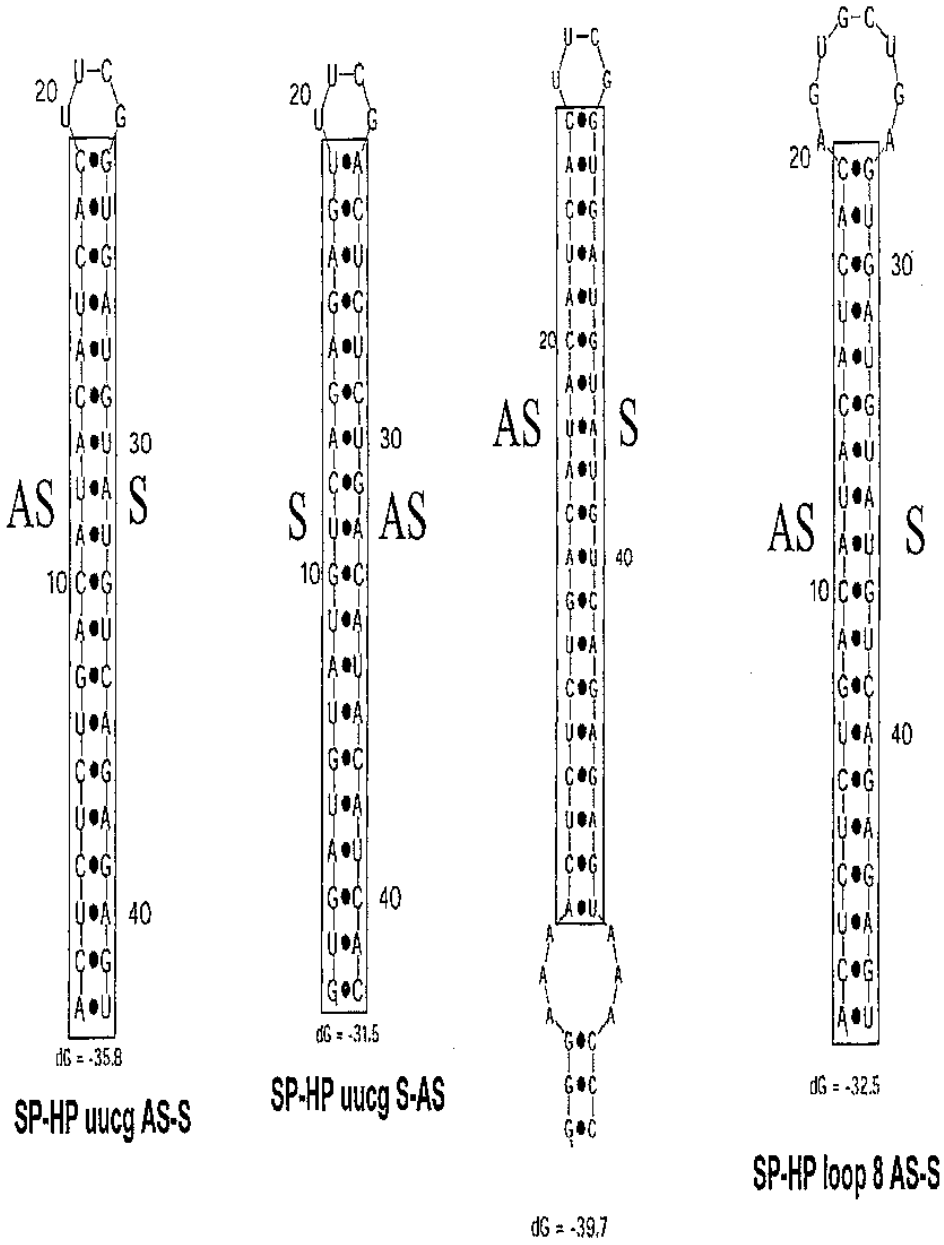


Figure 24

Hairpin SiRNA triggers
% reduction
120
100
80
60
40
20
0
SP-HP loop 8 AS-S
SP-HP uucg S-AS
SP-HP uucg AS-S
SP-HP uucg AS-S + 5' ext
2217
Control

Figure 25

SP-17-s UGAUGUAUGUCAGAGAGTT SP-17-as TTACUACAUACAGUCUCUC	SP-19-s GUGAUGUAUGUCAGAGAGUTT SP-17-as TTACUACAUACAGUCUCUC	SP-21-s UGUGAUGUAUGUCAGAGAGUGTT SP-17-as TTACUACAUACAGUCUCUC
SP-17-s UGAUGUAUGUCAGAGAGTT SP-19-as TTCACUACAUACAGUCUCUCA	SP-19-s GUGAUGUAUGUCAGAGAGUTT SP-19-as TTCACUACAUACAGUCUCUCA	SP-21-s UGUGAUGUAUGUCAGAGAGUGTT SP-19-as TTCACUACAUACAGUCUCUCA
SP-17-s UGAUGUAUGUCAGAGAGTT SP-21-as TTACACUACAUACAGUCUCUCAC	SP-19-s GUGAUGUAUGUCAGAGAGUTT SP-21-as TTACACUACAUACAGUCUCUCAC	SP-21-s UGUGAUGUAUGUCAGAGAGUGTT SP-21-as TTACACUACAUACAGUCUCUCAC
SP-17-s UGAUGUAUGUCAGAGAGTT SP-23-as TTGACACUACAUACAGUCUCUCACA	SP-19-s GUGAUGUAUGUCAGAGAGUTT SP-23-as TTGACACUACAUACAGUCUCUCACA	SP-21-s UGUGAUGUAUGUCAGAGAGUGTT SP-23-as TTGACACUACAUACAGUCUCUCACA
SP-17-s UGAUGUAUGUCAGAGAGTT SP-25-as TTCGACACUACAUACAGUCUCUCACAA	SP-19-s GUGAUGUAUGUCAGAGAGUTT SP-25-as TTCGACACUACAUACAGUCUCUCACAA	SP-21-s UGUGAUGUAUGUCAGAGAGUGTT SP-25-as TTCGACACUACAUACAGUCUCUCACAA
SP-17-s UGAUGUAUGUCAGAGAGTT SP-19 RNA-as GACACUACAUACAGUCUCUCA	SP-19-s GUGAUGUAUGUCAGAGAGUTT SP-19 RNA-as GACACUACAUACAGUCUCUCA	SP-21-s UGUGAUGUAUGUCAGAGAGUGTT SP-19 RNA-as GACACUACAUACAGUCUCUCA
SP-17-s UGAUGUAUGUCAGAGAGTT SP-19-5'-as CACUACAUACAGUCUCUCACA	SP-19-s GUGAUGUAUGUCAGAGAGUTT SP-19-5'-as CACUACAUACAGUCUCUCACA	SP-21-s UGUGAUGUAUGUCAGAGAGUGTT SP-19-5'-as CACUACAUACAGUCUCUCACA
SP-17-s UGAUGUAUGUCAGAGAGTT SP-19blun-as CACUACAUACAGUCUCUCA	SP-19-s GUGAUGUAUGUCAGAGAGUTT SP-19blun-as CACUACAUACAGUCUCUCA	SP-21-s UGUGAUGUAUGUCAGAGAGUGTT SP-19blun-as CACUACAUACAGUCUCUCA

Figure 26A

SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT SP-17-as TTACUACAUACAGUCUCUC	SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT SP-17-as TTACUACAUACAGUCUCUC
SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT SP-19-as TTCACUACAUACAGUCUCUCA	SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT SP-19-as TTCACUACAUACAGUCUCUCA
SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT SP-21-as TTACACUACAUACAGUCUCUCAC	SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT SP-21-as TTACACUACAUACAGUCUCUCAC
SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT SP-23-as TTGACACUACAUACAGUCUCUCACA	SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT SP-23-as TTGACACUACAUACAGUCUCUCACA
SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT SP-25-as TTCGACACUACAUACAGUCUCUCACAA	SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT SP-25-as TTCGACACUACAUACAGUCUCUCACAA
SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT SP-19 RNA-as GACACUACAUACAGUCUCUCA	SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT SP-19 RNA-as GACACUACAUACAGUCUCUCA
SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT SP-19-5'-as CACUACAUACAGUCUCUCACA	SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT SP-19-5'-as CACUACAUACAGUCUCUCACA
SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT SP-19blunt-as CACUACAUACAGUCUCUCA	SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT SP-19blunt-as CACUACAUACAGUCUCUCA

Figure 26B

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SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU SP-17-as TTACUACAUACAGUCUCUC	SP-19-5′-s CUGUGAUGUAUGUCAGAGAGT SP-17-as TTACUACAUACAGUCUCUC	SP-19blunt-s GUGAUGUAUGUCAGAGAGT SP-17-as TTACUACAUACAGUCUCUC
SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU SP-19-as TTCACUACAUACAGUCUCUCA	SP-19-5′-s CUGUGAUGUAUGUCAGAGAGT SP-19-as TTCACUACAUACAGUCUCUCA	SP-19blunt-s GUGAUGUAUGUCAGAGAGT SP-19-as TTCACUACAUACAGUCUCUCA
SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU SP-21-as TTACACUACAUACAGUCUCUCAC	SP-19-5′-s CUGUGAUGUAUGUCAGAGAGT SP-21-as TTACACUACAUACAGUCUCUCAC	SP-19blunt-s GUGAUGUAUGUCAGAGAGT SP-21-as TTACACUACAUACAGUCUCUCAC
SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU SP-23-as TTGACACUACAUACAGUCUCUCACA	SP-19-5′-s CUGUGAUGUAUGUCAGAGAGT SP-23-as TTGACACUACAUACAGUCUCUCACA	SP-19blunt-s GUGAUGUAUGUCAGAGAGT SP-23-as TTGACACUACAUACAGUCUCUCACA
SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU SP-25-as TTCGACACUACAUACAGUCUCUCACAA	SP-19-5′-s CUGUGAUGUAUGUCAGAGAGT SP-25-as TTCGACACUACAUACAGUCUCUCACAA	SP-19blunt-s GUGAUGUAUGUCAGAGAGT SP-25-as TTCGACACUACAUACAGUCUCUCACAA
SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU SP-19 RNA-as GACACUACAUACAGUCUCUCA	SP-19-5′-s CUGUGAUGUAUGUCAGAGAGT SP-19 RNA-as GACACUACAUACAGUCUCUCA	SP-19blunt-s GUGAUGUAUGUCAGAGAGT SP-19 RNA-as GACACUACAUACAGUCUCUCA
SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU SP-19-5′-as CACUACAUACAGUCUCUCACA	SP-19-5′-s CUGUGAUGUAUGUCAGAGAGT SP-19-5′-as CACUACAUACAGUCUCUCACA	SP-19blunt-s GUGAUGUAUGUCAGAGAGT SP-19-5′-as CACUACAUACAGUCUCUCACA
SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU SP-19blunt-as CACUACAUACAGUCUCUCA	SP-19-5′-s CUGUGAUGUAUGUCAGAGAGT SP-19blunt-as CACUACAUACAGUCUCUCA	SP-19blunt-s GUGAUGUAUGUCAGAGAGT SP-19blunt-as CACUACAUACAGUCUCUCA

Figure 26C

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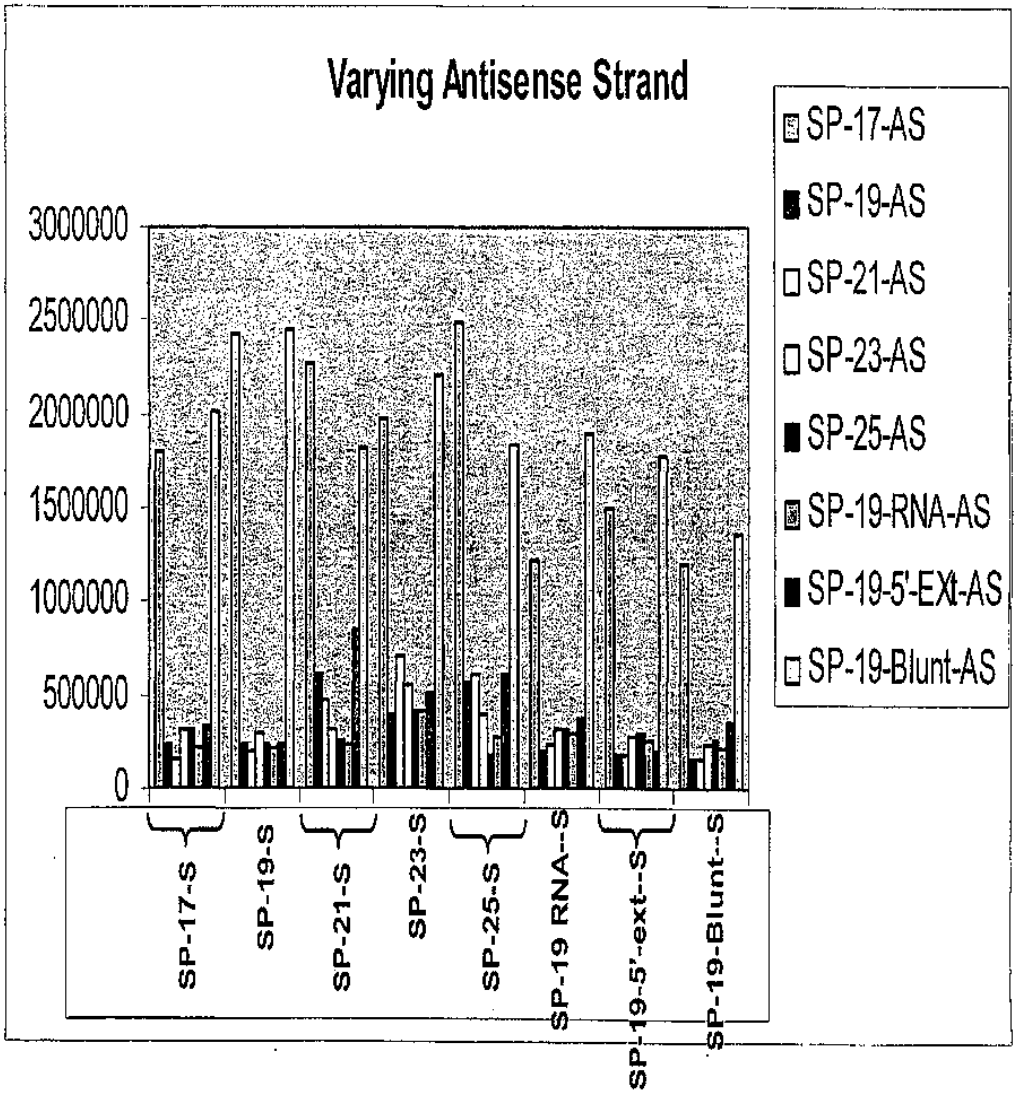

Varying Antisense Strand
SP-17-AS
SP-19-AS
SP-21-AS
SP-23-AS
SP-25-AS
SP-19-RNA-AS
SP-19-5'-EXt-AS
SP-19-Blunt-AS
3000000
2500000
2000000
1500000
1000000
500000
0
SP-17-S
SP-19-S
SP-21-S
SP-23-S
SP-25-S
SP-19 RNA--S
SP-19-5'-ext--S
SP-19-Blunt--S

Figure 27

SP-17-s UGAUGUAUGUCAGAGAGTT SP-17-as TTACUACAUACAGUCUCUC	SP-17-s UGAUGUAUGUCAGAGAGTT SP-19-as TTCACUACAUACAGUCUCUCA	SP-17-s UGAUGUAUGUCAGAGAGTT SP-21-as TTACACUACAUACAGUCUCUCAC
SP-19-s GUGAUGUAUGUCAGAGAGUTT SP-17-as TTACUACAUACAGUCUCUC	SP-19-s GUGAUGUAUGUCAGAGAGUTT SP-19-as TTCACUACAUACAGUCUCUCA	SP-19-s GUGAUGUAUGUCAGAGAGUTT SP-21-as TTACACUACAUACAGUCUCUCAC
SP-21-s UGUGAUGUAUGUCAGAGAGUGTT SP-17-as TTACUACAUACAGUCUCUC	SP-21-s UGUGAUGUAUGUCAGAGAGUGTT SP-19-as TTCACUACAUACAGUCUCUCA	SP-21-s UGUGAUGUAUGUCAGAGAGUGTT SP-21-as TTACACUACAUACAGUCUCUCAC
SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT SP-17-as TTACUACAUACAGUCUCUC	SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT SP-19-as TTCACUACAUACAGUCUCUCA	SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT SP-21-as TTACACUACAUACAGUCUCUCAC
SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT SP-17-as TTACUACAUACAGUCUCUC	SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT SP-19-as TTCACUACAUACAGUCUCUCA	SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT SP-21-as TTACACUACAUACAGUCUCUCAC
SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU SP-17-as TTACUACAUACAGUCUCUC	SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU SP-19-as TTCACUACAUACAGUCUCUCA	SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU SP-21-as TTACACUACAUACAGUCUCUCAC
SP-19-5'-s CUGUGAUGUAUGUCAGAGAGT SP-17-as TTACUACAUACAGUCUCUC	SP-19-5'-s CUGUGAUGUAUGUCAGAGAGT SP-19-as TTCACUACAUACAGUCUCUCA	SP-19-5'-s CUGUGAUGUAUGUCAGAGAGT SP-21-as TTACACUACAUACAGUCUCUCAC
SP-19blunt-s GUGAUGUAUGUCAGAGAGT SP-17-as TTACUACAUACAGUCUCUC	SP-19blunt-s GUGAUGUAUGUCAGAGAGT SP-19-as TTCACUACAUACAGUCUCUCA	SP-19blunt-s GUGAUGUAUGUCAGAGAGT SP-21-as TTACACUACAUACAGUCUCUCAC

Figure 28A

10x Genomics, Inc. Exhibit 2005
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SP-17-s UGAUGUAUGUCAGAGAGTT	SP-17-s UGAUGUAUGUCAGAGAGTT	SP-17-s UGAUGUAUGUCAGAGAGTT
SP-23-as TTGACACUACAUACAGUCUCUCACA	SP-25-as TTCGACACUACAUACAGUCUCUCACAA	SP-19 RNA-as GACACUACAUACAGUCUCUCA
SP-19-s GUGAUGUAUGUCAGAGAGUTT	SP-19-s GUGAUGUAUGUCAGAGAGUTT	SP-19-s GUGAUGUAUGUCAGAGAGUTT
SP-23-as TTGACACUACAUACAGUCUCUCACA	SP-25-as TTCGACACUACAUACAGUCUCUCACAA	SP-19 RNA-as GACACUACAUACAGUCUCUCA
SP-21-s UGUGAUGUAUGUCAGAGAGUGTT	SP-21-s UGUGAUGUAUGUCAGAGAGUGTT	SP-21-s UGUGAUGUAUGUCAGAGAGUGTT
SP-23-as TTGACACUACAUACAGUCUCUCACA	SP-25-as TTCGACACUACAUACAGUCUCUCACAA	SP-19 RNA-as GACACUACAUACAGUCUCUCA
SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT	SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT	SP-23-s CUGUGAUGUAUGUCAGAGAGUGUTT
SP-23-as TTGACACUACAUACAGUCUCUCACA	SP-25-as TTCGACACUACAUACAGUCUCUCACAA	SP-19 RNA-as GACACUACAUACAGUCUCUCA
SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT	SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT	SP-25-s GCUGUGAUGUAUGUCAGAGAGUGUUTT
SP-23-as TTGACACUACAUACAGUCUCUCACA	SP-25-as TTCGACACUACAUACAGUCUCUCACAA	SP-19 RNA-as GACACUACAUACAGUCUCUCA
SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU	SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU	SP-19 RNA-s GUGAUGUAUGUCAGAGAGUGU
SP-23-as TTGACACUACAUACAGUCUCUCACA	SP-25-as TTCGACACUACAUACAGUCUCUCACAA	SP-19 RNA-as GACACUACAUACAGUCUCUCA
SP-19-5'-s CUGUGAUGUAUGUCAGAGAGT	SP-19-5'-s CUGUGAUGUAUGUCAGAGAGT	SP-19-5'-s CUGUGAUGUAUGUCAGAGAGT
SP-23-as TTGACACUACAUACAGUCUCUCACA	SP-25-as TTCGACACUACAUACAGUCUCUCACAA	SP-19 RNA-as GACACUACAUACAGUCUCUCA
SP-19blunt-s GUGAUGUAUGUCAGAGAGT	SP-19blunt-s GUGAUGUAUGUCAGAGAGT	SP-19blunt-s GUGAUGUAUGUCAGAGAGT
SP-23-as TTGACACUACAUACAGUCUCUCACA	SP-25-as TTCGACACUACAUACAGUCUCUCACAA	SP-19 RNA-as GACACUACAUACAGUCUCUCA

Figure 28B

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SP-17-s	UGAUGUAUGUCAGAGAGTT
SP-19-5′-as	CACUACAUACAGUCUCUCACA
SP-19-s	GUGAUGUAUGUCAGAGAGUTT
SP-19-5′-as	CACUACAUACAGUCUCUCACA
SP-21-s	UGUGAUGUAUGUCAGAGAGUGTT
SP-19-5′-as	CACUACAUACAGUCUCUCACA
SP-23-s	CUGUGAUGUAUGUCAGAGAGUGUTT
SP-19-5′-as	CACUACAUACAGUCUCUCACA
SP-25-s	GCUGUGAUGUAUGUCAGAGAGUGUUTT
SP-19-5′-as	CACUACAUACAGUCUCUCACA
SP-19 RNA-s	GUGAUGUAUGUCAGAGAGUGU
SP-19-5′-as	CACUACAUACAGUCUCUCACA
SP-19-5′-s	CUGUGAUGUAUGUCAGAGAGT
SP-19-5′-as	CACUACAUACAGUCUCUCACA
SP-19blunt-s	GUGAUGUAUGUCAGAGAGT
SP-19-5′-as	CACUACAUACAGUCUCUCACA

SP-17-s	UGAUGUAUGUCAGAGAGTT
SP-19blunt-as	CACUACAUACAGUCUCUCA
SP-19-s	GUGAUGUAUGUCAGAGAGUTT
SP-19blunt-as	CACUACAUACAGUCUCUCA
SP-21-s	UGUGAUGUAUGUCAGAGAGUGTT
SP-19blunt-as	CACUACAUACAGUCUCUCA
SP-23-s	CUGUGAUGUAUGUCAGAGAGUGUTT
SP-19blunt-as	CACUACAUACAGUCUCUCA
SP-25-s	GCUGUGAUGUAUGUCAGAGAGUGUUTT
SP-19blunt-as	CACUACAUACAGUCUCUCA
SP-19 RNA-s	GUGAUGUAUGUCAGAGAGUGU
SP-19blunt-as	CACUACAUACAGUCUCUCA
SP-19-5′-s	CUGUGAUGUAUGUCAGAGAGT
SP-19blunt-as	CACUACAUACAGUCUCUCA
SP-19blunt-s	GUGAUGUAUGUCAGAGAGT
SP-19blunt-as	CACUACAUACAGUCUCUCA

Figure 28C

10x Genomics, Inc. Exhibit 2005
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Varying the sense strand
SP-17-Sense
SP-19-Sense
SP-21-Sense
SP-23-Sense
SP-25-Sense
SP-19-RNA-Sense
SP-19-5'-ext-Sense
SP-19-Blunt-Sense
3000000
2500000
2000000
1500000
1000000
500000
0
SP-17-AS
SP-19-AS
SP-21-AS
SP-23-AS
SP-25-AS
SP-19-RNA-AS
SP-19-5'-ext--AS
SP-19-Blunt--AS

Figure 29

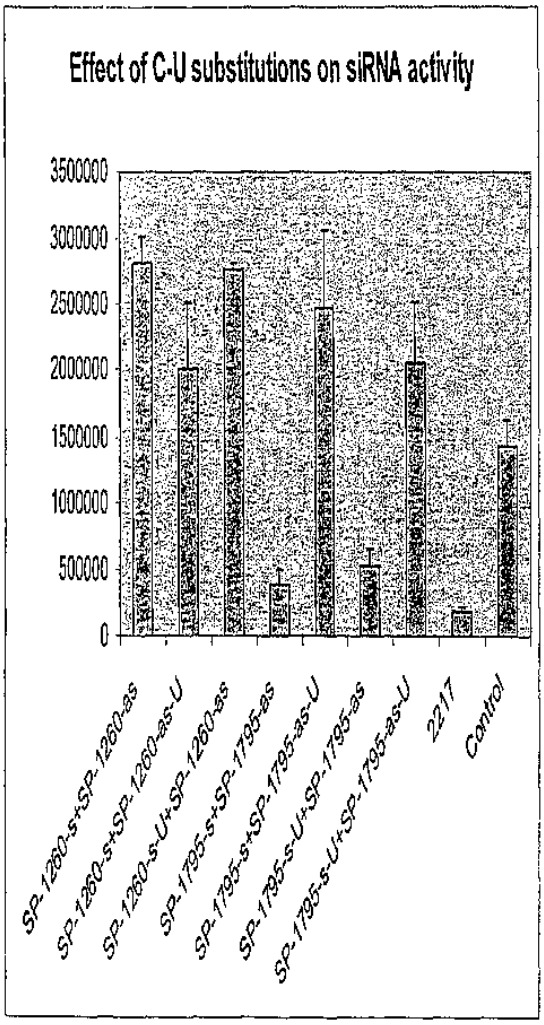


Figure 30

SP-1260-s	CGGCGCCCGGCCGGAUGUUdTdT
SP-1260-as	TdTdGCCGCGGGCCGGCCUACAA
SP-1260-s	CGGCGCCCGGCCGGAUGUUdTdT
SP-1260-as-U	TdTdGUUGUGGGUUGGUUUACAA
SP-1260-s-U	UGGUGUUUGGUUGGAUGUUdTdT
SP-1260-as	TdTdGCCGCGGGCCGGCCUACAA
SP-1795-s	GAGUUGUGGCCCGUUGUCAdTdT
SP-1795-as	TdTdCUCAACACCGGGCAACAGU
SP-1795-s	GAGUUGUGGCCCGUUGUCAdTdT
SP-1795-as-U	TdTdUUUAAUAUUGGGUAAUAGU
SP-1795-s-U	GAGUUGUGGUUUGUUGUUAdTdT
SP-1795-as	TdTdCUCAACACCGGGCAACAGU
SP-1795-s-U	GAGUUGUGGUUUGUUGUUAdTdT
SP-1795-as-U	TdTdUUUAAUAUUGGGUAAUAGU
SP-19 (2217)	GUGAUGUAUGUCAGAGAGUdTdT TdTdCACUACAUACAGUCUCUCA

ASO & ds-SiRNAs for PKCθ

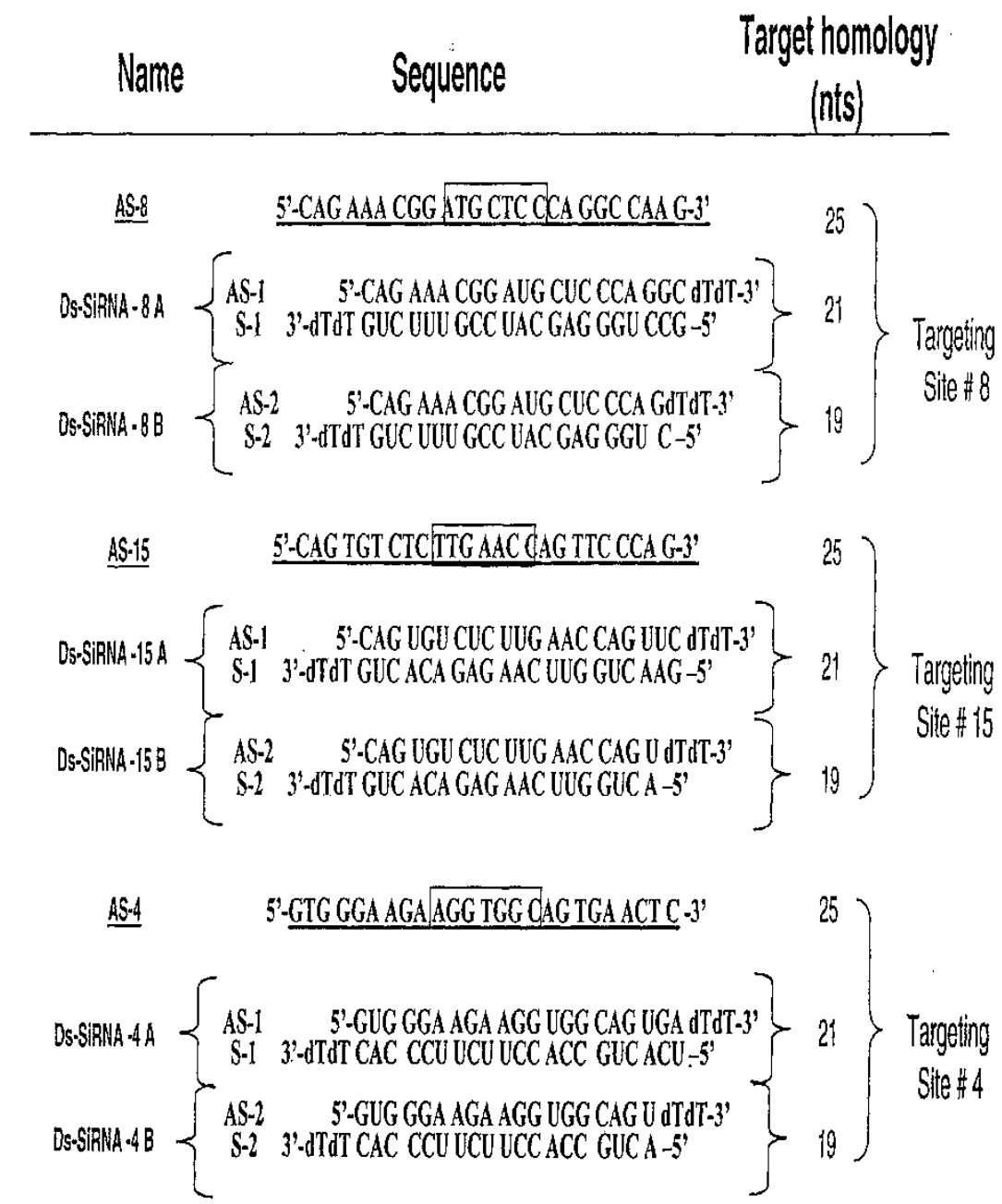


Figure - 31

10x Genomics, Inc. Exhibit 2005
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Unimolecular ss-SiRNAs for PKCθ

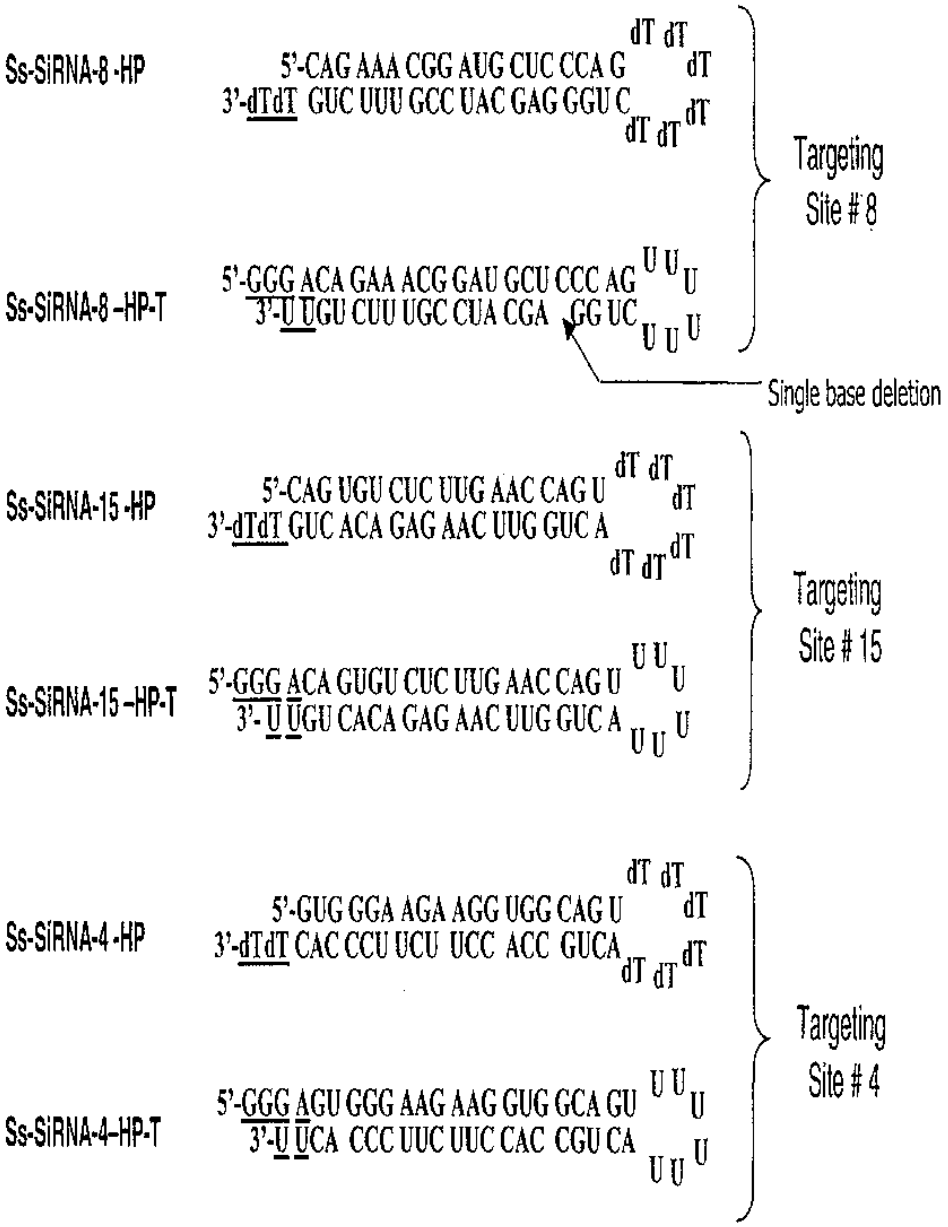


Figure - 32

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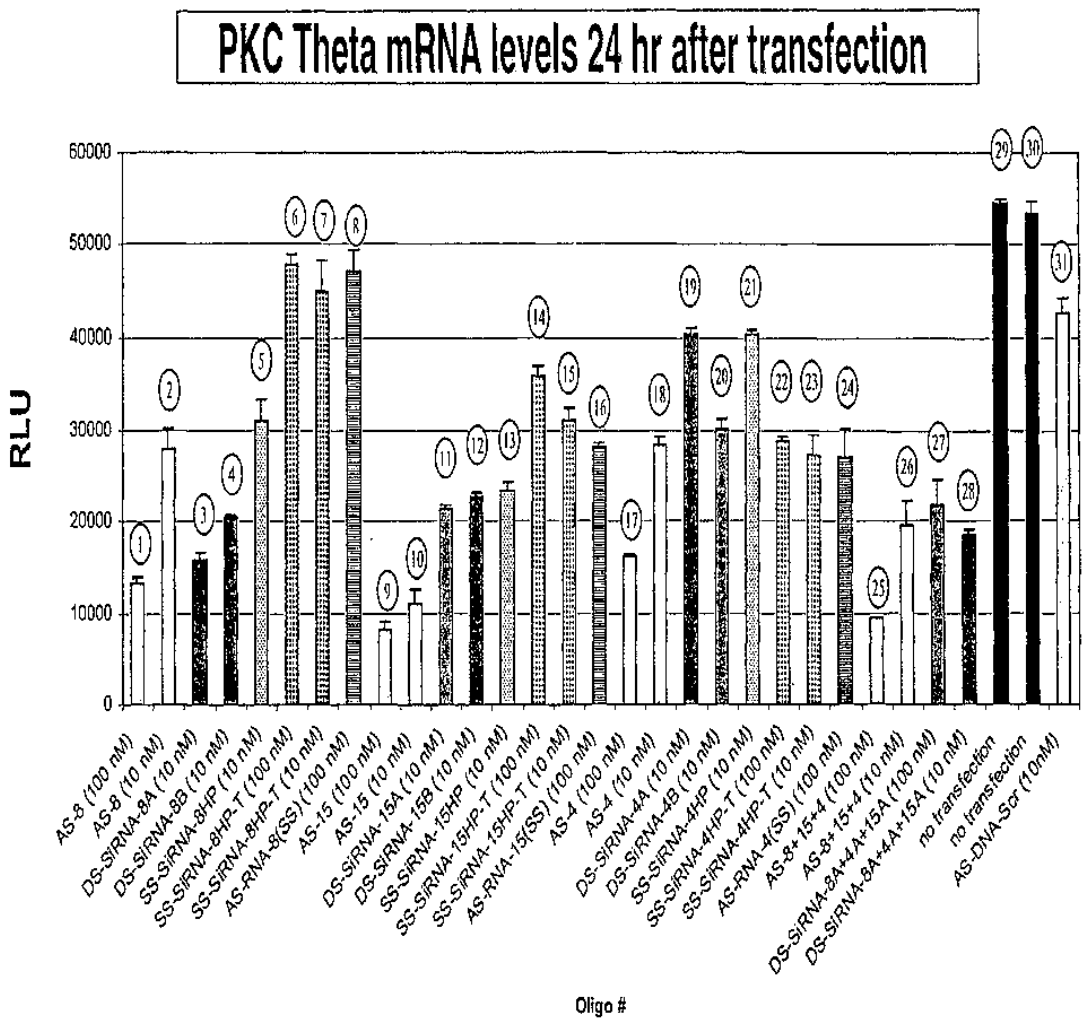

PKC Theta mRNA levels 24 hr after transfection
RLU
60000
50000
40000
30000
20000
10000
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
AS-8 (100 nM)
AS-8 (10 nM)
DS-SiRNA-8A (10 nM)
DS-SiRNA-8B (10 nM)
SS-SiRNA-8HP (10 nM)
SS-SiRNA-8HP-T (100 nM)
SS-SiRNA-8HP-T (10 nM)
AS-RNA-8(SS) (100 nM)
AS-15 (100 nM)
AS-15 (10 nM)
DS-SiRNA-15A (10 nM)
DS-SiRNA-15B (10 nM)
SS-SiRNA-15HP (10 nM)
SS-SiRNA-15HP-T (100 nM)
SS-SiRNA-15HP-T (10 nM)
AS-RNA-15(SS) (100 nM)
AS-4 (100 nM)
AS-4 (10 nM)
DS-SiRNA-4A (10 nM)
DS-SiRNA-4B (10 nM)
SS-SiRNA-4HP (10 nM)
SS-SiRNA-4HP-T (100 nM)
SS-SiRNA-4HP-T (10 nM)
AS-RNA-4(SS) (100 nM)
AS-8+15+4 (100 nM)
AS-8+15+4 (10 nM)
DS-SiRNA-8A+4A+15A (100 nM)
DS-SiRNA-8A+4A+15A (10 nM)
no transfection
no transfection
AS-DNA-Scr (10nM)
Oligo #

Figure - 33

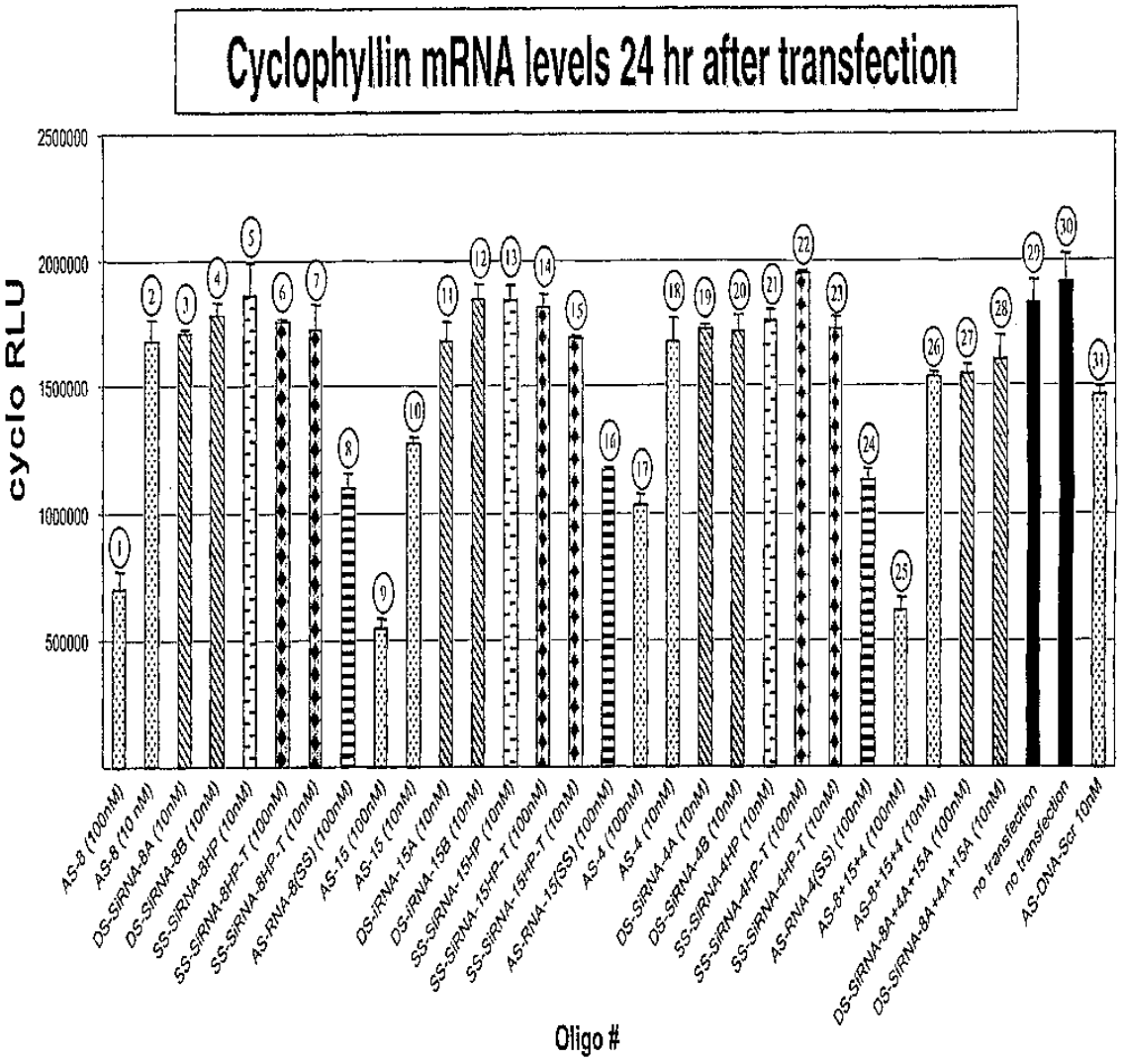

Cyclophyllin mRNA levels 24 hr after transfection
cyclo RLU
2500000
2000000
1500000
1000000
500000
Oligo #
AS-8 (100nM)
AS-8 (10 nM)
DS-SiRNA-8A (10nM)
DS-SiRNA-8B (10nM)
SS-SiRNA-8HP (10nM)
SS-SiRNA-8HP-T (100nM)
SS-SiRNA-8HP-T (10nM)
AS-RNA-8(SS) (100nM)
AS-15 (100nM)
AS-15 (10nM)
DS-iRNA-15A (10nM)
DS-iRNA-15B (10nM)
SS-SiRNA-15HP (10nM)
SS-SiRNA-15HP-T (100nM)
SS-SiRNA-15HP-T (10nM)
AS-RNA-15(SS) (100nM)
AS-4 (100nM)
AS-4 (10nM)
DS-SiRNA-4A (10nM)
DS-SiRNA-4B (10nM)
SS-SiRNA-4HP (10nM)
SS-SiRNA-4HP-T (100nM)
SS-SiRNA-4HP-T (10nM)
AS-RNA-4(SS) (100nM)
AS-8+15+4 (10nM)
AS-8+15+4 (100nM)
DS-SiRNA-8A+4A+15A (100nM)
DS-SiRNA-8A+4A+15A (10nM)
no transfection
no transfection
AS-DNA-Scr 10nM

Figure - 34

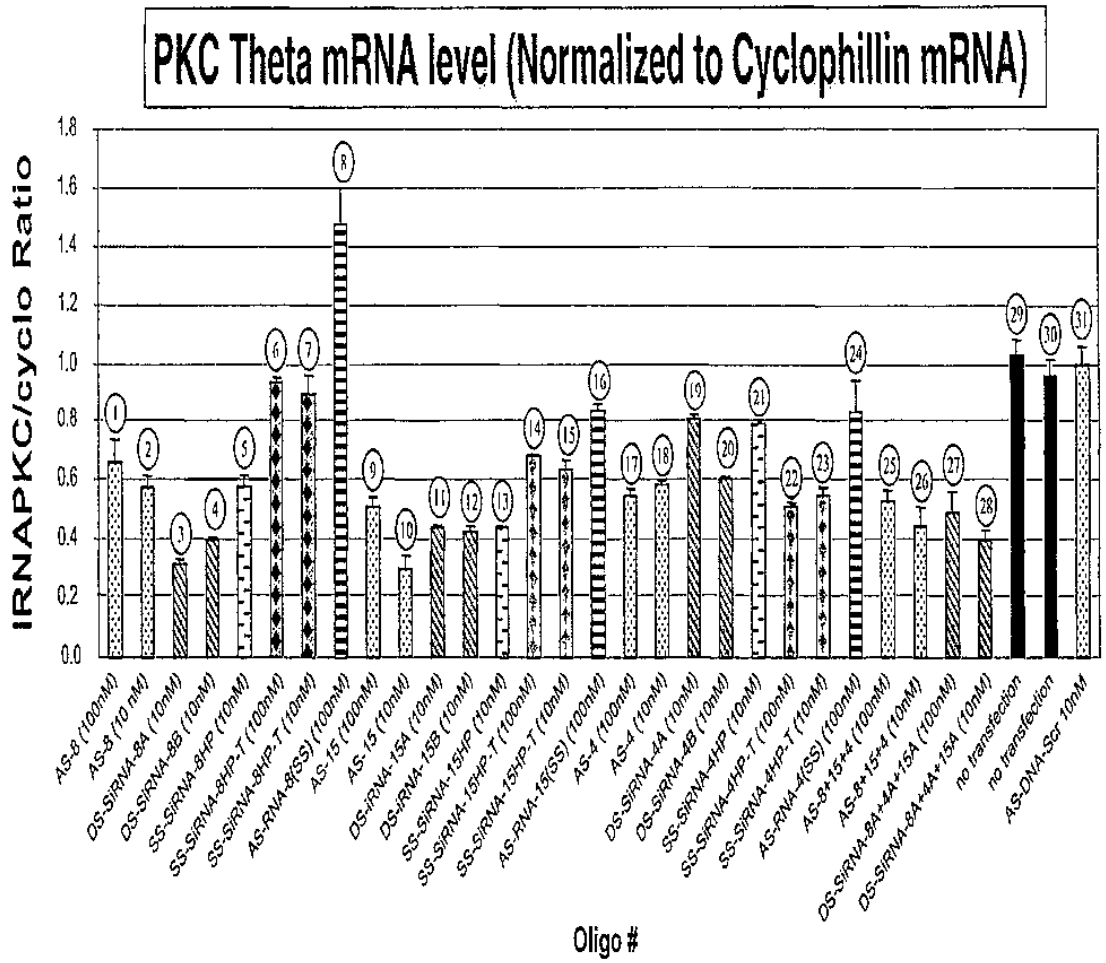

PKC Theta mRNA level (Normalized to Cyclophillin mRNA)
IRNAPKC/cyclo Ratio
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
AS-8 (100nM)
AS-8 (10 nM)
DS-SiRNA-8A (10nM)
DS-SiRNA-8B (10nM)
SS-SiRNA-8HP (10nM)
SS-SiRNA-8HP-T (100nM)
SS-SiRNA-8HP-T (10nM)
AS-RNA-8(SS) (100nM)
AS-15 (100nM)
AS-15 (10nM)
DS-iRNA-15A (10nM)
DS-iRNA-15B (10nM)
SS-SiRNA-15HP (10nM)
SS-SiRNA-15HP-T (100nM)
SS-SiRNA-15HP-T (10nM)
AS-RNA-15(SS) (100nM)
AS-4 (100nM)
AS-4 (10nM)
DS-SiRNA-4A (10nM)
DS-SiRNA-4B (10nM)
SS-SiRNA-4HP (10nM)
SS-SiRNA-4HP-T (100nM)
SS-SiRNA-4HP-T (10nM)
AS-RNA-4(SS) (100nM)
AS-8+15+4 (100nM)
AS-8+15+4 (10nM)
DS-SiRNA-8A+4A+15A (100nM)
DS-SiRNA-8A+4A+15A (10nM)
no transfection
no transfection
AS-DNA-Scr 10nM
Oligo #

Figure - 35

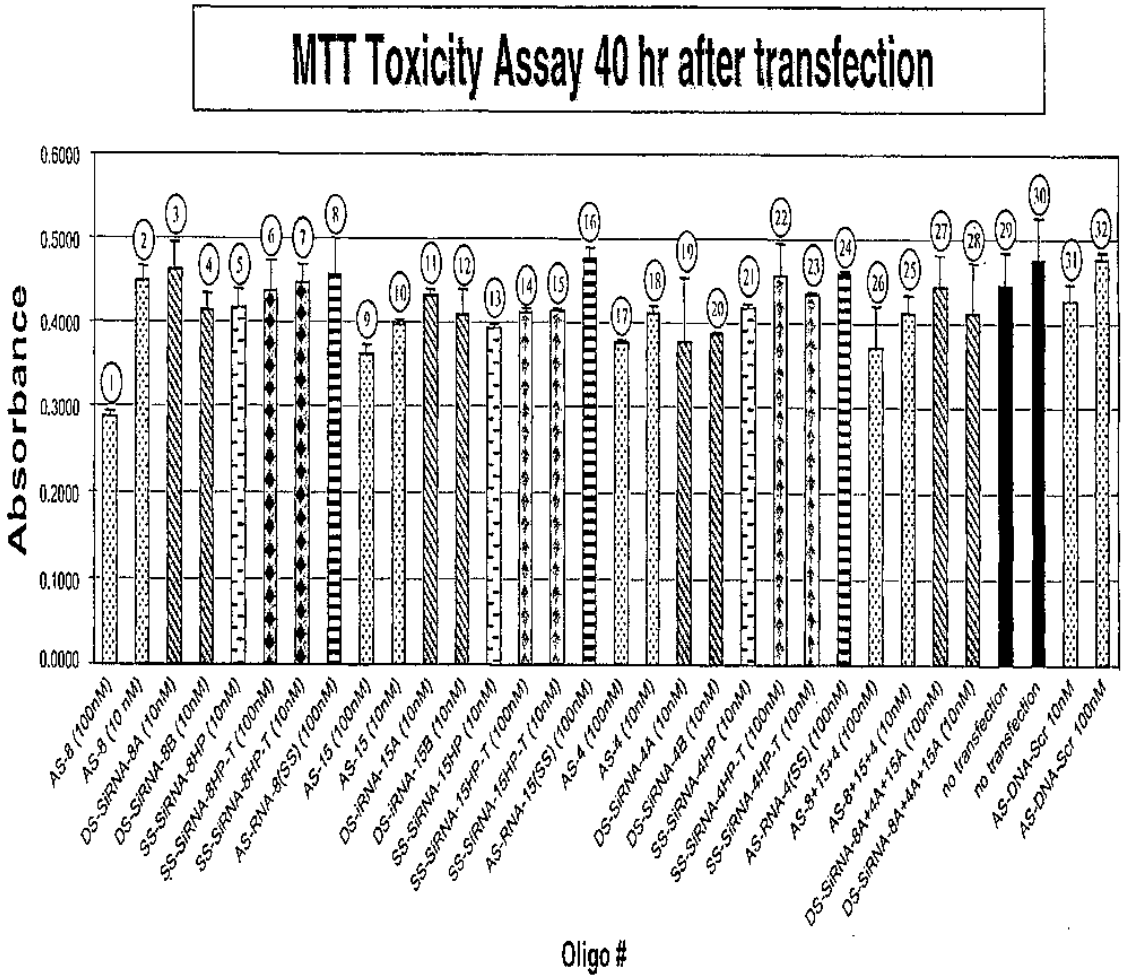

MTT Toxicity Assay 40 hr after transfection
Absorbance
0.6000
0.5000
0.4000
0.3000
0.2000
0.1000
0.0000
AS-8 (100nM)
AS-8 (10 nM)
DS-SiRNA-8A (10nM)
DS-SiRNA-8B (10nM)
SS-SiRNA-8HP (10nM)
SS-SiRNA-8HP-T (100nM)
SS-SiRNA-8HP-T (10nM)
AS-RNA-8(SS) (100nM)
AS-15 (100nM)
AS-15 (10nM)
DS-iRNA-15A (10nM)
DS-iRNA-15B (10nM)
SS-SiRNA-15HP (10nM)
SS-SiRNA-15HP-T (100nM)
SS-SiRNA-15HP-T (10nM)
AS-RNA-15(SS) (100nM)
AS-4 (100nM)
AS-4 (10nM)
DS-SiRNA-4A (10nM)
DS-SiRNA-4B (10nM)
SS-SiRNA-4HP (10nM)
SS-SiRNA-4HP-T (100nM)
SS-SiRNA-4HP-T (10nM)
AS-RNA-4(SS) (100nM)
AS-8+15+4 (100nM)
AS-8+15+4 (10nM)
DS-SiRNA-8A+4A+15A (100nM)
DS-SiRNA-8A+4A+15A (10nM)
no transfection
no transfection
AS-DNA-Scr 10nM
AS-DNA-Scr 100nM
Oligo #

Figure - 36

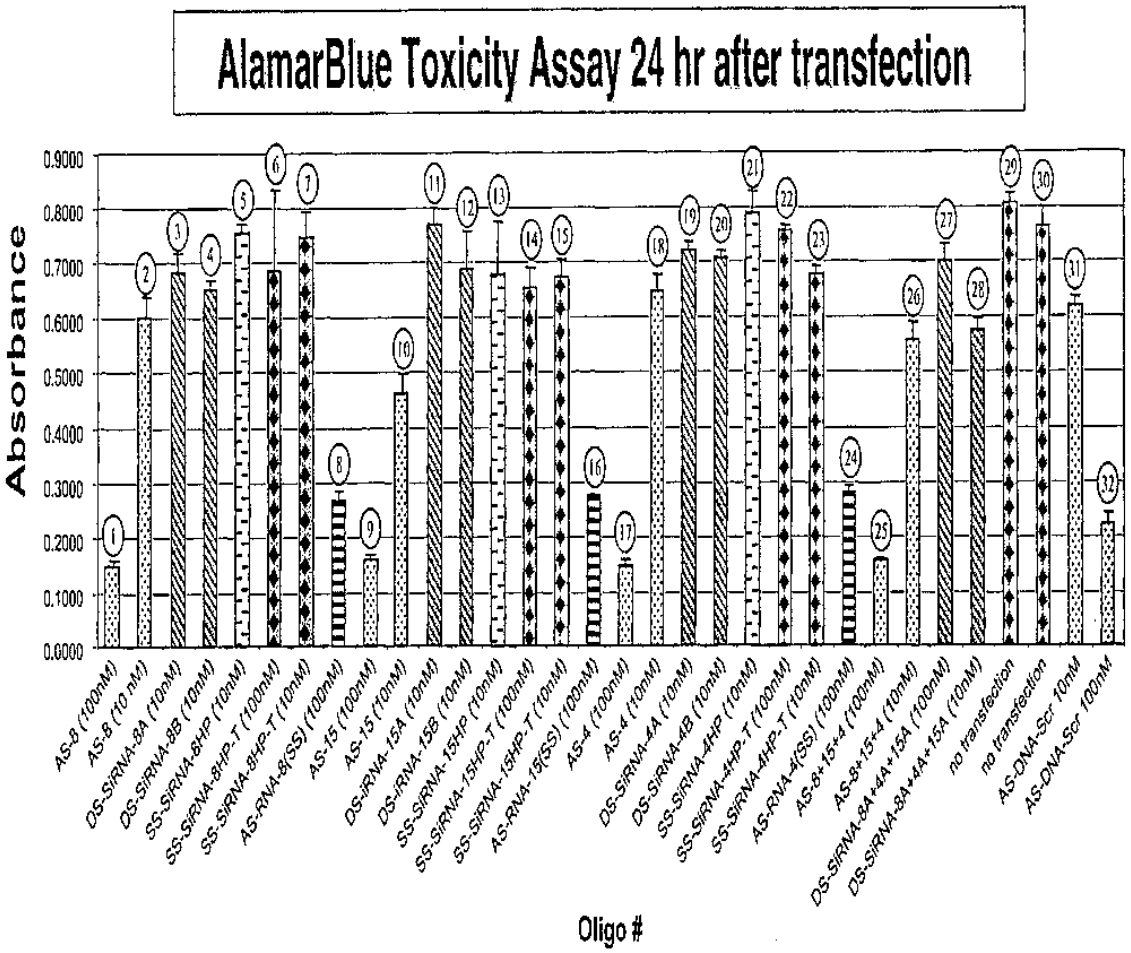
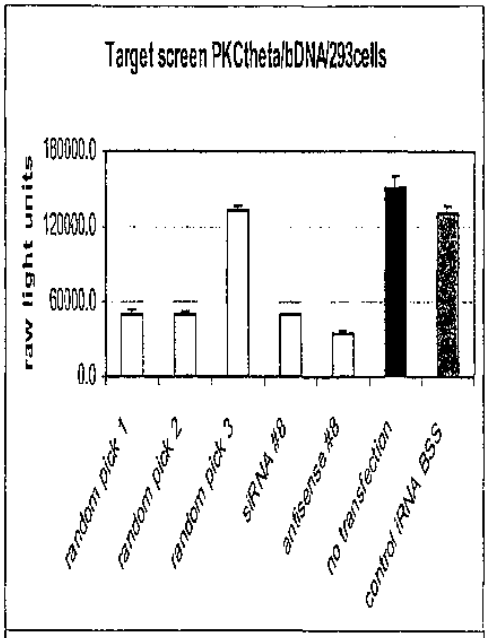

AlamarBlue Toxicity Assay 24 hr after transfection
Absorbance
0.9000
0.8000
0.7000
0.6000
0.5000
0.4000
0.3000
0.2000
0.1000
0.0000
AS-8 (100nM)
AS-8 (10 nM)
DS-SiRNA-8A (10nM)
DS-SiRNA-8B (10nM)
DS-SiRNA-8HP (10nM)
SS-SiRNA-8HP-T (100nM)
SS-SiRNA-8HP-T (10nM)
AS-RNA-8(SS) (100nM)
AS-15 (100nM)
AS-15 (10nM)
DS-iRNA-15A (10nM)
DS-iRNA-15B (10nM)
DS-iRNA-15HP (10nM)
SS-SiRNA-15HP-T (100nM)
SS-SiRNA-15HP-T (10nM)
SS-SiRNA-15(SS) (100nM)
AS-4 (100nM)
AS-4 (10nM)
DS-SiRNA-4A (10nM)
DS-SiRNA-4B (10nM)
DS-SiRNA-4HP (10nM)
SS-SiRNA-4HP-T (100nM)
SS-SiRNA-4HP-T (10nM)
AS-RNA-4(SS) (100nM)
AS-8+15+4 (100nM)
AS-8+15+4 (10nM)
DS-SiRNA-8A+4A+15A (100nM)
DS-SiRNA-8A+4A+15A (10nM)
no transfection
no transfection
AS-DNA-Scr 10nM
AS-DNA-Scr 100nM
Oligo #

Figure - 37

Reduction of PKCθ mRNA with randomly picked ds-SiRNAs

A



Randomly picked sequences

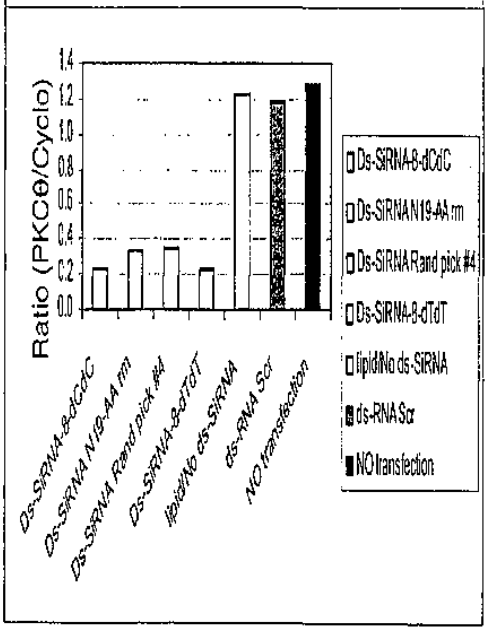
#1 5′-GGCUGUUAACCCUUACUGUGdTdT-3′
3′-dTdTCCGACAAUUGGGAAUGACAC

#2 5′-GCUGUUAACCCUUACUGUGCdTdT-3′
3′-dTdTCGACAAUUGGGAAUGACACG

#3 5′-UAACCCUUACUGUGCUGUGCdTdT-3′
3′-dTdTAUUGGGAAUGACACGACACG

#4 5′-AAGCACAACACAGUAAGGGdTdT-3′
3′-dTdTCGUGUUGUGUCAUUCCCAA

B



N19-AA-Rm

5′-ACAAUGAUCUGCAUGACUCdTdT-3′
3′-dTdTUUACUAGACGUACUGAGAA

#8-dCdC

5′-CAGAAACGGAUGCUCCCAGGCdCdC-3′
3′-dCdCGUCUUUGCCUACGAGGGUCCG

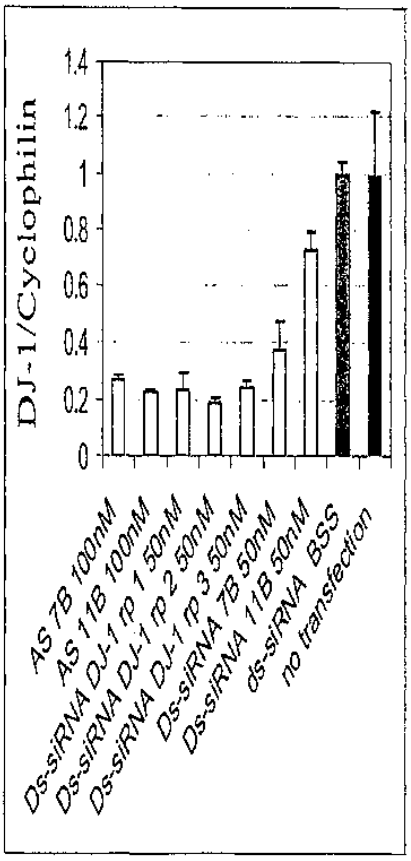
Ds-SiRNA BSS

5′- CUUCGCAUUGGCUCGCAAGCAdTdT

3′-dTdTGAAGCGUAACCGAGCGUUCGU

Figure - 38

Reduction of DJ-1 mRNA with ds-SiRNAs



Ds-SiRNA 7B

5′-UGGUCAUUUGUCCUGAUGCCdTdT-3′

3′-dTdTACCAGUAAACAGGACUACGG

Ds-SiRNA 11B

5′-UCUUCAAGGCUGGCAUCAGGAdTdT-3′

3′-dTdTAGAAGUUCCGACCGUAGUCCU

Ds-SiRNA DJ-1 rp-1

5′-UCUUCAAGGCUGGCAUCAGGAdTdT-3′

3′-dTdTAGAAGUUCCGACCGUAGUCCU

Ds-SiRNA DJ-1 rp-2

5′-AUUCUCAGAGUAGGUGUAAUGdTdT-3′

3′-dTdTUAAGAGUCUCAUCCACAUUAC

Ds-SiRNA DJ-1 rp-3

5′-GAACACAGUGAGAAUGGAUdTdT-3′

3′-dTdTCUUGUGUCACUCUUACCUA

AS 7B

5′-GGCATCAGGACAAATGACCACATCA-3′

AS 11B

5′-GACCTGCACAGATGGCGGCTATCAG-3′

Ds-SiRNA BSS

5′- CUUCGCAUUGGCUCGCAAGCAdTdT

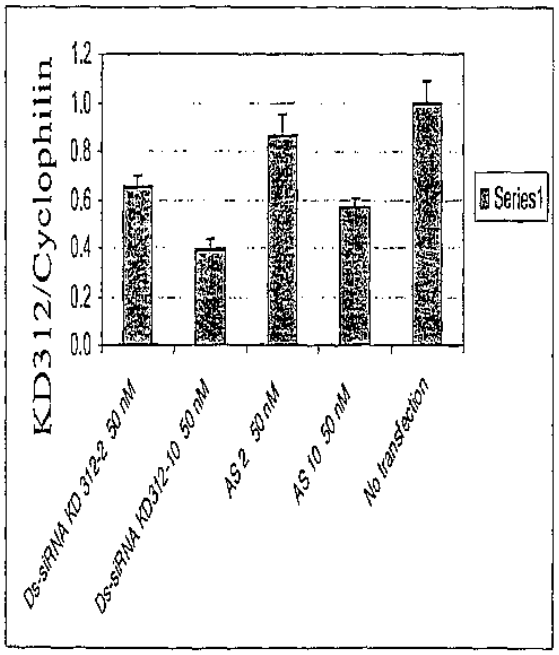
3′-dTdTGAAGCGUAACCGAGCGUUCGU

Figure - 39

WO 2004/061083
45/56
PCT/US2003/041377

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Reduction of KD312 mRNA with ds-SiRNAs



Ds-SiRNA KD312-2

5′-GCGGAUGGAGUAGAACUUCGGdTdT-3′
3′-dTdTCGCCUACCUCAUCUUGAACGC

Ds-SiRNA KD312-10

5′-CAGUACUGCACCGAGACCUUGdTdT-3′
3′-dTdTGUCAUGACGUGGCUCUGGAAC

AS 2

5′-GCGGATGGAGTAGAACTTGCGGTGG-3′

AS 10

5′-CAGTACTGCACCGAGACCTTGCGGT-3′

Figure - 40

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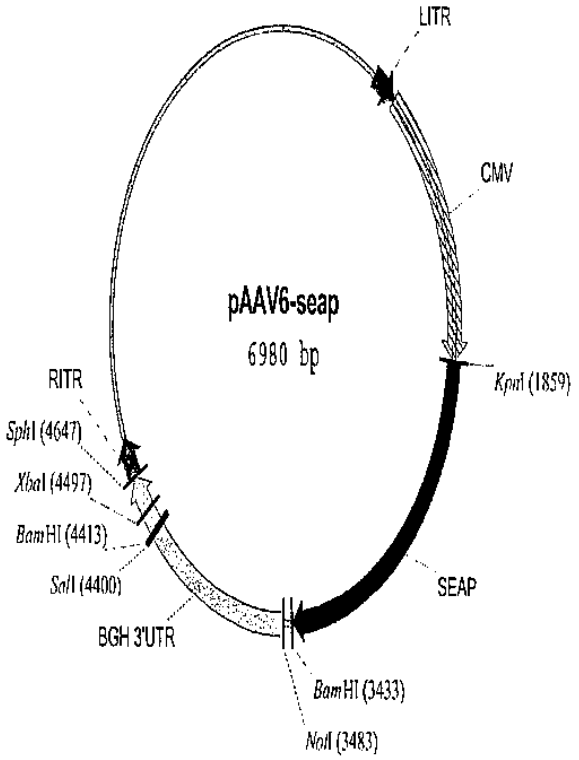
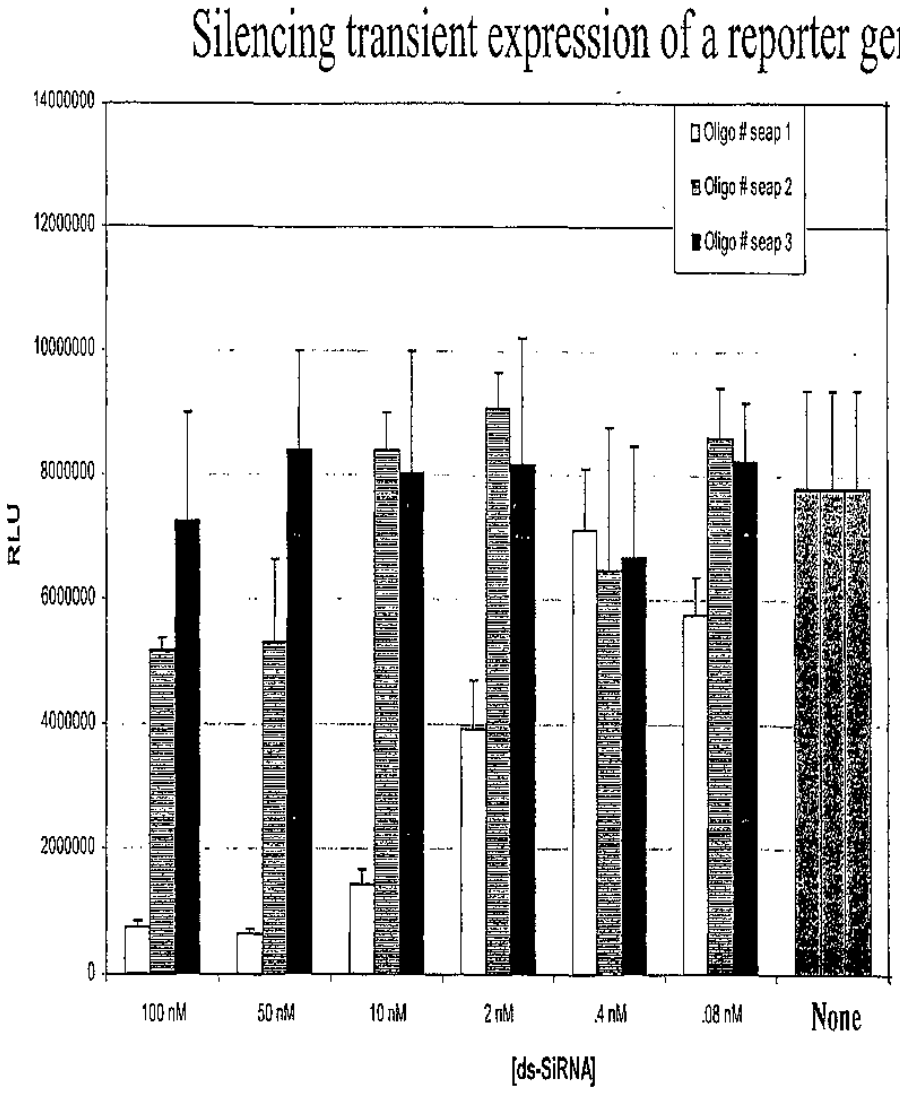

LITR
CMV
pAAV6-seap
6980 bp
KpnI (1859)
RITR
SphI (4647)
XbaI (4497)
BamHI (4413)
SalI (4400)
BGH 3'UTR
SEAP
BamHI (3433)
NotI (3483)

Figure - 41



Seap-1
5′-AGGAGAACCCGGACUUCUGdTdT-3′
3′-dTdTUCCUCUUGGGCCUGAAGAC

Seap-2
5′-GAGUGCAGCACGCCUCGCCdTdT-3′
3′-dTdTCUCACGUCGUGCGGAGCGG

Seap-3
5′-CACUGCCGACCACUCCCACdTdT-3′
3′-dTdTGUGACGGCUGGUGAGGGTG

Figure - 42

Persistence of RNAi

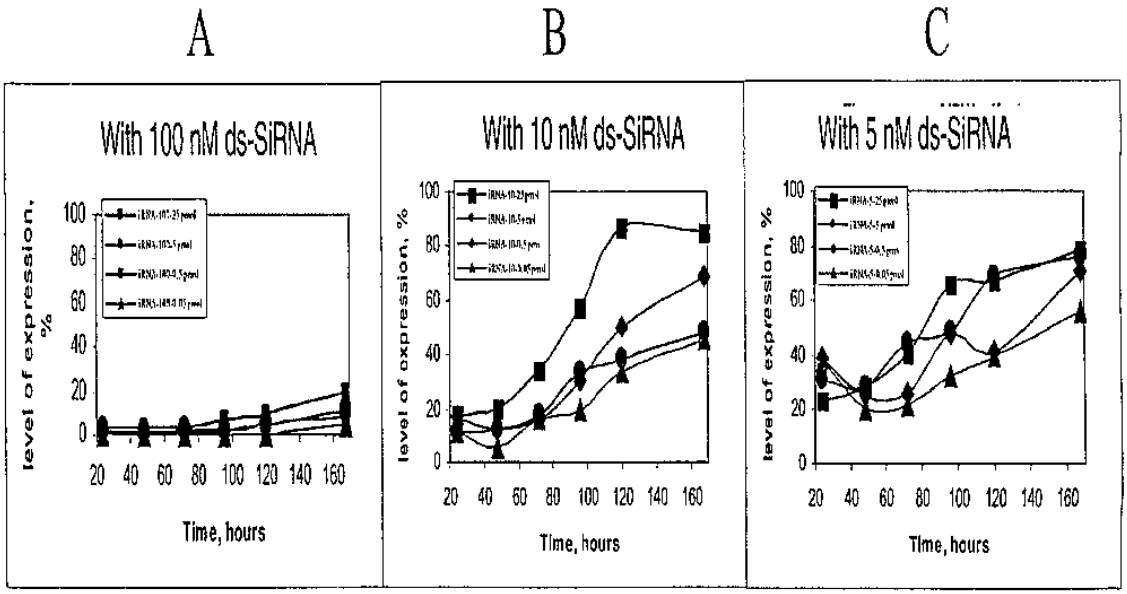


Figure - 43

Persistence of RNAi

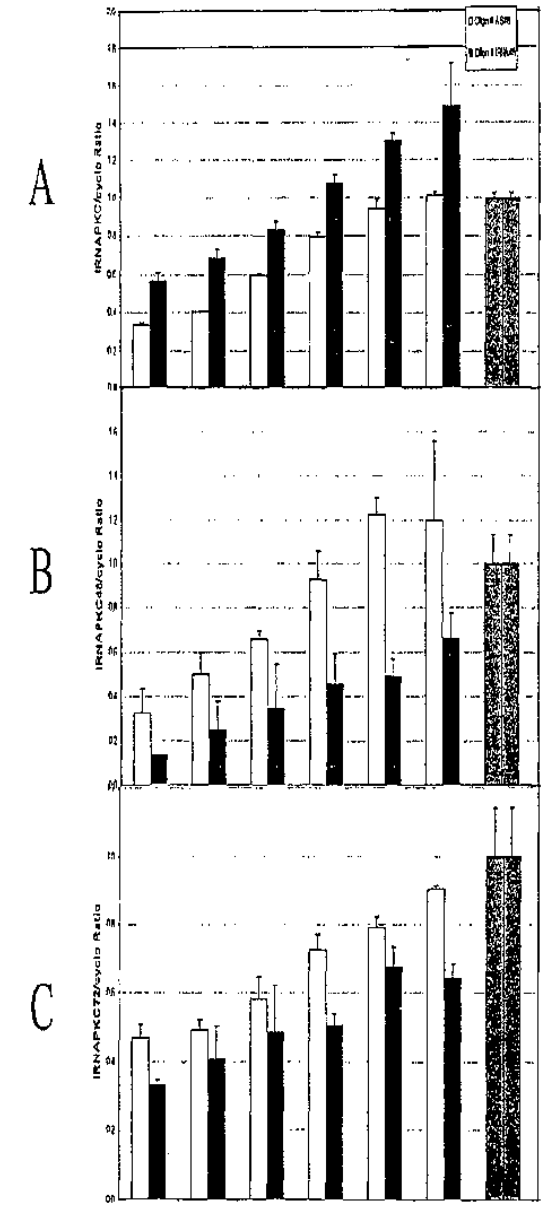


Figure - 44

Mut-1 5′-CAGAAACAGAUGCUCCCAGTT-3′
3′-TTGUCUUUGUCUACGAGGGUC-5′

Mut-2 5′-CAGAAAAGUAUGCUCCCAGTT-3′
3′-TTGUCUUUUCAUACGAGGGUC-5′

1. 5′-AAACGGAUGCUCTT-5′
3′-TTUUUGCCUACGAG-3′

2. 5′-GAAACGGAUGCUCCTT-5′
3′-TTCUUUGCCUACGAGG-3′

3. 5′-AGAAACGGAUGCUCCATT-5′
3′-TTUCUUUGCCUACGAGGU-3′

4. 5′-gAGAAACGGAUGCUCCCActt-3′
3′-ttcUCUUUGCCUACGAGGGUg-3′

5. 5′-guGAAACGGAUGCUCCCuctt-3′
3′-ttcaCUUUGCCUACGAGGGag-5′

6. 5′-gucAAACGGAUGCUCgguctt-3′
3′-ttcagUUUGCCUACGAGccag-5′

7. (Ds-SiRNA-8-A) 5′-CAGAAACGGAUGCUCCCAGTT-3′
3′-TTGUCUUUGCCUACGAGGGUC-5′

8. 5′-CAGAAACGGAUGCUCCCAGTT-3′
3′-TTGACUUUGCCUACGAGGGAC-5′

9. 5′-CAGAAACGGAUGCUCCCAGTT-3′
3′-TTGACUUUGCCUACGAGGGAC-5′

10. 5′-CAGAAACGGAUGCUCCCAGTT-3′
3′-TTGACUUUGCCUACGUGGGAC-5′

11. 5′-CAGAAACGGAUGCUCCCAGTT-3′
3′-TTGACUAUGCCUACGUGGGAC-5′

12. 5′-CAGAAACGGAUGCUCCCAGTT-3′
3′-TTGACUAUGCGUACGUGGGAC-5′

13. 5′-CAGAAACGGAUGCUCCCAGTT-3′
3′-TTGACUAUGCGUUCGUGGGAC

Figure - 45

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Understanding the nature of SiRNA trigger

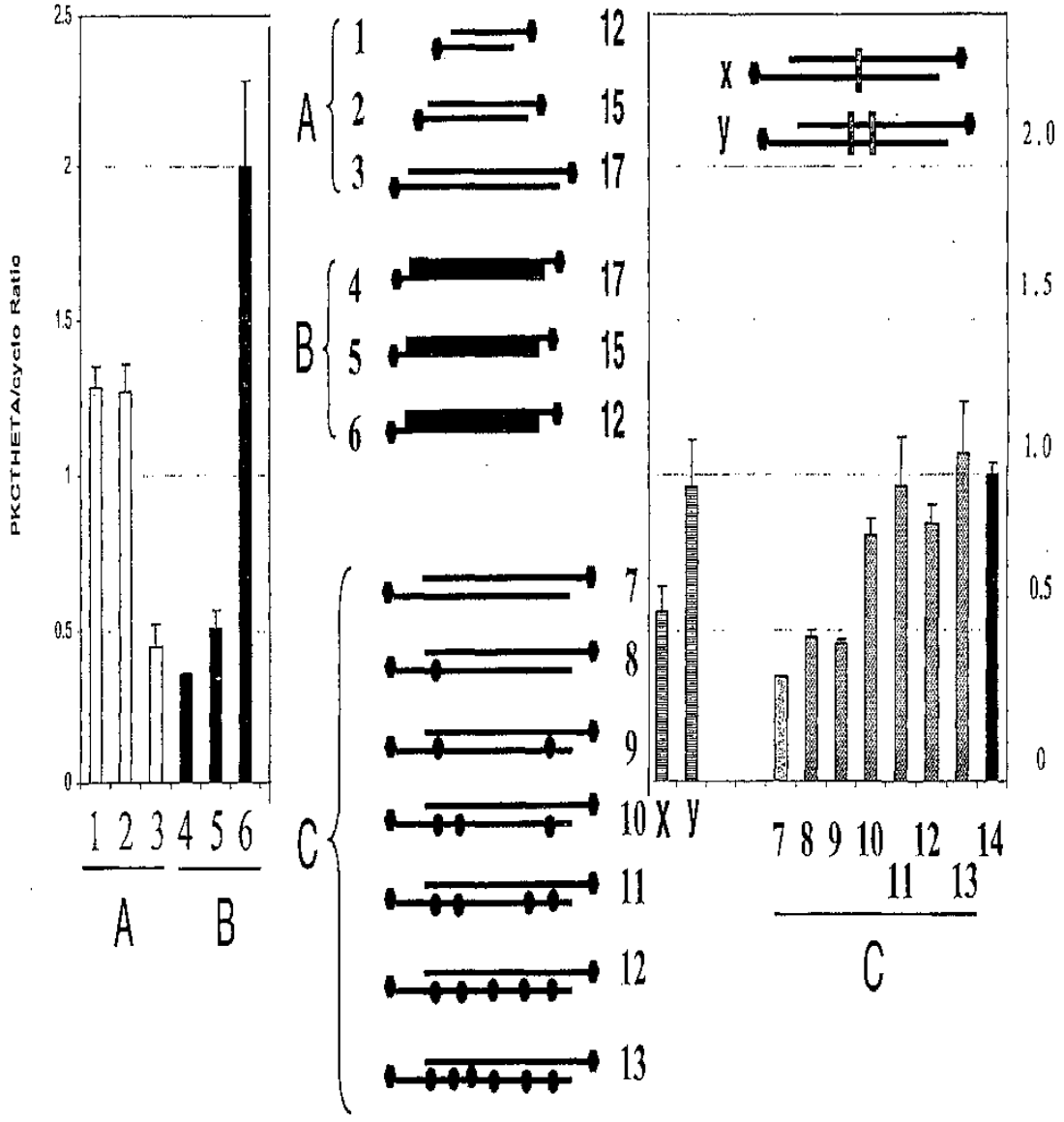


Figure - 46

Chemically-modified SiRNA

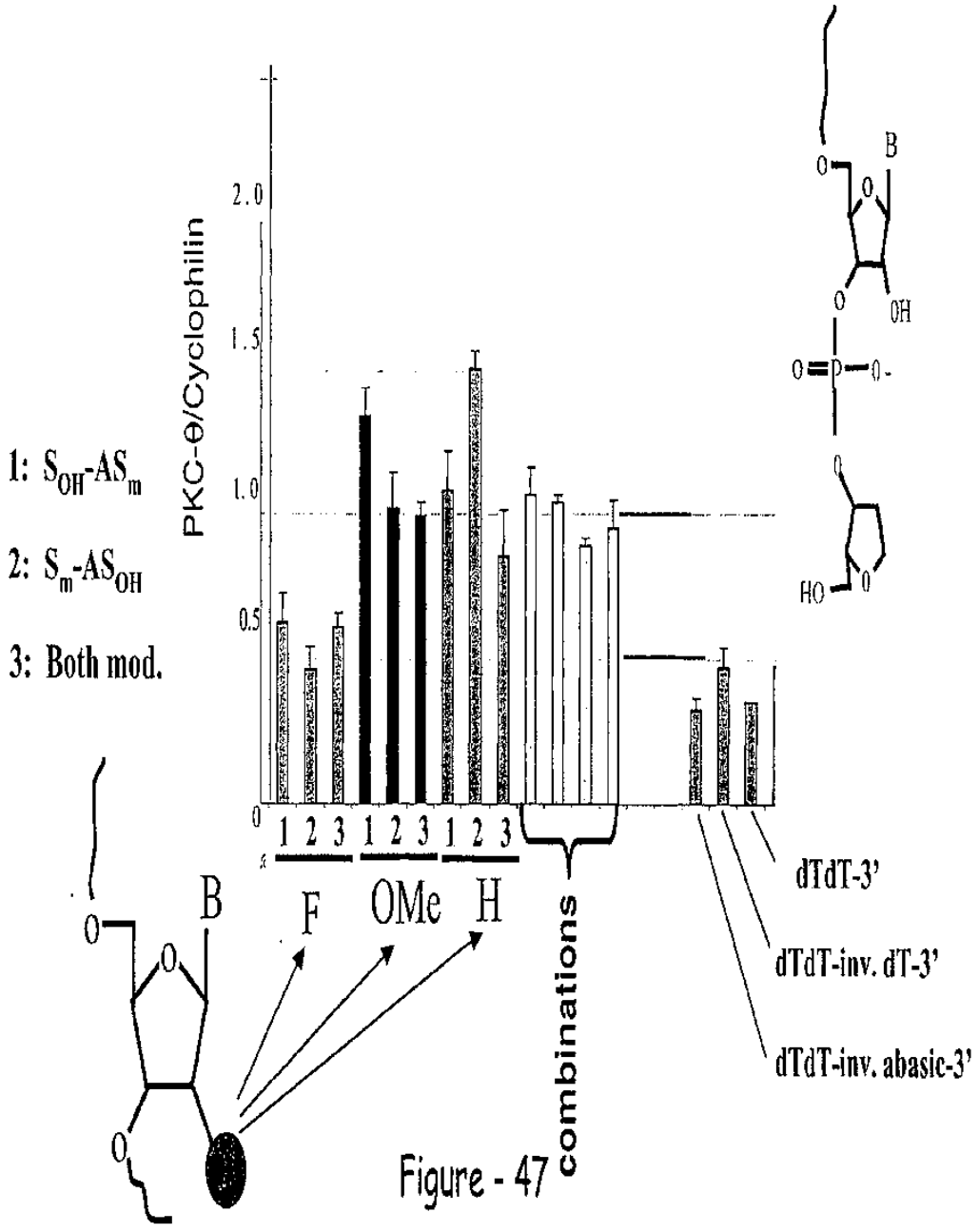
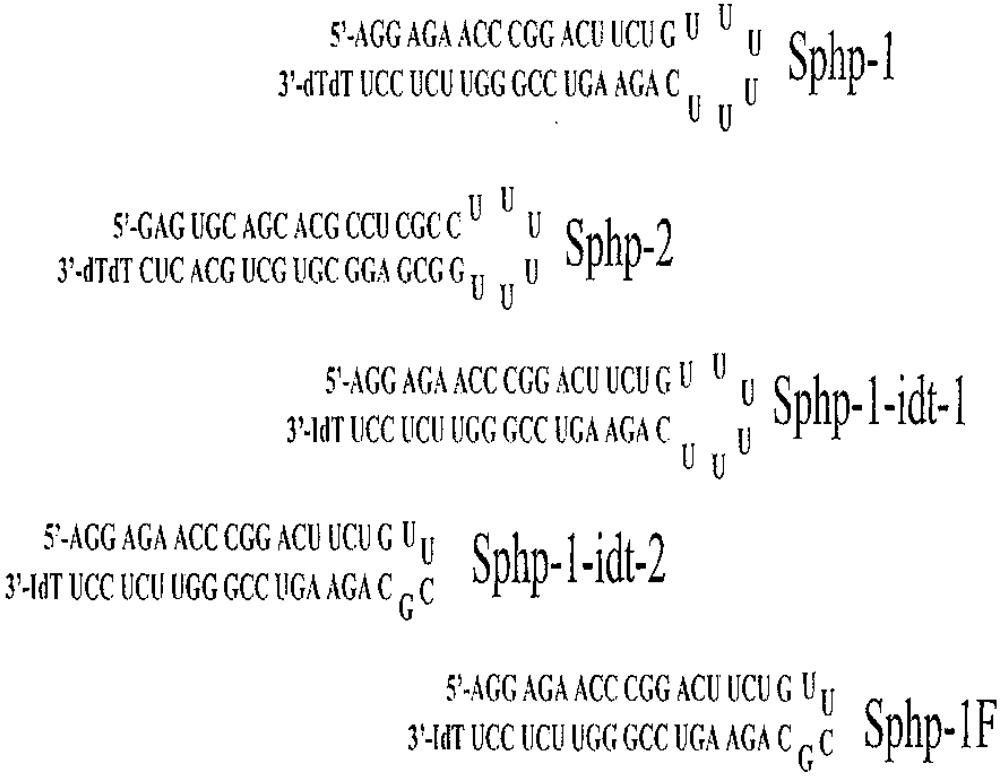


Figure - 47

Unimolecular & bimolecular SiRNAs for SEAP



3A: 5′-AGUAACCCUUACUGUGCUAdTdT-3′
3′-dTdTUCAUUGGGAAUGACACGAU-5′

3B: 5′-AGAUAACCCUUACUGUGCUdTdT-3′
3′-dTdTUCUAUUGGGAAUGACACGA-5′

Figure - 48

WO 2004/061083
54/56
PCT/US2003/041377

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Silencing transient expression of SEAP with Unimolecular & bimolecular SiRNAs

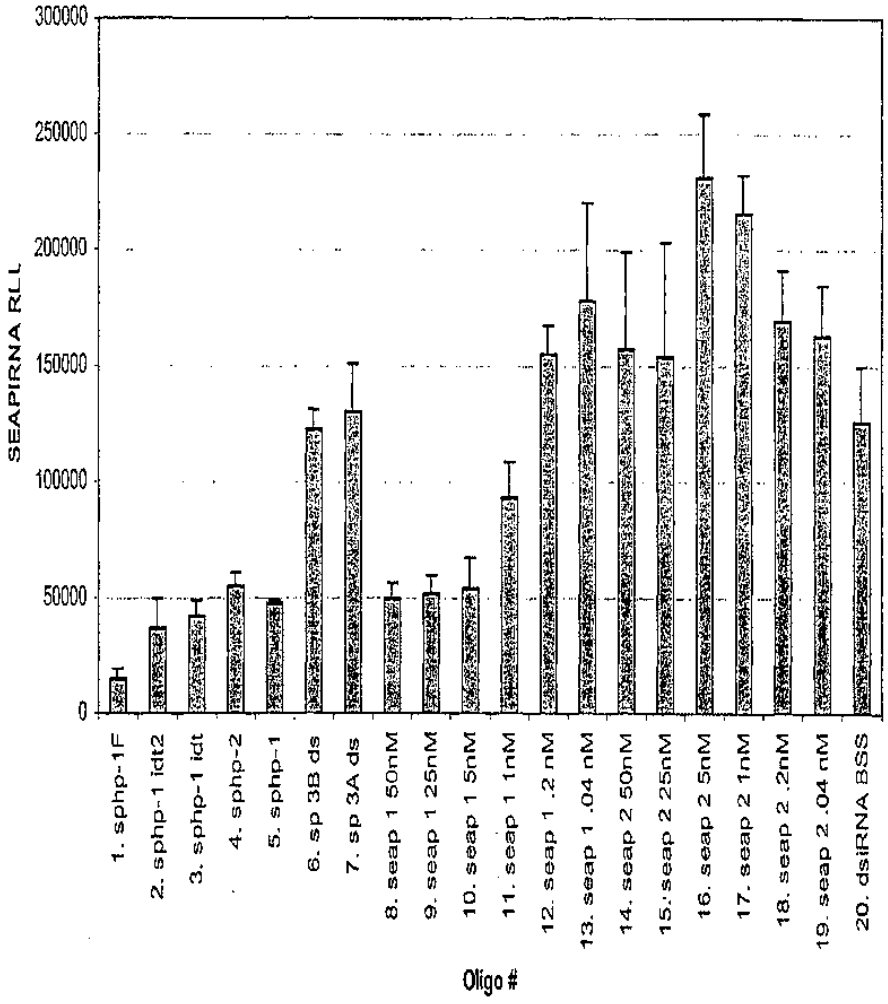


Figure - 49

Target mRNA	% G/C	# of Functional SiRNAs	Total # SiRNAs	% Success
PKC-θ	47.1	6	8	75
DJ-1	47.7	4	5	80
TELO	48.0	0	9	0
IGF1R	58.3	0	9	0
PKB-α	59.8	4	5	80
SEAP	60.6	2	6	33
mOPGL	61.2	0	5	0
KD312	63.7	1	2	50
TERT	75.5	0	9	0

Figure 50

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SEQUENCE LISTING

<110> AMGEN INC.

<120> RNA INTERFERENCE

<130> 06843.0062-00304

<140>
<141>

<150> 60/436,849
<151> 2002-12-27

<160> 151

<170> PatentIn Ver. 3.2

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<213> Artificial Sequence

<220>
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<210> 2
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<213> Artificial Sequence

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<210> 3
<211> 23
<212> DNA
<213> Artificial Sequence

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Synthetic oligonucleotide

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<210> 4
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Synthetic oligonucleotide

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<210> 7
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cagugucucu ugaaccaguu ctt 23

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gaacugguuc aagagacacu gtt 23

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<220>
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caguguucucu ugaaccagut t 21

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<220>
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gugggaagaa gguggcagug att 23

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Synthetic oligonucleotide

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Synthetic oligonucleotide

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<210> 15
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Synthetic oligonucleotide

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oligonucleotide

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acugccaccu ucuucccact t 21

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Synthetic oligonucleotide

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oligonucleotide

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<212> RNA
<213> Artificial Sequence

<220>
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<210> 18
<211> 46
<212> DNA
<213> Artificial Sequence

<220>
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Synthetic oligonucleotide

<220>
<223> Description of Artificial Sequence: Synthetic
oligonucleotide

<400> 18
caguguucucu ugaaccagut tttttacugg uucaagagac acugtt 46

<210> 19
<211> 50
<212> RNA
<213> Artificial Sequence

10x Genomics, Inc. Exhibit 2005
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<220>
<223> Description of Artificial Sequence: Synthetic oligonucleotide

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gggacagugu cucuugaacc aguuuuuuua cugguucaag agacacuguu 50

<210> 20
<211> 46
<212> DNA
<213> Artificial Sequence

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<220>
<223> Description of Artificial Sequence: Synthetic oligonucleotide

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<210> 21
<211> 50
<212> RNA
<213> Artificial Sequence

<220>
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gggaguggga agaagguggc aguuuuuuua cugccaccuu cuucccacuu 50

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tgc	cag	tct	tgt	cag	ggc	gag	gct	gtt	aac	cct	tac	tgt	96
Cys	Gln	Ser	Cys	Gln	Gly	Glu	Ala	Val	Asn	Pro	Tyr	Cys	
		20					25					30	
gtc	aaa	gag	tat	gtc	gaa	tca	gag	aac	ggg	cag	atg	tat	144
Val	Lys	Glu	Tyr	Val	Glu	Ser	Glu	Asn	Gly	Gln	Met	Tyr	
		35					40					45	

ggc	gag	gct	gtt	aac	cct	tac	tgt	gct	gtg	ctc			96
Gly	Glu	Ala	Val	Asn	Pro	Tyr	Cys	Ala	Val	Leu			
			25					30					
gtc	aaa	gag	tat	gtc	gaa	tca	gag	aac	ggg	cag	atg	tat	144
Val	Lys	Glu	Tyr	Val	Glu	Ser	Glu	Asn	Gly	Gln	Met	Tyr	
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35 40 45

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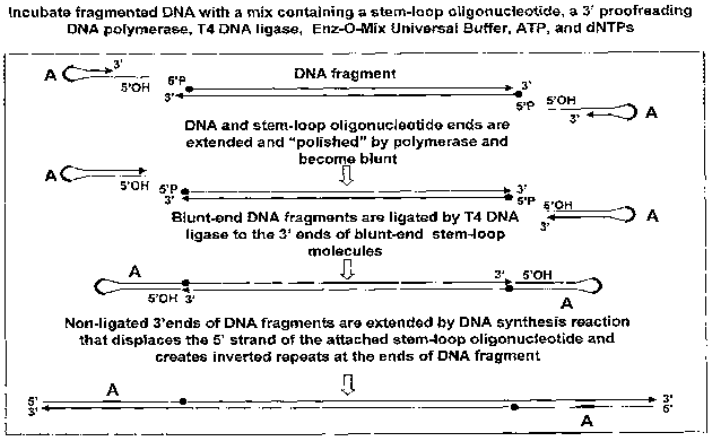
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(54) Title: COMPOSITIONS AND METHODS FOR PROCESSING AND AMPLIFICATION OF DNA, INCLUDING USING MULTIPLE ENZYMES IN A SINGLE REACTION



(57) Abstract: The present invention concerns preparation of DNA molecules, such as a library, using a stem-loop oligonucleotide. In particular embodiments, the invention employs a single reaction mixture and conditions. In particular, at least part of the inverted palindrome is removed during the preparation of the molecules to facilitate amplification of the molecules. Thus, in specific embodiments, the DNA molecules are suitable for amplification and are not hindered by the presence of the palindrome.

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COMPOSITIONS AND METHODS FOR PROCESSING AND AMPLIFICATION OF DNA, INCLUDING USING MULTIPLE ENZYMES IN A SINGLE REACTION

[0001] The present invention claims priority to U.S. Provisional Patent Application Serial No. 60/704,932, filed August 2, 2005, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[0002] The present invention generally concerns the fields of molecular biology and cellular biology. In particular, the present invention regards preparation and amplification of molecules, optionally utilizing a novel single-step reaction.

BACKGROUND OF THE INVENTION

[0003] Enzymatic reactions that involve DNA and RNA are numerous, and they play a central role in maintenance and propagation of living cells. Since the discovery of the DNA double helix structure in 1953, researchers have found, isolated and introduced into practice a multitude of different enzymes that can, for example, cut, nick, trim, join, unwind, phosphorylate, de-phosphorylate, methylate, de-methylate, recombine, replicate, transcribe, repair, and perform many other reactions with nucleic acid molecules. These enzymes are now actively used in many areas of biology, biotechnology and medicine to clone, amplify, sequence, identify mutations, quantify gene copy number, establish expression patterns, determine DNA methylation status, *etc.*

[0004] Frequently the process of DNA analysis involves multiple enzymatic reactions that are performed in a sequential manner, with intermediate purification steps between the reactions. Sometimes, the reactions are multiplexed to combine in one reaction the analysis of several DNA or RNA targets, and the nucleic acid processing or analysis may involve mutiplexing of two or three enzymatic processes in one reaction. Furthermore, DNA and RNA enzymatic reactions frequently utilize synthetic nucleic acid components, such as single stranded or double stranded oligonucleotides that function as PCR or sequencing primers, ligation adaptors, or fluorescent probes, for example.

Adaptors and Their Use for DNA Processing

[0005] Supplementing DNA ends with additional short polynucleotide sequences, referred to as an adaptor or linker, is used in many areas of molecular biology. The usefulness of

adapted DNA molecules is illustrated by but not limited to several examples, such as ligation-mediated locus-specific PCR, ligation-mediated whole genome amplification, adaptor-mediated DNA cloning, DNA affinity tagging, DNA labeling, *etc.*

Ligation-mediated amplification of unknown regions flanking known DNA sequence

[0006] Libraries generated by DNA fragmentation and addition of a universal adaptor to one or both DNA ends were used to amplify (by PCR) and sequence DNA regions adjacent to a previously established DNA sequence (see U.S. Patent No. 6,777,187 and references therein, for example, all of which are incorporated by reference herein in their entirety). The adaptor can be ligated to the 5' end, the 3' end, or both strands of DNA. The adaptor can have a 3' or 5' overhang, depending on the structure of the overhang generated by restriction enzyme digestion of DNA. It can also a have blunt end, especially in the cases when DNA ends are "polished" after enzymatic, mechanical, or chemical DNA fragmentation. Ligation-mediated PCR amplification is achieved by using a locus-specific primer (or several nested primers) and a universal primer complementary to the adaptor sequence.

Ligation-mediated whole genome amplification

[0007] Libraries generated by DNA fragmentation and subsequent attachment of a universal adaptor to both DNA ends were used to amplify whole genomic DNA (whole genome amplification, or WGA) (see U.S. Patent Applications Nos. 10/797,333 and 10/795,667 and references therein, for example, all of which are incorporated by reference herein in their entirety). The adaptor can be ligated to both strands of DNA or only to the 3' end followed by extension. The adaptor can have a 3' or 5' overhang, depending on the structure of the DNA end generated by restriction enzyme or other enzyme used to digest DNA. It can also have a blunt end, such as in the cases where DNA ends after enzymatic DNA cleavage are blunt or when the ends are repaired and "polished" after enzymatic, mechanical, or chemical DNA fragmentation. Whole genome PCR amplification is achieved by using one or two universal primers complementary to the adaptor sequence(s), in specific embodiments.

Adaptor-mediated DNA cloning

[0008] Adaptors (or linkers) are frequently used for DNA cloning (see Sambrook *et al.*, 1989, for example). Ligation of double stranded adaptors to DNA fragments produced by sonication, nebulization, or hydro-shearing process followed by restriction digestion within the adaptors allows production of DNA fragments with 3' or 5' protruding ends that can be efficiently introduced into a vector sequence and cloned.

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Use of Stem-Loop (Hairpin) DNA Oligonucleotides for Nucleic Acid Analysis

[0009] Stem-loop (also referred to as hairpin) oligonucleotides have been used in several applications for analysis and detection of nucleic acids. These applications include molecular beacons, stem-loop PCR primers, and stem-loop DNA probes, immobilized on microarrays (Broude, 2002).

Molecular beacons

[0010] A molecular beacon is a single-stranded oligonucleotide probe containing a sequence complementary to the target that is flanked by self-complementary termini, and carries a fluorophore and a quencher at the 3' and 5' ends, respectively (Tyagi and Kramer, 1996). In the absence of target the fluorophore and the quencher are in a close proximity, which quenches fluorescence. Upon hybridization with the target, the beacon changes its conformation so that the fluorophore and the quencher become separated, and fluorescence increases up to 100-200 times. Molecular beacons have found many applications for real-time monitoring of PCR (Tyagi *et al.*, 1998) and isothermal amplification (de Baar *et al.*, 2001), as microarray-immobilized probes (Liu *et al.*, 2000), as antisense probes for RNA detection *in vivo* (Sokol *et al.*, 1998), and as a probe to measure DNA polymerase activity (Summerer and Marx, 2002) and monitor conformational changes of DNA targets (Goddard *et al.*, 2000).

Stem-loop (Hairpin) PCR primers

[0011] PCR primers containing hairpin structures on their 5' ends with donor and acceptor moieties located in close proximity on the stem-loop stem have been proposed for homogeneous (a closed tube) format for amplification, real-time quantification and mismatch detection by PCR (Broude, 2002). A stem-loop primer with a fluorophore at the 5' end and a "scorpion" probe is simultaneously a molecular beacon and a PCR primer (Whitcombe *et al.*, 1999). It has a tail attached to its 5' end by a linker that prevents copying of the 5' extension. The probe element is designed so that it hybridizes to its target only when the target site has been incorporated into the same molecule by extension of the tailed primer. It was also shown that stem-loop probes can be used as primers for PCR to reduce primer-dimer formation and mispriming, thereby increasing its specificity (Kaboev *et al.*, 2000).

Stem-loop microarray probes

[0012] Stem-loop probes can also be used as capture devices if the loop is immobilized on a surface and dangling ends are used for stacking-hybridization, thus providing both faster kinetics and higher hybrid stability (Riccelli *et al.*, 2001). Immobilized molecular

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beacon probes can be used for direct detection of non-amplified target DNA and RNA molecules (Hamad-Schifferli *et al.*, 2002).

Multiplexed Reactions that Involve DNA and RNA Molecules

[0013] Several types of multiplexed reactions that involve DNA or RNA are described to date. The multiplexed reactions can be divided into two major categories: reactions where two or more DNA/RNA sequences are amplified or detected simultaneously in one enzymatic process, and reactions where several enzymatic processes occur simultaneously with one DNA or RNA template.

Several targets-one enzyme

[0014] Multiplex PCR and RT-PCR are examples of the first category of multiplexed reactions (Mackay *et al.*, 2000). In this case, several genomic or cDNA regions are amplified in one polymerase chain reaction carried out by one thermostable DNA polymerase. Whole genome or whole transcriptome amplification is another example of highly multiplexed DNA/RNA amplification reactions carried out by methophilic (Phi 29) or thermophylic (*Taq*) DNA polymerase (Sambrook *et al.*, 1989).

One target-several enzymes

[0015] "Long distance" PCR, nucleic acid sequence-based amplification (NASBA), its analog, transcription-mediated amplification (TMA), and DNA strand-displacement amplification (SDA) are examples of the second category of multiplexed DNA/RNA amplification reactions. In the "long distance" PCR method there is a mixture of *Taq* DNA polymerase and another thermo-stable DNA polymerase with 3'proofreading activity (Barns , 1994). TMA and NASBA methods utilize transcription-mediated amplification that involves three enzymes: T7 RNA polymerase, reverse transcriptase, and RNase H (Deiman *et al.*, 2001). In the SDA method, two enzymes (a DNA polymerase and a restriction endonuclease) are combined in one enzymatic step to amplify DNA (Hellyer and Naolean, 2004).

[0016] DNA nick-translation method is an example of a DNA labeling reaction that involves two enzymes. The method is based on simultaneous incubation of DNA with DNase I and DNA polymerase I. DNase I generates nicks in the DNA molecule, while DNA polymerase I incorporates labeled nucleotides by initiating DNA synthesis from the nicked sites (Sambrook *et al.*, 1989).

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[0017] dU-glycosylase (which is also referred to as Uracyl-DNA Glycosylase or UDG) and endonuclease VIII can be combined to produce the enzymatic mix, or the USER enzyme for fragmentation of DNA containing dU bases (New England Biolabs; Beverly, MA) may be employed. The fragmentation process occurs through enzymatic generation and cleavage of abasic sites at positions of dU bases.

Several targets-several enzymes

[0018] There is description of multiplexed amplification and detection of several nucleic acid sequences using three-enzyme TMA and NASBA methods (van Deursen *et al.*, 1999).

SUMMARY OF THE INVENTION

[0019] The present invention overcomes deficiencies in the art by providing novel methods and compositions for amplification of a plurality of double stranded DNA molecules by incorporating at least one sequence onto the ends of the DNA molecules. However, the invention avoids the disadvantages of other methods, such as the generation of primer dimers in polymerase chain reaction, for example.

[0020] In particular, the present invention allows for the amplification of molecules having at least one double stranded region by using adaptors that avoid the limitations of some adaptor molecules, such as those having the propensity to form adaptor dimers. In certain aspects, the present invention provides an inert oligonucleotide for attachment to a double stranded molecule such that it renders the oligonucleotide-ligated molecule capable of being modified, such as amplified, for example by polymerase chain reaction. Upon attachment of the inert adaptor to the molecule, the attached oligonucleotide becomes active and suitable for providing at least in part one or more sequences employable for amplification, while the non-attached, free adaptor remains inactive. For example, during polymerase chain reaction the free, non-attached inert adaptor can neither be primed nor used as a PCR primer (until it is intentionally and specifically fragmented and converted into a PCR primer); during amplification by transcription the promoter sequence of the non-attached, free adaptor does not initiate RNA synthesis; during amplification by strand displacement, the nicking enzyme recognition sequence of the non-attached adaptor remains uncut.

[0021] In particular aspects, the inert adaptor comprises a stem-loop oligonucleotide, wherein the inertness of the stem-loop oligonucleotide is at least in part a result

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of the dormant nature of its unique structure, biochemical propereties (one enzymatically active end), and/or physico-chemical characteristics (extremely high thermostability, for example). The oligonucleotide may comprise RNA, DNA, or both. The oligonucleotide may have one or more hairpins, and it may be further described as comprising any structure with multiple secondary structure and only one end. In specific embodiments, the oligonucleotide can change its functionality upon attachment to a double stranded nucleic acid molecule (DNA, RNA, or both). In other words, because of a conformational change of the oligonucleotide upon (such as following, for example) attachment to a double stranded nucleic acid molecule, one or more functional properties of the oligonucleotide become altered, such as one or more functional properties hidden in the oligonucleotide prior to attachment manifesting upon attachment.

[0022] In addition to the advantages provided by the adaptors of the invention, the invention further provides novel conditions for modification of DNA molecules with the adaptors, and subsequent amplification. In most cases in the art, the process of DNA analysis involves multiple enzymatic reactions that are performed in a sequential manner, frequently with intermediate purification steps between at least some of the reactions. For example, preparation of DNA libraries for subsequent amplification and analysis involves ligation of adaptor sequences to DNA ends to introduce a priming site for the PCR-mediated whole genome amplification and ligation-mediated PCR, a promoter element for the transcription-mediated DNA amplification, and/or cohesive ends to facilitate DNA integration into a vector molecule for cloning. Preparation of library from genomic or viral DNA by current adaptor attachment procedures typically occur in 6 steps (FIG. 1A):

[0023] Step 1 – fragmentation of high molecular weight (HMW) DNA to a size amenable to amplification or cloning;

[0024] Step 2 – DNA purification to remove nucleases and other reagents;

[0025] Step 3 – "polishing" of DNA ends by a 3' proofreading DNA polymerase(s) to generate blunt-ended DNA fragments with the 3' hydroxyl and 5' phosphate groups at the termini;

[0026] Step 4 – DNA purification to remove the polymerase and replace the buffer, or, alternatively, heat inactivation of the DNA polymerase;

[0027] Step 5 – adaptor ligation; and

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[0028] Step 6 –3' end extension (in the case when an adaptor is ligated only to one strand of DNA, specifically to the 5' phosphate).

[0029] However, such a multi-step process takes a considerable amount of time (1-2 days) and can be a major obstacle for high throughput and diagnostic-type applications, for example. The process becomes more complicated when library preparation involves additional enzymatic steps, such as DNA or library digestion with methylation-sensitive or methylation-specific endonucleases (for example, in the preparation of Methylome libraries, such as are described in U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety), or restriction nuclease cleavage within the adaptor sequence to produce sticky ends for DNA cloning.

[0030] The present invention satisfies a long-felt need in the art for obviating the requirement for multiple manipulations for nucleic acid processing. The current invention introduces and demonstrates new methods and compositions that allow reduction of several important multi-step enzymatic DNA processes to a single-step-reaction that is performed in one reaction volume (FIGS. 1B and 1C). Such simplification has been achieved by developing a highly multiplexed enzymatic method (Enz-O-Mix) that combines a substantial number (from 2 to 10) of enzymatic processes into one complex reaction mixture that occurs under universal buffer conditions.

[0031] Specifically, the current invention introduces novel processes of DNA library preparation and/or DNA amplification (FIG. 1C) that reduce the whole multi-step process to a single-step multiplexed enzymatic reaction involving as a minimum two **Enz**ymes, a stem-loop (hairpin) **O**ligonucleotide with a special base/bonds composition, and a universal buffer (**Mix**), which is herein referred to as **Enz-O-Mix**, that supports efficient functioning of all enzymatic activities present in the mixture. The invention demonstrates that the Enz-O-Mix approach can be used to prepare a DNA library for whole genome amplification (WGA Library), a Methylome library for amplification and analysis of methylated DNA regions, and/or even directly amplify DNA in a single multi-enzyme reaction, for example.

[0032] The Enz-O-Mix library/amplification has no limitations on the size and nature of DNA in the reaction. The method can be applied to high molecular weight DNA, such as is isolated from tissues or cell culture, for example, as well as highly degraded DNA, such as

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cell-free DNA from blood and urine and/or DNA extracted from formalin-fixed, paraffin-embedded tissues, for example.

[0033] The applications of the Enz-O-Mix method are numerous. The Enz-O-Mix method is easy to automate and use in clinical diagnostic and point-of-care applications, for example. In specific embodiments, different enzyme combinations are utilized for many novel kits and assays and utilized in such areas as biotechnology, the pharmaceutical industry, molecular diagnostics, forensics, pathology, bio-defense, and/or bio-computing, for example. The Enz-O-Mix approach is a simple and cost-effective alternative to the "lab-on-a chip" microfluidic approach that is currently attempting to solve the same problem (multi-step DNA processing) by reduction of reaction volumes and integration of multiple reactions into a small format (FIG. 2). The method can be used for *in vitro* as well as for *in vivo* nucleic acid amplification and/or detection.

[0034] In specific aspects, the present invention introduces a concept of multiplexing two or more enzymatic processes in one reaction, teaches how to optimize a highly multiplexed enzymatic process, and demonstrates in multiple specific examples the efficacy of this approach. In particular embodiments, the present invention is directed to compositions and methods for simultaneous processing of DNA molecules with a combination of enzymes in a one-step-one-tube reaction and producing either a collection of molecules suitable for amplification, or amplified DNA molecules.

[0035] In particular, the present invention greatly reduces the number of steps for library preparation by consolidating a series of steps into one step. For known whole genome amplification or whole methylome methods, these protocols greatly reduce the number of steps for library syntheses that utilize adaptors. That is, in the case of WGA library preparation from high molecular weight DNA, for example, the number of steps is reduced from about 6 to 1 (FIG. 1A and 1B). In the case of WMA library preparation from high molecular weight DNA, for example, the number of steps is reduced from about 7 to 1. In the case of WGA library preparation from fragmented DNA (plasma, serum, or urine, for example), the number of steps is reduced from about 4 to 1 (FIG. 1B). In the case of Methylome library preparation from fragmented DNA, for example, the number of steps is reduced from about 5 to 1 (FIG. 1B).

[0036] In particular embodiments, the methods of the invention can be easily applied to any type of fragmented double stranded DNA including but not limited to, for

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example, free DNA isolated from plasma, serum, and/or urine; apoptotic DNA from cells and/or tissues; DNA fragmented enzymatically *in vitro* (for example, by DNase I and/or restriction endonuclease); and/or DNA fragmented by mechanical forces (hydro-shear, sonication, nebulization, *etc.*).

[0037] In other embodiments, the invention can be easily applied to any high molecular weight double stranded DNA including, for example, DNA isolated from tissues, cell culture, bodily fluids, animal tissue, plant, bacteria, fungi, viruses, *etc.*

[0038] In one embodiment of the invention, there is incubation of DNA with an enzymatic mixture (Enz-O-Mix) comprising from about 2 to about 18 different enzymes in one buffer and a stem-loop (hairpin) oligonucleotide with a specific base/bonds composition.

[0039] In particular embodiments, there is a one-step enzymatic process including one or more of the following: a) "polishing" of DNA and stem-loop oligonucleotide ends by Klenow fragment of DNA polymerase I (or other proofreading DNA polymerase); b) ligation of the 3' end of stem-loop oligonucleotide to the 5' end of DNA, such as by a ligase, for example, by T4 DNA ligase; c) "fill-in" DNA synthesis that is initiated at the 3' end of DNA, propagates towards the end of the stem-loop oligonucleotide, and stops at a replication block or at the end of the oligonucleotide; and d) cleavage in the middle of a generated inverted repeat by a restriction nuclease; and e) restriction cleavage with a mixture of methylation-sensitive restriction enzymes or methylation-specific nuclease(s) (such as in embodiments concerning whole methylome amplification library), for example.

[0040] In particular aspects of the invention, an inverted repeat (palindrome) at the end of DNA molecules is not generated (or is generated and removed), because it may inhibit the amplification step (PCR), priming of the cDNA strand synthesis (amplification by transcription), or priming of the second DNA strand synthesis (amplification by strand displacement DNA synthesis). Thus, in specific embodiments at least part of the oligonucleotide sequence is eliminated, such as, by termination of replication in the middle of a stem-loop oligonucleotide or by cutting the generated inverted repeat internally, for example.

[0041] In one specific embodiment of this invention, a chemical modification, such as hexaethylene glycol (HEG) linker, a bulky base analog, or one or several abasic sites within the loop or adjacent to it, can be introduced during oligonucleotide synthesis. Such modifications will terminate the replication within the stem-loop oligonucleotide.

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[0042] In another specific embodiment, the replication block is generated during the reaction by incorporating one or more dU bases into the stem-loop oligonucleotide design and including dU-glycosylase (which is also referred to as Uracyl-DNA Glycosylase or UDG) in the reaction mix.

[0043] In some embodiments, a specific site for a cleavage enzyme is generated during "fill-in" DNA synthesis that is initiated at the 3' end of the DNA, propagates towards the end of the stem-loop oligonucleotide, and stops at the 5' end of the oligonucleotide. Such a site resides originally either completely or partially within the single-stranded loop of an oligonucleotide and becomes functional (cleavable) only after conversion of this loop into double-stranded form.

[0044] In one specific embodiment a cleavage enzyme is represented by a restriction endonuclease, in another by a homing endonuclease.

[0045] In some embodiments, the methods further comprise simultaneous digestion with an endonuclease or combination of endonucleases, such as restriction endonucleases, DNase I, benzonase, methylation-specific nuclease McrBC, apoptotic endonucleases, *etc.*, to generate in one step Whole Genome or Whole Methylome library even from high molecular weight (HMW) DNA.

[0046] In particular embodiments, a library generated by a one-step process is further amplified by PCR using universal primer complementary to the sequence introduced by attachment of a stem-loop oligonucleotide.

[0047] In particular embodiments, library generation and PCR amplification are combined into one integrated, closed-tube process in a thermocycler.

[0048] In one particular embodiment, an integrated, closed-tube library preparation/amplification process is supported by a universal PCR primer included in the original Enz-O-Mix.

[0049] In another particular embodiment, an integrated, closed-tube library preparation/amplification process is supported by a stem-loop oligonucleotide converted into a functional universal primer as a result of enzymatic and chemical reactions that occur before PCR amplification.

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[0050] In another embodiment, degradable stem-loop oligonucleotides are used as locus-specific primers for a hot start PCR process.

[0051] In another particular embodiment, a library generated by one-step process is further amplified by transcription utilizing a promoter sequence (T7, T3, SP6, *etc.*) introduced by attachment of a stem-loop oligonucleotide.

[0052] In particular embodiments, a stem-loop oligonucleotide design is employed for the one-step library synthesis reaction, although in alternative embodiments other adaptors may be utilized, such as a standard linear two-oligonucleotide adaptor, for example.

[0053] In particular embodiments, a stem-loop oligonucleotide provides a synthesized library with a new function that was present neither in the DNA molecule nor in the stem-loop oligonucleotide prior to its attachment. Examples include a functionally inactive T7 promoter sequence, a non-cleavable restriction site, and/or inactive nicking site residing at least partially within the loop or stem region of the stem-loop oligonucleotide and activated only by the attachment process and conversion of the single stranded loop into a double stranded molecule.

[0054] In particular embodiments, a stem-loop oligonucleotide provides a synthesized library with a 5' overhang that was not present either in the DNA molecule or in the stem-loop oligonucleotide prior to its attachment, and that can be used for covalent or non-covalent immobilization of a synthesized library on a solid support *via* hybridization or hybridization and ligation to a covalently attached oligonucleotide.

[0055] In a specific embodiment, the library synthesis and its immobilization within the tube (well) occurs simultaneously in a one Enz-O-Mix process. In this case, combinations of enzymes and stem-loop oliginucleotides are mixed together in a special tube with a covalently attached capture oligonucleotide to produce Enz-O-Mix-Immobilization kits for high throughput DNA processing and diagnostic applications in solid phase format.

[0056] In particular embodiments, combinations of enzymes and stem-loop oligonucleotides are mixed together to produce Enz-O-Mix kits for high throughput DNA processing and diagnostic applications. In particular embodiments, there is a Universal Enz-O-Mix Buffer that provides simultaneous activity of all enzymes used in the reaction.

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[0057] In particular embodiments, combinations of enzymes and stem-loop oligonucleotides are mixed together to produce integrated, closed-tube Enz-O-Mix kits for high throughput DNA fragmentation, amplification and labeling. In particular embodiments, there is a Universal Enz-O-Mix Buffer that supports simultaneous activities of all enzymes used in the reaction.

[0058] In a specific embodiment, there is a process for optimization of the Universal Buffer and other components of the multiplexed enzymatic reaction.

[0059] In particular aspects, the present invention is directed to a system and method for preparing a collection of molecules, particularly molecules suitable for amplification, such as amplification utilizing known sequences on the molecules. In specific embodiments, the oligonucleotide comprises a known sequence.

[0060] In an additional embodiment, there is a kit housed in a suitable container that comprises one or more compositions of the invention and/or comprises one or more compositions suitable for at least one method of the invention.

[0061] In one embodiment of the invention, there is a method of preparing a nucleic acid molecule, comprising: providing a double stranded nucleic acid molecule; and attaching one strand of a stem-loop oligonucleotide comprising an inverted repeat and a loop to the double stranded nucleic acid molecule to produce an oligonucleotide-attached nucleic acid molecule. The double stranded nucleic acid molecule may be a double stranded DNA molecule, in some embodiments. In specific embodiments, the attaching is further defined as attaching the oligonucleotide to the double stranded nucleic acid molecule under conditions to produce a non-covalent juntion, such as a nick, a gap, or a 5' flap structure, in the oligonucleotide-attached nucleic acid molecule. In particular aspects of the invention, the attaching is further defined as ligating. The method may further comprise displacing one strand of the oligonucleotide from the oligonucleotide-attached nucleic acid molecule by strand displacement or by nick translation polymerization. In a specific embodiment, at least part of the oligonucleotide-attached nucleic acid molecule is amplified, such as by polymerase chain reaction, RNA transcription, or strand displacement, for example. Methods of the invention may further comprise amplifying an oligonucleotide-attached nucleic acid molecule, wherein at least part of the inverted repeat is excluded from the amplified oligonucleotide-attached nucleic acid molecule.

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[0062] Ligating embodiments may be further defined as comprising: generating ligatable ends on the double stranded nucleic acid molecule; generating a ligatable end on the stem-loop oligonucleotide; and ligating one strand of the ligatable end of the stem-loop oligonucleotide to one strand of an end of the nucleic acid molecule, thereby generating a non-covalent junction, such as a nick, a gap, or a 5' flap structure, in the oligonucleotide-attached nucleic acid molecule. In further aspects, the methods comprise generating blunt ends on the nucleic acid molecule; generating a blunt end on the stem-loop oligonucleotide; and ligating one strand of the blunt end of the stem-loop oligonucleotide to one strand of a blunt end of the nucleic acid molecule, thereby generating a nick in the oligonucleotide-ligated nucleic acid molecule.

[0063] In specific aspect of the invention, the stem-loop oligonucleotide comprises a known sequence, for example a regulatory sequence, including a RNA polymerase promoter sequence. A regulatory sequence may reside at least in part within the stem of the stem-loop oligonucleotide, within the loop of the stem-loop oligonucleotide, or both.

[0064] Methods of the invention may further comprise digesting the DNA molecule with one or more endonucleases to produce DNA fragments; producing blunt ends on the DNA fragments; producing a blunt end on the stem-loop oligonucleotide; and ligating one strand of the blunt end of a stem-loop oligonucleotide to one strand of a blunt end of a DNA fragment, thereby generating a nick in an oligonucleotide-ligated DNA fragment. In a specific embodiment, the endonuclease is a restriction endonuclease, DNAse I, or an apoptotic endonuclease, or a mixture thereof. In particular embodiments, the restriction endonuclease is methylation-specific or methylation-sensitive.

[0065] In additional embodiments, the oligonucleotide-attached nucleic acid molecule comprises a nick having a 3' hydroxy group, wherein there is polymerization from the 3' hydroxy group of at least part of the oligonucleotide-attached nucleic acid molecule.

[0066] Strand displacement or nick translation polymerization may be further defined as polymerization that ceases at a non-replicable base or region in the loop or in a region of the stem adjacent to the loop.

[0067] In a specific aspect of the invention, the method further comprises the step of digesting the double stranded DNA molecule with an endonuclease to generate DNA fragments, wherein the oligonucleotide becomes ligated to one strand of the DNA fragment and

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wherein polymerization of an oligonucleotide-ligated DNA fragment excludes at least part of the inverted repeat by subjecting the oligonucleotide-ligated DNA fragment to strand displacement or nick translation polymerization that halts at a base or sequence in the loop or in a region of the stem adjacent to the loop.

[0068] In some embodiments, the stem-loop oligonucleotide is further defined as comprising a non-replicable base or sequence. In particular, in some cases at least part of the non-replicable base or sequence is present in the loop of the oligonucleotide or in a sequence of the stem adjacent to the loop. The non-replicable base or sequence may comprise an abasic site or sequence, hexaethylene glycol, and/or a bulky chemical moiety attached to the sugar-phosphate backbone or the base. In specific embodiments, the abasic site or sequence is introduced by one or more enzymes in the single solution. In a further specific embodiment, the loop of the stem-loop oligonucleotide comprises at least one deoxy-uridine.

[0069] In one particular embodiment, strand displacement or nick translation polymerization of the oligonucleotide-attached nucleic acid molecule generates an endonuclease site. In particular aspects, the methods further comprise the step of digesting the double stranded DNA molecule with an endonuclease to generate DNA fragments, wherein the oligonucleotide becomes blunt end ligated to one strand of the DNA fragment to produce an oligonucleotide-ligated DNA fragment, and wherein strand displacement or nick translation polymerization of the oligonucleotide-ligated DNA fragment generates an endonuclease site.

[0070] In particular aspects, the endonuclease site is a site-specific restriction endonuclease site and at least part of the inverted repeat is removed by cleavage with said restriction endonuclease. The endonuclease site may reside within the stem and/or loop regions. Examples of endonucleases include Eco NI or PacI. The endonuclease site may be a homing endonuclease site, such as I-Ceu I, I-Sce I, PI-Psp I, or PI-Sce I. In specific embodiments, RNA transcription is initiated from the regulatory sequence, thereby producing at least one transcribed polynucleotide.

[0071] In specific embodiments, a regulatory sequence resides at least in part within the stem of the stem-loop oligonucleotide, or at least in part within the loop of the stem-loop oligonucleotide. In a specific aspect, the regulatory sequence comprises a bacteriophage regulatory sequence, such as a T7 regulatory sequence, a T3 regulatory sequence, or a Sp6 regulatory sequence, for example. In specific aspects, a transcribed polynucleotide is replicated

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by a reverse transcriptase, which may be initiated by hybridization of the oligonucleotide complementary to the 3' end of the transcribed polynucleotide.

[0072] The stem-loop oligonucleotide may further comprise a recognition sequence for a nicking endonuclease and the oligonucleotide-attached nucleic acid molecule is amplified by strand displacement synthesis and second strand synthesis, in particular aspects of the invention. In a specific aspect, the recognition sequence for a nicking endonuclease resides at least in part within the stem of the stem-loop oligonucleotide. The recognition sequence for a nicking endonuclease may reside at least in part within the loop of the stem-loop oligonucleotide. In specific embodiments, the nicking endonuclease is N.Alw I, N.Bbv CIA, N.Bbv CIB, Nb.Bpu10I, Nb.BsmI, N.BstNBI, or N.Bst 9I.

[0073] In specific aspects, a 5' end of the stem-loop oligonucleotide lacks a phosphate.

[0074] In particular embodiments, the oligonucleotide-attached nucleic acid molecule is further modified, such as by cloning, including by incorporation of the modified molecule into a vector, said incorporation occuring at ends in the modified molecule generated by endonuclease cleavage within the inverted repeat.

[0075] In additional embodiments of the invention, methods of the invention occur in a single suitable solution, wherein the process occurs in the absence of exogenous manipulation. The method may occur at one temperature, such as from about 10°C to about 75°C. . In particular embodiments, the solution comprises one or more of the following: ligase; DNA polymerase; one or more endonucleases; RNA polymerase; reverse transcriptase; RNase H; deoxy-uridine glycosylase (which is also referred to as Uracyl-DNA Glycosylase or UDG); nickase; thermophilic DNA polymerase; ATP; rNTPs; and dNTPs.

[0076] In one aspect of the invention, there is a kit, housed in a suitable container, comprising: a stem-loop oligonucleotide; and a solution suitable for ligation of the oligonucleotide onto a double stranded molecule; and, optionally, one or more of the following: ligase; DNA polymerase one or more restriction enzymes; RNA polymerase; reverse transcriptase; RNase H; nickase; thermophilic DNA polymerase; ATP; rNTPs; and dNTPs. In specific embodiments, the oligonucleotide of the kit comprises a RNA polymerase promoter sequence, such as a T7 RNA polymerase promoter sequence, for example. The one or more

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endonucleases may be methylation-specific, methylation-sensitive, or may be a homing endonuclease.

[0077] In specific embodiments, methods of the invention occur in a single suitable solution, wherein the process of preparing and amplifying of a oligonucleotide-attached nucleic acid molecule occurs in the absence of exogenous manipulation. The method may be further defined as occurring at one temperature, such as between about 10°C to about 85°C or between about 10°C to about 85°C. In specific embodiments, the method is further defined as occurring at least two different temperatures, such as wherein at least one of the temperatures is from about 10°C to about 100°C.

[0078] In specific embodiments, the oligonucleotide-attached nucleic acid molecule is immobilized to a solid support, such as non-covalently or covalently.

[0079] Some methods of the invention further comprise the step of digesting the DNA molecule with an endonuclease to generate DNA fragments, wherein the oligonucleotide becomes attached to one strand of the DNA fragment, wherein strand displacement polymerization of the oligonucleotide-ligated DNA fragment and its arrest at a base or sequence in the loop or in a region of the stem adjacent to the loop generates a 5' overhang, and wherein the single stranded 5' overhang hybridizes to a complementary oligonucleotide covalently immobilized to a solid support.

[0080] Additional embodiments of the invention include a library of DNA molecules prepared by the methods of the invention.

[0081] In some embodiments of the invention, there is a method of preparing a DNA molecule, comprising: providing an oligonucleotide; and mixing the oligonucleotide with a double stranded DNA molecule, such that upon attachment of the oligonucleotide to the double stranded DNA molecule, the oligonucleotide attached to the DNA molecule is capable of demonstrating a function that it was incapable of before the attachment to the double stranded DNA molecule, thereby producing an oligonucleotide-attached DNA molecule suitable for modification. In specific aspects, the method occurs in a single suitable solution, wherein the process occurs in the absence of exogenous manipulation. Exemplary modifications include site-specific nicking, site-specific double-strand cleavage, transcription, recombination, amplification, polymerization, nick translation, strand displacement, immobilization or a combination thereof.

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[0082] In an additional embodiment of the invention, there is a composition comprising a stem-loop oligonucleotide, said oligonucleotide comprising an inverted repeat, a loop and at least one thermo-sensitive site or site capable of becoming a thermo-sensitive site, wherein the oligonucleotide is capable of producing a suitable primer upon exposure to heat. In additional embodiments, the composition further comprises a repair enzyme, such as one capable of converting damaged bases into an abasic site. Examples of DNA repair enzymes include a DNA glycosylase, such as dU-glycosylase, for example. In a specific aspect, the thermo-sensitive site is an abasic site, such as an apyrimidine site or an apurine site, for example. In an additional specific embodiment, the site capable of becoming a thermo-sensitive site is further defined as a base that can be enzymatically converted to a thermo-sensitive site. The base that can be enzymatically converted to a thermo-sensitive site may be deoxy-uridine and the enzyme may be dU-glycosylase (which is also referred to as Uracyl-DNA Glycosylase or UDG). In a specific aspect, the thermo-sensitive site is introduced into the oligonucleotide during synthesis, such as chemical synthesis. The composition may be capable of comprising a breakage of a strand of the oligonucleotide at one or more deoxy-uridine bases upon exposure to heat. The thermosensitive site or site capable of becoming a thermo-sensitive site may be located in the stem, in the loop, or both. The oligonucleotide may be blunt ended or have a 5' overhang.

[0083] In an additional embodiment, there is a method of producing a primer, comprising providing a composition of the invention and subjecting the composition to heat such that the primer is produced. In a specific aspect, the composition further comprises a DNA repair enzyme and the subjecting to heat is further defined as subjecting to two or more exposures of heat. One exposure to heat may comprise about 37°C and another exposure to heat may comprise about 95°C. One exposure to heat may comprise about 10°C to about 100°C whereas another exposure to heat may comprise about 80°C to about 100°C. The method may further comprise amplification of a DNA molecule utilizing said composition.

[0084] The foregoing has outlined rather broadly the features and technical advantages of the present invention in order that the detailed description of the invention that follows may be better understood. Additional features and advantages of the invention will be described hereinafter which form the subject of the claims of the invention. It should be appreciated by those skilled in the art that the conception and specific embodiment disclosed may be readily utilized as a basis for modifying or designing other structures for carrying out the same purposes of the present invention. It should also be realized by those skilled in the art that

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such equivalent constructions do not depart from the spirit and scope of the invention as set forth in the appended claims. The novel features that are believed to be characteristic of the invention, both as to its organization and method of operation, together with further objects and advantages will be better understood from the following description when considered in connection with the accompanying figures. It is to be expressly understood, however, that each of the figures is provided for the purpose of illustration and description only and is not intended as a definition of the limits of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0085] For a more complete understanding of the present invention, reference is now made to the following descriptions taken in conjunction with the accompanying drawings.

[0086] FIG. 1A shows a standard multi-step DNA adaptor attachment process.

[0087] FIG. 1B compares preparation processes for WGA and WMA libraries from degraded serum and urine DNA described in the present invention and in previously filed patent applications (U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005).

[0088] FIG. 1C is a general representation of the invention that can be described as a reduction of several important multi-step enzymatic DNA processes to a single-step multiplexed enzymatic reaction that is performed in one reaction volume (Enz-O-Mix Method).

[0089] FIG. 2 shows that the Enz-O-Mix approach can be viewed as a simple and cost-effective alternative to the "lab-on-a chip" microfluidic approach that is currently attempting to solve the same problem by reduction of reaction volumes and integration of multiple reactions into a small format.

[0090] FIG. 3 is a schematic description and composition of specific components of the Enz-O-Mix method and reagents involved in the attachment of stem-loop oligonucleotides to DNA ends. Four enzymatic reactions are taking place nearly simultaneously: "polishing" of the DNA ends and the hairpin double-stranded stem-region; ligation of the stem-loop oligonucleotide 3' end to the 5' phosphate of the DNA, leaving a nick between the 3' end of DNA and the 5' end of the hairpin double-stranded stem-region; polymerase extension of the 3' DNA end that propagates toward the end of stem-loop oligonucleotide; and by strand-displacement reaction within the oligonucleotide stem region. The process results in the library of DNA fragments with inverted repeat sequences at their ends.

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[0091] FIG. 4 shows three original secondary structures of the stem-loop oligonucleotide with the 3' or 5' protruding, or blunt end, and its final (blunt end) structure within the Enz-O-Mix.

[0092] FIGS. 5A-5B are schematic descriptions of the amplification of a library of DNA fragments with inverted repeat sequences at their ends by RNA transcription. Promoter sequence can be added to a DNA end either as a part of the oligonucleotide stem-region (A), or as a part of the oligonucleotide loop region (B). The products of the amplification are RNA molecules in this embodiment.

[0093] FIG. 6A illustrates the components and enzymatic reactions involved in the one-step DNA amplification by transcription using Enz-O-Mix reagent (Enz-O-Mix 5 in FIG. 19B, for example). The process linearly amplifies DNA and produces single stranded RNA molecules.

[0094] FIG. 6B illustrates additional components and enzymatic reactions involved in the one-step DNA amplification by transcription accompanied by the synthesis of complementary cDNA strand. The transcription (replication) block (see Sections E-H and FIGS. 8-10) is introduced into the stem-loop oligonucleotide upstream of the T7 promoter region to prevent formation of an inverted repeat at the 3' end of RNA molecules and thus increase the efficiency of the oligo T7 priming (the transcription block is shown as a black square). The process can linearly amplify DNA and produce double stranded DNA/RNA hybrids.

[0095] FIG. 7 is an illustration of the inhibitory effect on PCR of inverted repeats attached to both ends of DNA fragments. Heating and replication generates DNA molecules refractory to melting, priming, and PCR amplification either using the universal primer A or any internal site-specific primer pair.

[0096] FIG. 8 is a schematic description of the one-step Enz-O-Mix attachment process for a stem-loop oligonucleotide with non-replicable linker. The following enzymatic reactions are taking place nearly simultaneously: "polishing" of the DNA ends and the stem-loop oligonucleotide double-stranded stem-region; ligation of the oligonucleotide 3' end to the 5' phosphate of the DNA, leaving a nick between the 3' end of DNA and the 5' end of the hairpin double-stranded stem-region; polymerase extension of the 3' DNA end that propagates toward the end of oligonucleotide and stops at the replication block (non-replicable linker) within the loop or outside the loop but no more than about six bases away from the loop. The process

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results in the library of DNA fragments with universal sequence A at the ends and an inverted repeat attached only to the 5' end of DNA.

[0097] FIG. 9 is an illustration of the absence of an inhibitory effect on PCR of inverted repeats attached to only 5' ends of DNA fragments. Heating and replication generates DNA molecules with the universal sequence A and no inverted repeats at the ends (shown by dash lines) that can be successfully amplified by PCR.

[0098] FIG. 10A shows the structure of a stem-loop oligonucleotide with non-replicable linker introduced chemically during oligonucleotide synthesis and detailed events occurring at a DNA end during the multi-enzyme attachment process. The process occurs as described in FIG. 8.

[0099] FIG. 10B shows the structure of a stem-loop oligonucleotide with non-replicable linker introduced enzymatically during the attachment reaction and detailed events occurring at a DNA end during the multi-enzyme attachment process. Specifically, dU-glycosylase generates abasic sites within the loop and upper strand of the stem region of the stem-loop oligonucleotide. The abasic sites within a loop generate a non-replicable region, while the sites in a stem destabilize the duplex and facilitate strand displacement reaction. The process results in a library of DNA fragments with universal sequence at the ends and an inverted repeat attached only to the 5' end of DNA. Heating at 95°C during PCR generates breaks at abasic sites and completely eliminates the 5' portion of a stem-loop oligonucleotide.

[0100] FIG. 11A shows a standard adaptor formed by two different oligonucleotides, wherein one of them has a 5' phosphate group, and possible products formed when such adaptor is used in the multi-enzyme one-step ligation process.

[0101] FIG. 11B shows a standard adaptor formed by two different oligonucleotides without phosphate group and two products formed when such an adaptor is used in the multi-enzyme one-step ligation process. Only 50% of formed molecules are desirable products with correct orientation of the adaptor, whereas the other 50% of molecules have inverse adaptor orientation.

[0102] FIG. 11C shows a standard adaptor formed by two different oligonucleotides without a phosphate group, a protective group at the 3' end of one oligonucleotide, and products formed when such an adaptor is used in the multi-enzyme one-step

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ligation process. As in the case described on FIG. 11B, only 50% of formed molecules are desirable products with correct orientation of the adaptor, whereas the other 50% of molecules have inversed adaptor orientation.

[0103] FIG. 12 is a schematic description of the one-step Enz-O-Mix attachment process for a stem-loop oligonucleotide with an enzymatically cleavable site generated during the multi-enzyme reaction. Specifically, the endonuclease recognizes and cuts the oligonucleotide specific oligo-sequence R when it adopts a double-stranded conformation as a result of the attachment process. The reaction results in a library of DNA fragments with universal sequence A at the ends and no inverted repeat attached to the ends of DNA fragments.

[0104] FIG. 13A shows the stem-loop oligonucleotide with the recognition sequence for the restriction endonuclease Eco NI located at the loop region. The enzyme can not cut the CCTNNNNNAGG (SEQ ID NO: 20) region within the stem-loop oligonucleotide (not a recognizable structure) but it can cut it efficiently when the oligonucleotide is attached to a DNA end and adopts a canonical (double-stranded) Watson-Crick conformation.

[0105] FIG. 13B shows the stem-loop oligonucleotide with the recognition sequence for the restriction endonuclease PacI located within the loop region. The enzyme can not cut the single stranded TTAATTAA region within the stem-loop oligonucleotide, but it cuts it efficiently when the oligonucleotide is attached to a DNA end and adopts a canonical (double-strand) Watson-Crick conformation.

[0106] FIG. 13C shows the stem-loop oligonucleotide with the recognition sequence for the exemplary homing endonuclease I-Ceu I located within the loop region. The enzyme can not cut the 26-base single-stranded region within the hairpin loop, but it cuts efficiently when the oligonucleotide is attached to a DNA end and adopts a canonical (double-stranded) Watson-Crick conformation.

[0107] FIG. 14 is a schematic description of the one-step Enz-O-Mix attachment process for a stem-loop oligonucleotide with a very short stem. The process results in the library of DNA fragments with universal sequence A at the ends and very short inverted repeat attached to the ends of DNA fragments. Amplification by PCR is possible, because the melting temperature for terminal hairpins is low enough to prevent folding, self-priming and formation of long hairpin molecules, as shown on FIG. 7.

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[0108] FIG. 15 illustrates the components and enzymatic reactions involved in exemplary one-step or two-step (after library synthesis) DNA amplification by strand displacement DNA synthesis using Enz-O-Mix reagents (Enz-O-Mix 3 and Enz-O-Mix 6 in FIGS. 19A and 19B, for example). The amplification is initiated and further maintained by the generation of a nick within the attached oligonucleotide sequence. The process linearly amplifies DNA and produces double stranded DNA molecules.

[0109] FIG. 16A shows the stem-loop oligonucleotide with the recognition sequence for the nicking endonuclease N. Bbv CIA located at the loop region. The enzyme can not cut the single stranded GCTGAGG region within the stem-loop oligonucleotide (not a recognizable structure) but it can nick it efficiently when the oligonucleotide is attached to a DNA end and adopts a canonical (double-stranded) Watson-Crick conformation.

[0110] FIG. 16B shows a stem-loop oligonucleotide with the recognition sequence for the nicking endonuclease N. Bbv CIA located at the end of a stem region. The enzyme can not nick efficiently the GCTGAGG region within the stem-loop oligonucleotide (it is too close to the end) but it can nick it efficiently when the oligonucleotide is attached to a DNA end; the second site generated by attachment is not a good substrate for nicking (it is too close to the end).

[0111] FIG. 16C shows two exemplary multifunctional stem-loop oligonucleotides with several regulatory elements within a single (1) or multiple (2) loop regions: T7 promoter sequence (that includes all regions necessary for transcription), recognition sequence N for a nicking endonuclease, recognition sequence for a restriction endonuclease, and a replication/transcription block region (a chemical modification or a string of 2-4 dU bases). A multifunctional stem loop oligonucleotide can support different types of DNA amplification, including PCR, isothermal amplification by transcription, isothermal amplification by strand displacement synthesis, or even a combination thereof and it can also facilitate other modifications, including DNA immobilization, DNA recombination, DNA cloning, and a combination thereof, for example.

[0112] FIG. 17 shows generalization of the Enz-O-Mix method and demonstrates specific but exemplary applications.

[0113] FIG. 18 shows composition of five exemplary different Stem-Loop Oligo Attachment Master Mixes of the invention that are used for the one-step nucleic acid library synthesis.

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[0114] FIG. 19A schematically shows compositions of several Enz-O-Mix reagents, specifically, Enz-O-Mix 1, designed to convert fragmented DNA into a Whole Genome Library that can be amplified by PCR in an open, or close-tube format.. Enz-O-Mix 2 and Enz-O-Mix 3 are designed to convert fragmented DNA into a Whole Genome Library that can be amplified after or during the Whole Genome Library synthesis by transcription and strand displacement, respectively.

[0115] FIG. 19B shows schematically compositions of several Enz-O-Mix reagents, specifically, Enz-O-Mix 4, designed to convert HMW DNA into a Whole Genome Library that can be amplified by PCR in an open, or close-tube format.. Enz-O-Mix 5 and Enz-O-Mix 6 are designed to convert fragmented DNA into a Whole Genome Library that can be amplified after or during the Whole Genome Library synthesis by transcription and strand displacement, respectively.

[0116] FIG. 19C shows schematically compositions of several Enz-O-Mix reagents, specifically, Enz-O-Mix 7, designed to convert fragmented DNA into a Whole Methylome Library that can be amplified by PCR.. Enz-O-Mix 8 and Enz-O-Mix 9 are designed to convert fragmented DNA into a Whole Methylome Library that can be amplified after or during the Whole Methylome Library synthesis by transcription and strand displacement, respectively.

[0117] FIG. 19D shows schematically compositions of several Enz-O-Mix reagents, specifically, Enz-O-Mix 10, designed to convert HMW DNA into a Whole Methylome Library that can be amplified by PCR. Enz-O-Mix 11 and Enz-O-Mix 12 are designed to convert fragmented DNA into a Whole Methylome Library that can be amplified after or during the Whole Methylome Library synthesis by transcription and strand displacement, respectively.

[0118] FIG. 19E shows schematically compositions of Enz-O-Mix reagents 13 and 14 designed to convert fragmented and HMW DNA, respectively, into a Whole Genome Library that can be directly cloned into an appropriate vector.

[0119] FIG. 20 illustrates assembly, storage and use of Enz-O-Mix reagent(s) as a kit in high throughput applications and clinical diagnostics, for example.

[0120] FIG. 21A demonstrates the optimization process for Enz-O-Mix Universal Buffer using real-time PCR to monitor amplification of the generated library.

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[0121] FIG. 21B demonstrates the optimization process for Enz-O-Mix incubation temperature using real-time PCR to monitor amplification of the generated library.

[0122] FIG. 21C demonstrates the optimization process for Enz-O-Mix reagent (N) concentration using real-time PCR to monitor amplification of the generated library.

[0123] FIG. 22 shows real-time amplification curves of whole genome libraries prepared in a single step by ligation of a stem-loop oligonucleotide with EcoNI resrtiction site generated after ligation and strand-displacement extension of blunt-end AluI restriction fragments in the presence or in the absence of EcoNI. Excision of the terminal inverted repeat by EcoNI results in high amplification efficiency that exceeds amplification efficiency of the library with inverted repeats (in the absence of the Eco NI cleavage) by two orders of magnitude.

[0124] FIG. 23 shows real-time amplification curves of libraries prepared in a single step by ligation of blunt-end AluI restriction fragments to the stem-loop oligonucleotide comprising hexa-ethylene glycol replication stop (HEG Stem-Loop Oligo), a stretch of 5 ribonucleosides (Ribo Stem-Loop Oligo), or just deoxyribonucleosides (Stem-Loop Oligo) in their loop regions. Unlike the libraries made with HEG-containing oligonucleotide, the libraries with ribonucleosides in the loop region are not amplified efficiently, suggesting that ribonucleosides in the template strand do not arrest DNA replication carried by the Klenow fragment of DNA polymerase I.

[0125] FIGS. 24A, 24B, and 24C show the real-time PCR amplification curves of 3 exemplary human STS markers used for evaluation of genomic sequence representation in WGA libraries prepared in a single step by ligation of blunt-end AluI restriction fragments to a stem-loop oligonucleotide containing within a loop either the hexa-ethylene glycol linker (HEG) as a replication stop, or the EcoNI recognition sequence that becomes functional (cleavable) after ligation and strand-displacement extension. Twenty nanograms of purified material of each library is compared to 1 ng of human genomic DNA randomly fragmented to an average size of 1.5 Kb using Hydro Shear device (gDNA). In FIG. 24A, there is PCR using STS # 4 primers. In FIG. 24B, there is PCR using STS # 19 primers. In FIG. 24C, there is PCR using STS # 35 primers (Table II). Results show little or no representational bias for all 3 STS sequences.

[0126] FIG. 25 shows the real-time amplification curves of libraries prepared in a single step by ligation of blunt-end AluI restriction fragments to the stem-loop oligonucleotide containing deoxy-uridine in the 5' stem region and in the loop with T4 DNA ligase and Klenow

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fragment of DNA polymerase I, in the presence or in the absence Uracil-DNA glycosylase (which is also referred to as UDG or dU glycosylase). Amplification is conducted by either adding universal primer M_U or using the primer released as a result of the UDG activity, followed by heat-induced degradation of the stem-loop oligonucleotide.

[0127] FIG. 26 illustrates a one-step genomic DNA restriction digestion and whole genome amplification using degradable stem-loop oligonucleotide containing deoxy-uridine in the 5' stem region and in the loop. Human genomic DNA in amounts ranging from 20 ng to 10 pg or a blank sample (no DNA control) are incubated with the stem-loop oligonucleotide in the presence of 4 exemplary enzymatic activities: AluI restriction endonuclease, T4 DNA ligase, Klenow fragment of DNA polymerase I, and Uracil-DNA glycosylase (UDG) for 1 hour at 37°C and amplified by PCR using universal primer M_U.

[0128] FIG. 27 shows PCR amplification curves of specific promoter sites from amplified libraries prepared from non-methylated (N) and artificially methylated (M) cell-free urine DNA from a healthy donor. Libraries were prepared by ligation of a degradable stem-loop oligonucleotide containing deoxy-uridine with (N+, M+) or without (N-, M-) simultaneous cleavage with methylation-sensitive restriction enzymes. Promoter sites from non-methylated cleaved DNA (N+) amplify with significant (at least 10 cycles) delay as compared to uncut DNA (N-, M-) unlike methylated DNA which is refractory to cleavage (M+ versus M-).

[0129] FIG. 28 shows the products of simultaneous whole bacteriophage DNA library synthesis and isothermal amplification by T7 polymerase transcription of lambda DNA digested with restriction endonuclease Bst EII on an agarose gel in the presence (+T4 ligase) or absence of DNA ligase (-T4 ligase).

[0130] FIG. 29 shows the products of simultaneous library synthesis and isothermal amplification of cell-free urine DNA using the nicking activity of the nuclease N.BbvC IB and strand displacement DNA synthesis.

[0131] FIGS. 30A-30B compare two Enz-O-Mix library synthesis / PCR amplification processes (Enz-O-Mix 1 and Enz-O-Mix 4 from FIGS. 19A and 19B). FIG. 30A shows a 2-step, opened-tube protocol. In this case, Enz-O-Mix library synthesis / amplification is performed in two operational steps: step 1- a tube contatining DNA is supplemented with the library (WGA or WMA) synthesis reagents and incubated at 37 oC for 1 h, and step 2 – the tube is opened, supplemented with PCR amplification buffer / reagents and subjected to temperature

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cycling. FIG. 30B shows a 1-step, closed-tube protocol. In this case, Enz-O-Mix library synthesis / amplification is performed in one operational step when a tube containing DNA is supplemented with the library synthesis / amplification (PCR) reagents and universal buffer, and subjected to programmed temperature conditions within a thermocycler.

[0132] FIG. 31 shows an expected accumulation of DNA in a tube (a) and a typical temperature profile (b) for a one-step, closed tube Enz-O-Mix PCR-based WGA or WMA.

[0133] FIG. 32 illustrates heat-induced conversion of a stem-loop oligonucleotide adaptor containing dU-bases within the loop and the 5' stem region into a functional universal PCR primer.

[0134] FIG. 33 shows effect of DMSO and magnesium concentration on the whole genome amplification of HMW human DNA isolated from blood using integrated one-step, closed-tube Enz-O-Mix protocol illustrated in FIG. 30, 31, and 32, and real-time PCR detection.

[0135] FIG. 34 illustrate application of the one-step, closed tube Enz-O-Mix WGA process (DNA fragmentation, amplification, and labeling) for micro-array analysis.

[0136] FIGS. 35A and 35B show the one-step process for simultaneous GenomePlex or MethylPlex library synthesis and immobilization. In FIG. 35A, there is non-covalent library immobilization by hybridization of the exposed 5' stem region to an oligo S covalently attached to a tube/well surface. In FIG. 35B, there is a covalent library immobilization by hybridization and ligation of the exposed 5' stem region to an oligo S covalently attached to a tube/well surface.

[0137] FIG. 36 shows tubes, plates, micro-slides, and micro-beads manufactured for one-step GenomePlex or MethylPlex library synthesis and immobilization.

[0138] FIG. 37 shows a hypothetical fluidic device that utilizes the one-step GenomePlex or MethylPlex library synthesis and immobilization process.

[0139] FIG. 38 illustrates hot start locus-specific PCR amplification of DNA using degradable stem-loop primers.

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[0140] FIG. 39 shows the specific events that lead to a conversion of the inactive stem-loop oligonucleotides into the active PCR primers.

[0141] FIG. 40 shows accumulation of a specific PCR product (A) and a typical temperature profile (B) for a hot start PCR using degradable stem-loop primers

DETAILED DESCRIPTION OF THE INVENTION

[0142] The present application incorporates by reference herein in its entirety U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005. Also incorporated by reference herein in its entirety is U.S. Patent Application Serial No. unknown, filed March 2, 2006, entitled "Isolation of CpG Islands by Thermal Segregation and Enzymatic Selection-Amplification Method," which itself claims priority to U.S. Provisional Patent Application Serial No. 60/704,541, filed August 2, 2005.

I. Definitions

[0143] In keeping with long-standing patent law convention, the words "a" and "an" when used in the present specification in concert with the word comprising, including the claims, denote "one or more." Some embodiments of the invention may consist of or consist essentially of one or more elements, method steps, and/or methods of the invention. It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

[0144] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and so forth which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., Sambrook, Fritsch, and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition (1989), OLIGONUCLEOTIDE SYNTHESIS (M. J. Gait Ed., 1984), ANIMAL CELL CULTURE (R. I. Freshney, Ed., 1987), the series METHODS IN ENZYMOLOGY (Academic Press, Inc.); GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J. M. Miller and M. P. Calos eds. 1987), HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, (D. M. Weir and C. C. Blackwell, Eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Siedman, J. A. Smith, and K. Struhl, eds., 1987), CURRENT PROTOCOLS IN IMMUNOLOGY (J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach and W. Strober, eds., 1991); ANNUAL REVIEW OF IMMUNOLOGY; as well as monographs in

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journals such as ADVANCES IN IMMUNOLOGY. All patents, patent applications, and publications mentioned herein, both supra and infra, are hereby incorporated herein by reference.

[0145] A skilled artisan recognizes that there is a conventional single letter code in the art to represent a selection of nucleotides for a particular nucleotide site. For example, R refers to A or G; Y refers to C or T; M refers to A or C; K refers to G or T; S refers to C or G; W refers to A or T; H refers to A or C or T; B refers to C or G or T; V refers to A or C or G; D refers to A or G or T; and N refers to A or C or G or T.

[0146] The term "blunt end" as used herein refers to the end of a dsDNA molecule having 5' and 3' ends, wherein the 5' and 3' ends terminate at the same nucleotide position. Thus, the blunt end comprises no 5' or 3' overhang.

[0147] The term "double stranded molecule" as used herein refers to a molecule that is double stranded at least in part.

[0148] The term "homing endonuclease" as used herein refers to proteins that are encoded by polynucleotides having mobile, self-splicing introns. Homing endonucleases promote the movement of at least part of the DNA sequences that encode them from one polynucleotide location to another by generating a site-specific double-stranded break at a target site in an allele that lacks the corresponding mobile intron. Examples include but are not limited to at least the following: I-Ceu I, I-Sce I, PI-Psp I, PI-Sce I, or a mixture thereof.

[0149] The terms "hairpin" and "stem-loop oligonucleotide" as used herein refer to a structure formed by an oligonucleotide comprised of 5' and 3' terminal regions that are inverted repeats and a non-self-complementary central region, wherein the self-complementary inverted repeats form a double-stranded stem and the non-self-complementary central region forms a single-stranded loop.

[0150] The term "in the absence of exogenous manipulation" as used herein refers to there being modification of a DNA molecule without changing the solution in which the DNA molecule is being modified. In specific embodiments, it occurs in the absence of the hand of man or in the absence of a machine that changes solution conditions, which may also be referred to as buffer conditions. In further specific embodiments, changes in temperature occur during the modification.

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[0151] The term "nonidentical function" as used herein refers to two or more enzymes that do not comprise the same activity. For example, two restriction endonucleases would be considered to have identical function, although a restriction endonuclease and a ligase would be considered to have nonidentical function. Further, endonucleases are defined as enzymes that cut double stranded DNA and therefore that have identical function, although this may be performed in a variety of ways. For example, some restriction endonucleases cut frequently enough that they may be considered to have a DNA fragmentation function (such as to reduce an average DNA size to the size appropriate for efficient WGA, for example), and other restriction endonucleases have a function of cleaving unmethylated restriction sites, for example to generate a Methylome library (such as cleavage that would not reduce an average DNA size), for example.

[0152] The term "polished" as used herein refers to the repair of dsDNA fragment termini that may be enzymatically repaired, wherein the repair constitutes the fill in of recessed 3' ends or the exonuclease activity trimming back of 5' ends to form a "blunt end" compatible with adaptor ligation.

II. Specific Embodiments of the Invention

[0153] The present invention may employ particular compositions and methods as described in the following exemplary embodiments.

A. One-step attachment of double-stranded inverted repeat DNA sequences to DNA fragments using stem-loop oligonucleotides

[0154] In this embodiment of the present invention, as illustrated in FIG. 3, DNA is incubated with an exemplary mixture comprising a stem-loop oligonucleotide with 3' recessed, 3' protruding, or blunt end (FIG. 4); a 3'proofreading DNA polymerase (Klenow fragment of the DNA polymerase I, T4 DNA polymerase, *etc.*); T4 DNA ligase; Enz-O-Mix Universal Buffer; ATP; and dNTPs. Four exemplary enzymatic reactions are taking place simultaneously: "polishing" of the DNA ends and the oligonucleotide double-stranded stem-region; ligation of the oligonucleotide 3' end to the 5' phosphate of the DNA leaving a nick between the 3' end of DNA and the 5' end of the oligonucleotide double-stranded stem-region; polymerase extension of the 3' DNA end that propagates toward the end of the stem-loop oligonucleotide; and a strand-displacement reaction within the oligonucleotide stem region. The process results in a library of DNA fragments with inverted repeat adaptors at their ends.

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B. Transcription-mediated amplification of DNA library with attached promoter sequence

[0155] In this embodiment of the present invention, as illustrated in FIG. 5, a DNA library produced by incubation with an exemplary mixture comprising a stem-loop oligonucleotide with 3' recessed, 3' protruding, or blunt end (FIG. 4) and T7 promoter sequence within the stem or loop region; a 3'proofreading DNA polymerase (Klenow fragment of the DNA polymerase I, T4 DNA polymerase, *etc.*); T4 DNA ligase; Enz-O-Mix Universal Buffer; ATP; and dNTPs (as shown in FIG. 3), is used as a template for the transcription-mediated amplification. In FIG. 5A, promoter sequence resides within the stem region of a stem-loop oligonucleotide, and there are two inversely oriented promoters at the ends of all DNA fragments: internally located promoter that initiates synthesis of long RNA molecules, and terminal promoter that initiates synthesis of very short (<10 bases) RNA initiation products (not shown in FIG. 5A) (similar products will be produced from the non-attached stem-loop oligonucleotides present in the mixture). In FIG. 5B, the promoter sequence resides within the loop region of a stem-loop oligonucleotide, and there is only one active promoter element that supports synthesis of long RNA products. The transcription-mediated amplification is achieved by incubation of the synthesized DNA library (see above) with T7 RNA polymerase in the presence of ribonucleotides (rNTPs). The products of this reaction are RNA molecules.

[0156] Supplementation of a transcription-mediated amplification reaction with additional ingredients, such as a reverse transcriptase polymerase (M-MuLV reverse transcriptase, AMV reverse transcriptase, *etc.*) and a primer complementary to the T7 promoter or the internal stem region at the 3' end of the RNA molecule (see FIG. 6B), would allow the synthesis of a complementary cDNA. Efficient priming at the 3' ends of RNA is achieved by blocking the transcription-mediated RNA synthesis within the adaptor region to prevent formation of a folded structure at the 3' ends of RNA. Transcriptional arrest can be achieved by using the same chemical or enzymatic modifications of a stem-loop oligonucleotide as described herein in Sections E, F, G, and H and shown in FIGS. 8, 10, and 16C. The products of such a reaction are double stranded DNA/RNA hybrid molecules.

C. Enz-O-Mix reagents and procedures for one-step library synthesis and linear amplification by transcription

[0157] This embodiment is shown in FIG. 6A and illustrates exemplary components and enzymatic reactions involved in the process catalyzed by Enz-O-Mix reagent (Enz-O-Mix 5, FIG. 19B). This reagent simultaneously synthesizes a DNA library with T7

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promoter at the ends and amplifies it linearly by transcription. The Enz-O-Mix reagent 5 comprises a stem-loop oligonucleotide with T7 promoter sequence in the loop region; a DNA fragmentation endonuclease (a restriction enzyme, DNase I, methylation-specific nuclease McrBC, benzonase, apoptotic endonuclease, *etc.*); a 3' proofreading DNA polymerase; T4 DNA ligase; T7 RNA polymerase; Enz-O-Mix Universal Buffer; rNTP; and dNTPs. First, HMW DNA is fragmented by an endonuclease to produce 100-3,000 bp DNA fragments. The T4 DNA polymerase "polishes" the DNA ends and the stem-loop oligonucleotide double-stranded stem-region, and T4 ligase ligates the 3' end of the oligonucleotide to the 5' phosphate of the DNA leaving a nick between the 3' end of DNA and the 5' end of the oligonucleotide double-stranded stem-region. DNA polymerase extends the available 3' DNA end toward the end of a stem-loop oligonucleotide and generates a library of DNA fragments with active conformation of T7 promoter at their ends. RNA polymerase transcribes the library, linearly amplifying DNA and producing single stranded RNA molecules.

[0158] In the case of fragmented DNA (for example, cell-free DNA from blood and/or urine) the reaction does not require a fragmentation endonuclease (Enz-O-Mix 2, FIG. 19A); all other components may be the same as described above.

[0159] In another embodiment, as shown in FIG. 6B, supplementation of a transcription-mediated amplification reaction with the additional ingredients, such as a reverse transcriptase polymerase (M-MuLV reverse transcriptase, AMV reverse transcriptase, *etc.*) and T7 primer, allows the synthesis of a complementary cDNA. Efficient priming at the 3' ends of RNA is achieved by blocking the transcription-mediated RNA synthesis within the adaptor region to prevent formation of a folded structure at the 3' ends of RNA. Transcriptional arrest can be achieved by using the same chemical or enzymatic modifications of a stem-loop oligonucleotide as described herein in Sections E, F, G, and H and shown in FIGS 8, 10, and 16C. The products of such a reaction are double stranded DNA/RNA hybrid molecules.

D. Inhibitory effect of inverted repeats at DNA ends on whole genome amplification and locus-specific PCR

[0160] As shown in FIG. 7, inverted repeats attached to both ends of DNA fragments have an inhibitory effect on PCR process. Heating to 95°C during a first PCR cycle denatures DNA strands and generates hairpin-like structures at both 3' and 5' ends of DNA fragments due to the folding of terminal inverted repeat sequences (the melting temperature for these structures is usually more than 100°C). Subsequent cooling activates a thermostable DNA

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polymerase and results in extension of the self-primed 3' ends and creation of long hairpin DNA molecules refractory to melting, priming, and PCR amplification. Neither the universal primer A nor any internal, site-specific primer pair would produce any detectable PCR product.

E. Library created by Enz-O-Mix attachment process with a stem-loop oligonucleotide with a non-replicable linker

[0161] This embodiment is illustrated in FIG. 8 and describes the one-step Enz-O-Mix attachment process for a stem-loop oligonucleotide with a non-replicable linker. The exemplary reaction mix comprises fragmented DNA; a stem-loop oligonucleotide with 3' recessed, 3' protruding or blunt end (FIG. 4), and a non-replicable linker (such as about in the central part of the oligonucleotide); a 3' proofreading DNA polymerase (Klenow fragment of the DNA polymerase I, T4 DNA polymerase, *etc.*); T4 DNA ligase; Enz-O-Mix Universal Buffer; ATP; and dNTPs. At least the following enzymatic reactions are taking place: "polishing" of the DNA ends and the stem-loop oligonucleotide double-stranded stem-region; ligation of the oligonucleotide 3' end to the 5' phosphate of the DNA, leaving a nick between the 3' end of DNA and the 5' end of the oligonucleotide double-stranded stem-region; polymerase extension of the 3' DNA end that propagates toward the end of stem-loop oligonucleotide and stops somewhere within the loop or close to the loop region at the replication block, for example. The process results in the library of DNA fragments with universal sequence A at the ends and an inverted repeat attached only to the 5' end of DNA.

F. Libraries produced by the Enz-O-Mix attachment process with a stem-loop oligonucleotide comprising a non-replicable linker have no inhibitory effect on PCR

[0162] As shown in FIG. 9, a DNA library produced by the Enz-O-Mix (Enz-O-Mix 1 in the case of fragmented DNA, FIG. 19A, or Enz-O-Mix 4 in the case of HMW DNA, FIG. 19B) attachment process with a stem-loop oligonucleotide with a non-replicable linker has no inhibitory effect on WGA and PCR. Heating to 95°C during first PCR cycle denatures DNA strands and generates hairpin-like structures only at the 5' ends of DNA fragments. At a lower PCR temperature, the universal primer A anneals to the A' region at the 3' ends of DNA fragments, and a thermostable DNA polymerase replicates DNA until it reaches a non-replicable linker in the loop or in the region adjacent to the loop stem region of the stem-loop oligonucleotide attached to the 5' DNA end. Replication of the hairpin region is accompanied by a strand-displacement reaction within the oligonucleotide stem region. Synthesized DNA

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molecules with the universal sequence A and no inverted repeats at the ends (shown by dashed lines) can then successfully be amplified by PCR.

G. Stem-loop oligonucleotide with a non-replicable linker introduced during chemical synthesis

[0163] FIG. 10A shows the structure of a stem-loop oligonucleotide with a non-replicable linker introduced chemically during oligonucleotide synthesis and shows detailed events occurring at DNA end during the exemplary multi-enzyme attachment process. Many structural modifications within the oligonucleotide, such as the exemplary hexaethelene glycol linker, the abasic site, or a cluster of abasic sites (a gap), a bulky group within the base or backbone, *etc.* can block propagation of the DNA replication. The exemplary process occurs as described in FIG. 8.

H. Stem-loop oligonucleotide with a non-replicable abasic gap generated enzymatically by dU-glycosylase during the attachment process

[0164] This embodiment is illustrated in FIG. 10B and shows the structure of a stem-loop oligonucleotide with non-replicable linker introduced enzymatically during the attachment reaction and shows detailed events occurring at a DNA end during the exemplary multi-enzyme attachment process. The exemplary reaction mix comprises fragmented DNA; a stem-loop oligonucleotide with 3' recessed, 3' protruding or blunt end (FIG. 4), and dU bases within the loop and stem regions (optional); a dU-glycosylase; a 3' proofreading DNA polymerase; T4 DNA ligase; Enz-O-Mix Universal Buffer; ATP; and dNTPs. dU-glycosylase creates abasic sites within the loop and upper strand of the stem region of the oligonucleotide; DNA polymerase "polishes" the ends of DNA and the stem-loop oligonucleotide and generates blunt ends; T4 DNA ligase attaches the 3' end of the oligonucleotide and the 5' end of DNA fragment, leaving a nick between the 3' end of DNA and the 5' end of the hairpin double-stranded stem-region; a DNA polymerase extends the 3' end of DNA toward the end of the stem-loop oligonucleotide and stops somewhere within the loop or close to the loop region of the oligonucleotide, such as at the replication block generated by a contiguous abasic site region. The role of abasic sites in the about central stem region is to destabilize base pairing and facilitate the strand displacement reaction. The process results in a library of DNA fragments with universal sequence at both ends and an inverted repeat attached only to the 5' end of DNA, thereby suitable for PCR amplification. Heating at 95°C during PCR generates breaks at abasic sites and completely eliminates the 5' portion of a stem-loop oligonucleotide.

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I. Stem-loop oligonucleotides are advantageous for the Enz-O-Mix attachment process

[0165] This embodiment illustrates the low efficiency and problems associated with use of standard adaptors in the Enz-O-Mix attachment process, although in alternative embodiments standard adaptors may be employed.

[0166] FIG. 11A shows a standard adaptor formed by two different oligonucleotides, wherein one of them has the 5' phosphate group, and possible products formed when such an adaptor is used in the multi-enzyme one-step ligation process. The adaptor should be present at high concentration to provide efficient ligation to DNA ends. Because both ends of a standard adaptor can be ligated to DNA and form adaptor-adaptor conjugates (a dominating product of the reaction), a number of undesirable products may be high, and the yield of desirable molecules may be low, in certain aspects.

[0167] FIG. 11B shows a standard adaptor formed by two different oligonucleotides without phosphate group and two products formed when such an adaptor is used in the multi-enzyme one-step ligation process. Only 50% of formed molecules are desirable products with correct orientation of the adaptor, while the other 50% of molecules have inversed adaptor orientation.

[0168] FIG. 11C shows a standard adaptor formed by two different oligonucleotides without phosphate group but with a protective group at the 3' end of one oligonucleotide and the products formed when such adaptor is used in the multi-enzyme one-step ligation process. A blocked 3' end or recessive 3' end or both are well-known in the art and have been used to prevent formation of products with inversed adaptor orientation during ligation reaction. Presence of a 3' proofreading DNA polymerase in the mix will replace the terminal 3' blocked nucleotide with normal nucleotide (a), and repair and extend the recessed, blocked 3' end (b). As in the case described for FIG. 11B, only 50% of formed molecules are desirable products with correct orientation of the adaptor, while the other 50% of molecules have inversed adaptor orientation.

J. Library generated by the Enz-O-Mix attachment process using a stem-loop oligonucleotide with a cleavable site generated during the multi-enzyme reaction

[0169] In this embodiment of the present invention, as illustrated in FIG. 12, there is a schematic description of the one-step Enz-O-Mix attachment process for a stem-loop

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oligonucleotide with an enzymatically cleavable site generated during the multi-enzyme reaction. The reaction mix contains fragmented DNA, a stem-loop oligonucleotide with 3' recessed, 3' protruding or blunt end (FIG. 4), and a specific DNA sequence R within the loop or in the loop and adjacent stem region; a 3'proofreading DNA polymerase (Klenow fragment of the DNA polymerase I, T4 DNA polymerase, *etc.*); T4 DNA ligase; an endonuclease that recognizes and cuts the oligonucleotide specific oligo-sequence R when it adopts a double-stranded conformation; Enz-O-Mix Universal Buffer; ATP; and dNTPs. Four enzymatic reactions are taking place nearly simultaneously: "polishing" of the DNA ends and the stem-loop oligonucleotide double-stranded stem-region; ligation of the oligonucleotide 3' end to the 5' phosphate of the DNA leaving a nick between the 3' end of DNA and the 5' end of the hairpin double-stranded stem-region, polymerase extension of the 3' DNA end that propagates toward the end of stem-loop oligonucleotide and generates inverted repeats at the ends of DNA fragments; and, finally, cleavage of inverted repeats by an endonuclease at the R sites. The process results in a library of DNA fragments with universal sequence A at the ends, and no inverted repeat attached to the ends of DNA fragments, which is thereby competent for PCR amplification.

K. Examples of stem-loop adaptors with a cleavable restriction site generated by DNA synthesis

[0170] FIG. 13A shows an exemplary stem-loop oligonucleotide with recognition sequence for the restriction endonuclease Eco NI located at the loop region. The enzyme can not cut the CCTNNNNNAGG (SEQ ID NO: 20) region within the stem-loop oligonucleotide (an unrecognizable structure) but it will cut it efficiently when the oligonucleotide is attached to a DNA end and adopts a canonical (double-stranded) Watson-Crick conformation. Cleavage with Eco NI eliminates inverted repeats that inhibit PCR applications.

[0171] FIG. 13B shows an exemplary stem-loop oligonucleotide with recognition sequence for the restriction endonuclease Pac I located within the loop region. The enzyme can not cut the TTAATTAAregion within the stem-loop oligonucleotide, but it will cut it efficiently when the oligonucleotide is attached to a DNA end and adopts a canonical (double-stranded) Watson-Crick conformation. Cleavage eliminates inverted repeats that inhibit PCR applications. Use of a rare-cutting restriction enzyme such as Pac I reduces the number of DNA fragments affected by the enzyme cleavage.

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[0172] FIG. 13C shows the stem-loop oligonucleotide with recognition sequence for the homing endonuclease I-Ceu I located within the loop region. The enzyme can not cut the 26-base single-stranded region within the loop, but it cuts efficiently when the stem-loop oligonucleotide is attached to a DNA end and adopts a canonical (double-stranded) Watson-Crick conformation. Cleavage eliminates inverted repeats that inhibit PCR applications. Use of a homing endonuclease dramatically reduces and practically eliminates cleavage of genomic DNA.

L. Library generated by the Enz-O-Mix attachment process with a stem-loop oligonucleotide with a short stem

[0173] Amplifiable libraries can be synthesized in the presence of but not limited to a stem-loop oligonucleotide with a non-replicable or cleavable linker. FIG. 14 shows a schematic description of an exemplary one-step Enz-O-Mix attachment process using a stem-loop oligonucleotide with a very short stem. The exemplary reaction mix comprises fragmented DNA, a stem-loop oligonucleotide with 3' recessed, 3' protruding or blunt end (FIG. 4), and a short stem region (5-8 bases); a 3'proofreading DNA polymerase (Klenow fragment of the DNA polymerase I, T4 DNA polymerase, *etc.*); T4 DNA ligase; Enz-O-Mix Universal Buffer; ATP; and dNTPs. Three enzymatic reactions are taking place: "polishing" of the DNA ends and the stem-loop oligonucleotide double-stranded stem-region; ligation of the oligonucleotide 3' end to the 5' phosphate of the DNA, leaving a nick between the 3' end of DNA and the 5' end of the hairpin double-stranded stem-region; and polymerase extension of the 3' DNA end that propagates toward the end of the stem-loop oligonucleotide and generates short inverted repeats at the ends of DNA fragments. The process results in a library of DNA fragments with universal sequence A at the ends and a very short inverted repeat attached to the ends of DNA fragments (FIG. 14, shown in bold). PCR is possible because the melting temperature for terminal hairpins is low enough to prevent folding, self-priming and formation of long hairpin molecules, as shown on FIG. 7. Primer A (representing the 3' portion of a stem-loop oligonucleotide without the complementary 5' region) or even the whole stem-loop oligonucleotide can be utilized as a primer for PCR amplification.

M. Enz-O-Mix reagents and procedures for one-step library synthesis and one-step library synthesis/amplification utilizing strand displacement DNA amplification

[0174] This embodiment is shown in FIG. 15 and illustrates exemplary components and enzymatic reactions involved in the process catalyzed by Enz-O-Mix reagents (Enz-O-Mix 3 and Enz-O-Mix 6, FIGS. 19A and 19B). The reagent Enz-O-Mix 3 can use fragmented (cell-free serum DNA or urine DNA, for example), while the reagent Enz-O-Mix 6 can utilize HMW

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DNA and synthesize a library of DNA fragments with the recognition sites for a nicking endonuclease (N.AlwI, N.BbvCIA, N.BbvCIB, Nb.Bpu10I, Nb.BsmI, N.Bst9I, N.BstNBI, *etc.*) at their ends to allow their subsequent amplification by strand displacement DNA synthesis. The presence of nicking endonuclease and strand displacing polymerase in the reagents Enz-O-Mix 3 and Enx-O-Mix 6 allows one to simultaneously synthesize and amplify DNA library by strand displacement DNA synthesis.

[0175] For example, the Enz-O-Mix reagent 3 (suitable for fragmented DNA) comprises a stem-loop oligonucleotide with a recognition sequence for a nicking nuclease; a primer P complementary at least partially to the stem region of a stem-loop oligonucleotide; a 3' proofreading DNA polymerase; a strand displacing DNA polymerase; T4 DNA ligase; a nicking endonuclease; Enz-O-Mix Universal Buffer; and dNTPs. T4 DNA polymerase "polishes" the DNA ends and the stem-loop oligonucleotide double-stranded stem-region. T4 ligase ligates the 3' end of the oligonucleotide to the 5' phosphate of the DNA, leaving a nick between the 3' end of DNA and the 5' end of the oligonucleotide double-stranded stem-region. DNA polymerase extends the available 3' DNA end toward the end of a stem-loop oligonucleotide and generates a library of DNA fragments with functional recognition site for a nicking endonuclease (N.AlwI, N.BbvCIA, N.BbvCIB, Nb.Bpu10I, Nb.BsmI, N.Bst9I, N.BstNBI, *etc.*) at their ends. DNA polymerase initiates multiple rounds of strand displacement DNA synthesis at nicks generated by a nicking nuclease, followed by synthesis of a second DNA strand (primed by primer P), producing double stranded DNA molecules and linearly amplifying DNA.

[0176] In the case of HMW DNA (Enz-O-Mix 6, FIG. 19B), the reaction requires a DNA fragmentation endonuclease (a restriction enzyme, DNase I, methylation-specific nuclease McrBC, benzonase, apoptotic endonuclease *etc.*), and all other components may be the same as described above for Enz-O-Mix 3.

N. Examples of stem-loop adaptors with a nicking endonuclease recognition site generated by DNA synthesis

[0177] FIG. 16A shows the stem-loop oligonucleotide with recognition sequence for the exemplary nicking endonuclease N.Bbv CIA located at the loop region. The enzyme can not nick the single stranded GCTGAGG region within the stem-loop oligonucleotide (an unrecognizable structure), but it will nick it efficiently when the oligonucleotide is attached to a DNA end and adopts a canonical (double-stranded) Watson-Crick conformation.

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[0178] FIG. 16B shows an exemplary stem-loop oligonucleotide with recognition sequence for the nicking endonuclease N.Bbv CIA located at the end of the stem region. The enzyme can not nick efficiently the terminally located TTAATTAA region within the stem-loop oligonucleotide, but it will nick it efficiently when the oligonucleotide is attached to a DNA end and the site acquires an internal location. A second nicking site, generated by the oligonucleotide attachment process, would have a terminal location and low nicking efficiency.

[0179] FIG. 16C shows an exemplary stem-loop oligonucleotides 1 and 2 that contains several functional elements within the loop region including the recognition sequence for a nicking endonuclease. Nultifunctional stem-loop oligonucleotide have several regulatory elements within the loop region: T7 promoter sequence (that includes all regions necessary for transcription); recognition sequence N for a nicking endonuclease (N.AlwI, N.BbvCIA, N.BbvCIB, Nb.Bpu10I, Nb.BsmI, N.Bst9I, N.BstNBI, *etc.*); recognition sequence for a restriction endonuclease; and a replication/transcription block region (a chemical modification or a string of 2-4 dU bases). The regulatory elements can reside within a single loop region (L1 in the stem-loop oligonucleotide 1), or be distributed across several loop regions (L1, L2, and L3 in the stem-loop oligonucleotide 2). A multifunctional stem-loop oligonucleotide can support different types of DNA amplification, including PCR, isothermal amplification by transcription, isothermal amplification by strand displacement synthesis, or even a combination thereof and can also facilitate modification such as DNA immobilization, DNA recombination, DNA cloning, or a combination thereof. Activation of the RNA transcription, DNA nicking, DNA double-stranded cleavage, and generation of the 5' overhang functions of the stem-loop adaptor occurs only upon its enzymatic processing and attachment to DNA ends.

O. Enz-O-Mix is a general method for synthesis and amplification of DNA libraries

[0180] This embodiment of the present invention, as illustrated in FIG. 17, shows generalization of the Enz-O-Mix method and demonstrates its specific applications. When Enz-O-Mix reagent is combined with DNA and incubated at optimal temperature (for example, 37°C) for a time necessary to complete the reaction (for example, 1 hour), although it can be more or less, it results in either a DNA library that can be amplified or cloned in a separate reaction, or a DNA library that is amplified isothermally during the incubation with the Enz-O-Mix reagent. For example, the exemplary products of reaction could be as follows: 1) a library of DNA fragments with universal sequence or sequences attached to the ends of DNA fragments so that it can be amplified by PCR (PCR WGA Library); 2) a library of DNA fragments with a promoter

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sequence attached at the ends of DNA fragments so that it can be amplified by transcription (Transcription WGA Library); 3) a Methylome Library, *i.e.* the library of adapted DNA fragments where all non-methylated CpG islands are eliminated (such as are described in U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety); 4) a library of DNA fragments with ready-to-clone sticky-end adaptors at the ends; 5) an amplified DNA library where the product is DNA or RNA; and/or 6) an amplified Methylome library where the product is DNA or RNA.

P. Stem-loop Oligo Attachment Master Mix is a key component of all Enz-O-Mix reactions

[0181] This embodiment is shown in FIG. 18 and illustrates the components of four different Stem-Loop Oligo Attachment Master Mixes described in the invention and utilized in different Enz-O-Mix reactions involved in the DNA library synthesis. The Stem-Loop Oligo Attachment Master Mix is a key component of the Enz-O-Mix reaction, and it is present in all Enz-O-Mix reagents.

[0182] The exemplary Stem-Loop Oligo Attachment Master Mix I comprises a stem-loop oligonucleotide; a 3' proofreading DNA polymerase; a DNA ligase; Enz-O-Mix Universal Buffer; ATP; and dNTPs (see also FIG. 3 and FIG. 14).

[0183] The exemplary Stem-Loop Oligo Attachment Master Mix II comprises a stem-loop oligonucleotide with a non-replicable chemically introduced linker; a 3' proofreading DNA polymerase; a DNA ligase; Enz-O-Mix Universal Buffer; ATP; and dNTPs (see also FIG. 10A).

[0184] An exemplary Stem-Loop Oligo Attachment Master Mix III comprises a stem-loop oligonucleotide with a non-replicable enzymatically-introduced abasic linker; dU-glycosylase; a 3' proofreading DNA polymerase; a DNA ligase; Enz-O-Mix Universal Buffer; ATP; and dNTPs (see also FIG. 10B).

[0185] An exemplary Stem-Loop Oligo Attachment Master Mix IV comprises a stem-loop oligonucleotide with a cleavable site introduced by DNA replication; a site-specific endonuclease; a 3' proofreading DNA polymerase; a DNA ligase; Enz-O-Mix Universal Buffer; ATP; and dNTPs (see also FIG. 12 and FIGS. 14A, 14B, and 14C).

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Q. Enz-O-Mix reagents and procedures for the one-step Whole Genome Libray synthesis and combined Whole Genome Library synthesis-amplification

[0186] Whole Genome Amplification (WGA) is a process that is used to amplify total genomic DNA for many important research and diagnostic applications when the amount of DNA is limited. WGA is frequently preceded by a multi-step WGA library synthesis process. These embodiments describe the Enz-O-Mix reagents and procedures for the one-step Whole Genome Library synthesis and combined one-step, close-tube Whole Genome Library synthesis-amplification processes.

[0187] FIG. 19A shows schematically compositions of three Enz-O-Mix reagents, specifically Enz-O-Mix 1, Enz-O-Mix 2, and Enz-O-Mix 3 designed to convert fragmented DNA into a Whole Genome Library competent for amplification by PCR, transcription, or strand displacement amplification, respectively.

[0188] Reagent Enz-O-Mix 1 may comprise Stem-Loop Oligo Attachment Master Mixes shown in FIG. 18, and it could be also supplemented with a thermophilic DNA polymerase. The library prepared by the Enz-O-Mix 1 process can be used for PCR-mediated WGA in a open-tube (two-step process) or a close-tube (one-step process) formats (as shown in FIG. 30).

[0189] Reagent Enz-O-Mix 2 may comprise Stem-Loop Oligo Attachment Master Mixes shown in FIG. 18 (in this case a stem-loop oligonucleotide has the T7 promoter sequence in the loop or stem region in addition to other necessary base/backbone modifications), and it could be also supplemented with the T7 RNA polymerase and rNTPs. The Enz-O-Mix 2 can be used for a two-step transcription-mediated Whole Genome Amplification (when the library is prepared first, and then amplified) or for a one-step ithothermal amplification process when the Whole Genome library synthesis and its amplification by transcription occurs simultaneously (as shown in FIG. 6).

[0190] Reagent Enz-O-Mix 3 may comprise Stem-Loop Oligo Attachment Master Mixes shown in FIG. 18 (in this case, a stem-loop oligonucleotide has a recognition sequence for a nicking endonuclease in the loop or stem region in addition to other necessary base/backbone modifications). It could be also supplemented with a nicking endonuclease (N.AlwI, N.BbvCIA, N.BbvCIB, Nb.Bpu10I, Nb.BsmI, N.Bst9I, N.BstNBI, *etc.*), a strand displacing DNA polymerase (Klenow exo-, Phi 29, Bst I, *etc.*), and primer P (FIG. 15). The Enz-O-Mix 3 can be used for a two-step strand displacement-mediated Whole Genome Amplification (when the

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library is prepared first, and then amplified) or for a one-step ithothermal amplification process when the Whole Genome library synthesis and its amplification by a strand displacement synthesis occurs simultaneously (as shown in FIG. 15).

[0191] FIG. 19B shows schematically compositions of three Enz-O-Mix reagents, specifically Enz-O-Mix 4, Enz-O-Mix 5, and Enz-O-Mix 6 designed to convert high molecular DNA into a Whole Genome Library competent for amplification by PCR, transcription, or strand displacement amplification, respectively.

[0192] Reagent Enz-O-Mix 4 may comprise Stem-Loop Oligo Attachment Master Mixes shown in FIG. 18; it is also supplemented with a DNA fragmentation endonuclease such as restriction enzyme DNase I, Benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease, etc.; and it could be also supplemented with a thermophilic DNA polymerase. The library prepared by the Enz-O-Mix 4 process can be used for PCR-mediated WGA in a open-tube (two-step process) or a close-tube (one-step process) formats (as shown in FIG. 30).

[0193] Reagent Enz-O-Mix 5 may comprise Stem-Loop Oligo Attachment Master Mixes shown in FIG. 18 (in this case, a stem-loop oligonucleotide has the T7 promoter sequence in the loop or stem region in addition to other necessary base/backbone modifications), and it is also supplemented with a DNA fragmentation endonuclease such as restriction enzyme DNase I, benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease, *etc.*; and it could be also supplemented with RNA polymerase and rNTPs. The Enz-O-Mix 5 can be used for a two-step transcription-mediated Whole Genome Amplification of HMW DNA (when the library is prepared first, and then amplified) or for a one-step ithothermal amplification process when the Whole Genome library synthesis and its amplification by transcription occurs simultaneously (as shown in FIG. 6).

[0194] Reagent Enz-O-Mix 6 may comprise Stem-Loop Oligo Attachment Master Mixes shown in FIG. 18 (in this case, a stem-loop oligonucleotide has a recognition sequence for a nicking endonuclease in the loop or stem region in addition to other necessary base/backbone modifications), and it is supplemented with a DNA fragmentation endonuclease such as restriction enzyme, DNase I, benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease. It could also be supplemented with a nicking endonuclease (N.AlwI, N.BbvCIA, N.BbvCIB, Nb.Bpu10I, Nb.BsmI, N.Bst9I, N.BstNBI, *etc.*); a strand displacing DNA polymerase (Klenow exo-, Phi 29, Bst I, *etc.*); and primer P (FIG. 15). The Enz-O-Mix 6 can be

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used for a two-step strand displacement-mediated Whole Genome Amplification of HMW DNA (when the library is prepared first, and then amplified) or for a one-step ithothermal amplification process when the Whole Genome library synthesis and its amplification by a strand displacement synthesis occurs simultaneously (as shown in FIG. 15).

R. Enz-O-Mix reagents and procedures for one-step Whole Methylome Library synthesis and combined Whole Methylome Library synthesis-amplification

[0195] Whole Methylome Amplification (WMA) is a process that was recently developed to amplify methylated DNA and suppress amplification of non-methylated genomic (promoter) regions for many important research and diagnostic applications, especially when the amount of DNA is limited (see, for example, U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety). In a standard protocol, WMA is preceded by a multi-step WMA library synthesis process. This embodiment describes the exemplary Enz-O-Mix reagents and procedures for the one-step Whole Methylome Library synthesis and combined Whole Methylome Library synthesis-amplification processes.

[0196] FIG. 19C shows schematically compositions of three Enz-O-Mix reagents, Enx-O-Mix 7,Enx-O-Mix 8, and Enx-O-Mix 9, designed to convert fragmented DNA into a Whole Methylome Library competent for amplification by PCR, transcription, or strand displacement, for example.

[0197] Reagent Enz-O-Mix 7 may comprise one of Stem-Loop Oligo Attachment Master Mixes shown in FIG. 18, and it is also supplemented with a mixture of several (about 5-about 12) methylation-sensitive restriction enzymes, such as Aci I, Acc II, Asp LE I, Ava I, Bce AI, Bsa HI, Bsh 1236 I, Bsi E1, Bsi SI, Bst FN I, Bst HH I, Bst UI, Cfo I, Hap II, Hga I, Hha I, HinP1 I, Hin 6I, Hpa II, Hpy 99I, Hpy CH4 IV, Hsp AI, Mvn I, and Ssi I,, to produce a substantial fragmentation of all non-methylated CpG islands (see, for example, U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety). Reagent Enz-O-Mix 7 could be also supplemented with a thermophilic DNA polymerase. The Methylome library prepared by the Enz-O-Mix 7 process can be used for PCR-mediated WMA in a open-tube (two-step process) or a close-tube (one-step process) formats (as shown in FIG. 30).

[0198] Reagent Enz-O-Mix 8 may comprise Stem-Loop Oligo Attachment Master Mixes shown in FIG. 18 (in this case a stem-loop oligonucleotide has the T7 promoter sequence

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in the loop or stem region in addition to other necessary base/backbone modifications), and it is also supplemented with a mixture of several (about 5-about 12) methylation-sensitive restriction enzymes, such as Aci I, Acc II, Asp LE I, Ava I, Bce AI, Bsa HI, Bsh 1236 I, Bsi E1, Bsi SI, Bst FN I, Bst HH I, Bst UI, Cfo I, Hap II, Hga I, Hha I, HinP1 I, Hin 6I, Hpa II, Hpy 99I, Hpy CH4 IV, Hsp AI, Mvn I, and Ssi I,, to produce a substantial fragmentation of all non-methylated CpG islands (U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety). Reagent Enz-O-Mix 8 could be also supplemented with T7 RNA polymerase and rNTPs. The Enz-O-Mix 8 can be used for a two-step transcription-mediated Whole Methylome Amplification (when the Whole Methylome library is prepared first, and then amplified) or for a one-step ithothermal amplification process when the Methylome library synthesis and its amplification by transcription occurs simultaneously.

[0199] Reagent Enz-O-Mix 9 may comprise Stem-Loop Oligo Attachment Master Mixes shown in FIG. 18 (in this case, a stem-loop oligonucleotide has a recognition sequence for a nicking endonuclease in the loop or stem region in addition to other necessary base/backbone modifications), and it is also supplemented with a mixture of several (about 5-12) methylation-sensitive restriction enzymes, such as Aci I, Acc II, Asp LE I, Ava I, Bce AI, Bsa HI, Bsh 1236 I, Bsi E1, Bsi SI, Bst FN I, Bst HH I, Bst UI, Cfo I, Hap II, Hga I, Hha I, HinP1 I, Hin 6I, Hpa II, Hpy 99I, Hpy CH4 IV, Hsp AI, Mvn I, and Ssi I,, to produce a substantial fragmentation of all non-methylated CpG islands (see, for example, U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety). Reagent Enz-O-Mix 9 could be also supplemented with nicking endonuclease (N.AlwI, N.BbvCIA, N.BbvCIB, Nb.Bpu10I, Nb.BsmI, N.Bst9I, N.BstNBI, etc.), a strand displacing DNA polymerase (Klenow exo-, Phi 29, Bst I, etc.), and primer P (FIG. 15). The Enz-O-Mix 9 can be used for a two-step strand displacement-mediated Whole Methylome Amplification (when the library is prepared first, and then amplified) or for a one-step ithothermal amplification process when the Methylome library synthesis and its amplification by a strand displacement synthesis occurs simultaneously (as shown in FIG. 15).

[0200] FIG. 19D show schematically compositions of another three Enz-O-Mix reagents, Enx-O-Mix 10, Enx-O-Mix 11, and Enx-O-Mix 12, designed to convert HMW DNA into a Whole Methylome Library competent for amplification by PCR, transcription, or strand displacement, for example.

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[0201] Reagent Enz-O-Mix 10 may comprise one of Stem-Loop Oligo Attachment Master Mixes shown in FIG. 18, and it is also supplemented with a DNA fragmentation endonuclease such as restriction enzyme, DNase I, benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease, and a mixture of several (about 5-12) methylation-sensitive restriction enzymes, such as Aci I, Acc II, Asp LE I, Ava I, Bce AI, Bsa HI, Bsh 1236 I, Bsi E1, Bsi SI, Bst FN I, Bst HH I, Bst UI, Cfo I, Hap II, Hga I, Hha I, HinP1 I, Hin 6I, Hpa II, Hpy 99I, Hpy CH4 IV, Hsp AI, Mvn I, and Ssi I,, to produce a substantial fragmentation of all non-methylated CpG islands (see, for example, U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety). It could be also supplemented with a thermophilic DNA polymerase. The Methylome library prepared by the Enz-O-Mix 10 process can be used for PCR-mediated WMA in a open-tube (two-step process) or a close-tube (one-step process) formats (as shown in FIG. 30).

[0202] Reagent Enz-O-Mix 11 may comprise Stem-Loop Oligo Attachment Master Mixes shown in FIG. 18 (in this case a stem-loop oligonucleotide has the T7 promoter sequence in the loop or stem region in addition to other necessary base/backbone modifications), and it is also supplemented with a DNA fragmentation endonuclease, such as restriction enzyme, DNase I, benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease, *etc.*;; and a mixture of several (about 5-about 12) methylation-sensitive restriction enzymes, such as Aci I, Acc II, Asp LE I, Ava I, Bce AI, Bsa HI, Bsh 1236 I, Bsi E1, Bsi SI, Bst FN I, Bst HH I, Bst UI, Cfo I, Hap II, Hga I, Hha I, HinP1 I, Hin 6I, Hpa II, Hpy 99I, Hpy CH4 IV, Hsp AI, Mvn I, and Ssi I,, to produce a substantial fragmentation at all non-methylated CpG islands (see, for example, U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety). Reagent Enz-O-Mix 11 could be also supplemented with T7 RNA polymerase and rNTPs. The Enz-O-Mix 11 can be used for a two-step transcription-mediated Whole Methylome Amplification (when the Whole Methylome library is prepared first, and then amplified) or for a one-step ithothermal amplification process when the Methylome library synthesis and its amplification by transcription occurs simultaneously.

[0203] Reagent Enz-O-Mix 12 may comprise Stem-Loop Oligo Attachment Master Mixes shown in FIG. 18 (in this case, a stem-loop oligonucleotide has a recognition sequence for a nicking endonuclease in the loop or stem region in addition to other necessary base/backbone modifications), and it is also supplemented with a DNA fragmentation endonuclease, such as restriction enzyme, DNase I, benzonase, methylation-specific nuclease McrBC, apoptotic

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endonuclease; and a mixture of several (about 5-12) methylation-sensitive restriction enzymes, such as Aci I, Acc II, Asp LE I, Ava I, Bce AI, Bsa HI, Bsh 1236 I, Bsi E1, Bsi SI, Bst FN I, Bst HH I, Bst UI, Cfo I, Hap II, Hga I, Hha I, HinP1 I, Hin 6I, Hpa II, Hpy 99I, Hpy CH4 IV, Hsp AI, Mvn I, and Ssi I,, to produce a substantial fragmentation of all non-methylated CpG islands (see, for example, U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety). Reagent Enz-O-Mix 12 could be also supplemented with nicking endonuclease (N.AlwI, N.BbvCIA, N.BbvCIB, Nb.Bpu10I, Nb.BsmI, N.Bst9I, N.BstNBI, etc.), a strand displacing DNA polymerase (Klenow exo-, Phi 29, Bst I, etc.), and primer P (FIG. 15). The Enz-O-Mix 12 can be used for a two-step strand displacement-mediated Whole Methylome Amplification (when the library is prepared first, and then amplified) or for a one-step ithothermal amplification process when the Methylome library synthesis and its amplification by a strand displacement synthesis occurs simultaneously (as shown in FIG. 15).

S. Enz-O-Mix reagents and procedures for the one-step Whole Genome Libray synthesis for cloning

[0204] DNA cloning is a powerful tool for whole genome DNA sequencing and analysis of cDNA libraries, for example. In a standard process, DNA is randomly fragmented, repaired, attached to linkers, and then integrated into a vector. This embodiment describes the Enz-O-Mix reagents for the one-step preparation of DNA fragments with sticky ends that is easy to clone.

[0205] FIG. 19E shows schematically exemplary compositions of two Enz-O-Mix reagents designed to convert fragmented (Enz-O-Mix 13) or HMW DNA (Enz-O-Mix 14) into a Whole Genome Library for cloning.

[0206] Reagent Enz-O-Mix 13 may comprise a Stem-Loop Oligo Attachment Master Mix IV shown in FIG. 18, and it is the only component of the Enz-O-Mix 13 reagent. The library generated by the Enz-O-Mix 13 process can only utilize a fragmented DNA (for example, cell-free DNA from blood and/or urine, or DNA fragmented enzymatically or mechanically, for example) and be used for cloning.

[0207] Reagent Enz-O-Mix 14 may comprise a Stem-Loop Oligo Attachment Master Mix IV shown in FIG. 18 that is supplemented with a DNA fragmentation endonuclease, such as restriction enzyme, DNase I, benzonase, methylation-specific nuclease McrBC, apoptotic

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endonuclease, *etc.* The library generated by the Enz-O-Mix 14 process can utilize HMW DNA from any type of cells and tissues and be used for cloning.

T. Enz-O-Mix reagent kits and their use in high-throughput applications and clinical diagnostics

[0208] In this embodiment of the present invention, as illustrated in FIG. 20, pre-assembled Enz-O-Mix reagents can be stored at -20°C and used as ready-to-use kits.

[0209] Exemplary pre-assembled Enz-O-Mix 1 comprises all major components of the reaction, such as a stem-loop oligonucleotide; DNA polymerase; a DNA ligase; dU-glycosylase (when the hairpin non-replicable region is generated by abasic sites); a hairpin-specific endonuclease (when the hairpin has a cleavable site generated during oligonucleotide attachment process); thermophilic DNA polymerase (close-tube WGA library synthesis / PCR-mediated amplification reaction); ATP; and dNTPs.

[0210] Exemplary pre-assembled Enz-O-Mix 2 comprises a stem-loop oligonucleotide with a promoter sequence in a stem or loop region; DNA polymerase; a DNA ligase; dU-glycosylase (when the non-replicable region is formed by abasic sites); a site-specific endonuclease (when the stem-loop oligonucleotide has a cleavable site created during oligonucleotide attachment process); ATP; and dNTPs; In the case of close-tube, isothermal library synthesis / transcription-mediated amplification reaction it also comprises RNA polymerase and rNTPs.

[0211] Exemplary pre-assembled Enz-O-Mix 3 may comprise a stem-loop oligonucleotide with a nicking endonuclease sequence in a stem or a loop region; DNA polymerase; a DNA ligase; dU-glycosylase (when the non-replicable region is formed by abasic sites); a site-specific endonuclease (when the stem-loop oligonucleotide has a cleavable site generated during oligonucleotide attachment process); a DNA fragmentation endonuclease, such as restriction enzyme(s), DNase I, Benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease *etc.*; ATP; and dNTP, In the case of close-tube, isothermal library synthesis / strand displacement-mediated amplification reactiuon it also comprises a nicking endonuclease (N.AlwI, N.BbvCIA, N.BbvCIB, Nb.Bpu10I, Nb.BsmI, N.Bst9I, N.BstNBI, *etc.*); a strand displacing DNA polymerase (Klenow exo-, Phi 29, Bst I, *etc.*); and a primer.

[0212] Exemplary pre-assembled Enz-O-Mix 4 may comprise all major components of the reaction, such as a stem-loop oligonucleotide; DNA polymerase; a DNA

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ligase; dU-glycosylase (when the hairpin non-replicable region is created by abasic sites); a hairpin-specific endonuclease (when the hairpin has a cleavable site created during oligonucleotide attachment process); a DNA fragmentation endonuclease such as restriction enzyme, DNase I, Benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease ; thermophilic DNA polymerase (close-tube WGA library synthesis / PCR-mediated amplification reaction); ATP; and dNTPs.

[0213] Exemplary pre-assembled Enz-O-Mix 5 may comprise a stem-loop oligonucleotide with a promoter sequence in a stem or loop region; DNA polymerase; DNA ligase; dU-glycosylase (when the non-replicable region is formed by abasic sites); a site-specific endonuclease (when the stem-loop oligonucleotide has a cleavable site created during the oligonucleotide attachment process); a DNA fragmentation endonuclease, such as restriction enzyme, DNase I, benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease, *etc.*; ATP; and dNTPs. In the case of close-tube, isothermal library synthesis / transcription-mediated amplification reaction it also comprises RNA polymerase and rNTPs..

[0214] Exemplary pre-assembled Enz-O-Mix 6 comprises a stem-loop oligonucleotide with a nicking endonuclease sequence in a stem or a loop region, DNA polymerase, a DNA ligase, dU-glycosylase (when the non-replicable region is formed by abasic sites), a site-specific endonuclease (when the stem-loop oligonucleotide has a cleavable site created during oligonucleotide attachment process), a DNA fragmentation endonuclease such as restriction enzyme(s), DNase I, Benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease; ATP; and dNTPs. In the case of close-tube, isothermal library synthesis / strand displacement-mediated amplification reactiuon it also comprises a nicking endonuclease (N.AlwI, N.BbvCIA, N.BbvCIB, Nb.Bpu10I, Nb.BsmI, N.Bst9I, N.BstNBI, *etc.*); a strand displacing DNA polymerase (Klenow exo-, Phi 29, Bst I, *etc.*); and a primer.

[0215] Exemplary pre-assembled Enz-O-Mix 7 may comprise all major components of the reaction, such as a stem-loop oligonucleotide ; DNA polymerase; T4 DNA ligase; dU-glycosylase (when the hairpin non-replicable region is created by abasic sites); a hairpin-specific endonuclease (when the hairpin has a cleavable site created during oligonucleotide attachment process); a mixture of several (about 5-about 12) methylation-sensitive restriction enzymes, such as Aci I, Acc II, Asp LE I, Ava I, Bce AI, Bsa HI, Bsh 1236 I, Bsi E1, Bsi SI, Bst FN I, Bst HH I, Bst UI, Cfo I, Hap II, Hga I, Hha I, HinP1 I, Hin 6I, Hpa II, Hpy 99I, Hpy CH4 IV, Hsp AI, Mvn I, and Ssi I,, to produce a substantial fragmentation at all

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non-methylated CpG islands (see, for example, U.S. Patent Application Serial No. U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety); thermophilic DNA polymerase (close-tube WMA library synthesis / PCR-mediated amplification reaction); ATP; and dNTPs.

[0216] Exemplary pre-assembled Enz-O-Mix 8 may comprise a stem-loop oligonucleotide with a promoter sequence in a stem or loop region; DNA polymerase; a DNA ligase; dU-glycosylase (when the non-replicable region is formed by abasic sites); a site-specific endonuclease (when the stem-loop oligonucleotide has a cleavable site created during the oligonucleotide attachment process); a mixture of several (about 5-about 12) methylation-sensitive restriction enzymes, such as Aci I, Acc II, Asp LE I, Ava I, Bce AI, Bsa HI, Bsh 1236 I, Bsi E1, Bsi SI, Bst FN I, Bst HH I, Bst UI, Cfo I, Hap II, Hga I, Hha I, HinP1 I, Hin 6I, Hpa II, Hpy 99I, Hpy CH4 IV, Hsp AI, Mvn I, and Ssi I,, to produce a substantial fragmentation at all non-methylated CpG islands (see, for example, U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety); ATP; and dNTPs. In the case of close-tube, isothermal library synthesis / transcription-mediated amplification reaction it also comprises RNA polymeraseand rNTPs.

[0217] Exemplary pre-assembled Enz-O-Mix 9 may comprise a stem-loop oligonucleotide with a nicking endonuclease sequence in a stem or a loop region; DNA polymerase; a DNA ligase; dU-glycosylase (when the non-replicable region is formed by abasic sites); a site-specific endonuclease (when the stem-loop oligonucleotide has a cleavable site created during oligonucleotide attachment process); a DNA fragmentation endonuclease, such as restriction enzyme(s), DNase I, Benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease, *etc.*; a mixture of several (about 5-about 12) methylation-sensitive restriction enzymes, such as Aci I, Acc II, Asp LE I, Ava I, Bce AI, Bsa HI, Bsh 1236 I, Bsi E1, Bsi SI, Bst FN I, Bst HH I, Bst UI, Cfo I, Hap II, Hga I, Hha I, HinP1 I, Hin 6I, Hpa II, Hpy 99I, Hpy CH4 IV, Hsp AI, Mvn I, and Ssi I,, to produce a substantial fragmentation at all non-methylated CpG islands (see, for example, U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety); ATP; and dNTPs. In the case of close-tube, isothermal library synthesis / strand displacement-mediated amplification reactiuon it also comprises a nicking endonuclease (N.AlwI, N.BbvCIA, N.BbvCIB, Nb.Bpu10I, Nb.BsmI, N.Bst9I, N.BstNBI, *etc.*); a strand displacing DNA polymerase (Klenow exo-, Phi 29, Bst I, *etc.*); and a primer.

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[0218] Exemplary pre-assembled Enz-O-Mix 10 may comprise all major components of the reaction, such as a stem-loop oligonucleotide; DNA polymerase; a DNA ligase; dU-glycosylase (when the hairpin non-replicable region is created by abasic sites); a hairpin-specific endonuclease (when the hairpin has a cleavable site created during oligonucleotide attachment process); a DNA fragmentation endonuclease, such as restriction enzyme, DNase I, Benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease, *etc.*; a mixture of several (about 5-about 12) methylation-sensitive restriction enzymes, such as Aci I, Acc II, Asp LE I, Ava I, Bce AI, Bsa HI, Bsh 1236 I, Bsi E1, Bsi SI, Bst FN I, Bst HH I, Bst UI, Cfo I, Hap II, Hga I, Hha I, HinP1 I, Hin 6I, Hpa II, Hpy 99I, Hpy CH4 IV, Hsp AI, Mvn I, and Ssi I,, to produce a substantial fragmentation at all non-methylated CpG islands (see, for example, U.S. Patent Application Serial No. No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety); thermophilic DNA polymerase (close-tube WGA library synthesis / PCR-mediated amplification reaction); ATP; and dNTPs.

[0219] Exemplary pre-assembled Enz-O-Mix 11 may comprise a stem-loop oligonucleotide with a promoter sequence in a stem or loop region; DNA polymerase; a DNA ligase; dU-glycosylase (when the non-replicable region is formed by abasic sites); a site-specific endonuclease (when the stem-loop oligonucleotide has a cleavable site created during oligonucleotide attachment process); a DNA fragmentation endonuclease, such as restriction enzyme, DNase I, benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease; a mixture of several (about 5-12) methylation-sensitive restriction enzymes, such as Aci I, Acc II, Asp LE I, Ava I, Bce AI, Bsa HI, Bsh 1236 I, Bsi E1, Bsi SI, Bst FN I, Bst HH I, Bst UI, Cfo I, Hap II, Hga I, Hha I, HinP1 I, Hin 6I, Hpa II, Hpy 99I, Hpy CH4 IV, Hsp AI, Mvn I, and Ssi I,, to produce a substantial fragmentation at all non-methylated CpG islands (see, for example, U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety); ATP; and dNTPs. In the case of close-tube, isothermal library synthesis / transcription-mediated amplification reaction it also comprises RNA polymerase and rNTPs..

[0220] Exemplary pre-assembled Enz-O-Mix 12 may comprise a stem-loop oligonucleotide with a nicking endonuclease sequence in a stem or a loop region; DNA polymerase; a DNA ligase; dU-glycosylase (when the non-replicable region is formed by abasic sites); a site-specific endonuclease (when the stem-loop oligonucleotide has a cleavable site created during oligonucleotide attachment process); a DNA fragmentation endonuclease, such as

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restriction enzyme(s), DNase I, benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease; a mixture of several (about 5-about 12) methylation-sensitive restriction enzymes, such as Aci I, Acc II, Asp LE I, Ava I, Bce AI, Bsa HI, Bsh 1236 I, Bsi E1, Bsi SI, Bst FN I, Bst HH I, Bst UI, Cfo I, Hap II, Hga I, Hha I, HinP1 I, Hin 6I, Hpa II, Hpy 99I, Hpy CH4 IV, Hsp AI, Mvn I, and Ssi I,, to produce a substantial fragmentation at all non-methylated CpG islands (see, for example, U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005, which is incorporated by reference herein in its entirety); ATP; and dNTPs. In the case of close-tube, isothermal library synthesis / strand displacement-mediated amplification reactiuon it also comprises a nicking endonuclease (N.AlwI, N.BbvCIA, N.BbvCIB, Nb.Bpu10I, Nb.BsmI, N.Bst9I, N.BstNBI, *etc.*) a strand displacing DNA polymerase (Klenow exo-, Phi 29, Bst I, *etc.*); and a primer.

[0221] Kits comprising the exemplary Enz-O-Mixes described above may be supplied with the Enz-O-Mix Universal Buffer. When used in high-throughput applications and clinical diagnostics, the Enz-O-Mix reagent is first diluted by the Enz-O-Mix Universal Buffer, aliquoted into microplate wells, pre-incubated at the reaction temperature (for example, about 37°C) for a few minutes, supplemented with DNA, and incubated at the reaction temperature for about 1-about 16 h.

[0222] FIG. 20 demonstrates an exemplary application of the Enz-O-Mix kits for high-throughput library preparation / amplification in the 96- or 384-well format.

U. Enz-O-Mix reaction optimization procedures

[0223] In this embodiment, as shown in FIG. 21, there are exemplary procedures for identifying the most optimal parameters for any Enz-O-Mix process using real-time PCR to monitor amplification of the generated library, or a direct fluorescent detection of the amplified product in the case of combined isothermal library synthesis-amplification process.

[0224] FIG. 21A illustrates the procedure for optimization of the Enz-O-Mix Universal Buffer. Three specific but exemplary buffers are utilized, including Universal Buffers 1, 2 and 3. Buffer 2 results in the early WGA amplification curve, suggesting that the multi-enzymatic reaction was more efficient in this buffer, and the Universal Buffer 2 is particularly suitable for the Enz-O-Mix reaction.

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[0225] FIG. 21B illustrates the procedure for concentration optimization of the Enz-O-Mix incubation temperature. Three temperatures are employed; specifically, T_1, T_2 and T_3. Temperature T_3 results in the early WGA amplification curve, suggesting that the multi-enzymatic reaction was more efficient at this temperature and that the parameter T_3 should be selected as a more suitable condition for the Enz-O-Mix reaction.

[0226] FIG. 21C illustrates the procedure for optimization of the Enz-O-Mix component N. Three concentrations of the component N; specifically, C^N_1, C^N_2 and C^N_3, were utilized. Concentration C^N_1 results in the early WGA amplification curve, suggesting that the multi-enzymatic reaction was more efficient at this component N concentration, and that the parameter C^N_1 is a particularly suitable condition for the Enz-O-Mix reaction.

V. High potential for the Enz-O-Mix approach in future biotechnology and diagnostic medicine

[0227] The applications of the Enz-O-Mix method are numerous. The Enz-O-Mix method is easy to automate and use in clinical diagnostic and point-of-care applications. Different enzyme combinations could lead to many novel kits and assays, and applications in such areas as biotechnology, the pharmaceutical industry, molecular diagnostics, forensics, pathology, bio-defense, and bio-computing are contemplated The Enz-O-Mix approach can be viewed as a simple and cost-effective alternative to the "lab-on-a chip" micro-fluidic approach that is currently attempting to solve the same problem (multi-step DNA processing) by reduction of reaction volumes and integration of multiple reactions into a small format.

[0228] Exemplary applications for the invention include but are not limited to the following:

[0229] a) A one-step, closed tube preparation and amplification of genomic libraries (WGA) from highly degraded serum, plasma, and/or urine (such as the supernatant fraction) DNA. DNA amplification and re-amplification can be used as an *in vitro* "immortalization" process to maintain and generate necessary quantities of valuable but limited DNA samples for gene association studies, mutation and microsatellite instability detection in cancer diagnostics, etc.

[0230] b) A one-step, closed tube preparation and amplification of genomic libraries (WGA) from formalin fixed, paraffin embedded tissues for cancer diagnostics and research applications.

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[0231] c) A one-step, closed tube preparation and amplification of Methylome libraries;

[0232] d) A one-step preparation and simultaneous immobilization of prepared DNA libraries on a solid support.

EXAMPLES

[0233] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1: ONE STEP WHOLE GENOME LIBRARY AMPLIFICATION USING HAIRPIN ADAPTOR WITH EcoNI RESRTICTION SITE GENERATED AFTER LIGATION AND STRAND-DISPLACEMENT EXTENSION

[0234] In this example, a single step WGA process is described wherein a partial hairpin oligonucleotide adaptor containing EcoNI recognition half sites in its stem is extended by a DNA polymerase to form a blunt end hairpin molecule and is ligated *via* its free 3' end to the 5' phosphate of blunt-ended DNA restriction fragments by T4 DNA ligase. Due to the strand-displacement activity of the DNA polymerase, the free 3' end of the restriction fragments are extended using as template the ligated 3' end of the adaptor, thus opening up the hairpin structure and generating functional EcoNI cleavage site (FIG. 12 and 13A). Following cleavage with EcoNI present in the same reaction mix, the DNA library molecules have their terminal inverted repeats excised and become amplifiable by PCR using a primer complementary to the 5' stem portion of the adaptor sequence. The whole process takes place in a single tube and in one step in just about 1 hour, in specific embodiments.

[0235] Human genomic DNA isolated from the peripheral blood of a healthy donor by standard procedures was digested with 10 units of AluI restriction enzyme (NEB) for 1 hour following the manufacturer's protocol.

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[0236] For library preparation, 5 nanograms of AluI-digested DNA were incubated in a reaction mixture comprising 1x NEBuffer 4 (20 mM Tris-acetate, 10 mM Mg acetate, 50 mM potassium acetate, 1 mM dithiothreitol, pH 7.9), 1 μM EcoNI Ext Adaptor oligonucleotide (Table I, SEQ ID NO:1), 1 mM ATP, 120 μM dNTPs, 2 Units of Klenow fragment of DNA polymerase I, 400 units of T4 DNA ligase (New England Biolabs, NEB; Beverly, MA), and 10 units of EcoNI restriction enzyme (NEB) in a final volume of 15 μl for 1 hour at 37°C. Enzymes were inactivated for 10 min at 75 °C. Control reaction that contained no EcoNI restriction enzyme was also run in parallel.

[0237] The resulting library was amplified by real-time PCR in a reaction mixture containing: 1 x Titanium *Taq* reaction buffer (Clontech; Mountain View, CA); 200 μM each dNTP; 1:10,000 dilutions of fluorescein and SybrGreen I (Molecular Probes; Carlsbad, CA); 1 μM of K_U primer (Table I, SEQ ID NO:2); and 5 units of Titanium *Taq* polymerase (Clontech; Mountain View, CA) in a reaction volume of 75 μl. PCR amplification was carried out for 15 cycles at 95°C for 15 sec and 65°C for 2 min on i-Cycler real-time PCR instrument (Bio-Rad; Hercules, CA). FIG. 22 shows the amplification curves of the libraries prepared in the presence or in the absence of EcoNI. Cleavage of the terminal inverted repeat by EcoNI improved the efficiency of amplification by 7 cycles, *i.e.* over two orders of magnitude.

[0238] Preliminary representation analysis of the library prepared in the presence of EcoNI was conducted using three exemplary human genomic STS markers (Table II). The material amplified by PCR with the universal K_U primer was purified with Qiaquick filters (Qiagen; Valencia, CA), and 20 ng aliquots were analyzed in real-time PCR. Reactions were carried out for 45 cycles at 94°C for 15 sec and 68°C for 1 min on i-Cycler (Bio-Rad; Hercules, CA). The PCR reaction mixture comprised the following: 1 x Titanium *Taq* reaction buffer (Clontech; Mountain View, CA); 200 μM each dNTP; 1:10, 000 dilutions of fluorescein and SybrGreen I (Molecular Probes); 0.2 μM each forward and reverse STS primer (Table II); 5 units of Titanium *Taq* polymerase (Clontech; Mountain View, CA); and 20 ng library DNA. A control reaction comprising 1 ng of human genomic DNA randomly fragmented to an average size of 1.5 Kb using Hydro Shear device (Gene Machines; Palo Alto, CA) was performed. FIGS. 24A-24C show unbiased representation of the analyzed human STS sequences in the EcoNI - treated library.

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EXAMPLE 2: ONE STEP WHOLE GENOME AMPLIFICATION USING HAIRPIN ADAPTOR WITH NON-REPLICABLE 18-ATOM HEXA-ETHYLENEGLYCOL SPACER

[0239] In this example, a single step WGA process is described wherein a hairpin oligonucleotide adaptor containing non-replicable 18-atom hexa-ethyleneglycol (HEG) spacer in its loop is ligated *via* its free 3' end to the 5' phosphate of DNA restriction fragments in the presence of T4 DNA ligase and a DNA polymerase. Due to the strand-displacement activity of the polymerase, the free 3' ends of the restriction fragments are extended until the replication stop is reached (see FIGS. 8 and 10A). This process results in truncated 3' termini of the resulting WGA library such that they do not contain a terminal inverted repeat. The library molecules are then amplified by PCR using a primer complementary to the universal sequence of the extended 3' termini. The whole process takes place in a single tube in one step and is completed in just about 1 hour, in specific embodiments.

[0240] In control experiments, hairpin adaptor molecules that either do not comprise a replication stop or modified bases or that comprise ribo-nucleosides in their loop region are compared for their ability to participate in WGA along with the hexa-ethyleneglycol adaptor described above.

[0241] Human genomic DNA isolated from the peripheral blood of a healthy donor by standard procedures was digested with 10 units of AluI restriction enzyme (NEB) for 1 hour following the manufacturer's protocol.

[0242] Five nanograms of AluI digested DNA were incubated in a reaction mixture comprising 1x NEBuffer 4; 1 μM HEG Adaptor oligonucleotide (Table I, SEQ ID NO:3) or control hairpin adaptors (see below); 1 mM ATP; 120 μM dNTPs; 2 Units of Klenow fragment of DNA polymerase I; and 400 units of T4 DNA ligase (NEB) in a final volume of 15 μl for 1 hour at 37°C. Enzymes were inactivated for 10 min at 75 °C.

[0243] To test the effect of the hexa-ethyleneglycol replication stop, control reactions containing partial hairpin oligonucleotides extended by Klenow to form blunt end hairpin adaptors that either do not contain replication stop (Table I, Hairpin Adaptor, SEQ ID NO:5), or containing ribonucleosides in their loop region (Table I, Hairpin Ribo Adaptor, SEQ ID NO:6) were substituted for the HEG Adaptor (Table I, SEQ ID NO:3) and run in parallel as described above.

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[0244] The resulting libraries were amplified by real-time PCR in a reaction mixture comprising the following: 1 x Titanium *Taq* reaction buffer (Clontech; Mountain View, CA); 200 μM each dNTP; 1:10,000 dilutions of fluorescein and SybrGreen I (Molecular Probes; Carlsbad, CA); 1 μM of universal primer (Y_U primer, Table I SEQ ID NO:4 in the case of the HEG adaptor, or K_U primer, Table I, SEQ ID NO:2, in the case of control hairpin adaptors); and 5 units of Titanium *Taq* polymerase (Clontech; Mountain View, CA) in a reaction volume of 75 μl. PCR amplification was carried out at 95°C for 15 sec and 65°C for 2 min on i-Cycler real-time PCR instrument (Bio-Rad; Hercules, CA). As shown on FIG. 23, amplification of the library prepared by ligation of hexa-ethyleneglycol adaptor was 9 cycles more efficient than a library made with adaptor not containing a replication stop in its loop structure. The results shown on FIG. 23 also indicate that an array of 5 consecutive ribonucleosides in the adaptor's loop is not a preferable replication stop for Klenow fragment of DNA polymerase I, since amplification of a library prepared by ligation of such an adaptor amplified only 2 cycles earlier than a library made with adaptor containing only deoxyribonucleosides.

[0245] Preliminary representation analysis of the library prepared by ligation of HEG adaptor was done using three exemplary human genomic STS markers (Table II). The material amplified by PCR with the universal Y_U primer was purified with Qiaquick filters (Qiagen; Valencia, CA), and 20 ng aliquots were analyzed in real-time PCR as described in Example 1. FIGS. 24A-24C show unbiased representation of the analyzed human STS sequences in this exemplary library.

EXAMPLE 3: ONE STEP WHOLE GENOME LIBRARY PREPARATION USING DEGRADABLE HAIRPIN ADAPTOR COMPRISING DEOXY-URIDINE

[0246] In this example, a single step WGA process is described wherein a hairpin oligonucleotide adaptor comprising deoxy-uridine in both its 5' stem region and in its loop (FIG. 10B) is ligated *via* its free 3' end to the 5' phosphates of DNA restriction fragments in the presence of 3 enzymatic activities: T4 DNA ligase, DNA polymerase, and Uracil-DNA glycosylase (which is also referred to as UDG or dU glycosylase). UDG catalyzes the release of free uracil and generates abasic sites in the adaptor's loop region and the 5' half of the hairpin. The strand-displacement activity of the DNA polymerase extends the free 3' end of the restriction fragments until an abasic site is reached, serving as a replication stop (FIG. 10B). This process results in truncated 3' ends of the resulting WGA library fragments such that they do not have terminal inverted repeats. Prior to amplification, samples are heated in order to break

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the phosphodiester bonds of the generated abasic sites, and the adaptor molecules are degraded into 2 small fragments and one intact 18 base oligonucleotide complementary to the universal sequence of the extended 3' termini (FIG. 10B). The released 18 base oligonucleotide then serves as a primer in subsequent PCR amplification, or alternatively it can be exogenously supplied. The entire process takes place in a single tube in one step and is completed in just 1 hour.

[0247] Human genomic DNA isolated from the peripheral blood of a healthy donor by standard procedures was digested with 10 units of AluI restriction enzyme (New England Biolabs; Beverly, MA) for 1 hour following the manufacturer's protocol.

[0248] For library preparation, five nanograms of AluI-digested genomic DNA were incubated in an exemplary reaction mixture comprising 1x NEBuffer 4; 2 μM dU Hairpin Adaptor oligonucleotide (Table I, SEQ ID NO:7); 1 mM ATP; 120 μM dNTPs; 2 Units of Klenow fragment of DNA polymerase I; 400 units of T4 DNA ligase; and 2 Units of UDG (New England Biolabs; Beverly, MA) in a final volume of 15 μl for 1 hour at 37°C. Enzymes were inactivated at 75 °C for 10 min. A control reaction containing no UDG was run in parallel.

[0249] The resulting library was amplified by real-time PCR in a reaction mixture comprising the following: 1 x Titanium *Taq* reaction buffer (Clontech, Mountain View, CA); 200 μM each dNTP; 1:10,000 dilutions of fluorescein and SybrGreen I (Molecular Probes; Carlsbad, CA); 1 μM of universal M_U primer (Table I, SEQ ID NO:8); and 5 units of Titanium *Taq* polymerase (Clontech; in a reaction volume of 75 μl). PCR amplifications were carried out at 95°C for 15 sec and 65°C for 2 min on i-Cycler real-time PCR instrument (Bio-Rad; Hercules, CA). In a separate amplification reaction, no external universal M_U primer was supplied to test if endogenously-generated primer is sufficient for library amplification. FIG. 25 shows that virtually identical amplification curves were generated whether or not the universal primer was added exogenously or was generated as a result of the UDG activity followed by heat-induced degradation of the adaptor. FIG. 25 also shows that if UDG was not present during library preparation, this caused an 8 cycle-delay in amplification. This latter result once again indicates the preference of removal of the terminal inverted repeat of a hairpin adaptor for efficient library priming and amplification.

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EXAMPLE 4: ONE STEP GENOMIC DNA RESTRICTION DIGESTION AND WHOLE GENOME LIBRARY PREPARATION USING DEGRADABLE HAIRPIN ADAPTOR COMPRISING DEOXY-URIDINE

[0250] In this example, a single step WGA process is described wherein a hairpin oligonucleotide adaptor comprising deoxy-uridine described in Example 3 is ligated *via* its free 3' end to the 5' phosphates of DNA restriction fragments generated from intact genomic DNA in a single exemplary reaction mix comprising 4 exemplary enzymatic activities: AluI restriction endonuclease, T4 DNA ligase, DNA polymerase, and Uracil-DNA glycosylase (UDG) (FIG. 19A, Enz-O-Mix 3). UDG catalyses the release of free uracil and generates abasic sites in the adaptor's loop region and the 5' half of the hairpin. AluI digests the target DNA into restriction fragments. T4 DNA ligase generates a phosphodiester bond between the 3' ends of the adaptor molecules and the 5' phosphates of the restriction fragments. Finally, the strand-displacement activity of the DNA polymerase extends the free 3' end of the restriction fragments using as template the ligated 3' end of the hairpin stem until an abasic site is reached that serves as a replication stop (FIG. 10B). Prior to amplification, samples are heated to degrade the abasic sites of the adaptor, and the resulting library is amplified using the universal primer M_U. The entire process takes place in a single tube in one step and is completed in just 1 hour.

[0251] For library preparation, 20, 10, 3, 1, 0.3, 0.1, 0.03, 0.01, or 0 nanograms of human genomic DNA isolated from the peripheral blood of a healthy donor by standard procedures were incubated in an exemplary reaction mix comprising: 1x NEBuffer4; 2 μM dU Hairpin Adaptor oligonucleotide (Table I, SEQ ID NO:7); 1 mM ATP; 120 μM dNTPs; 2 Units of Klenow fragment of DNA polymerase I; 400 units of T4 DNA ligase; 2 Units of UDG; and 10 units of AluI restriction endonuclease (New England Biolabs; Beverly, MA) in a final volume of 15 μl for 1 hour at 37°C. Enzymes were inactivated for 10 min at 75 °C.

[0252] The resulting libraries were amplified by real-time PCR in the exemplary reaction mixture comprising the following: 1 x Titanium *Taq* reaction buffer (Clontech; Mountain View, CA); 200 μM each dNTP; 1:10,000 dilutions of fluorescein and SybrGreen I (Molecular Probes; Carlsbad, CA); 1 μM of universal M_U primer (Table I, SEQ ID NO:8); and 5 units of Titanium *Taq* polymerase (Clontech; Mountain View, CA) in a reaction volume of 75 μl. PCR amplifications were carried out at 95°C for 15 sec and 65°C for 2 min on i-Cycler real-time PCR instrument (Bio-Rad; Hercules, CA). FIG. 26 shows the amplification curves of the resulting libraries. As shown, a series of titration curves was generated in a concentration-dependent manner. The smallest amount of DNA corresponding to less than two genome

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equivalents was still discernible from the control reaction with no DNA, indicating that the described single closed tube process is an efficient and sensitive way of whole genome amplification.

EXAMPLE 5: SIMPLIFIED PROTOCOL COMBINING PREPARATION OF METHYLOME LIBRARIES FROM CELL-FREE URINE DNA AND CLEAVAGE WITH METHYLATION-SENSITIVE RESTRICTION ENZYMES IN ONE STEP

[0253] In this example, the preparation of methylome libraries from cell-free urine DNA by ligation of hairpin oligonucleotide adaptor comprising deoxy-uridine as described in Example 3 is combined with the simultaneous cleavage with a mix of methylation-sensitive restriction enzymes in a single step.

[0254] Cell-free DNA was isolated from urine of healthy donors collected in 50 ml Falcon tubes and stabilized for storage by adding 0.1 volume of 0.5 M EDTA. Urine samples were centrifuged at 1,800 x g for 10 min at ambient temperature to sediment cells, and supernatant was transferred carefully to a fresh tube. Equal volume of 6 M solution of guanidine thiocyanate was added to each sample followed by 1/6 vol of Wizard Miniprep resin (Promega catalog # A7141; Madison, WI). DNA was bound to the resin by rotation for 1 hour at ambient temperature. The resin was then sedimented by brief centrifugation at 500 x g and loaded on Wizard minicolumns (Promega catalog #A7211; Madison, WI) using syringe barrel extensions after carefully decanting out the supernatant. Resin was washed with 5 ml of wash buffer (Promega catalog # A8102; Madison, WI) using Qiagen QIAvac-24 vacuum manifold. Minicolumns were then centrifuged for 2 min at 10,000 x g to remove residual wash buffer and bound DNA was eluted with 50 µl of DNAse-free water at 10,000 x g for 1 min. Eluted DNA was buffered by adding 0.1 vol of 10x TE-L buffer and quantified on a fluorescent spectrophotometer using Pico Green (Molecular Probes; Carlsbad, CA) and l phage D.

[0255] Aliquots of 200 ng of cell-free DNA were artificially methylated by incubation in 20 µl of NEBuffer 2 (New England Biolabs; Beverly, MA) with 4 units of M.SssI CpG methylase (New England Biolabs; Beverly, MA) in the presence of 160 µM SAM for 1 hour at 37°C.

[0256] Twenty five nanograms of methylated or non-methylated DNA were incubated in 1x NEBuffer 4 (New England Biolabs; Beverly, MA) comprising 0.35 units of T4 DNA polymerase (New England Biolabs; Beverly, MA); 1.5 µM of dU-Hairpin Adaptor (Table

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I, SEQ ID NO:7); 0.5 units of UDG (New England Biolabs; Beverly, MA); 400 units of T4 DNA ligase (New England Biolabs; Beverly, MA); 30 μM dNTPs, 0.75 mM ATP; 75 μg/ml BSA; 16.7 units of AciI; 16.7 units of HhaI; 8.3 units each of BstUI, HpaII, and Hinp1I (New England Biolabs; Beverly, MA) in a final volume of 20 μl for 1 hour at 37°C. A second aliquot of 25 ng of methylated or non-methylated DNA was incubated in parallel with all the ingredients described above but without the restriction enzymes ("uncut" control).

[0257] One half of each sample (12.5 ng) was then amplified by quantitative PCR in an exemplary reaction mix comprising 1 x Titanium *Taq* reaction buffer (Clontech; Mountain View, CA); 200 μM of each dNTP; fluorescein calibration dye (1:100,000) and SYBR Green I (1:100,000); 1 μM universal primer M_U-1 (Table I, SEQ ID NO:8); 4% DMSO; 200 μM 7-deaza-dGTP (Sigma); and 5 units of Titanium *Taq* polymerase (Clontech) in a final volume of 75 μl. Samples were pre-heated at 72°C for 15 min followed by 95°C for 5 min and 12 cycles at 94°C for 15 sec and 65°C for 2 min on an I-Cycler real-time PCR instrument (Bio-Rad; Hercules, CA). Amplified libraries were purified using MultiScreen PCR cleanup system (Millipore) and quantified by optical density reading.

[0258] Methylation analysis of promoter sites was performed by real-time PCR using aliquots of 160 ng of each digested or non-digested amplified library DNA incubated in exemplary reaction mixtures comprising the following: 1x Titanium *Taq* reaction buffer (Clontech; Mountain View, CA); 200 μM of each dNTP; 4% DMSO; 0.5 M betaine; FCD (1:100,000) and SYBR Green I (1:100,000); 200 nM each forward and reverse primer (Table II, SEQ ID NO:11 and SEQ ID NO:12 for GSTP-1 promoter, SEQ ID NO:13 and SEQ ID NO:14 for MDR-1 promoter, SEQ ID NO:9 and SEQ ID NO:10 for EDNRB promoter, and SEQ ID NO:15 and SEQ ID NO:16 for PTGS-2 promoter); and approximately 1.5 units of Titanium *Taq* polymerase (Clontech; Mountain View, CA) in a final volume of 15μl at 95°C for 3 min followed by 50 cycles at 94°C for 15 sec and 68°C for 1 min.

[0259] FIG. 27 shows PCR amplification curves of specific promoter sites in amplified libraries prepared from methylated (M) or non-methylated (N) urine DNA in the presence (+) or in the absence (-) of methylation-sensitive restriction enzymes. As expected, promoter sites from non-methylated cleaved DNA (N+) amplified with significant (at least 10 cycles) delay as compared to uncut DNA (N-, M-) for all four promoter sites tested. On the other hand, methylated DNA was completely refractory to cleavage (M+). These results demonstrate

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that the method disclosed in the present invention can be applied as a simple one-step non-invasive high-throughput diagnostic procedure for detection of aberrant methylation in cancer.

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TABLE I OLIGONUCLEOTIDE SEQUENCES

No Code	Sequence 5' – 3'
1. EcoNI Ext Adaptor	CCAAACACACCCAACACA**CCT**AAAAA**AGG**TGT (SEQ ID NO:1)*
2. K_U	TGTGTTGGGTGTGTTTGG (SEQ ID NO:2)
3. HEG Adaptor	AAGAGAGAGGGAAGGAAGAA/HEG/AACTTCCTTCCCTCTC TCTT (SEQ ID NO:3)**
4. Y_U	CTTCCTTCCCTCTCTCTT (SEQ ID NO:4)
5. Hairpin Adaptor	CCAAAC ACACCCAACACAAAAAGTGTTG (SEQ ID NO:5)
6. Hairpin Ribo Adaptor	CCAAAC ACACCCAACACrArArArArAGTGTTG (SEQ ID NO:6)***
7. dU Hairpin Adaptor	TGTGTTGGGdUGdUGTGTGGdUdUdUdUdUCCACACACAC CCAACACA (SEQ ID NO:7)****
8. M_U	CCACACACACCCAACACA (SEQ ID NO:8)
9. EDNRB	GGAGGAGTCTTTCGAGTTCAA (SEQ ID NO:9) Forward CGGGAGGAATACAGACACGTCTT (SEQ ID NO:10) Reverse
10. GSTP-1	GGAAAGAGGGAAAGGCTTC (SEQ ID NO:11) Forward CCCCAGTGCTGAGTCACGG (SEQ ID NO:12) Reverse
11. MDR-1	GGGTGGGAGGAAGCATCGTC (SEQ ID NO:13) Forward GGTCTCCAGCATCTCCACGAA (SEQ ID NO:14) Reverse
12. PTGS-2	AGAACTGGCTCTCGGAAGCG (SEQ ID NO:15) Forward GGGAGCAGAGGGGGTAGTC (SEQ ID NO:16) Reverse
13.dUT7 Hairpin Adaptor	TGTGTTGGGdUGdUGTGTGGdUdUdUdUdUATTTAATACG ACTCACTATAGGGAGACCACACACACCCAACACA (SEQ ID NO:17)****
14.dU_N.BbvC IB_Nick Hairpin Adaptor	TGTGTTGGGdUGdUGTGTGGdUdUdUdUdUATTTAATACG AC**CCTCAGC**ACCACACACACCCAACACA (SEQ ID NO:18)*****

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15. dU_N.BbvC IB_Nick ATTTAATACGACCCTCAGCACCAC (SEQ ID NO:19)***** Primer

* Underlined sequences indicate EcoNI palindrome

**HEG = hexa-ethyleneglycol

*** rA = Adenosine

**** dU = deoxy-Uridine

***** Underlined sequence indicates N.BbvC IB nicking endonuclease recognition site

TABLE II. HUMAN STS MARKERS USED FOR REPRESENTATION ANALYSIS BY QUANTITATIVE REAL-TIME PCR

STS #	UniSTS Database Name*
4	SHGC-149956
8	csnpmnat1-pcr1-1
35	SHGC-146602

* Unique names of STS marker sequences from the National Center for Biotechnology Information UniSTS database. Sequences of the STS regions as well as the forward and backward primers used in quantitative real-time PCR can be found in the UniSTS database at the National Center for Biotechnology Information website.

EXAMPLE 6: ENZ-O-MIX DNA LIBRARYPREPARATION AND SIMULTANEOUS AMPLIFICATION BY TRANSCRIPTION

[0260] This example demonstrates an exemplary preparation of a library from lambda phage BstEII restriction digest by ligation of hairpin oligonucleotide adaptor comprising deoxy-uridine and T7 promoter sequence combined with the simultaneous isothermal amplification by *in vitro* transcription with T7 RNA polymerase (see FIG. 6A and FIG. 19A, Enz-O-Mix 2). The adaptor oligonucleotide used in this example (Table I, SEQ ID NO:9) is based on the adaptor sequence described in Example 3 (Table I, SEQ ID NO:8), except that it has the consensus T7 phage promoter sequence incorporated in the loop region. Four enzymatic activities are present in the mix: T4 DNA ligase, T4 DNA polymerase, Uracil-DNA glycosylase (UDG), and T7 RNA polymerase. UDG creates abasic sites by destabilizing the 5' end of the adaptor stem. T4 DNA polymerase generates blunt ends at the restriction fragments leaving intact 5' phosphate groups that are necessary for ligation to the free 3' end of the adaptor by the T4 DNA ligase. T4 DNA polymerase then extends the 3' ends of the newly ligated restriction fragments into the adaptor, thereby displacing the 5' end of the adaptor's stem until an abasic site created by UDG is reached that stops further extension. By doing so, the T4 DNA polymerase

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generates a fully functional double-stranded T7 promoter, and *in vitro* transcription is initiated by T7 RNA polymerase.

[0261] One hundred nanograms of a lambda phage BstEII restriction DNA fragments (NEB Cat. # M0208S; New England Biolabs; Beverly, MA) were incubated in 1x NEBuffer 4 (NEB) with 2 μM adaptor oligonucleotide comprising deoxy-uridine and T7 promoter sequence (Table I, SEQ ID NO:9); 1 mM ATP; 40 μM each dNTP; 500 μM each rNTP; 200 μg/ml BSA; 5.6 mM DTT; 1 unit of uracil-DNA glycosylase (New England Biolabs; Beverly, MA); 0.18 unit of T4 DNA polymerase (New England Biolabs; Beverly, MA); 600 units of T4 DNA ligase (New England Biolabs; Beverly, MA); and 37.5 units of T7 RNA polymerase (Epicentre; Madison, WI) for 2 hours at 37°C in a final volume of 15 μl. A negative control containing no T4 DNA ligase was also run in parallel. Products of the library amplification were analyzed by gel electrophoresis on 1.5% agarose gel after staining with Sybr Gold (Molecular Probes; Carlsbad, CA).

[0262] FIG. 28 shows the products of an exemplary *in vitro* transcription amplification. As shown, in the presence of all four enzymatic activities the lambda restriction fragments are converted into a library amplified as RNA products, whereas in the absence of T4 DNA ligase no accumulation of RNA occurs. This demonstrates that library preparation and isothermal amplification can be combined into a single-step process.

EXAMPLE 7: ENZ-O-MIX DNA LIBRARY PREPARATION AND SIMULTANEOUS IMMOBILIZATION ON SOLID SUPPORT

[0263] This embodiment is illustrated in FIG. 29 and describes the one-step Enz-O-Mix attachment process for a stem-loop oligonucleotide with a non-replicable linker that is accompanied by immobilization of the synthesized library to the surface of a vessel where the reaction occurs (for example, a tube, micro-plate well, glass slide, micro-beads, *etc.*). The exemplary reaction mix comprises HMW DNA; a stem-loop oligonucleotide with 3' recessed, 3' protruding or blunt end (FIG. 4), and a non-replicable linker somewhere in the central part of the oligonucleotide; a DNA fragmentation endonuclease, such as restriction enzyme, DNase I, benzonase, methylation-specific nuclease McrBC, apoptotic endonuclease, *etc.*; a 3'proofreading DNA polymerase (Klenow fragment of the DNA polymerase I, T4 DNA polymerase, *etc.*); T4 DNA ligase; Enz-O-Mix Universal Buffer; ATP; and dNTPs. In specific embodiments, the reaction occurs inside a tube or a micro-plate well containing a hybridization-capture oligonucleotide (HCO) covalently attached to the inner surface of the reaction vessel.

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Oligonucleotide HCO has a sequence that is complementary to a portion of the stem-loop oligonucleotide located between the 5' end and the non-replicable linker. Four enzymatic reactions and one hybridization reaction are taking place as follows: (1) DNA fragmentation by a nuclease(s); (2) "polishing" of the DNA ends and the stem-loop oligonucleotide double-stranded stem-region; (3) ligation of the oligonucleotide 3' end to the 5' phosphate of the DNA, leaving a nick between the 3' end of DNA and the 5' end of the oligonucleotide double-stranded stem-region; (4) polymerase extension of the 3' DNA end that propagates toward the end of stem-loop oligonucleotide, displaces the 5' portion of the stem-loop oligonucleotide, stops somewhere within the loop or close to the loop region, at the replication block, and generates single stranded overhangs at the 5' ends of DNA molecules; and (5) immobilization of DNA fragments through a hybridization of the generated 5' overhangs to the surface-attached capture oligonucleotide HCO.

[0264] Library immobilization can be non-covalent (as shown on FIG. 29A), or covalent (as shown on FIG. 29B). The first case (non-covalent immobilization) relies solely on the hybridization between the 5' overhang (produced from the stem-loop oligonucleotide during its ligation to DNA ends and subsequent replication) and the capture oligo HCO. The second case (covalent immobilization) involves hybridization as an initial step, but it also involves additional enzymatic steps, such as site-specific cleavage within the single stranded 5' overhang and ligation of the 5' end of DNA to the ligation-capture oligonucleotide construct LCO. The purpose of the cleavage reaction is to generate a 5' phosphate group for the ligation reaction. Such cleavage can be achieved, for example, by incubation with the USER enzyme (a mixture of Uracil DNA glycosylase (UDG) and the DNA glycosylase-lyase Endonuclease VIII; New England Biolabs, Beverly, MA). In that case, UDG catalyses the excision of a uracil base (located somewhere at the 5' portion of the stem-loop oligonucleotide), forming an abasic (apyrimidinic) site while leaving the phosphodiester backbone intact. The lyase activity of Endonuclease VIII breaks the phosphodiester backbone at the 3′ and 5′ sides of the abasic site leaving a 5′ phosphate. A generated phosphate group at the end of the 5' overhang is then ligated by a DNA ligase to the 3' end of the ligation-capture oligonucleotide construct LCO (see FIG. 29B).

[0265] Library synthesis and immobilization can occur within the specifically designed tubes, micro-well plates, on the surface of micro-slides, or micro-beads (FIG. 30). In all of these cases, the hybridization capture oligonucleotide HCO, or the covalent ligation capture

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oligo construct LCO, is covalently attached to the inner surface of reaction tubes and plates, spotted on the glass (plastic) surface to create a micro-array, or cover the surface of micro-beads.

[0266] The synthesized and surface-immobilized Whole Genome Library (as described above), or Whole Methylome Library (if the methylation-sensitive restriction nucleases, such as Aci I, Acc II, Asp LE I, Ava I, Bce AI, Bsa HI, Bsh 1236 I, Bsi E1, Bsi SI, Bst FN I, Bst HH I, Bst UI, Cfo I, Hap II, Hga I, Hha I, HinP1 I, Hin 6I, Hpa II, Hpy 99I, Hpy CH4 IV, Hsp AI, Mvn I, and Ssi I,, or the methylation-specific nucleases, such as McrBC, are included into the Enx-O-Mix or used after the library synthesis) can be further processed enzymatically, washed, and amplified or stored in an immobilized format. FIG. 31 illustrates a specific but exemplary use of the one-step library synthesis and immobilization in a hypothetical fluidic device. The process starts by a library synthesis (I), is continued by a library immobilization at the bottom of a reaction vessel (II), and is completed by removal of the first reagent mix, washing, and introduction of a new reagent mix (for example, PCR components).

EXAMPLE 8: ENZ-O-MIX DNA LIBRARY PREPARATION AND SIMULTANEOUS AMPLIFICATION BY STRAND DISPLACEMENT SYNTHESIS

[0267] This example demonstrates an exemplary preparation of a library from cell-free urine DNA by ligation of hairpin oligonucleotide adaptor comprising deoxy-uridine and N.BbvC IB nicking endonuclease recognition site located at the loop region as illustrated in FIG. 16A followed by isothermal amplification with a strand-dispalcing DNA polymerase (see FIG. 6A and FIG. 19A, Enz-O-Mix 3). The adaptor oligonucleotide used in this example (Table I, SEQ ID NO:18) is based on the adaptor sequence described in Example 3 (Table I, SEQ ID NO:8), except that it has recognition sequence 5'-CCTCAGC-3' for N.BbvC IB nicking endonuclease in the loop region. In the first step, a stem-loop adaptor attachment is accomplished by using a mix (FIG. 18, Master Mix III) with 3 enzymatic activities: T4 DNA ligase, T4 DNA polymerase, and Uracil-DNA glycosylase. UDG creates abasic sites by destabilizing the 5' end of the adaptor stem. T4 DNA polymerase generates blunt ends at the restriction fragments leaving intact 5' phosphate groups that are necessary for ligation to the free 3' end of the adaptor by the T4 DNA ligase. T4 DNA polymerase then extends the 3' ends of the newly ligated restriction fragments into the adaptor, thereby displacing the 5' end of the adaptor's stem until an abasic site generated by UDG is reached that stops further extension. By doing so, the T4 DNA polymerase generates a functional double-stranded N.BbvC IB nicking endonuclease recognition site. In the second step, following thermal inactivation of T4 DNA polymerase and T4 DNA ligas, the mix is supplemented with two additional enzymatic activities,

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N.BbvC IB nicking endonuclease generating single-starnded nicks at the adaptor (and internal) sites, and the strand-displacing Klenow fragment of DNA polymerase I, initiating strand displacement from the nicks. The newly synthesized strand is extended until a nick or abasic site at the template strand is encountered. In order to fill-in a second strand and to recreate intact N.BbvC IB recognition sites, an oligonucleotide primer comprising the adaptor's loop sequence at its 5'-end plus five bases complementary to the adaptor's stem at its 3'-end (Table I, SEQ ID NO:19) is also added to the mix. As a result, a self-sustained isothermal DNA amplification process is accomplished.

[0268] Cell-free DNA was isolated from urine of healthy donors as described in Example 5. Aliquots of 50 nanograms of DNA were incubated in 1x NEBuffer 4 (New England Biolabs; Beverly, MA) with 2 μM adaptor oligonucleotide comprising deoxy-uridine and N.BbvC IB nicking endonuclease recognition site (Table I, SEQ ID NO:18); 1 mM ATP; 40 μM each dNTP; 200 μg/ml BSA; 1 unit of uracil-DNA glycosylase (New England Biolabs; Beverly, MA); 0.18 unit of T4 DNA polymerase (New England Biolabs; Beverly, MA); and 600 units of T4 DNA ligase (New England Biolabs; Beverly, MA) for 1 hour at 37°C in a final volume of 15 μl. A negative control containing no T4 DNA ligase was run in parallel. Samples were heated at 65°C for 15 min, cooled to 37°C and supplemented with 10 units of N.BbvC IB nicking endonuclease (New England Biolabs; Beverly, MA); 27 ng/μl of *E. coli* Single Stranded Binding Protein (SSB protein, USB Corporation, Clevelang, OH); 5 units of Klenow Exo- fragment of DNA polymerase I (USB Corporation, Clevelang, OH); 0.5 μM of dU_N.BbvC IB_Nick Primer (Table I, SEQ ID NO:19); and 200 μM of each dNTP in a final volume of 30 μl of 1x NEBuffer 4 (New England Biolabs; Beverly, MA). To test the effect of additives on the representation of GC-rich sequences such as promoter sites, reactions containing 4% of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO) and 7deaza-dGTP (Roche Diagnostics, Indianapolis, IN) were run in parallel. A control reaction containing no N.BbvC IB nicking endonuclease was also included. Samples were incubated for 1 to 3 hours at 37 °C and reactions were stopped by adding 50 mM of EDTA. Products of the library amplification were analyzed by gel electrophoresis on 20 % acrylamide TBE gel (Invitrogen Corporation, Carlsbad, CA), after staining with Sybr Gold (Molecular Probes; Carlsbad, CA).

[0269] FIG. 29 shows the products of an exemplary isothermal DNA amplification after 1 hour incubation. As shown, in the presence of all five enzymatic activities the lambda

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restriction fragments are converted into a library amplified as DNA products, whereas in the absence of T4 DNA ligase or N.BbvC IB nicking endonuclease no accumulation of DNA occurs.

EXAMPLE 9: INTEGRATED WHOLE GENOME AMPLIFICATION IN A CLOSED-TUBE REACTION USING DEGRADABLE HAIRPIN ADAPTOR COMPRISING DEOXY-URIDINE

[0270] Traditionally, preparation of DNA libraries and their subsequent amplification by PCR involves multiple separate processes and different buffer systems. Previous embodiments and examples of this invention have demonstrated that Enz-O-Mx approach can substantially simplify these processes and reduce number of steps necessary for WGA and WMA library preparation to one step. However, in previous Examples the Enzo-O-Mix DNA libraries were first, prepared in small volume (15μl) of buffer A (NEB 4), and then amplified by PCR in a larger volume (75 ml) of buffer B (Titanium Taq reaction buffer), FIG. 30A. In all of these cases, Enz-O-Mix library synthesis / amplification is performed in two operational steps: step 1- a tube contatining DNA is supplemented with the library (WGA or WMA) synthesis reagents and incubated at 37 °C for 1 h, and step 2 – a tube is opened, supplemented with PCR amplification buffer / reagents and subjected to temperature cycling. Such an approach is referred to herein as a two-step, opened-tube protocol.

[0271] This example introduces further simplification in preparation and PCR-mediated amplification of WGA / WMA libraries when all the necessary synthesis / amplification reagents are introduced into the tube prior reaction, and library synthesis and subsequent amplification occur in the same volume and the same buffer without opening the tube within a pre-programmed thermocycler (FIG. 30B). Such an approach is referred to herein as an integrated, one-step, closed-tube protocol. Due to its simplicity and lack of any human intervention, the integrated, one-step, closed-tube Enz-O-Mix DNA amplification can be easily automated and used for high-throughput research applications and clinical diagnostics.

[0272] FIG. 31 shows a one possible temperature profile (b) and an envisioned DNA accumulation during amplification (a), where incubation at 37 °C for 1 h (DNA library synthesis) is followed by heating at 95 °C for 10 min (Taq DNA polymerase activation and stem-loop adaptor → universal PCR primer conversion), and then by thermocycling between 65°C and 95 °C for 1 h (PCR-mediated whole genome or whole methylome amplification).

[0273] FIG. 32 shows biochemical and physicochemical reactions involved in the transformation of the stem-loop adaptor oligonucleotide into a functional universal PCR primer

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adequate for efficient amplification of synthesized WGA / WMA libraries. Dual utilization of the stem-loop oligonucleotide as adaptor and PCR primer eliminate a necessity to introduce into reaction an additional single stranded oligo-primer (due to the 3' profreading activity of a DNA polymerase involved in the library synthesis process such primer should contain at least several nuclease-resistant bases at its 3' terminus).

[0274] In this example, a WGA process is described wherein a hairpin oligonucleotide adaptor comprising deoxy-uridine described in Example 3 is ligated *via* its free 3' end to the 5' phosphates of DNA restriction fragments generated from intact genomic DNA in a single enclosed container exemplary reaction mix comprising 6 exemplary enzymatic activities: AluI restriction endonuclease, RsaI restriction endonuclease, T4 DNA ligase, T4 DNA polymerase, a hot-start Taq DNA polymerase, and Uracil-DNA glycosylase (UDG). In an initial isothermal incubation UDG catalyses the release of free uracil and generates abasic sites in the adaptor's loop region and the 5' half of the hairpin. AluI and RsaI digest the target DNA into restriction fragments. T4 DNA polymerase generates blunt ends of the restriction fragments. T4 DNA ligase generates a phosphodiester bond between the 3' ends of the adaptor molecules and the 5' phosphates of the restriction fragments. T4 DNA polymerase extends the free 3' end of the restriction fragments using as template the ligated 3' end of the hairpin stem until an abasic site is reached that serves as a replication stop (FIG. 10B). Samples are heated to 72°C to inactivate all thermo-labile enzymes and to activate Taq polymerase, then to 95°C to degrade the abasic sites of the adaptor and to generate active primer from the remaining intact strand of the adaptor that is free of abasic sites. The resulting library is finally amplified by thermal cycling. The entire process takes place in a single enclosed reaction container under programmed temeparture control algorithm and without any intermediate liquid handling.

[0275] Ten nanogram aliquots of human genomic DNA isolated from the peripheral blood of a healthy donor by standard procedures were incubated in an exemplary reaction mix comprising: 1x Titanium Taq buffer (Clontech, Mountain View, CA); 0.6 µl of Titanium Taq (Clontech, Mountain View, CA); 2 µM dU Hairpin Adaptor oligonucleotide (Table I, SEQ ID NO:7); 1 mM ATP; 200 µM dNTPs; 0.36 Units of T4 DNA polymerase; 1200 units of T4 DNA ligase; 2 Units of UDG; 10 units each of AluI and RsaI restriction endonucleases (New England Biolabs; Beverly, MA), and 1:10,000 dilutions of fluorescein and SybrGreen I (Molecular Probes; Carlsbad, CA) in a final volume of 30 µl. To study the effect of Mg^{++} ions and DMSO, reactions supplemented with $MgCl_2$ at final concentrations of 5 mM and

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7.5 mM and with DMSO at a final concentration of 4% were also included (It should be noted that the 1x Titanium Taq buffer contains 3.5 mM $MgCl_2$). Reactions were incubated at 37°C for 1 hr, followed by 72°C for 10 min, 95 °C for 10 min, and 11 cycles of 94 °C for 20 sec and 65 °C for 2 min. on i-Cycler real-time PCR instrument (Bio-Rad; Hercules, CA). FIG. 33 shows the amplification curves of the resulting libraries. As shown, Mg^{++} concentrations of 5 mM and 7.5 mM in the reaction buffer supported the enzymatic activities present in the mix better than the basic Titanium Taq buffer containing 3.5 mM $MgCl_2$. The presence of 4% DMSO did not have significant effect on the WGA amplification when higher Mg^{++} concentrations were applied.

EXAMPLE 10: HOT START PCR USING DEGRADABLE STEM-LOOP PRIMERS

[0276] Specificity and the ability to amplify a single DNA or RNA target is one of the most important requirements for application of PCR in molecular diagnostics. Hot start PCR protocol was introduced to reduce the non-specific primer/template and primer/primer annealing events that occur at lower temperatures and subsequently result in non-specific amplification products. Several methods and corresponding commercial products for performing hot start PCR rely upon the physical separation of PCR reagents until the high temperature of the reaction has been reached. Those products include the following:

[0277] 1. Wax beads (Ampliwax PCR Gems, Perkin Elmer) that create a temporary barrier between dNTPs, buffer, and $MgCl_2$ on one side of the wax layer, and DNA template and DNA polymerase on another side.

[0278] 2. Small beads of wax with encapsulated Taq DNA polymerse (Taq Bead Hot Start Polymerase, Promega). In this case Taq DNA polymerase is released when the reaction reaches 60°C.

[0279] 3. Small beads of wax with encapsulated magnesium (StartaSpere, Stratagene).

[0280] 4. Taq DNA polymerase inactivated by antibody (JumStart Taq DNA polymerase, Sigma; TaqStart and TthStart, Clontech; AmpliTaq Gold, PE Biosystems; HotStarTaq, Qiage; *etc.*). Antibody binds to the polymerase and inactivates it at low temperature but denatures and releases the active polymerase at high temperature.

[0281] Example 9 introduced the idea of using a degradable, dU-containing stem-loop oligonucleotide-adaptor as a universal PCR primer (see FIG. 32) for whole genome

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amplification. This embodiment extends the use of degradable, dU-containing stem-loop oligonucleotides as "hot start" PCR primers for locus-specific DNA amplification.

[0282] As shown in FIG. 38 and FIG 39, both PCR primers are synthesized in a form of stem-loop ologonucleotides A and B. The 3' portion of the stem-loop oligonucleotides represents the primer sequence (no dU bases); the 5' portion represents the sequence complementary to the primer sequence and comprises one or more dU bases substituting dT bases; and the loop comprises several dT and dU bases.

[0283] PCR reaction is assembled by mixing together DNA, dNTPs, magnesium, PCR buffer, Taq DNA polymerase (or any other thermostable DNA polymerase), and dU-glycosylase (FIG. 38 and FIG. 40). A thermocycler is programmed to have the following conditions:

[0284] a) Incubation step at 37°C for 15 – 30 min that is necessary to convert all dU bases within the stem-loop oligonucleotides A and B into the abasic sites;

[0285] b) Incubation step at 95°C for 10min that is necessary to denature DNA, introduce breaks at the abasic sites within the stem-loop oligonucleotides A and B, and thus release the active primers A and B;

[0286] c) A regular PCR cycling mode that amplifies the DNA region defined by the primer A and B.

[0287] The conversion of the stem-loop oligonucleotides A and B into PCR primers A and B (see FIG. 39) results from several breaks introduced by heating the loop and the 5' portion of the oligonucleotides. At high temperature, the small oligonucleotides originated from the 5' stem regions can not form stable interaction with the remaining intact 3' primer regions and do not affect the PCR process. In general, the position of dU bases within the 5' stem region is dictated by the location of thymines in a DNA sequence, although they can be also introduced at another nucleotide position (thus generating a stem with one ore several mismatched bases). The number of dU bases within the 5' stem and the loop regions can vary from 1 to about 6, and an optimal number can be determined empirically.

[0288] The proposed hot start primer method can be used in PCR and other DNA/RNA amplification methods that utilize thermostable polymerases. It has several advantages over the hot start PCR methods that involve either non-degradable hairpin primers

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with short stem [Kaboev, O.K., et al., 2000; Ailenberg, M., and Silverman, M., 2000], or duplex primers [Kong, D., et al., 2004]: a) the hybridization kinetics are not compromised by the presence of a stem region (as it might be in the case of non-degradable stem-loop primer method); b) no inverted repeat is formed at the ends of PCR amplicons that could substantially reduce the efficiency of the PCR amplification process (as it happens in the case of non-degradable stem-loop primer method); and c) oligonucleotides complementary to the priming oligonucleotide (products of the stem-loop oligonucleotide fragmentation) are short, do not form stable interaction with the remaining intact 3' primer regions and, as a result, do not affect the PCR process (as it possible in the duplex primer method). Advantages over other existing hot-start methods include but are not limited to the following: a) no phase separation is necessary and all reaction components are originally present in the same reaction mix simplifying storage and reducing production cost; b) no expensive blocking antibodies are involved; c) no mutagenesis or chemical modification of amino acid reidues in the thermostabile DNA polymerase are required whatsoever.

REFERENCES

[0289] All patents and publications mentioned in the specification are indicative of the level of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

PATENTS AND PATENT APPLICATIONS

[0290] U.S. Patent No. 6,777,187

[0291] U.S. Patent Application No. 10/797,333

[0292] U.S. Patent Application No. 10/795,667

[0293] U.S. Patent Application No. 11/071,864

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[0312] Although the present invention and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the invention as defined by the appended claims. Moreover, the scope of the present application is not intended to be limited to the particular embodiments of the process, machine, manufacture, composition of matter, means, methods and steps described in the specification. As one of ordinary skill in the art will readily appreciate from the disclosure of the present invention, processes, machines, manufacture, compositions of matter, means, methods, or steps, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein may be utilized according to the present invention. Accordingly, the appended claims are intended to include within their scope such processes, machines, manufacture, compositions of matter, means, methods, or steps.

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CLAIMS

What is claimed is:

1. A method of preparing a nucleic acid molecule, comprising:

providing a double stranded nucleic acid molecule; and

attaching one strand of a stem-loop oligonucleotide comprising an inverted repeat and a loop to the double stranded nucleic acid molecule to produce an oligonucleotide-attached nucleic acid molecule.

2. The method of claim 1, wherein the double stranded nucleic acid molecule is a double stranded DNA molecule.

3. The method of claim 1, wherein the attaching is further defined as attaching the oligonucleotide to the double stranded nucleic acid molecule under conditions to produce a non-covalent junction in the oligonucleotide-attached nucleic acid molecule.

4. The method of claim 3, wherein the non-covalent junction comprises a nick, a gap, or a 5' flap structure.

5. The method of claim 1, wherein the attaching is further defined as ligating.

6. The method of claim 1, further comprising displacing one strand of the oligonucleotide from the oligonucleotide-attached nucleic acid molecule by strand displacement or by nick translation polymerization.

7. The method of claim 1, wherein at least part of the oligonucleotide-attached nucleic acid molecule is amplified.

8. The method of claim 7, wherein the amplification comprises polymerase chain reaction.

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9. The method of claim 7, wherein the amplification comprises RNA transcription.

10. The method of claim 7, wherein the amplification comprises strand displacement.

11. The method of claim 1, further comprising amplifying the oligonucleotide-attached nucleic acid molecule, wherein at least part of the inverted repeat is excluded from the amplified oligonucleotide-attached nucleic acid molecule.

12. The method of claim 5, wherein the ligating is further defined as comprising:

generating ligatable ends on the double stranded nucleic acid molecule;

generating a ligatable end on the stem-loop oligonucleotide; and

ligating one strand of the ligatable end of the stem-loop oligonucleotide to one strand of an end of the nucleic acid molecule, thereby generating a non-covalent junction in the oligonucleotide-attached nucleic acid molecule.

13. The method of claim 1, further defined as comprising:

generating blunt ends on the nucleic acid molecule;

generating a blunt end on the stem-loop oligonucleotide; and

ligating one strand of the blunt end of the stem-loop oligonucleotide to one strand of a blunt end of the nucleic acid molecule, thereby generating a nick in the oligonucleotide-ligated nucleic acid molecule.

14. The method of claim 1, wherein said stem-loop oligonucleotide comprises a known sequence.

15. The method of claim 1, wherein said stem-loop oligonucleotide comprises a regulatory sequence.

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16. The method of claim 2, further comprising:

digesting the DNA molecule with one or more endonucleases to produce DNA fragments;

producing blunt ends on the DNA fragments;

producing a blunt end on the stem-loop oligonucleotide; and

ligating one strand of the blunt end of a stem-loop oligonucleotide to one strand of a blunt end of a DNA fragment, thereby generating a nick in a oligonucleotide-ligated DNA fragment.

17. The method of claim 16, wherein the endonuclease is a restriction endonuclease, DNAse I, or an apoptotic endonuclease.

18. The method of claim 17, wherein the restriction endonuclease is methylation-specific or methylation-sensitive.

19. The method of claim 1, wherein the oligonucleotide-attached nucleic acid molecule comprises a nick having a 3' hydroxy group, wherein there is polymerization from the 3' hydroxy group of at least part of the oligonucleotide-attached nucleic acid molecule.

20. The method of claim 5, wherein the strand displacement or nick translation polymerization is further defined as polymerization that ceases at a non-replicable base or region in the loop or in a region of the stem adjacent to the loop.

21. The method of claim 2, further comprising the step of digesting the double stranded DNA molecule with an endonuclease to generate DNA fragments, wherein the oligonucleotide becomes ligated to one strand of the DNA fragment and wherein polymerization of an oligonucleotide-ligated DNA fragment excludes at least part of the inverted repeat by subjecting the oligonucleotide-ligated DNA fragment to strand displacement or nick translation polymerization that halts at a base or sequence in the loop or in a region of the stem adjacent to the loop.

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22. The method of claim 1, wherein the stem-loop oligonucleotide is further defined as comprising a non-replicable base or sequence.

23. The method of claim 22, wherein at least part of the non-replicable base or sequence is present in the loop of the oligonucleotide or in a sequence of the stem adjacent to the loop.

24. The method of claim 22, wherein the non-replicable base or sequence comprises an abasic site or sequence, hexaethylene glycol, or a bulky chemical moiety attached to the sugar-phosphate backbone or the base.

25. The method of claim 24, wherein the abasic site or sequence is introduced by one or more enzymes in the single solution.

26. The method of claim 1, wherein the loop of the stem-loop oligonucleotide comprises at least one deoxy-uridine.

27. The method of claim 6, wherein the strand displacement or nick translation polymerization of the oligonucleotide-attached nucleic acid molecule generates an endonuclease site.

28. The method of claim 2, further comprising the step of digesting the double stranded DNA molecule with an endonuclease to generate DNA fragments, wherein the oligonucleotide becomes ligated to one strand of the DNA fragment to produce an oligonucleotide-ligated DNA fragment, and wherein strand displacement or nick translation polymerization of the oligonucleotide-ligated DNA fragment generates an endonuclease site.

29. The method of claim 27, wherein the endonuclease site is a site-specific restriction endonuclease site and wherein at least part of the inverted repeat is removed by cleavage with said restriction endonuclease.

30. The method of claim 27, wherein the endonuclease site resides within the stem and loop regions.

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31. The method of claim 27, wherein the endonuclease site resides within the loop region.

32. The method of claim 27, wherein the endonuclease site resides within the stem region.

33. The method of claim 27, wherein the endonuclease site is a homing endonuclease site.

34. The method of claim 15, wherein RNA transcription is initiated from the regulatory sequence, thereby producing at least one transcribed polynucleotide.

35. The method of claim 34, wherein the transcribed polynucleotide is replicated by a reverse transcriptase.

36. The method of claim 35, wherein reverse transcription is initiated by hybridization of the oligonucleotide complementary to the 3' end of the transcribed polynucleotide.

37. The method of claim 1, wherein the stem-loop oligonucleotide further comprises a recognition sequence for a nicking endonuclease and wherein the oligonucleotide-attached nucleic acid molecule is amplified by strand displacement synthesis and second strand synthesis.

38. The method of claim 37, wherein the recognition sequence for a nicking endonuclease resides at least in part within the stem of the stem-loop oligonucleotide.

39. The method of claim 37, wherein the recognition sequence for a nicking endonuclease resides at least in part within the loop of the stem-loop oligonucleotide.

40. The method of claim 1, wherein a 5' end of the stem-loop oligonucleotide lacks a phosphate.

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41. The method of claim 1, wherein the oligonucleotide-attached nucleic acid molecule is further modified.

42. The method of claim 41, wherein the further modification comprises cloning.

43. The method of claim 42, further defined as comprising incorporation of the modified molecule into a vector, said incorporation occuring at ends in the modified molecule generated by endonuclease cleavage within the inverted repeat.

44. The method of claim 1, further defined as occuring in a single suitable solution, wherein the process occurs in the absence of exogenous manipulation.

45. The method of claim 44, further defined as occurring at one temperature.

46. The method of claim 44, wherein the solution comprises one or more of the following:

ligase;
DNA polymerase;
one or more endonucleases;
RNA polymerase;
reverse transcriptase;
RNase H;
deoxy-uridine glycosylase;
nickase;
thermophilic DNA polymerase;
ATP;
rNTPs; and
dNTPs.

47. The method of claim 7, further defined as occuring in a single suitable solution, wherein the process of preparing and amplifying

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of a oligonucleotide-attached nucleic acid molecule occurs in the absence of exogenous manipulation.

48. The method of claim 1, wherein the oligonucleotide-attached nucleic acid molecule is immobilized to a solid support.

49. The method of claim 48, wherein the molecule is immobilized non-covalently.

50. The method of claim 48, wherein the molecule is immobilized covalently.

51. The method of claim 2, further comprising the step of digesting the DNA molecule with an endonuclease to generate DNA fragments, wherein the oligonucleotide becomes attached to one strand of the DNA fragment, wherein strand displacement polymerization of the oligonucleotide-ligated DNA fragment and its arrest at a base or sequence in the loop or in a region of the stem adjacent to the loop generates a 5' overhang, and wherein the single stranded 5' overhang hybridizes to a complementary oligonucleotide covalently immobilized to a solid support.

52. A method of preparing a DNA molecule, comprising:

providing an oligonucleotide; and

mixing the oligonucleotide with a double stranded DNA molecule, such that upon attachment of the oligonucleotide to the double stranded DNA molecule, the oligonucleotide attached to the DNA molecule is capable of demonstrating a function that it was incapable of before the attachment to the double stranded DNA molecule, thereby producing an oligonucleotide-attached DNA molecule suitable for modification.

53. The method of claim 52, further defined as occurring in a single suitable solution, wherein the process occurs in the absence of exogenous manipulation.

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00323

54. The method of claim 52, wherein the modification comprises site-specific nicking, site-specific double-strand cleavage, transcription, recombination, amplification, polymerization, nick translation, strand displacement, immobilization or a combination thereof.

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00324

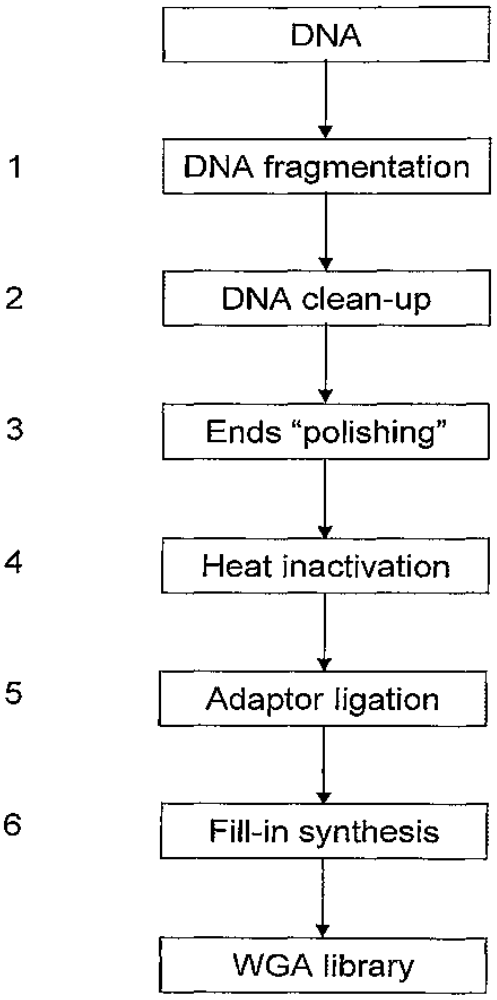

DNA
1
DNA fragmentation
2
DNA clean-up
3
Ends "polishing"
4
Heat inactivation
5
Adaptor ligation
6
Fill-in synthesis
WGA library

FIG. 1A

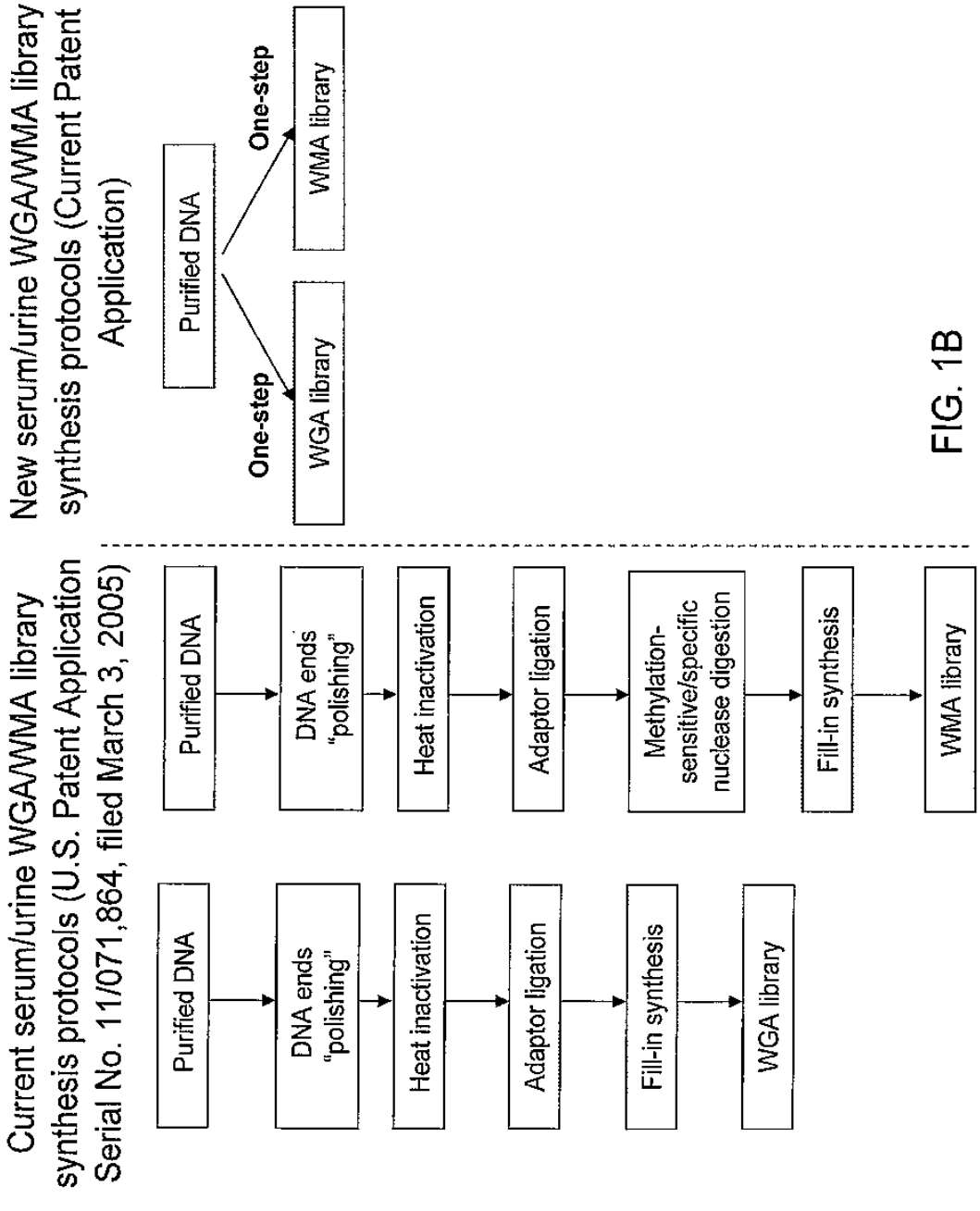

Current serum/urine WGA/WMA library synthesis protocols (U.S. Patent Application Serial No. 11/071,864, filed March 3, 2005)
Purified DNA
DNA ends "polishing"
Heat inactivation
Adaptor ligation
Fill-in synthesis
WGA library
Purified DNA
DNA ends "polishing"
Heat inactivation
Adaptor ligation
Methylation-sensitive/specific nuclease digestion
Fill-in synthesis
WMA library
New serum/urine WGA/WMA library synthesis protocols (Current Patent Application)
Purified DNA
One-step
WGA library
One-step
WMA library

FIG. 1B

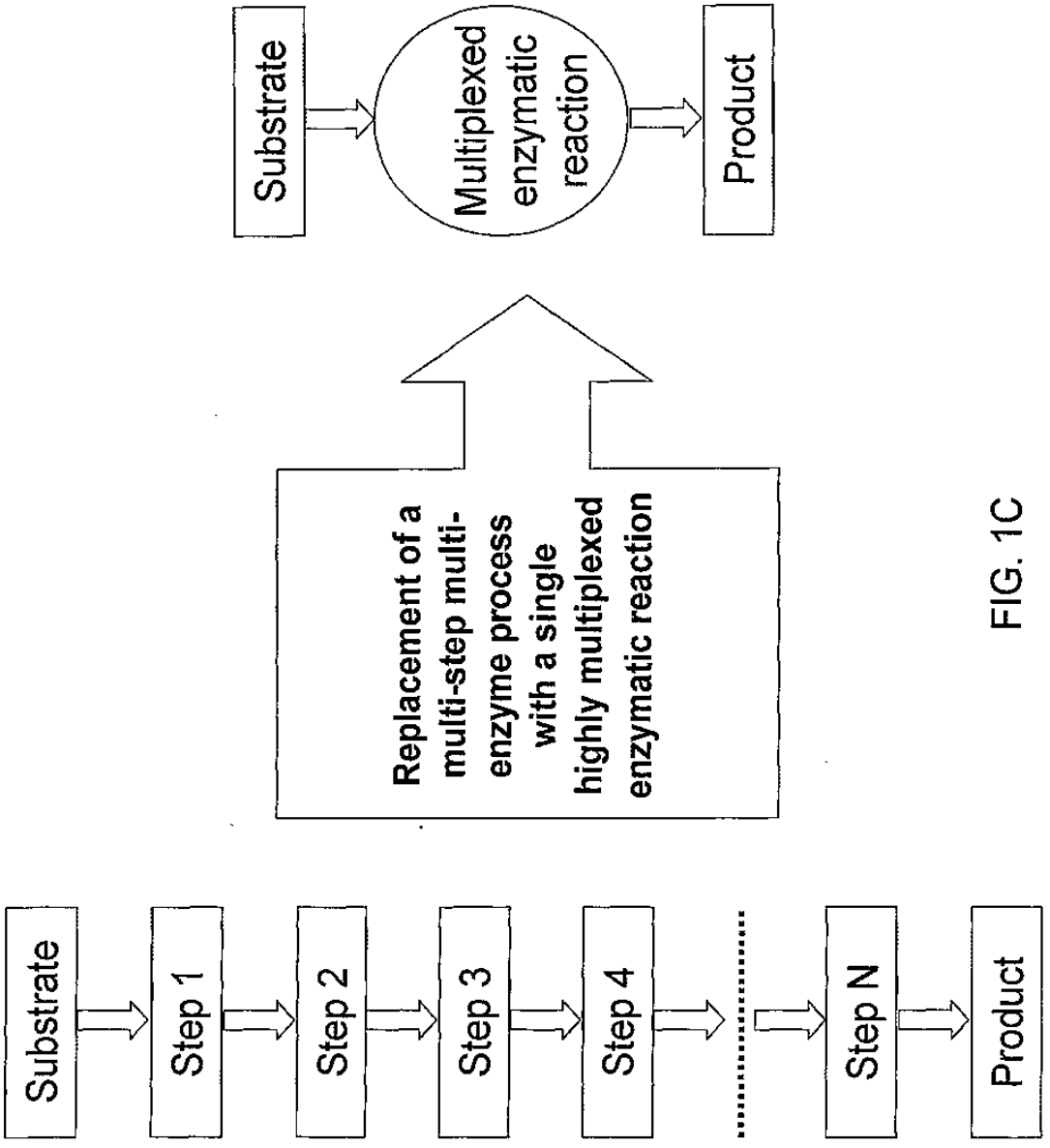

Substrate
Multiplexed enzymatic reaction
Product
Replacement of a multi-step multi-enzyme process with a single highly multiplexed enzymatic reaction
Substrate
Step 1
Step 2
Step 3
Step 4
Step N
Product

FIG. 1C

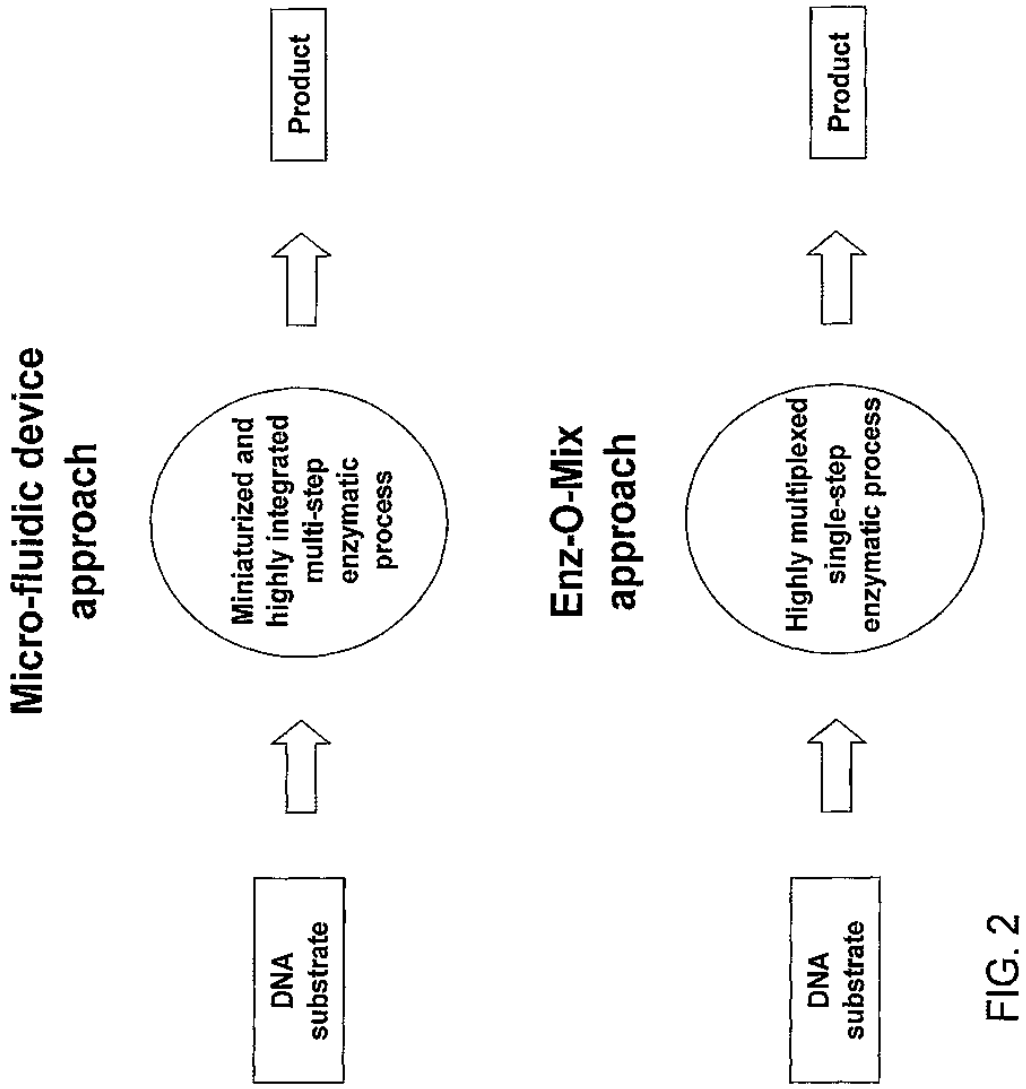

Micro-fluidic device approach
DNA substrate
Miniaturized and highly integrated multi-step enzymatic process
Product
Enz-O-Mix approach
DNA substrate
Highly multiplexed single-step enzymatic process
Product

FIG. 2

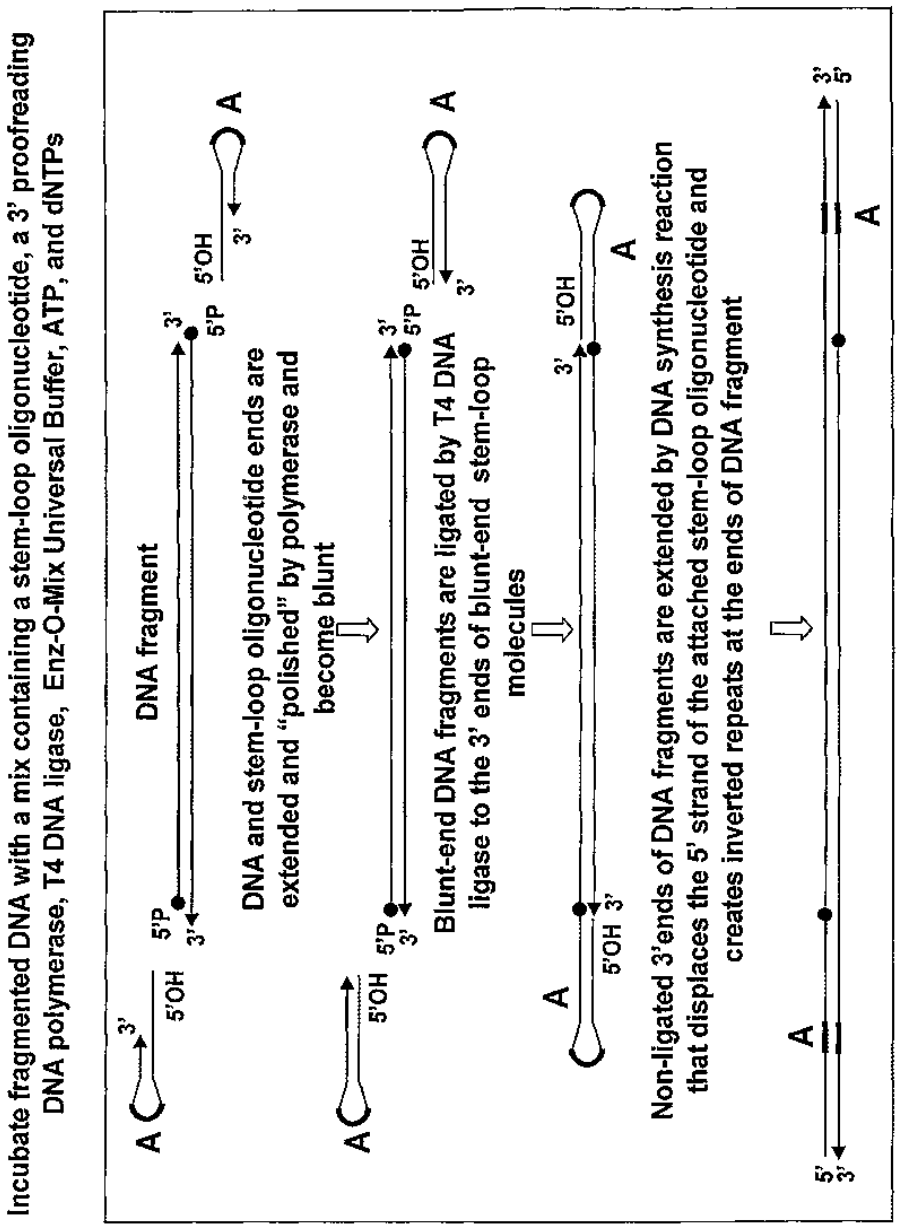

Incubate fragmented DNA with a mix containing a stem-loop oligonucleotide, a 3' proofreading DNA polymerase, T4 DNA ligase, Enz-O-Mix Universal Buffer, ATP, and dNTPs
DNA fragment
DNA and stem-loop oligonucleotide ends are extended and "polished" by polymerase and become blunt
Blunt-end DNA fragments are ligated by T4 DNA ligase to the 3' ends of blunt-end stem-loop molecules
Non-ligated 3'ends of DNA fragments are extended by DNA synthesis reaction that displaces the 5' strand of the attached stem-loop oligonucleotide and creates inverted repeats at the ends of DNA fragment
A
3'
5'OH
5'P
3'
5'OH
3'
5'P
3'
5'P
5'OH
3'
3'
5'OH
A
5'OH
3'
5'
3'
5'
3'
A

FIG. 3

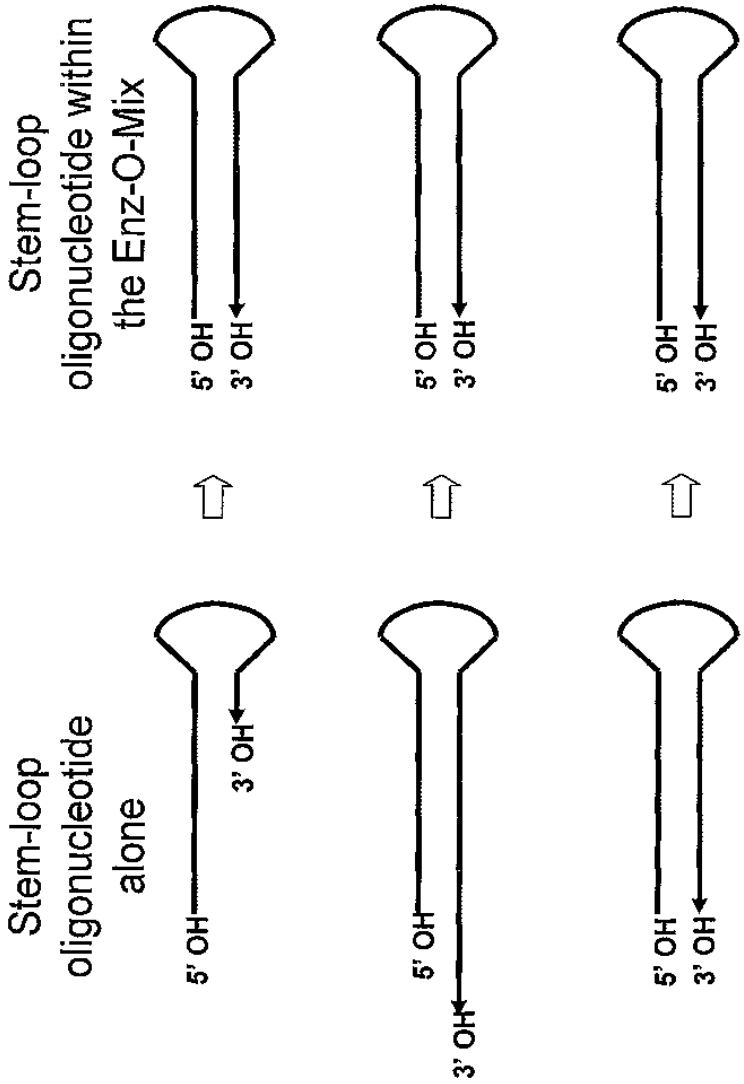

Stem-loop oligonucleotide alone
Stem-loop oligonucleotide within the Enz-O-Mix
5' OH
3' OH
DNA end "polishing" activity

FIG. 4

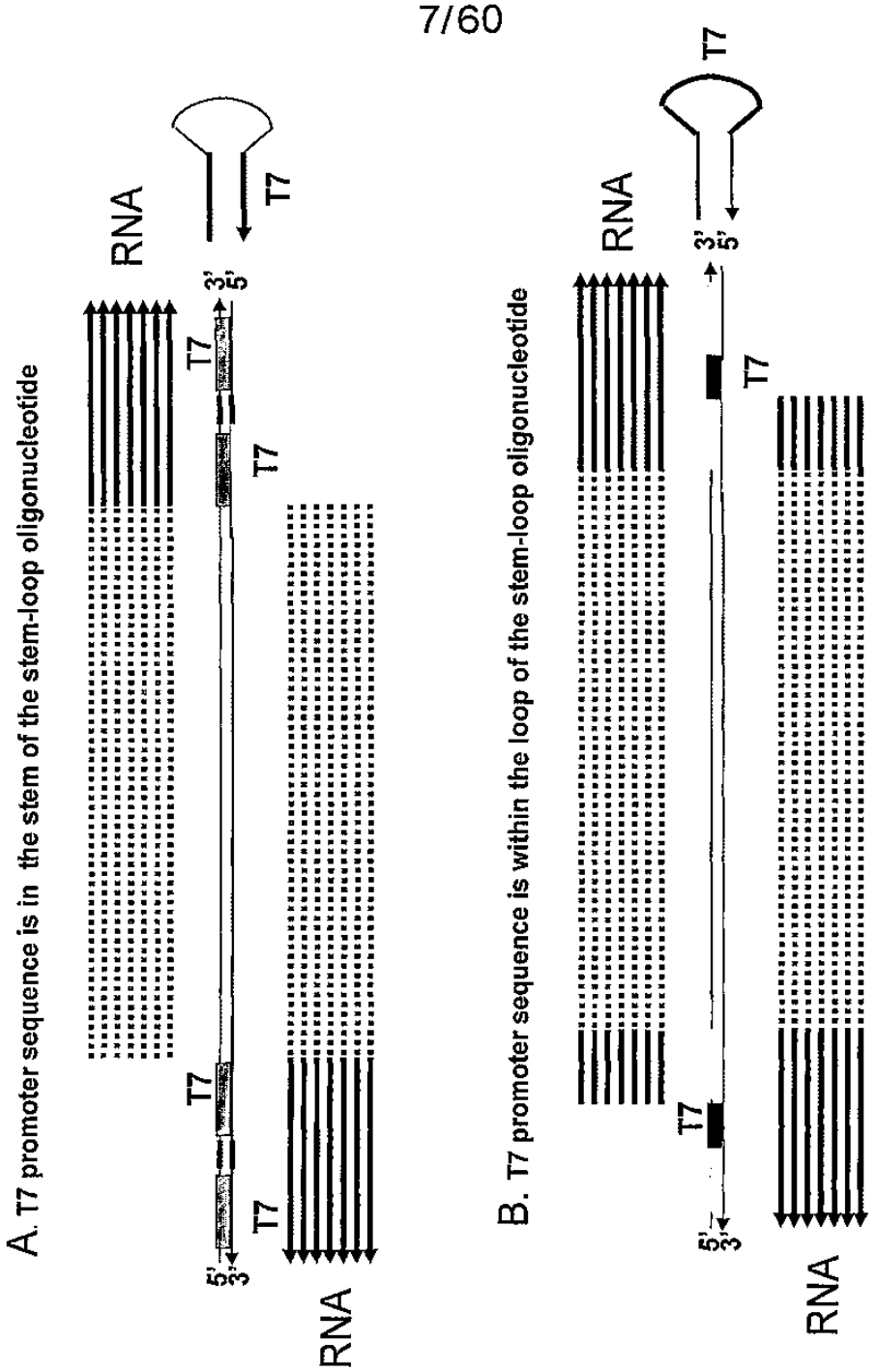

A. T7 promoter sequence is in the stem of the stem-loop oligonucleotide
RNA
T7
3'
5'
T7
T7
T7
5'
3'
RNA
B. T7 promoter sequence is within the loop of the stem-loop oligonucleotide
RNA
T7
3'
5'
T7
T7
5'
3'
RNA

FIG. 5

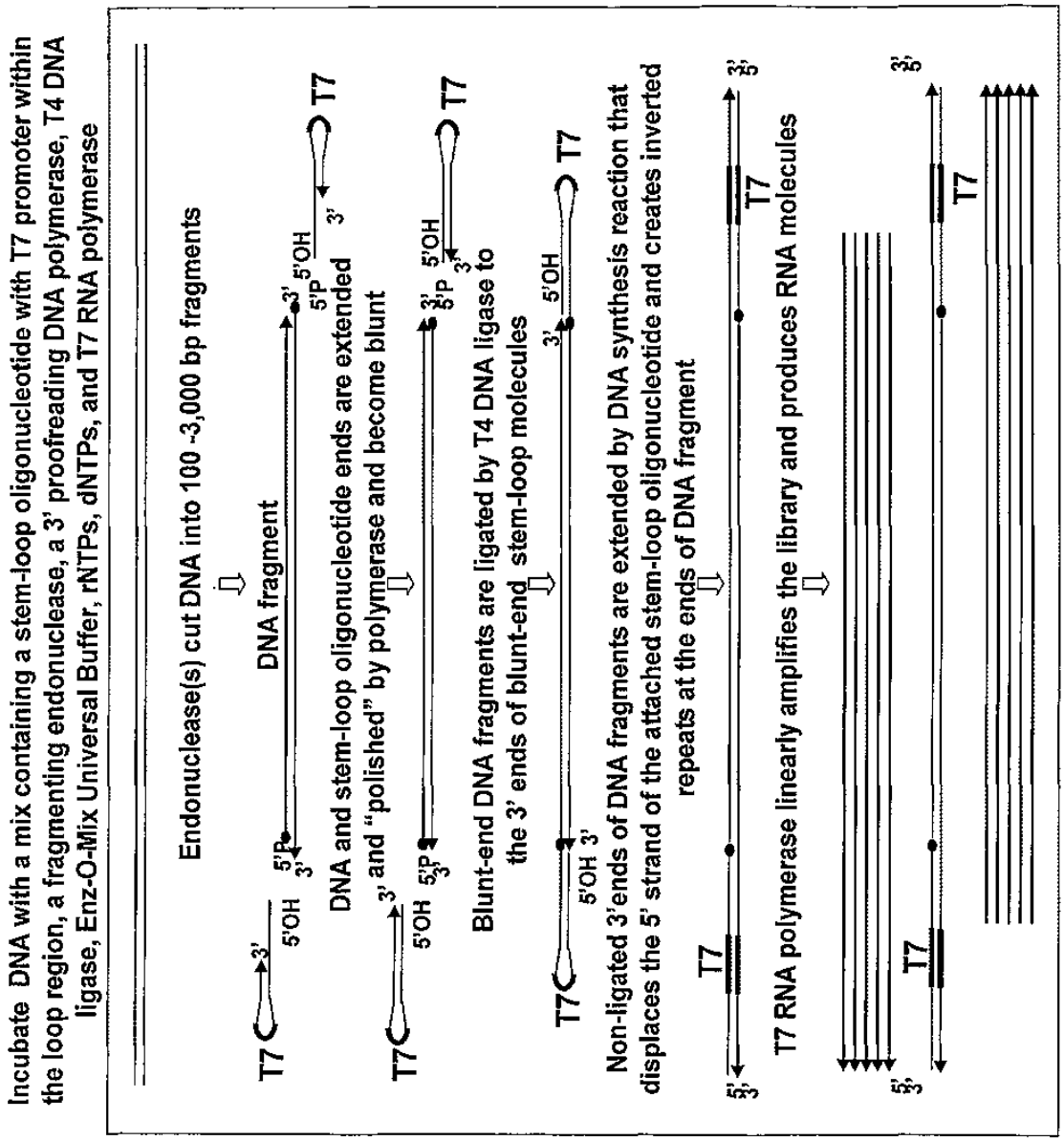

Incubate DNA with a mix containing a stem-loop oligonucleotide with T7 promoter within the loop region, a fragmenting endonuclease, a 3' proofreading DNA polymerase, T4 DNA ligase, Enz-O-Mix Universal Buffer, rNTPs, dNTPs, and T7 RNA polymerase
Endonuclease(s) cut DNA into 100 -3,000 bp fragments
DNA fragment
T7
5'OH
3'
5'P
DNA and stem-loop oligonucleotide ends are extended and become blunt
"polished" by polymerase
Blunt-end DNA fragments are ligated by T4 DNA ligase to the 3' ends of blunt-end stem-loop molecules
Non-ligated 3'ends of DNA fragments are extended by DNA synthesis reaction that displaces the 5' strand of the attached stem-loop oligonucleotide and creates inverted repeats at the ends of DNA fragment
T7 RNA polymerase linearly amplifies the library and produces RNA molecules

FIG. 6A

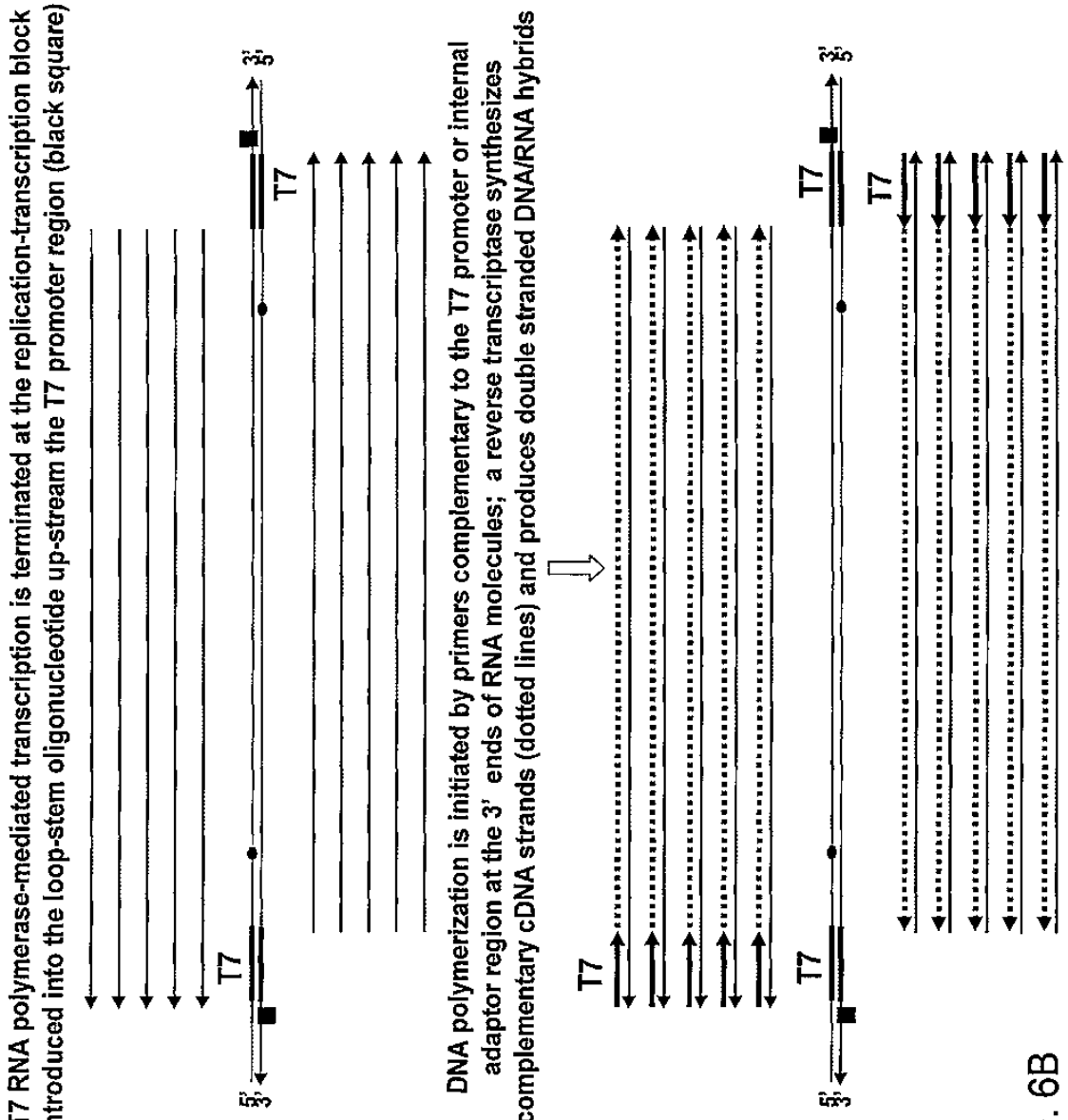

T7 RNA polymerase-mediated transcription is terminated at the replication-transcription block introduced into the loop-stem oligonucleotide up-stream the T7 promoter region (black square)
T7
5'
3'
3'
5'
DNA polymerization is initiated by primers complementary to the T7 promoter or internal adaptor region at the 3' ends of RNA molecules; a reverse transcriptase synthesizes complementary cDNA strands (dotted lines) and produces double stranded DNA/RNA hybrids

FIG. 6B

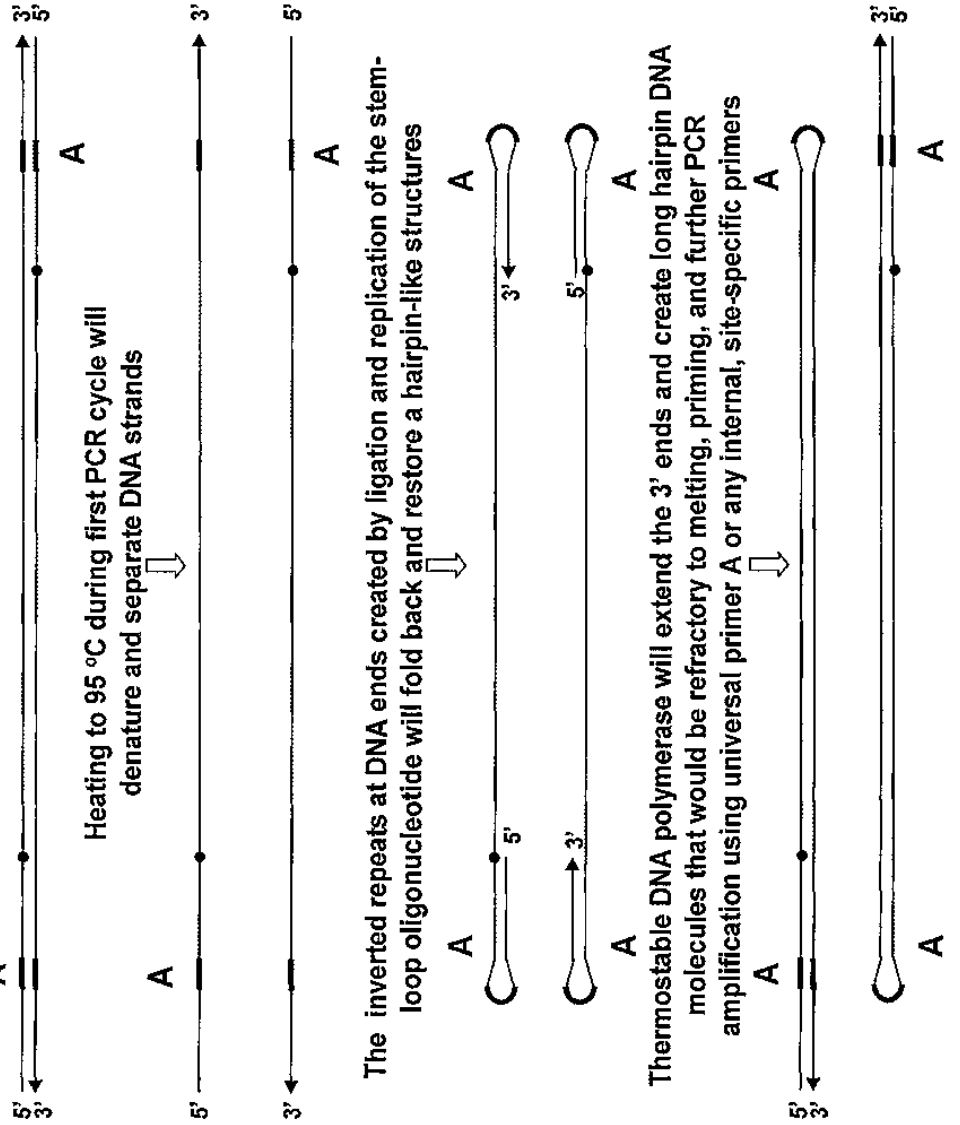

Heating to 95 °C during first PCR cycle will denature and separate DNA strands
The inverted repeats at DNA ends created by ligation and replication of the stem-loop oligonucleotide will fold back and restore a hairpin-like structures
Thermostable DNA polymerase will extend the 3' ends and create long hairpin DNA molecules that would be refractory to melting, priming, and further PCR amplification using universal primer A or any internal, site-specific primers
A
5'
3'

FIG. 7

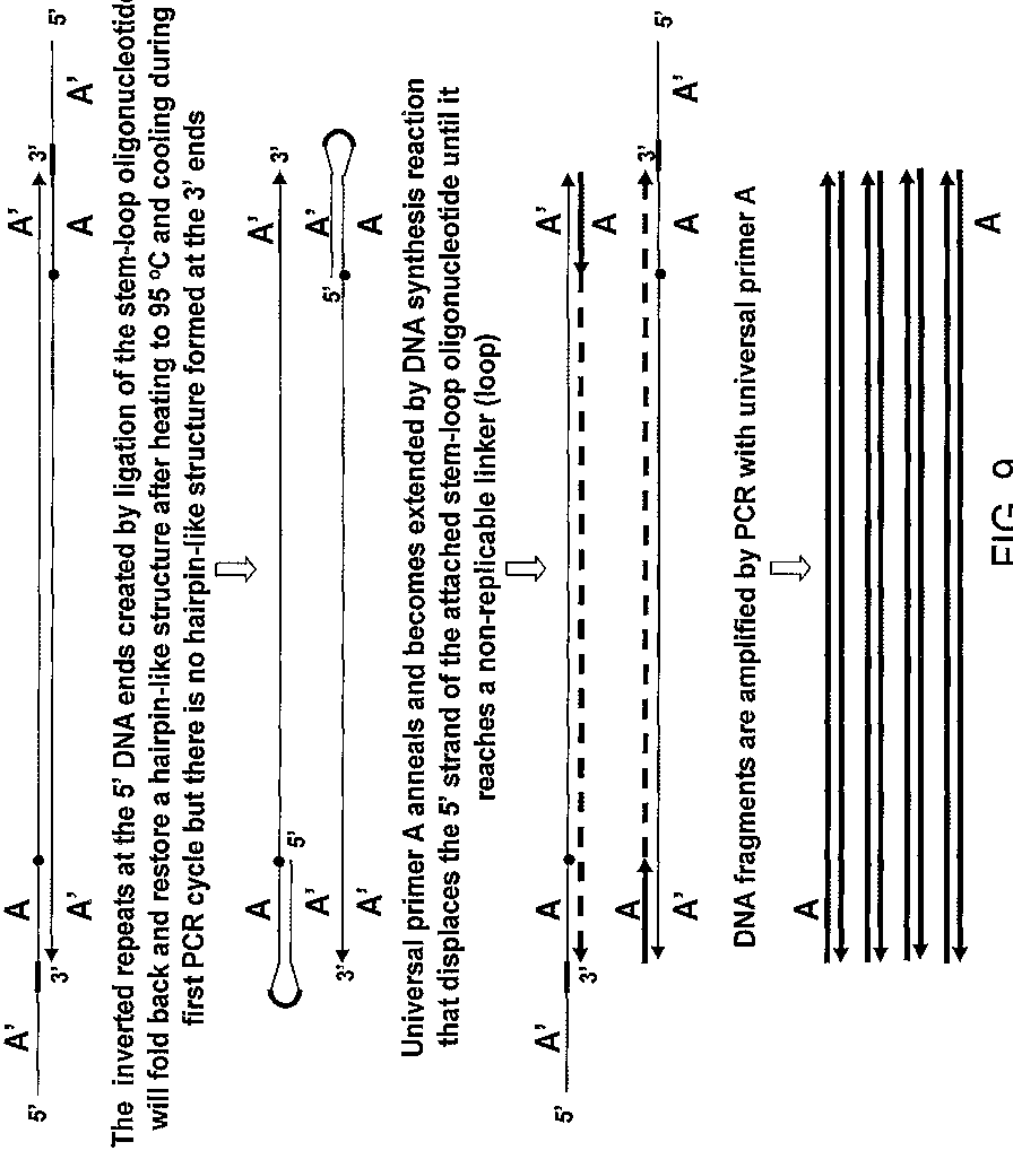

5'
A'
A
3'
A'
A
A'
5'
The inverted repeats at the 5' DNA ends created by ligation of the stem-loop oligonucleotide will fold back and restore a hairpin-like structure after heating to 95 °C and cooling during first PCR cycle but there is no hairpin-like structure formed at the 3' ends
Universal primer A anneals and becomes extended by DNA synthesis reaction that displaces the 5' strand of the attached stem-loop oligonucleotide until it reaches a non-replicable linker (loop)
DNA fragments are amplified by PCR with universal primer A

FIG. 9

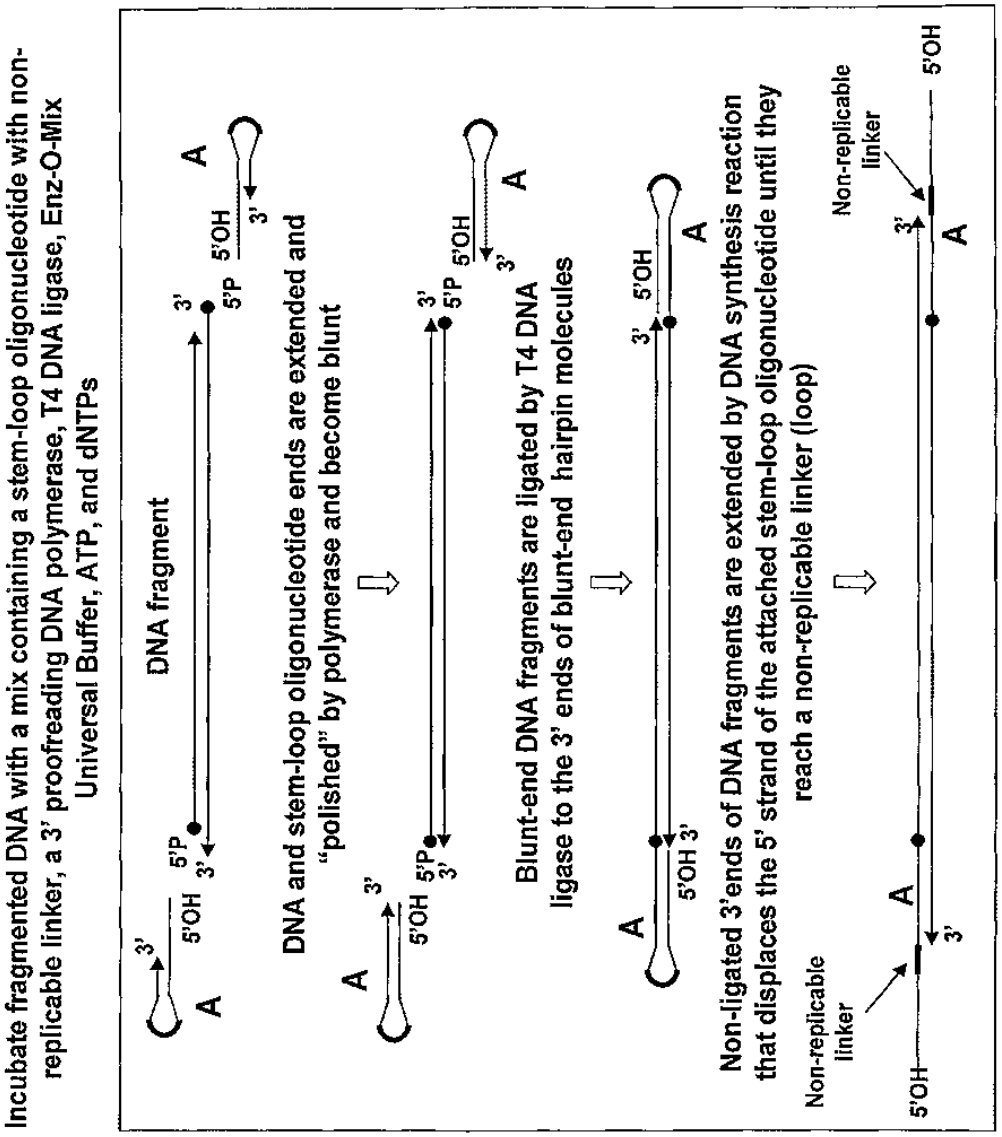

Incubate fragmented DNA with a mix containing a stem-loop oligonucleotide with non-replicable linker, a 3' proofreading DNA polymerase, T4 DNA ligase, Enz-O-Mix Universal Buffer, ATP, and dNTPs
DNA fragment
3'
5'OH
5'P
3'
A
DNA and stem-loop oligonucleotide ends are extended and "polished" by polymerase and become blunt
Blunt-end DNA fragments are ligated by T4 DNA ligase to the 3' ends of blunt-end hairpin molecules
Non-ligated 3'ends of DNA fragments are extended by DNA synthesis reaction that displaces the 5' strand of the attached stem-loop oligonucleotide until they reach a non-replicable linker (loop)
Non-replicable linker
5'OH 3'

FIG. 8

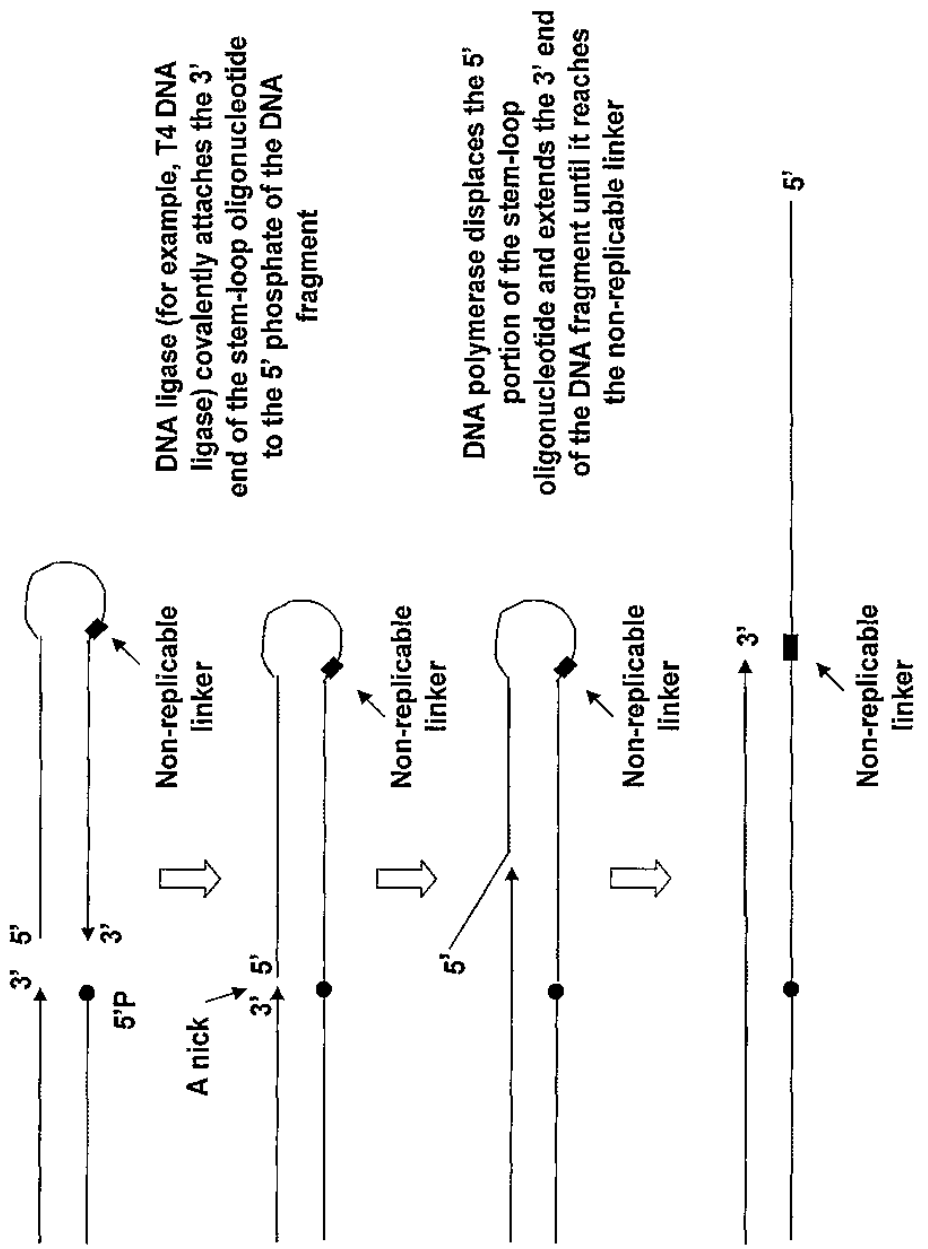

DNA ligase (for example, T4 DNA ligase) covalently attaches the 3' end of the stem-loop oligonucleotide to the 5' phosphate of the DNA fragment
DNA polymerase displaces the 5' portion of the stem-loop oligonucleotide and extends the 3' end of the DNA fragment until it reaches the non-replicable linker
Non-replicable linker
Non-replicable linker
Non-replicable linker
Non-replicable linker
5'
3'
5'P
A nick
3'
5'
5'
3'
5'

FIG. 10A

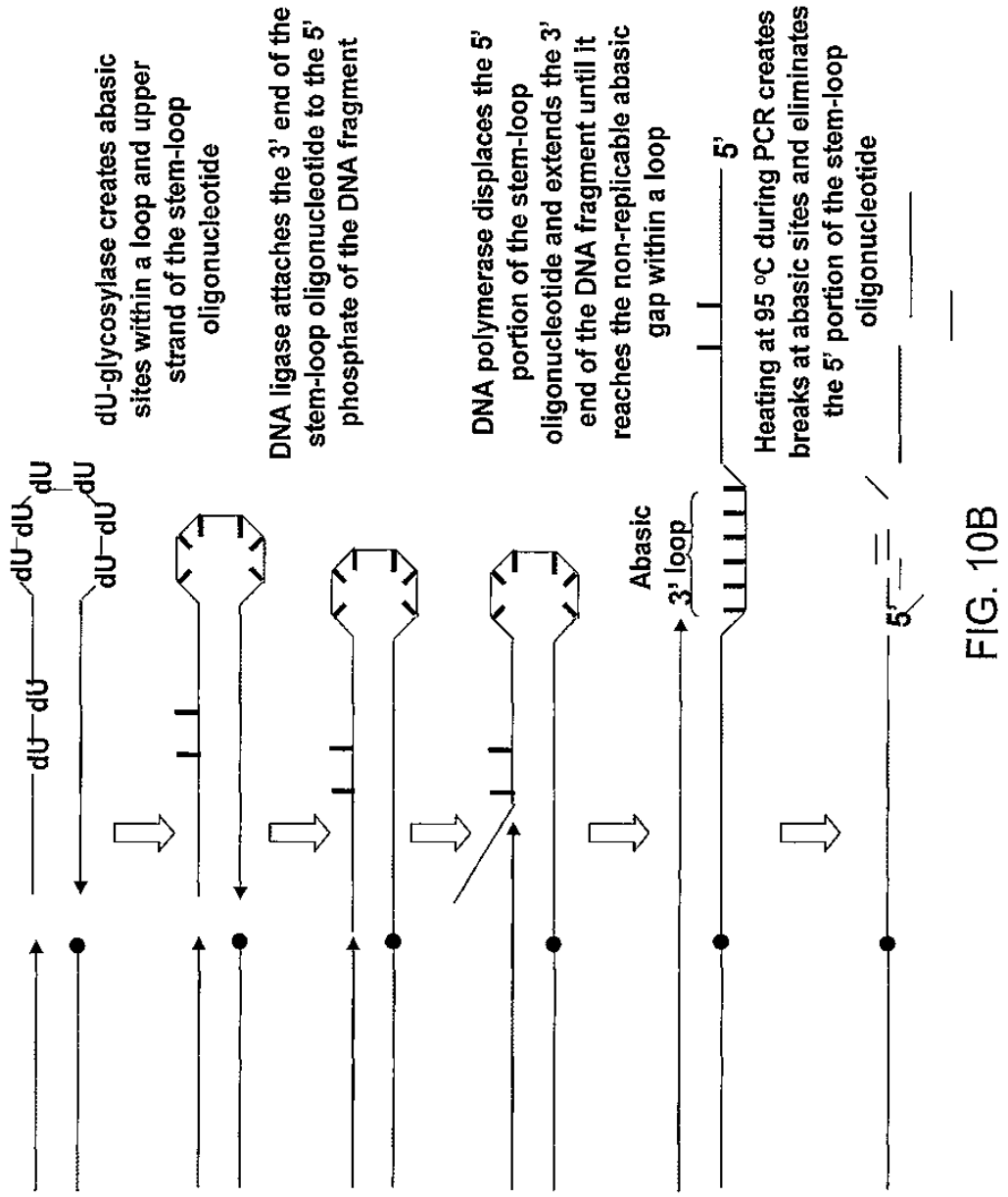

dU-glycosylase creates abasic sites within a loop and upper strand of the stem-loop oligonucleotide
DNA ligase attaches the 3' end of the stem-loop oligonucleotide to the 5' phosphate of the DNA fragment
DNA polymerase displaces the 5' portion of the stem-loop oligonucleotide and extends the 3' end of the DNA fragment until it reaches the non-replicable abasic gap within a loop
Heating at 95 °C during PCR creates breaks at abasic sites and eliminates the 5' portion of the stem-loop oligonucleotide
dU
Abasic
3' loop
5'
5'

FIG. 10B

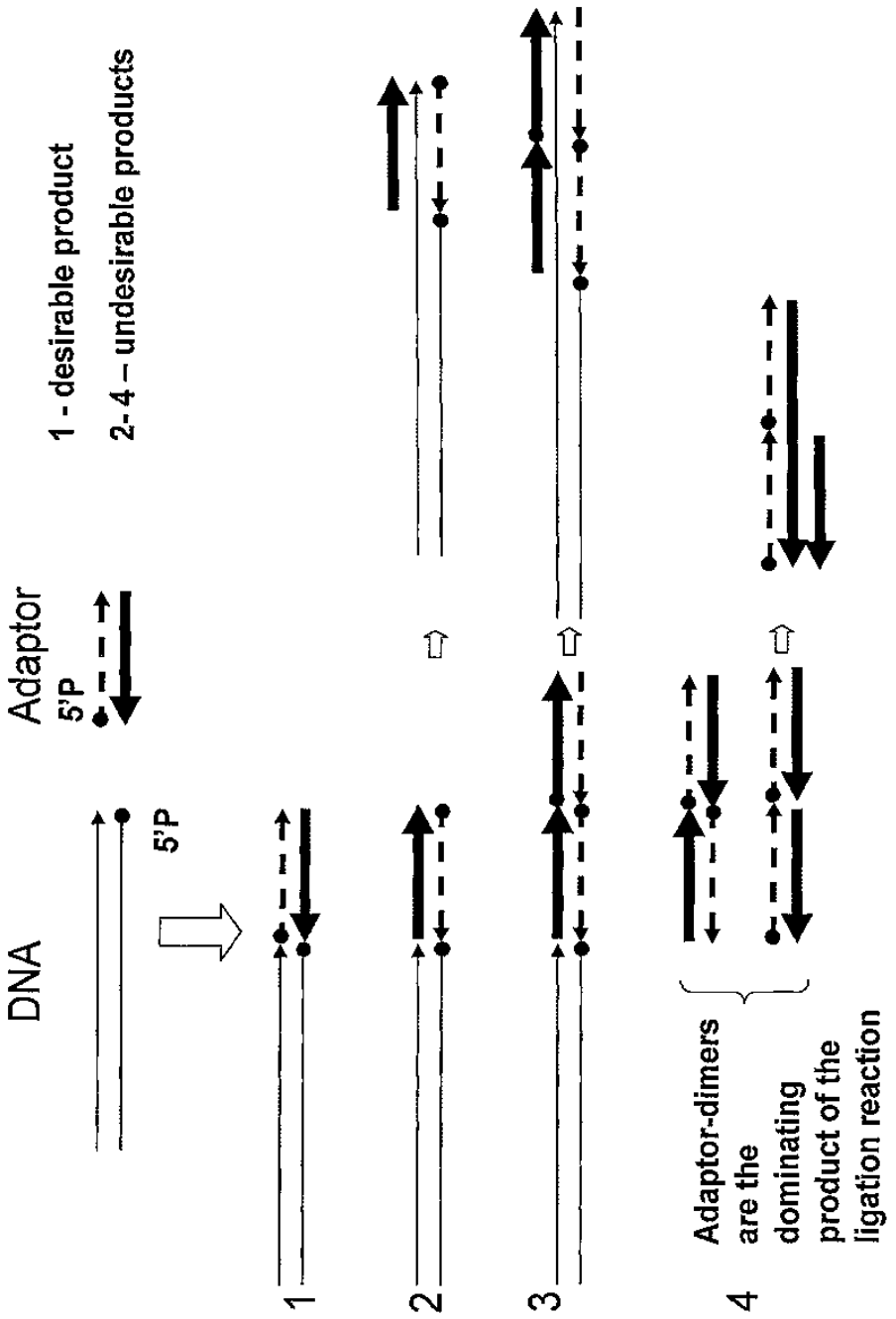

1 - desirable product
2-4 – undesirable products
Adaptor
5'P
DNA
5'P
1
2
3
4
Adaptor-dimers are the dominating product of the ligation reaction

FIG. 11A

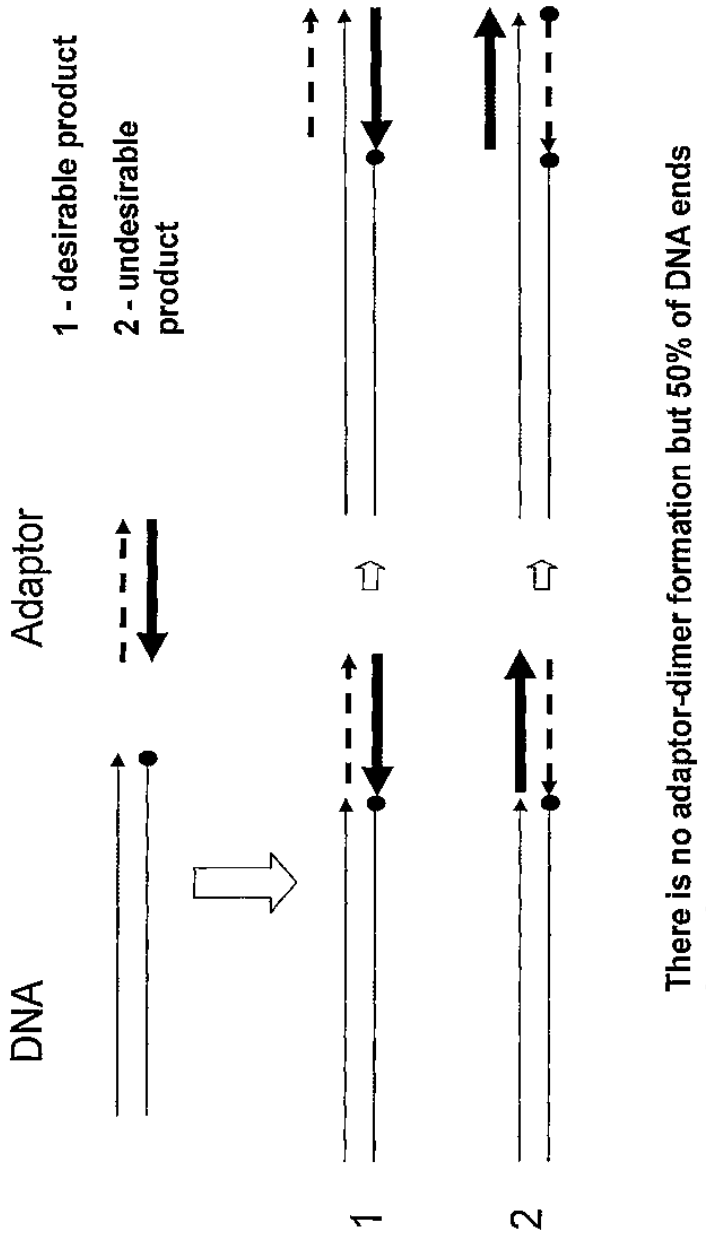
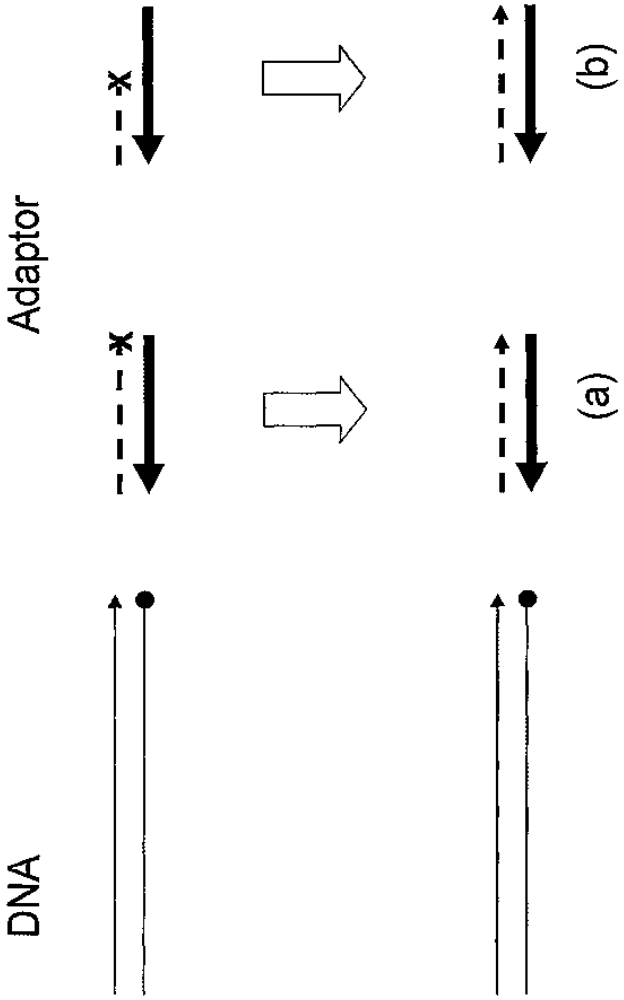

DNA
Adaptor
1 - desirable product
2 - undesirable product
1
2
There is no adaptor-dimer formation but 50% of DNA ends have inverse adaptor orientation

FIG. 11B



3'-blocked oligonucleotides within the standard 2-oligo adaptor will be repaired and extended by 3' proofreading polymerase present in the mix and ligated to DNA ends in two orientations

FIG. 11C

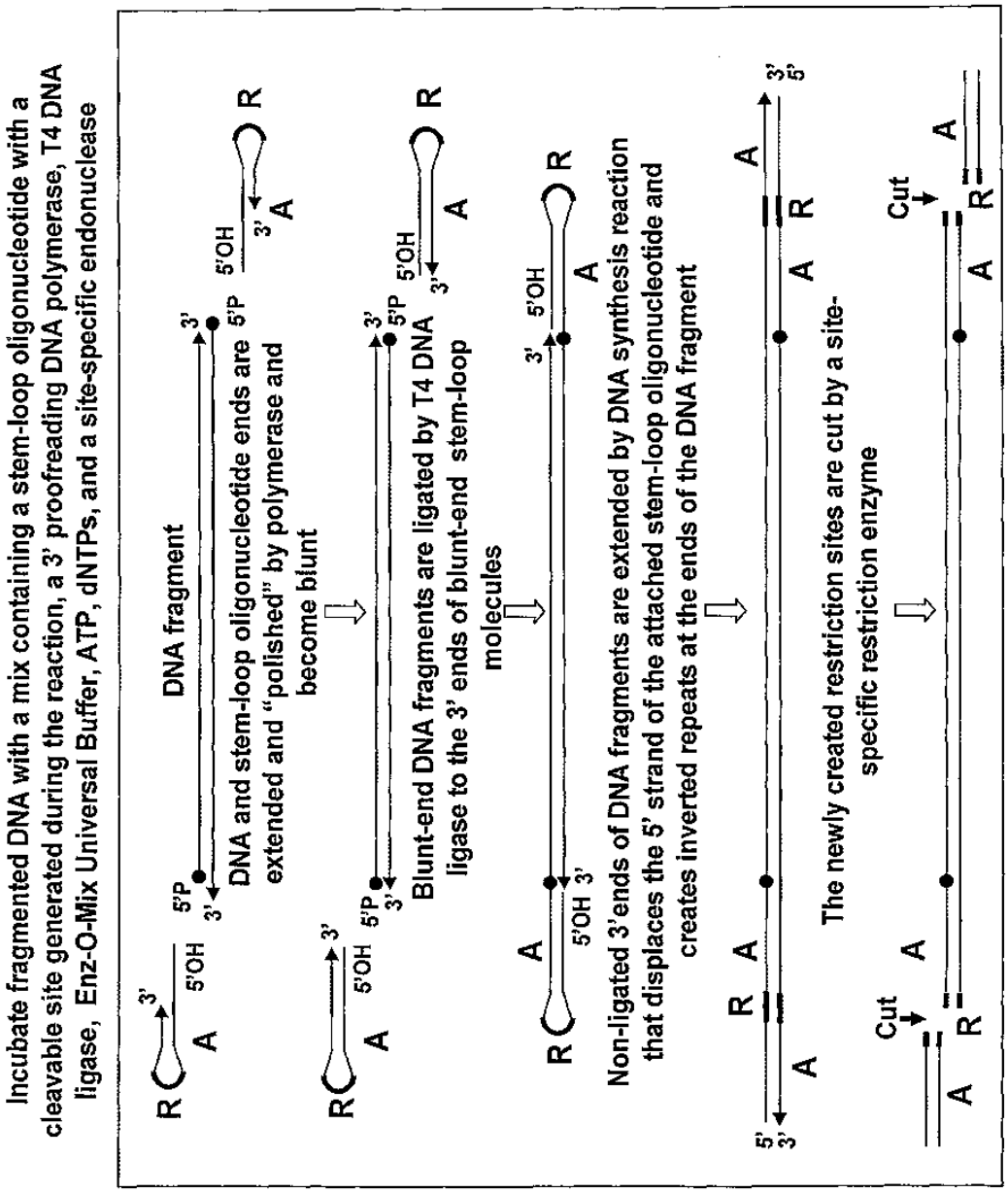

Incubate fragmented DNA with a mix containing a stem-loop oligonucleotide with a cleavable site generated during the reaction, a 3' proofreading DNA polymerase, T4 DNA ligase, Enz-O-Mix Universal Buffer, ATP, dNTPs, and a site-specific endonuclease
DNA fragment
DNA and stem-loop oligonucleotide ends are extended and "polished" by polymerase and become blunt
Blunt-end DNA fragments are ligated by T4 DNA ligase to the 3' ends of blunt-end stem-loop molecules
Non-ligated 3'ends of DNA fragments are extended by DNA synthesis reaction that displaces the 5' strand of the attached stem-loop oligonucleotide and creates inverted repeats at the ends of the DNA fragment
The newly created restriction sites are cut by a site-specific restriction enzyme
Cut
Cut

FIG. 12

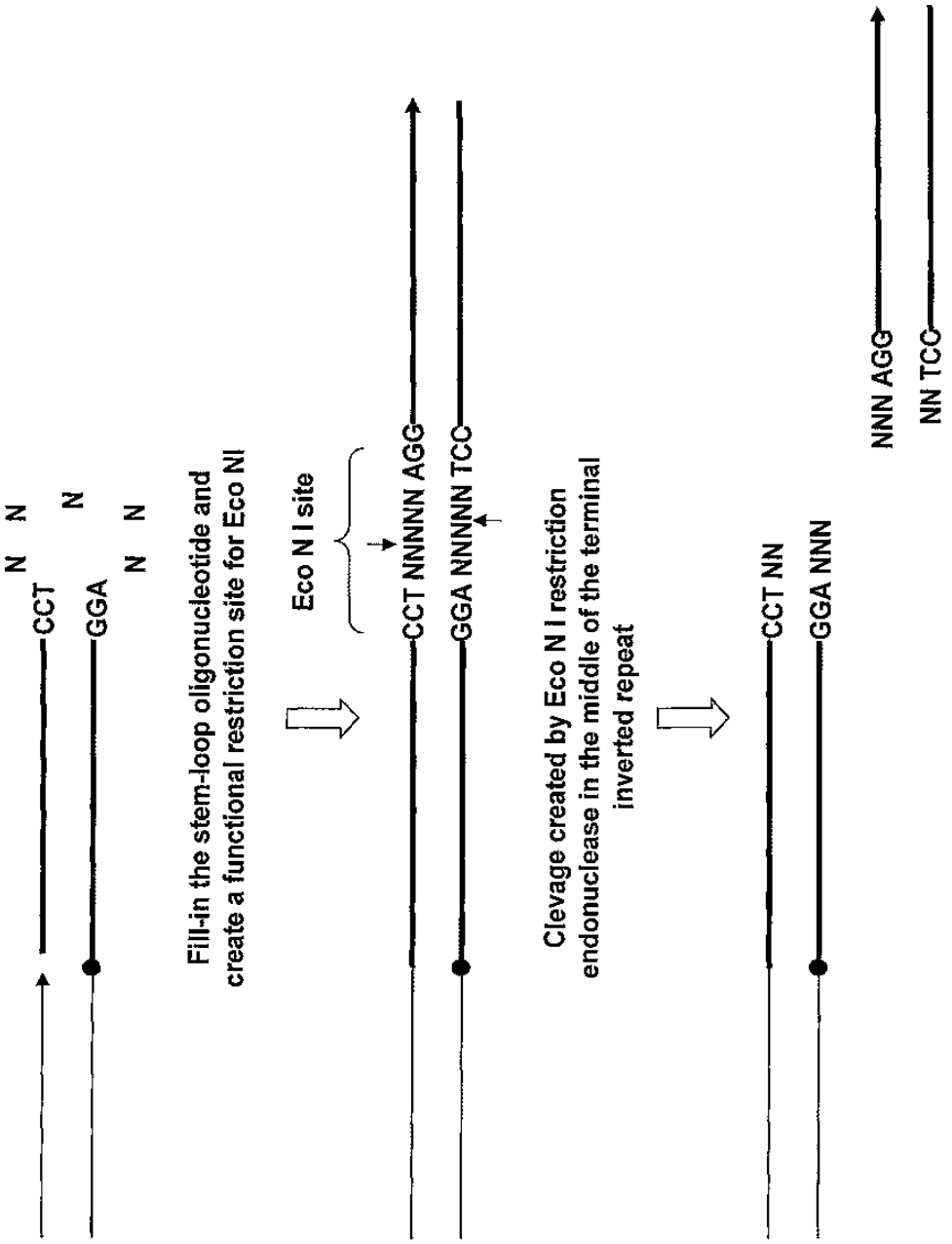

N N
CCT N
GGA
N N
Fill-in the stem-loop oligonucleotide and create a functional restriction site for Eco NI
Eco N I site
CCT NNNNN AGG
GGA NNNNN TCC
Clevage created by Eco N I restriction endonuclease in the middle of the terminal inverted repeat
CCT NN
GGA NNN
NNN AGG
NN TCC

FIG. 13A

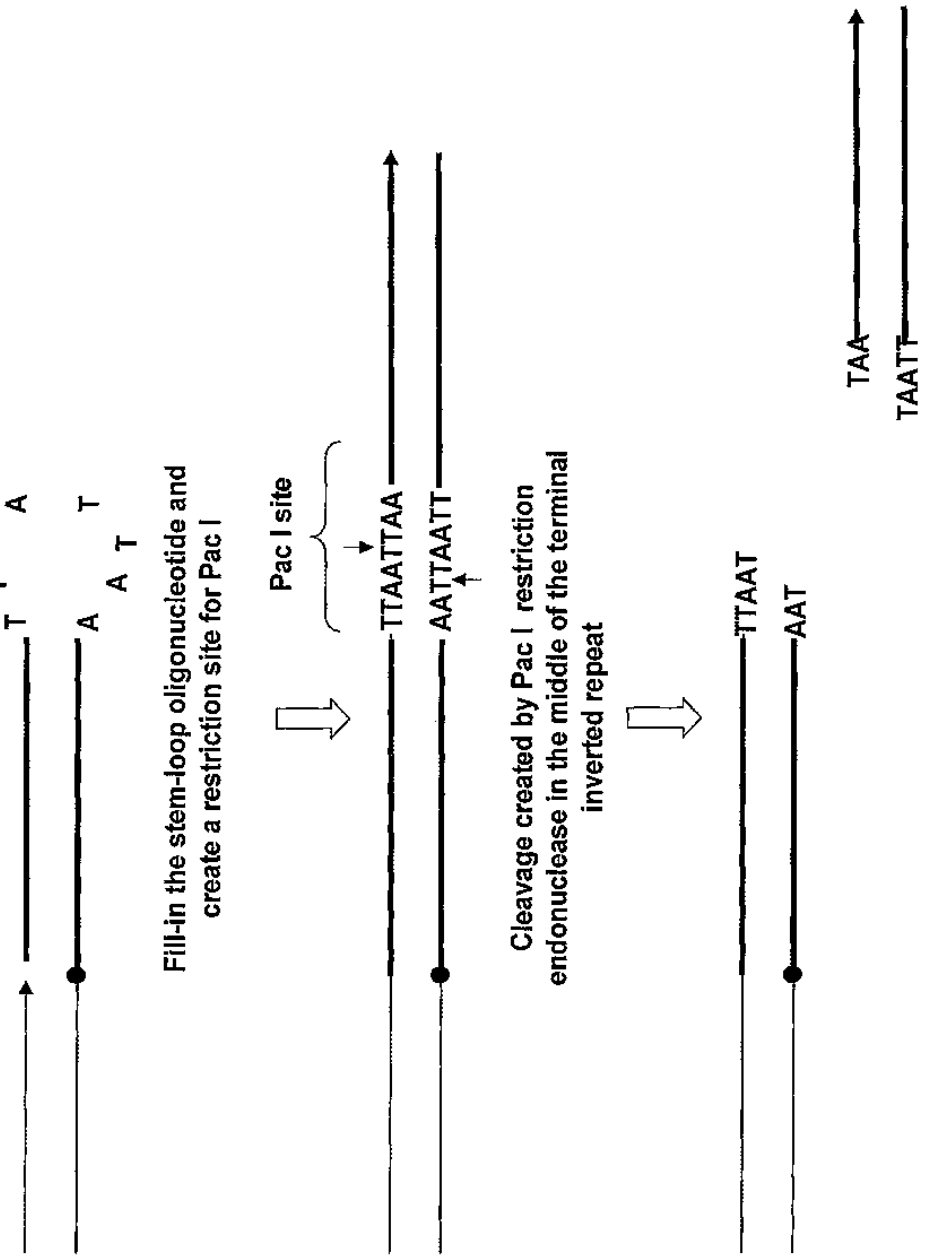

T T A A
A A T T
Fill-in the stem-loop oligonucleotide and create a restriction site for Pac I
Pac I site
TTAATTAA
AATTAATT
Cleavage created by Pac I restriction endonuclease in the middle of the terminal inverted repeat
TTAAT
AAT
TAA
TAATT

FIG. 13B

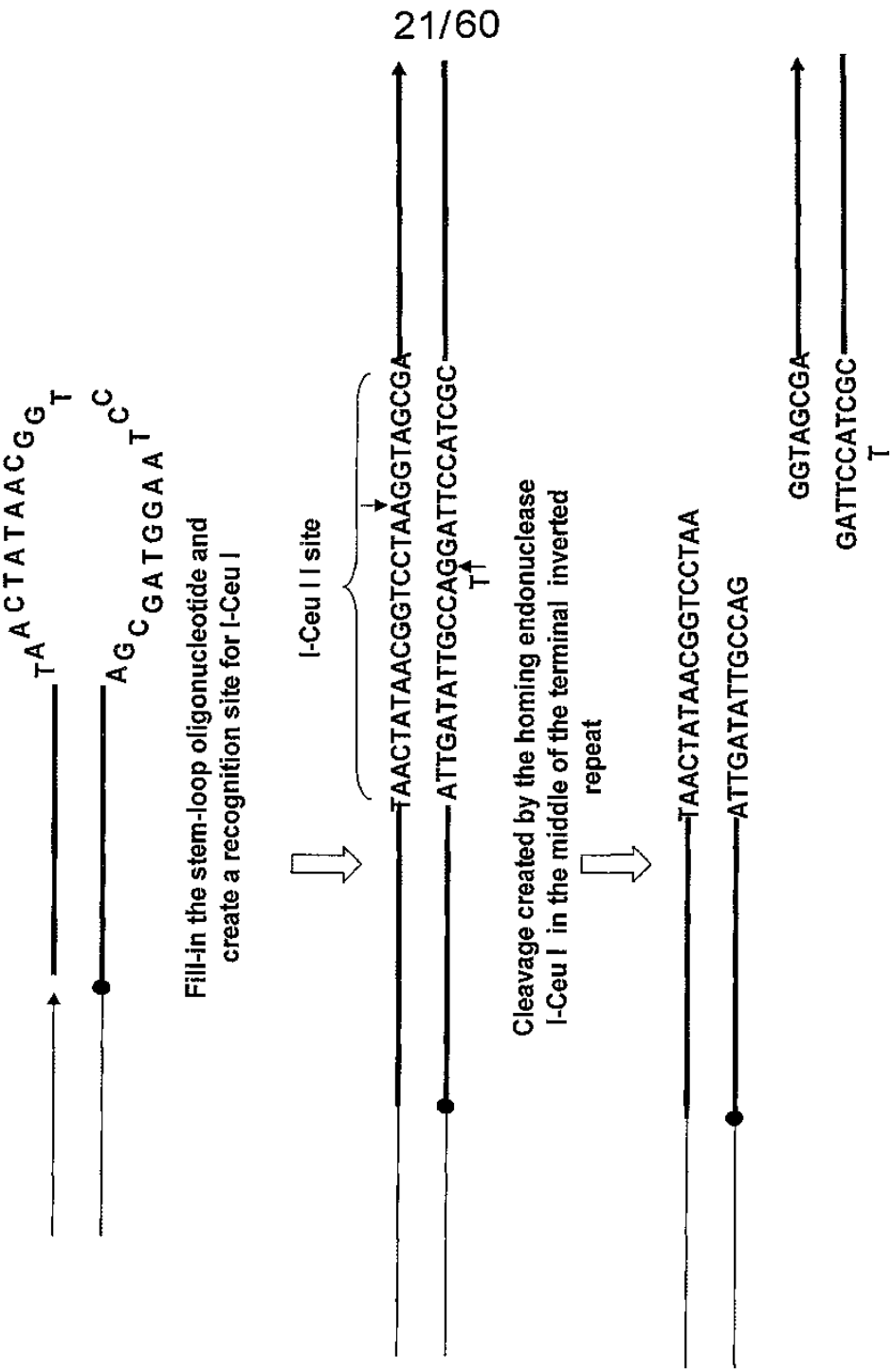

Fill-in the stem-loop oligonucleotide and create a recognition site for I-Ceu I
I-Ceu I I site
TAACTATAACGGTCCTAAGGTAGCGA
ATTGATATTGCCAGGATTCCATCGC
Cleavage created by the homing endonuclease I-Ceu I in the middle of the terminal inverted repeat
TAACTATAACGGTCCTAA
ATTGATATTGCCAG
GGTAGCGA
GATTCCATCGC

FIG. 13C

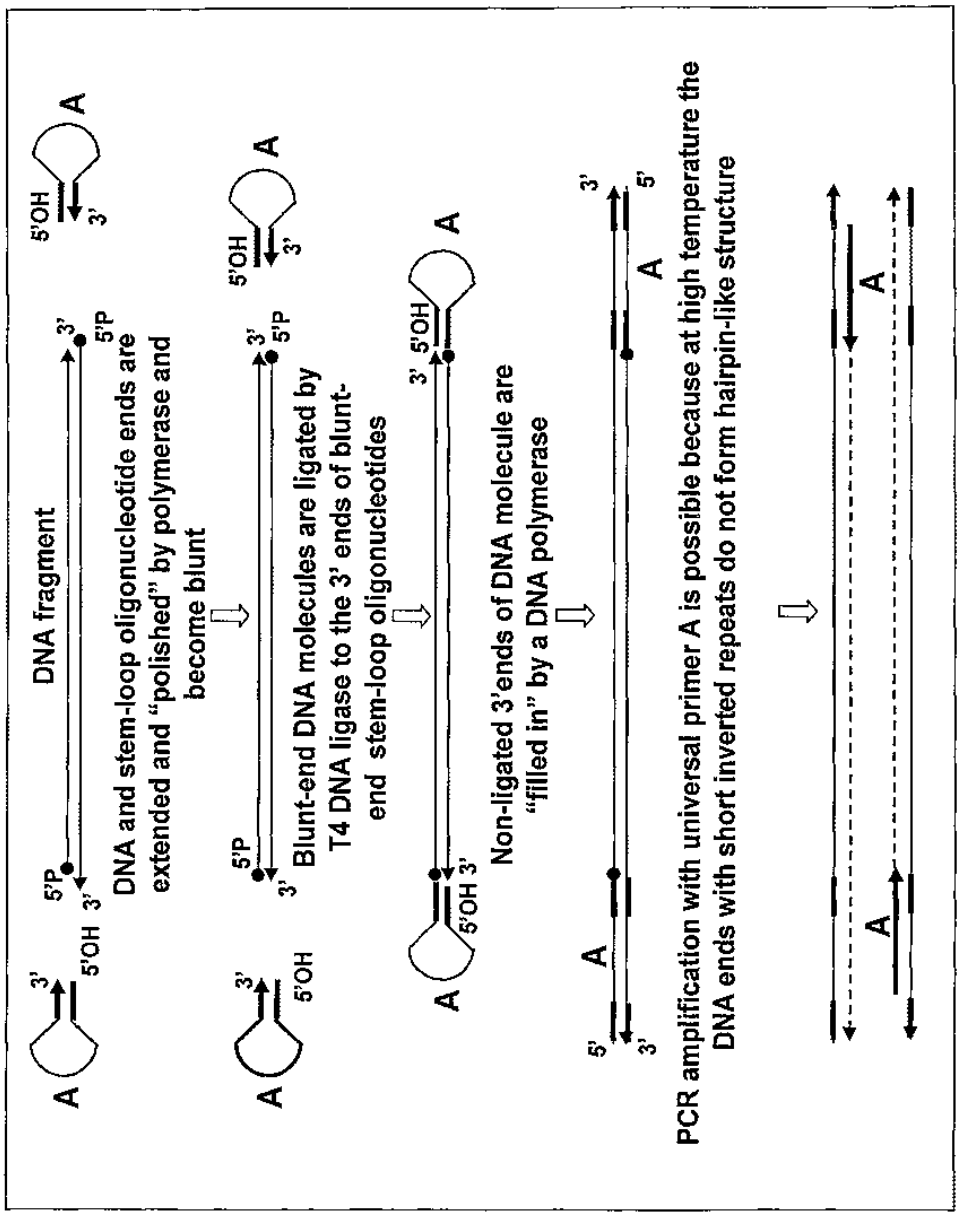

Incubate fragmented DNA with a mix containing a stem-loop oligonucleotide with very short stem (5-8 bases), a 3' proofreading DNA polymerase, T4 DNA ligase, Enz-O-Mix Universal Buffer, dNTP, and ATP
DNA fragment
DNA and stem-loop oligonucleotide ends are extended and "polished" by polymerase and become blunt
Blunt-end DNA molecules are ligated by T4 DNA ligase to the 3' ends of blunt-end stem-loop oligonucleotides
Non-ligated 3'ends of DNA molecule are "filled in" by a DNA polymerase
PCR amplification with universal primer A is possible because at high temperature the DNA ends with short inverted repeats do not form hairpin-like structure
A
5'OH
3'
5'P

FIG. 14

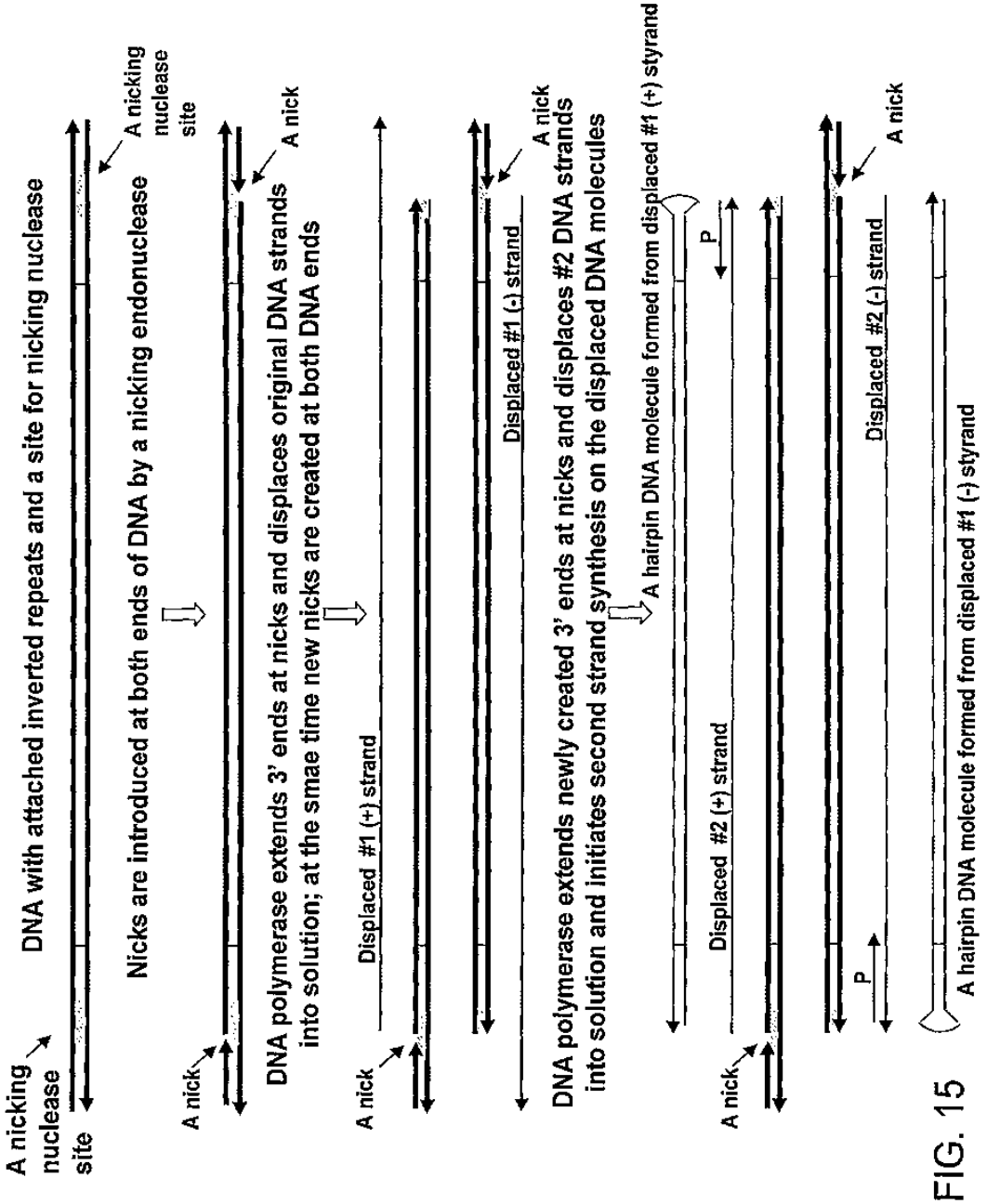

A nicking nuclease site
DNA with attached inverted repeats and a site for nicking nuclease
A nicking nuclease site
Nicks are introduced at both ends of DNA by a nicking endonuclease
A nick
A nick
DNA polymerase extends 3' ends at nicks and displaces original DNA strands into solution; at the smae time new nicks are created at both DNA ends
A nick
Displaced #1 (+) strand
Displaced #1 (-) strand
A nick
DNA polymerase extends newly created 3' ends at nicks and displaces #2 DNA strands into solution and initiates second strand synthesis on the displaced DNA molecules
A hairpin DNA molecule formed from displaced #1 (+) styrand
P
A nick
Displaced #2 (+) strand
A nick
P
Displaced #2 (-) strand
A hairpin DNA molecule formed from displaced #1 (-) styrand

FIG. 15

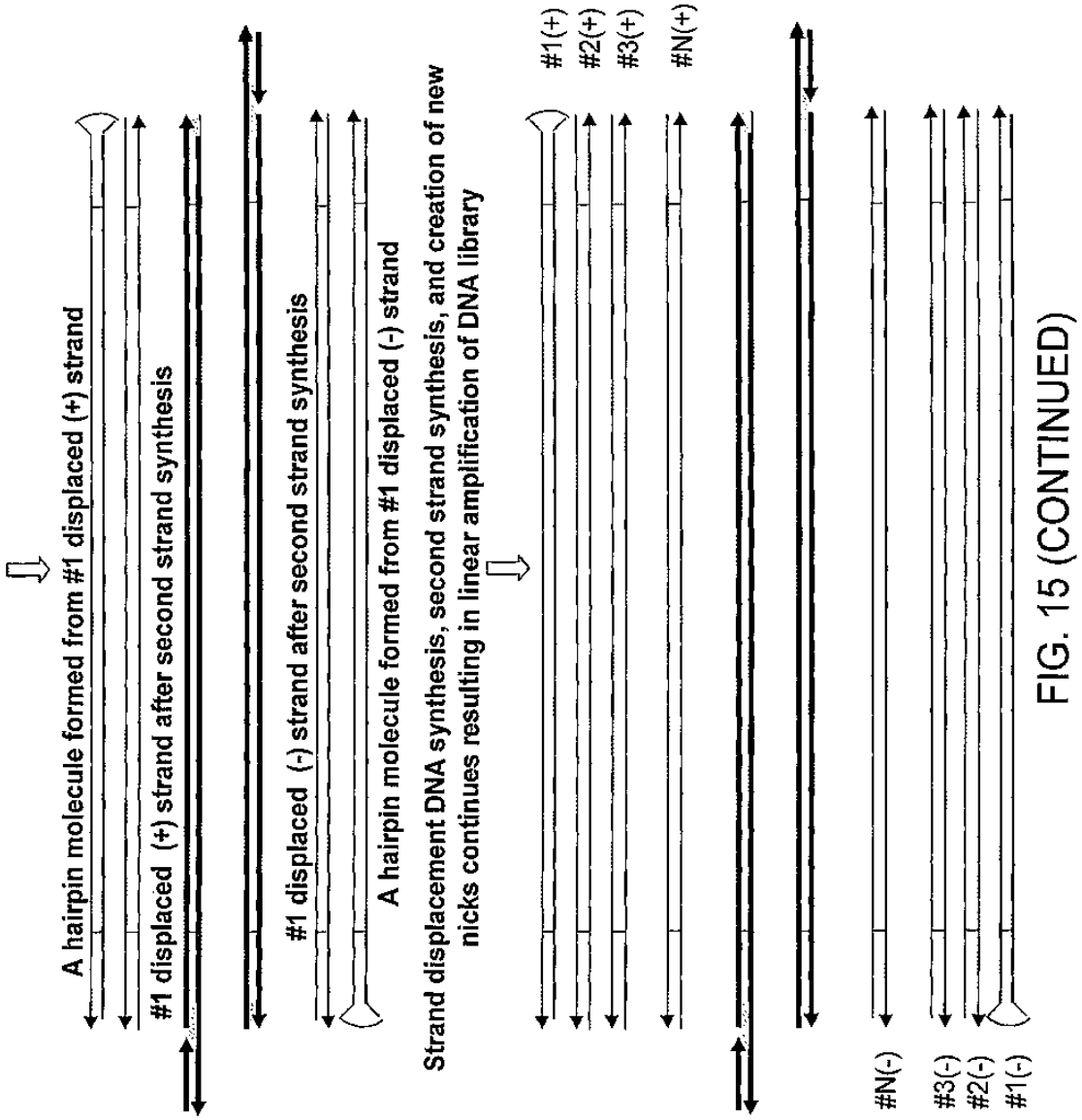


FIG. 15 (CONTINUED)

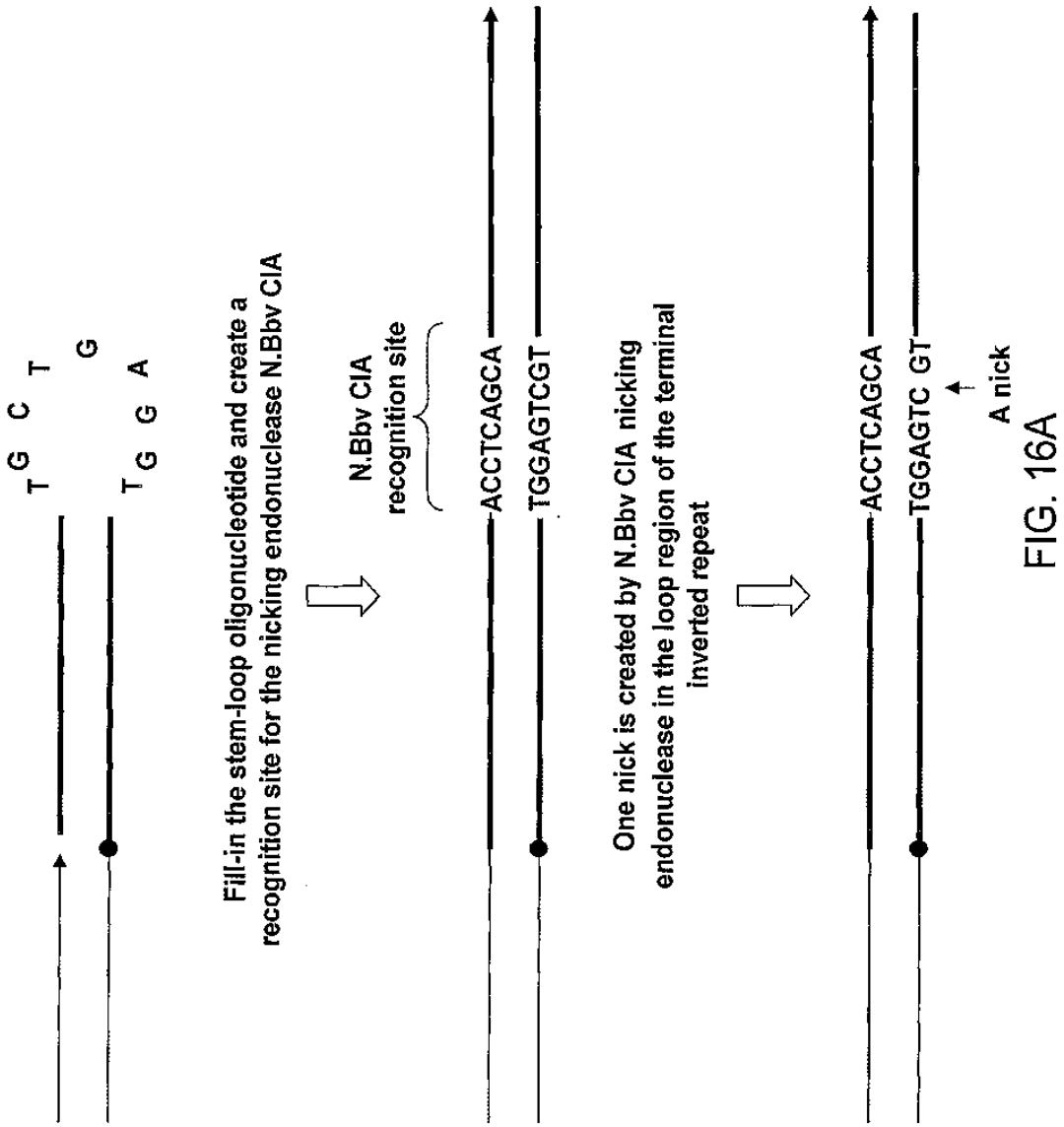

T G C T
G
T G G A
Fill-in the stem-loop oligonucleotide and create a recognition site for the nicking endonuclease N.Bbv CIA
N.Bbv CIA recognition site
ACCTCAGCA
TGGAGTCGT
One nick is created by N.Bbv CIA nicking endonuclease in the loop region of the terminal inverted repeat
ACCTCAGCA
TGGAGTC GT
A nick

FIG. 16A

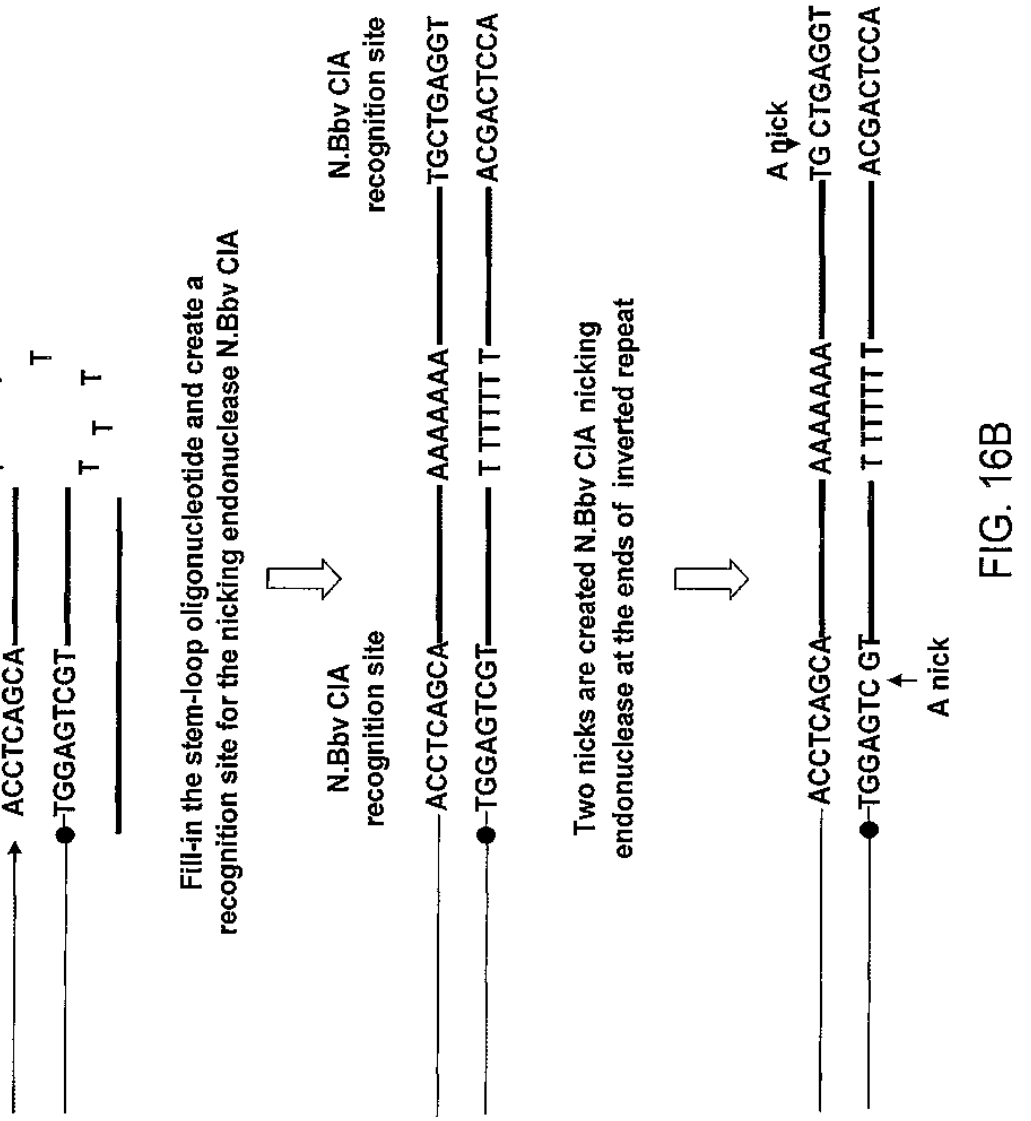
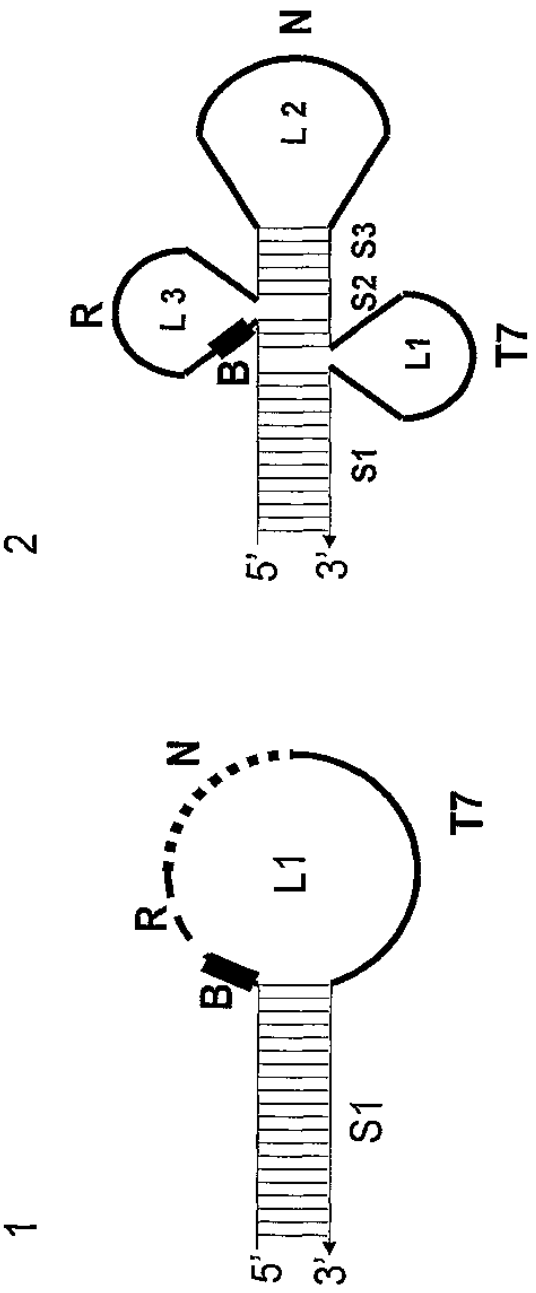

ACCTCAGCA
TGGAGTCGT
T T T
T T T
Fill-in the stem-loop oligonucleotide and create a recognition site for the nicking endonuclease N.Bbv CIA
N.Bbv CIA recognition site
ACCTCAGCA AAAAAAA TGCTGAGGT
TGGAGTCGT T TTTTT T ACGACTCCA
N.Bbv CIA recognition site
Two nicks are created N.Bbv CIA nicking endonuclease at the ends of inverted repeat
A nick
ACCTCAGCA AAAAAAA TG CTGAGGT
TGGAGTC GT T TTTTT T ACGACTCCA
A nick

FIG. 16B



T7 - T7 promoter sequence (including all necessary for efficient transcription downstream and upstream regions;

N - recognition sequence for a nicking endonuclease;

R - recognition sequence for a restriction endonuclease;

B - replication or transcription block (chemical group, abasic site or gap, etc.);

S1, S2, S3 – stem regions 1, 2, and 3;

L1, L2, L3 – loop regions 1, 2, and 3.

FIG. 16C

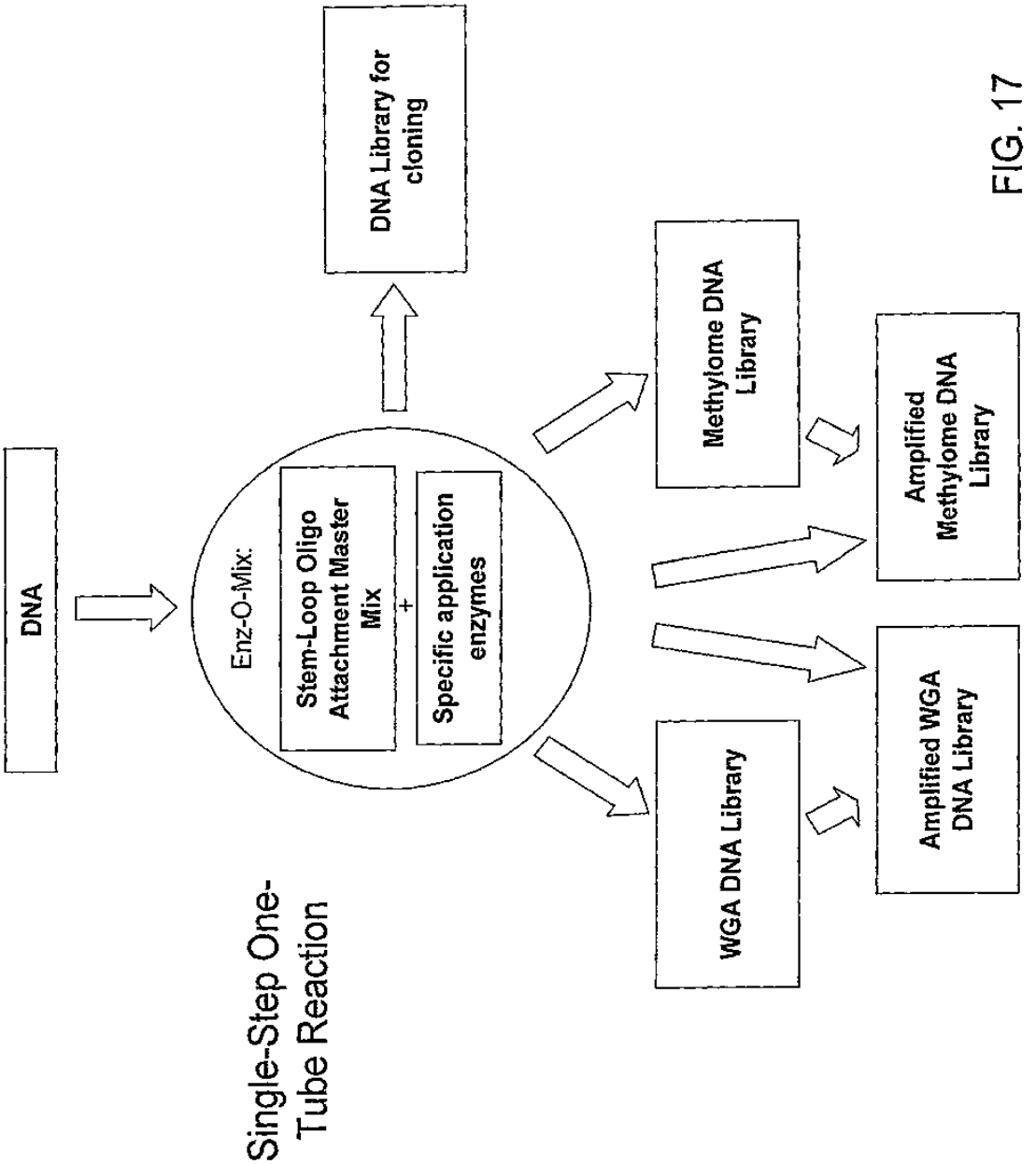

DNA
Enz-O-Mix:
Stem-Loop Oligo Attachment Master Mix
+
Specific application enzymes
DNA Library for cloning
Methylome DNA Library
Amplified Methylome DNA Library
WGA DNA Library
Amplified WGA DNA Library
Single-Step One-Tube Reaction

FIG. 17

Stem-Loop Oligo Attachment Master Mix I	Stem-Loop Oligo Attachment Master Mix II	Stem-Loop Oligo Attachment Master Mix III	Stem-Loop Oligo Attachment Master Mix IV
Stem-loop oligonucleotide 3' proofreading DNA polymerase T4 DNA ligase Universal Buffer ATP dNTPs	Stem-loop oligonucleotide with non-replicable linker (chemical) 3' proofreading DNA polymerase T4 DNA ligase Universal Buffer ATP dNTPs	Stem-loop oligonucleotide with non-replicable linker (abasic) dU-glycosylase 3' proofreading DNA polymerase T4 DNA ligase Universal Buffer ATP dNTPs	Stem-loop oligonucleotide with cleavable site created by replication Site-specific endonuclease 3' proofreading DNA polymerase T4 DNA ligase Universal Buffer ATP dNTPs

FIG. 18

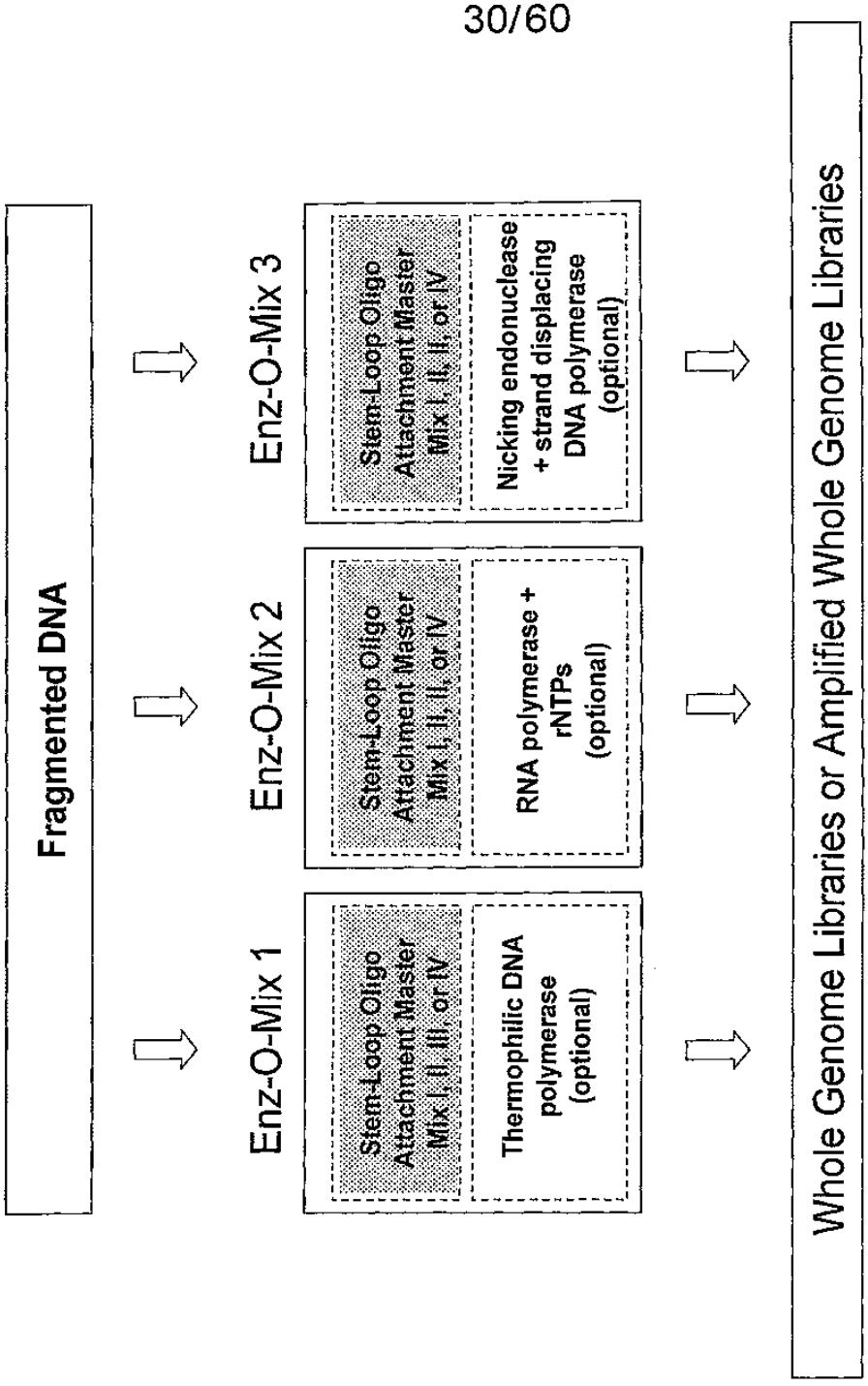

Fragmented DNA
Enz-O-Mix 1
Stem-Loop Oligo Attachment Master Mix I, II, III, or IV
Thermophilic DNA polymerase (optional)
Enz-O-Mix 2
Stem-Loop Oligo Attachment Master Mix I, II, II, or IV
RNA polymerase + rNTPs (optional)
Enz-O-Mix 3
Stem-Loop Oligo Attachment Master Mix I, II, II, or IV
Nicking endonuclease + strand displacing DNA polymerase (optional)
Whole Genome Libraries or Amplified Whole Genome Libraries

FIG. 19A

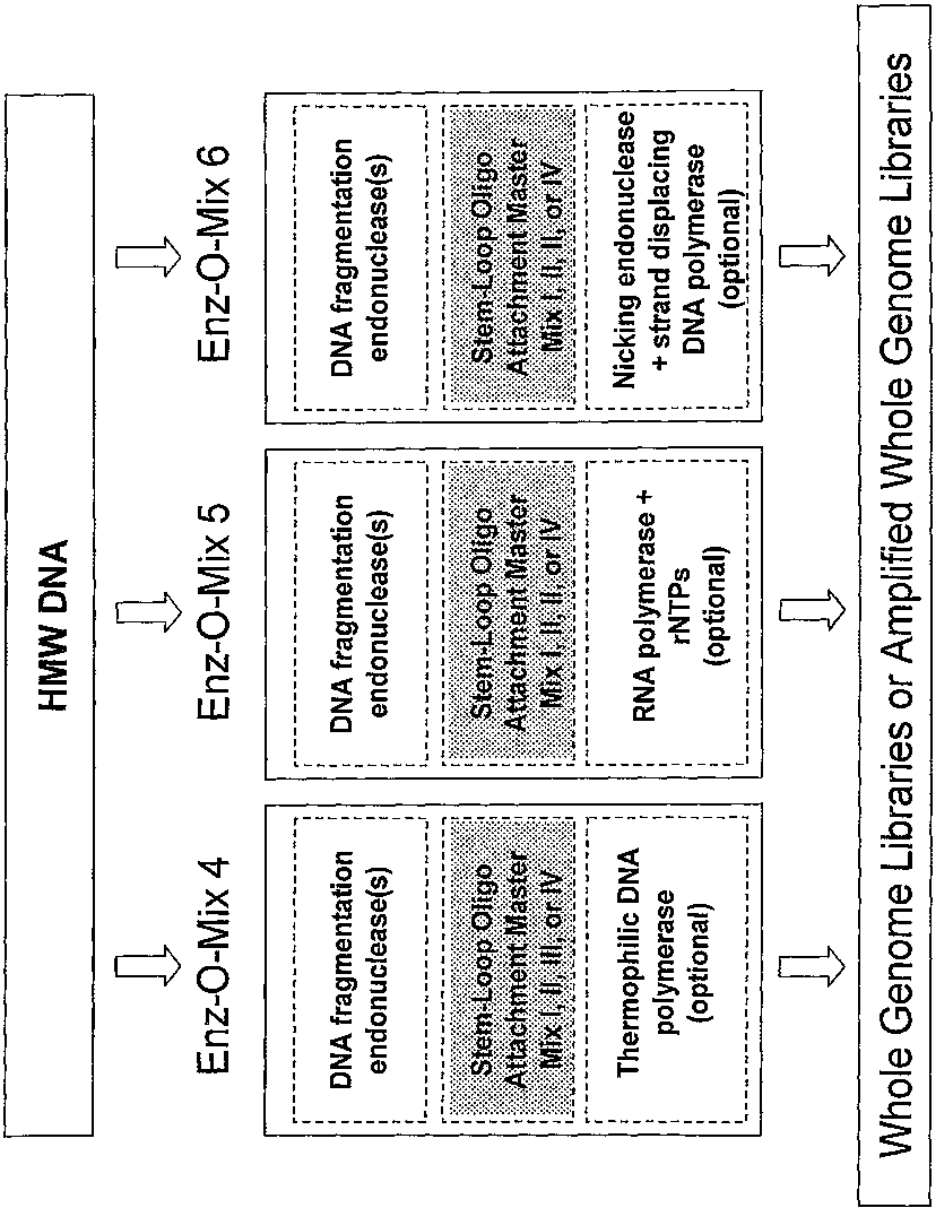

HMW DNA
Enz-O-Mix 4
DNA fragmentation endonuclease(s)
Stem-Loop Oligo Attachment Master Mix I, II, III, or IV
Thermophilic DNA polymerase (optional)
Enz-O-Mix 5
DNA fragmentation endonuclease(s)
Stem-Loop Oligo Attachment Master Mix I, II, II, or IV
RNA polymerase + rNTPs (optional)
Enz-O-Mix 6
DNA fragmentation endonuclease(s)
Stem-Loop Oligo Attachment Master Mix I, II, II, or IV
Nicking endonuclease + strand displacing DNA polymerase (optional)
Whole Genome Libraries or Amplified Whole Genome Libraries

FIG. 19B

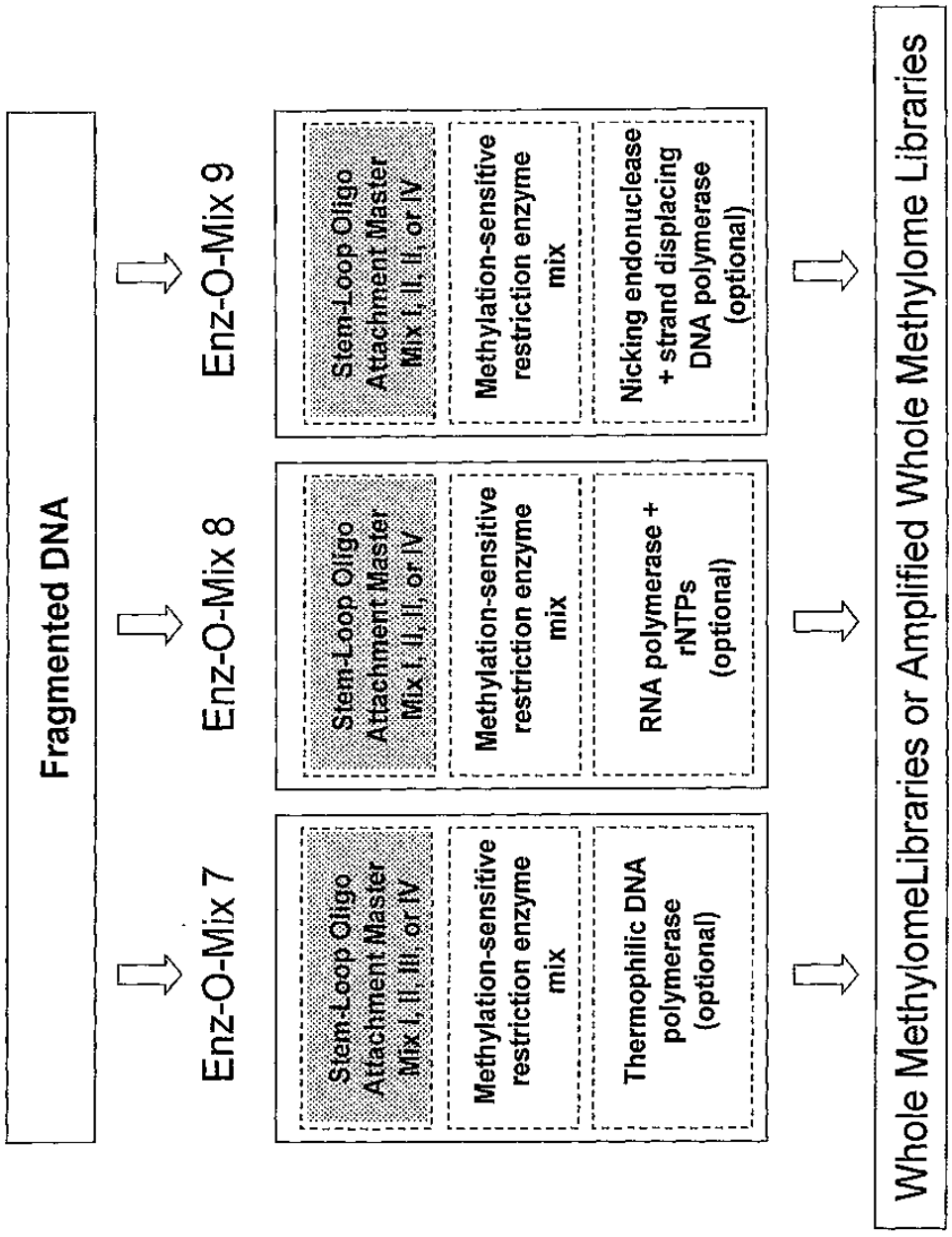

Fragmented DNA
Enz-O-Mix 7
Stem-Loop Oligo Attachment Master Mix I, II, III, or IV
Methylation-sensitive restriction enzyme mix
Thermophilic DNA polymerase (optional)
Enz-O-Mix 8
Stem-Loop Oligo Attachment Master Mix I, II, II, or IV
Methylation-sensitive restriction enzyme mix
RNA polymerase + rNTPs (optional)
Enz-O-Mix 9
Stem-Loop Oligo Attachment Master Mix I, II, II, or IV
Methylation-sensitive restriction enzyme mix
Nicking endonuclease + strand displacing DNA polymerase (optional)
Whole MethylomeLibraries or Amplified Whole Methylome Libraries

FIG. 19C

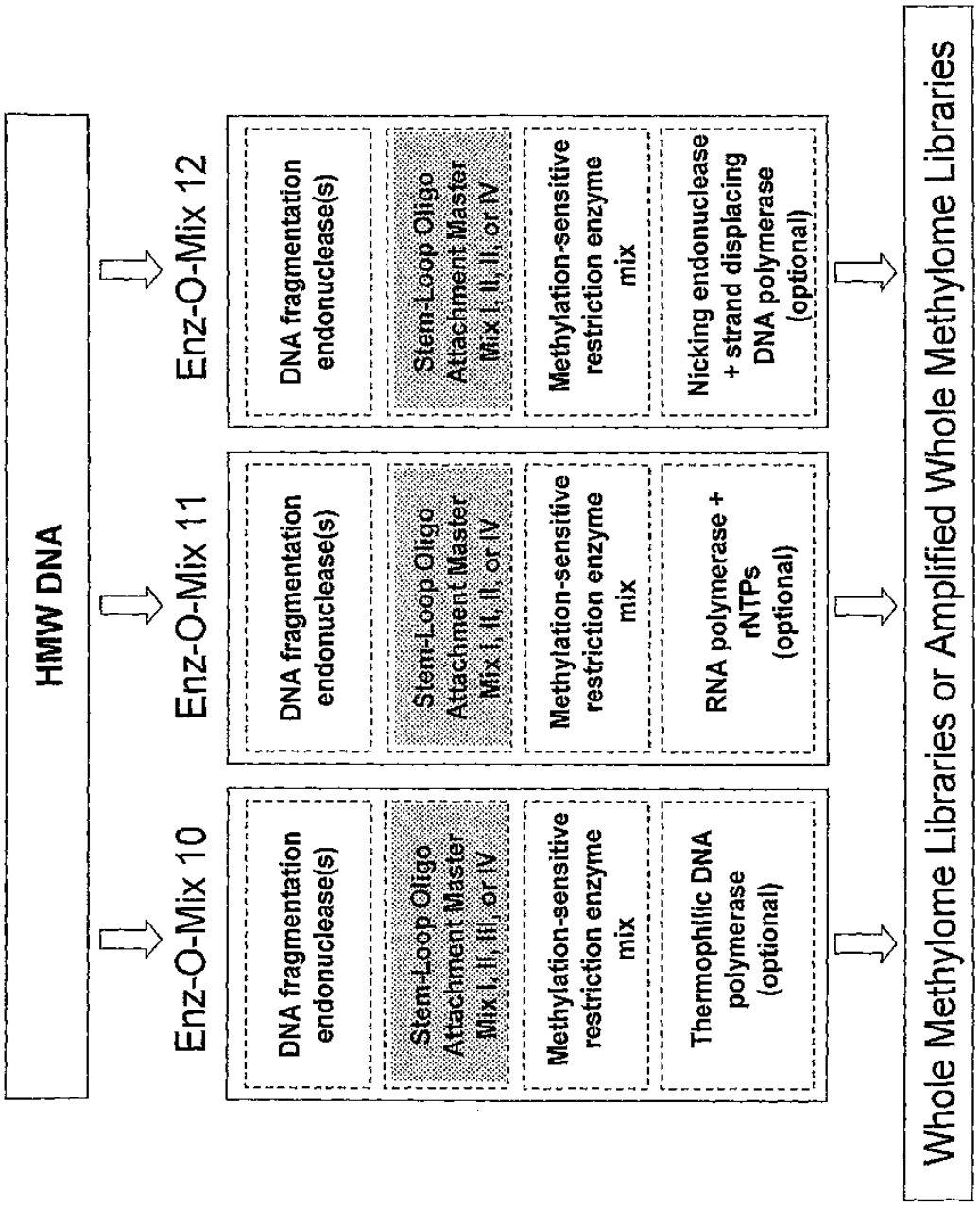

HMW DNA
Enz-O-Mix 10
DNA fragmentation endonuclease(s)
Stem-Loop Oligo Attachment Master Mix I, II, III, or IV
Methylation-sensitive restriction enzyme mix
Thermophilic DNA polymerase (optional)
Enz-O-Mix 11
DNA fragmentation endonuclease(s)
Stem-Loop Oligo Attachment Master Mix I, II, II, or IV
Methylation-sensitive restriction enzyme mix
RNA polymerase + rNTPs (optional)
Enz-O-Mix 12
DNA fragmentation endonuclease(s)
Stem-Loop Oligo Attachment Master Mix I, II, II, or IV
Methylation-sensitive restriction enzyme mix
Nicking endonuclease + strand displacing DNA polymerase (optional)
Whole Methylome Libraries or Amplified Whole Methylome Libraries

FIG. 19D

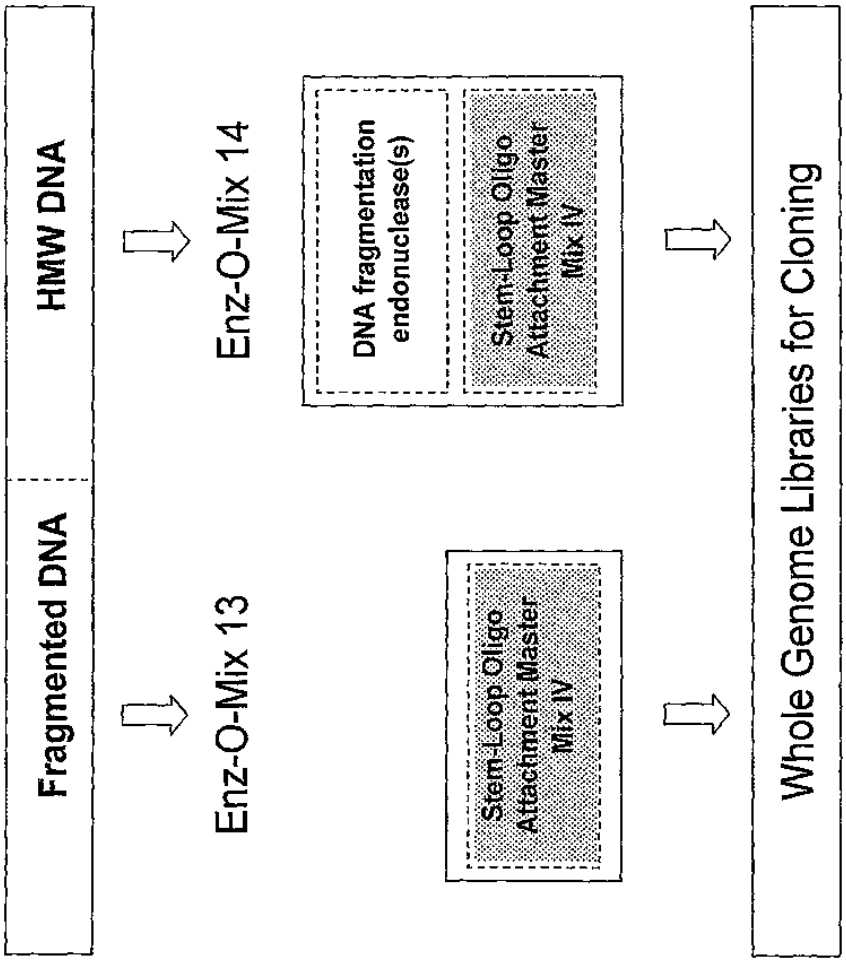

Fragmented DNA
HMW DNA
Enz-O-Mix 13
Enz-O-Mix 14
DNA fragmentation endonuclease(s)
Stem-Loop Oligo Attachment Master Mix IV
Stem-Loop Oligo Attachment Master Mix IV
Whole Genome Libraries for Cloning

FIG. 19E

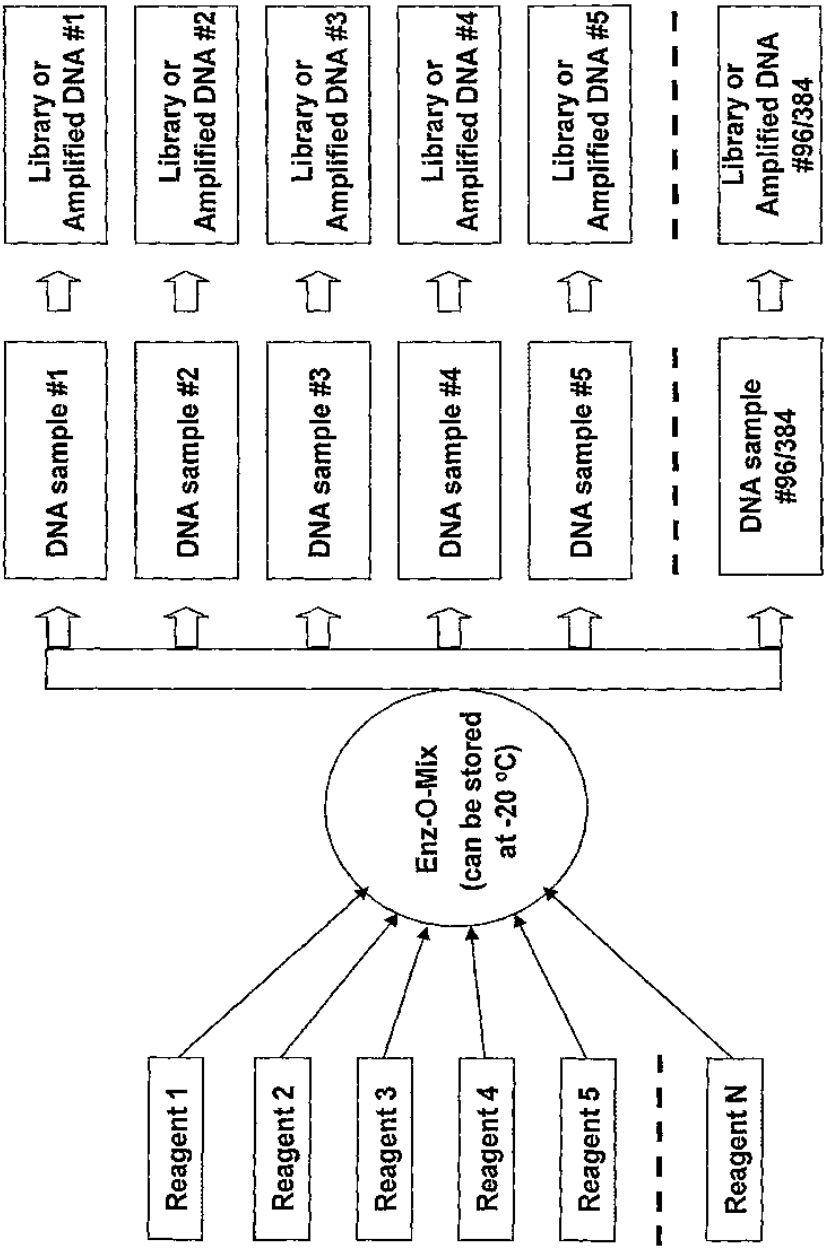

Library or Amplified DNA #1
Library or Amplified DNA #2
Library or Amplified DNA #3
Library or Amplified DNA #4
Library or Amplified DNA #5
Library or Amplified DNA #96/384
DNA sample #1
DNA sample #2
DNA sample #3
DNA sample #4
DNA sample #5
DNA sample #96/384
Enz-O-Mix (can be stored at -20 °C)
Reagent 1
Reagent 2
Reagent 3
Reagent 4
Reagent 5
Reagent N

FIG. 20

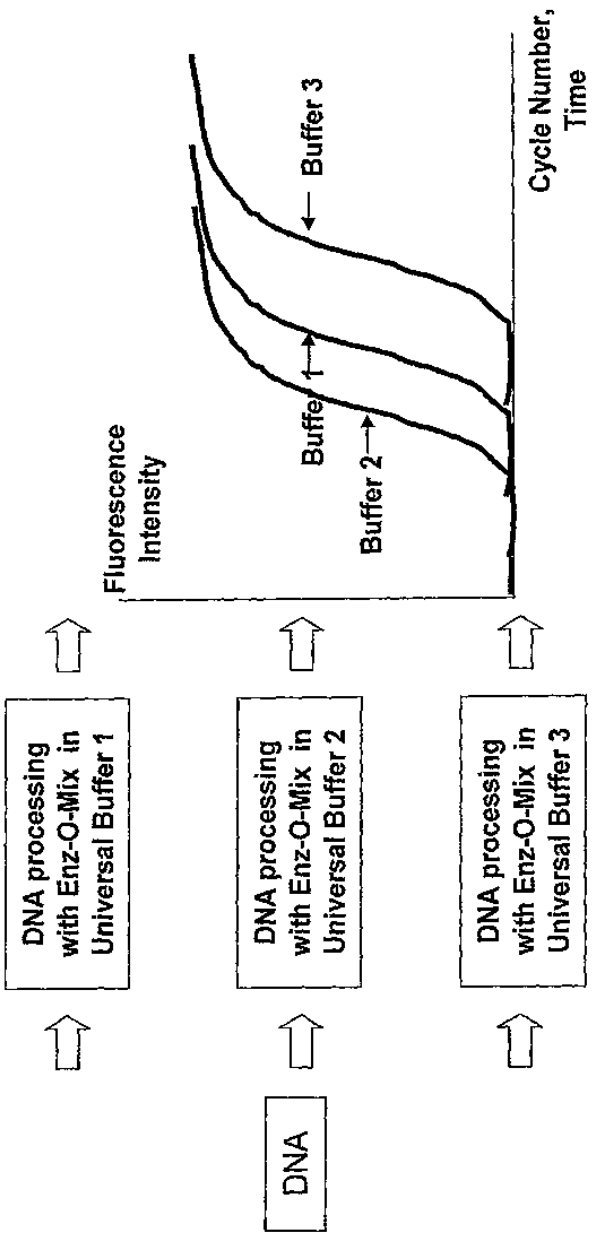

DNA
DNA processing with Enz-O-Mix in Universal Buffer 1
DNA processing with Enz-O-Mix in Universal Buffer 2
DNA processing with Enz-O-Mix in Universal Buffer 3
Fluorescence Intensity
Buffer 1
Buffer 2
Buffer 3
Cycle Number, Time

FIG. 21A

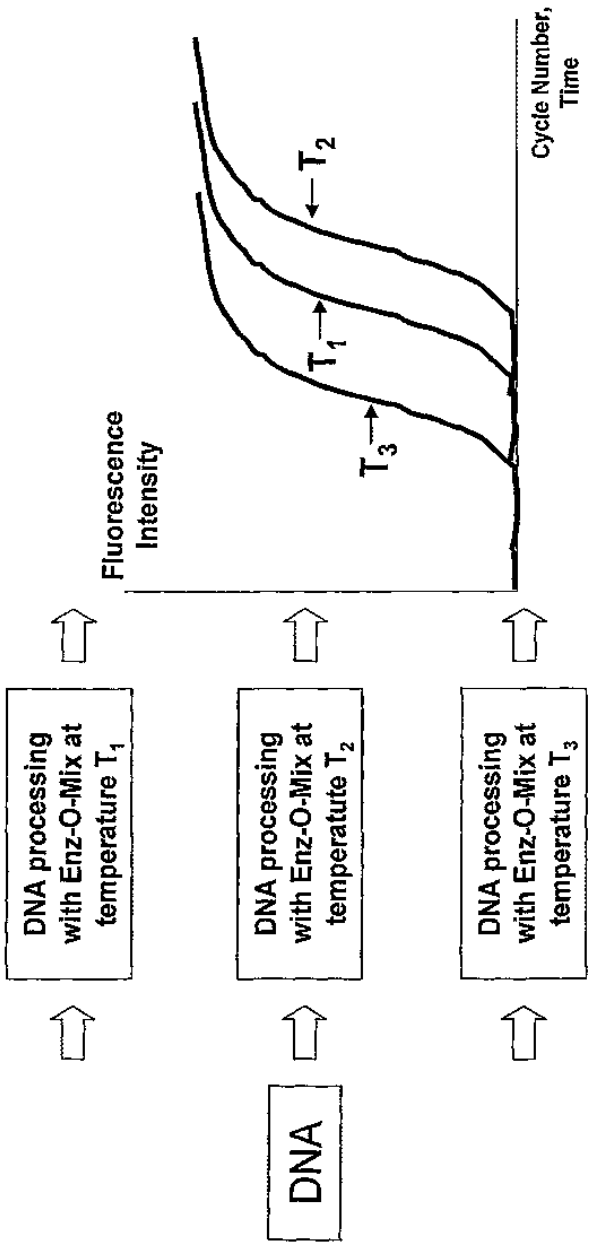

DNA
DNA processing with Enz-O-Mix at temperature T_1
DNA processing with Enz-O-Mix at temperatute T_2
DNA processing with Enz-O-Mix at temperature T_3
Fluorescence Intensity
Cycle Number, Time
T_1
T_2
T_3

FIG. 21B

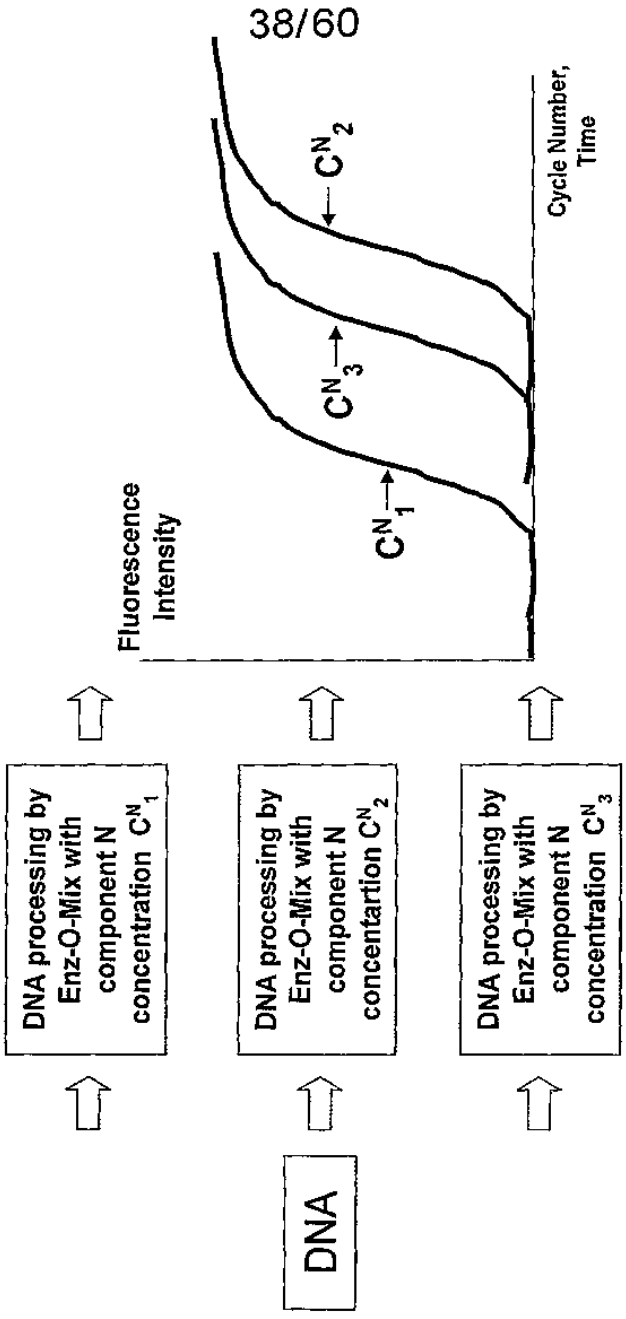

DNA
DNA processing by Enz-O-Mix with component N concentration C^N_1
DNA processing by Enz-O-Mix with component N concentartion C^N_2
DNA processing by Enz-O-Mix with component N concentration C^N_3
Fluorescence Intensity
C^N_1
C^N_3
C^N_2
Cycle Number, Time

FIG. 21C

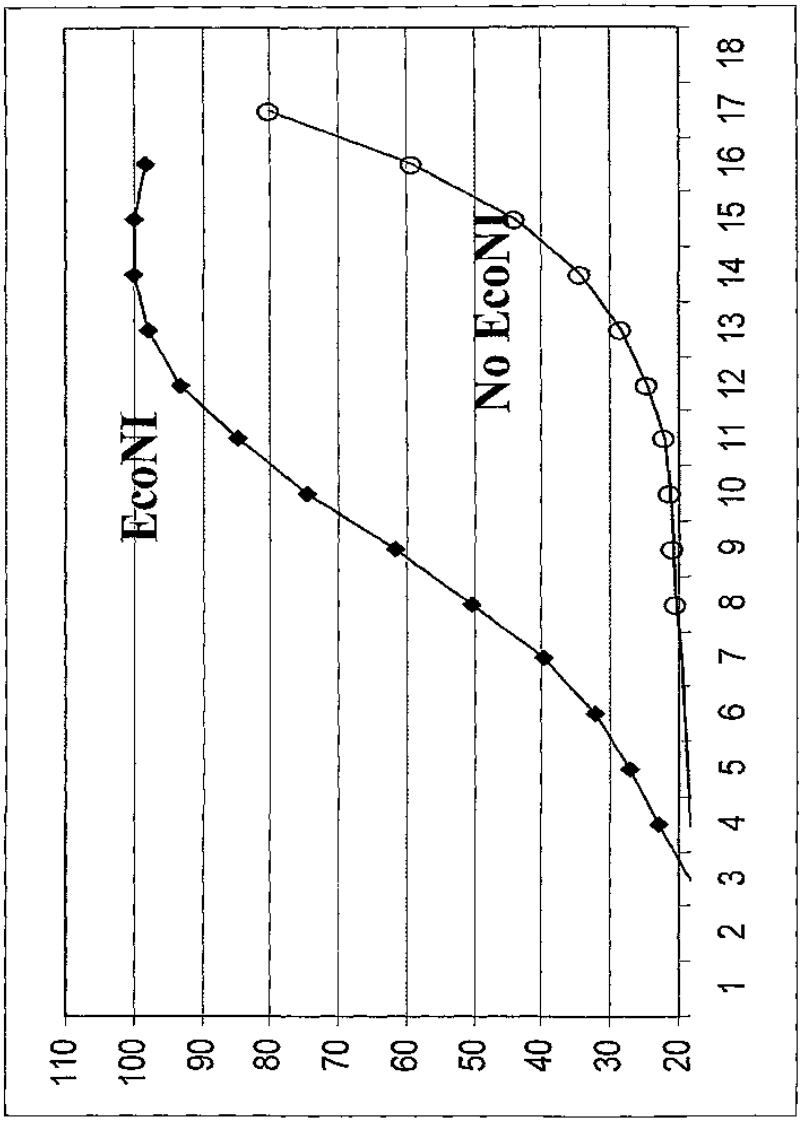

% Max Rfu
110
100
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Cycle Number
EcoNI
No EcoNI

FIG. 22

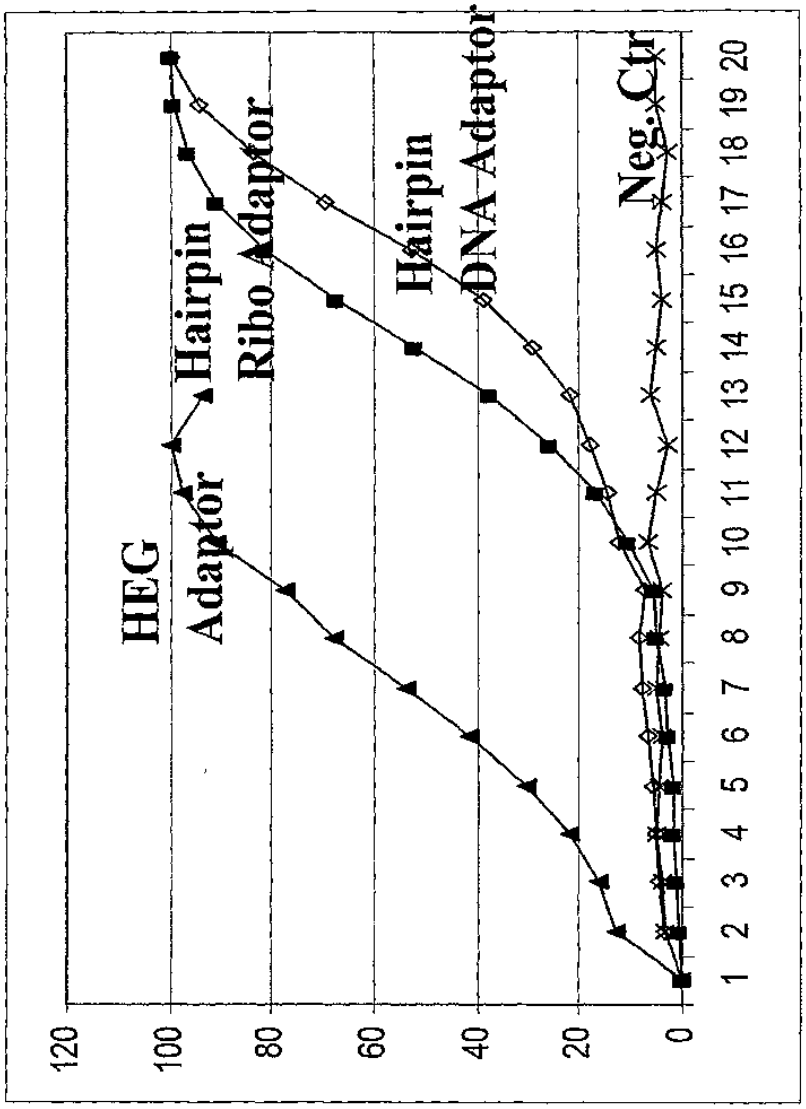

HEG
Adaptor
Hairpin
Ribo Adaptor
Hairpin
DNA Adaptor
Neg. Ctr
% Max Rfu
Cycle Number
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

FIG. 23

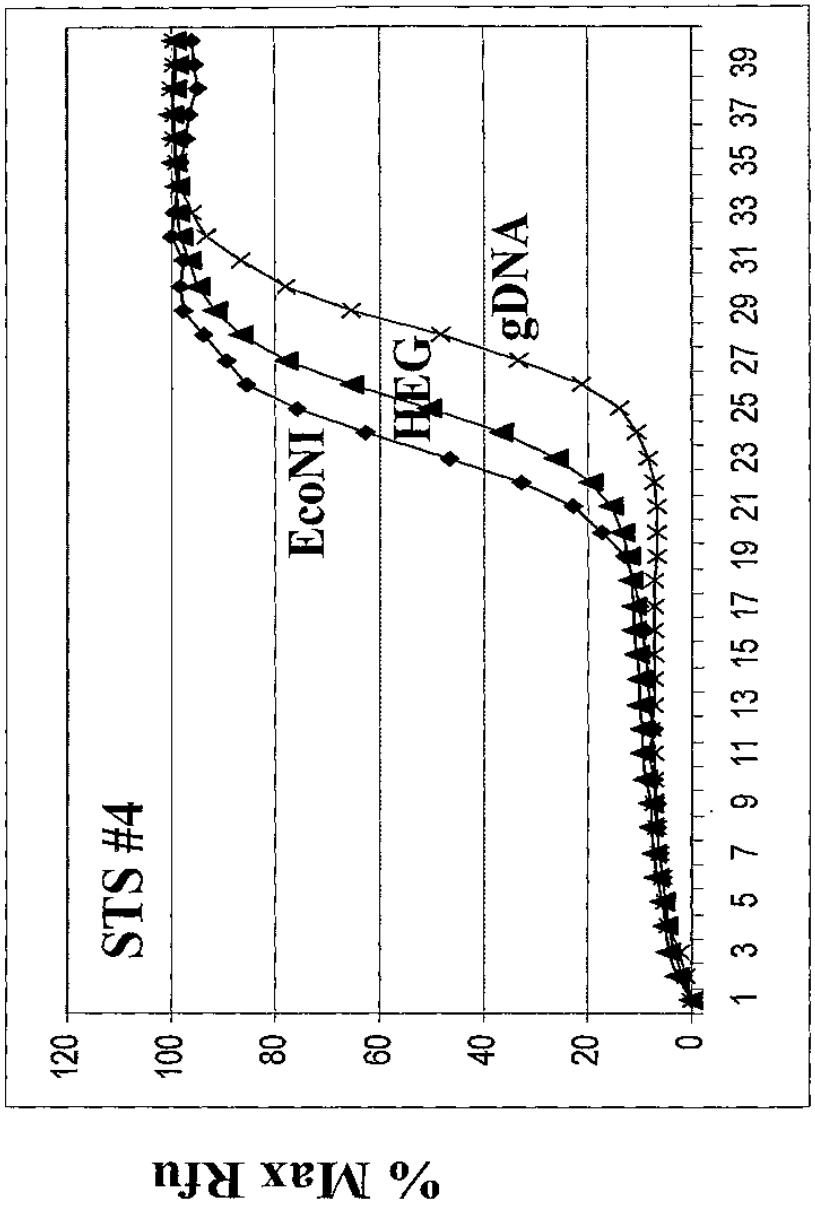

STS #4
EcoNI
HEG
gDNA
% Max Rfu
Cycle Number
0
20
40
60
80
100
120
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39

FIG. 24A

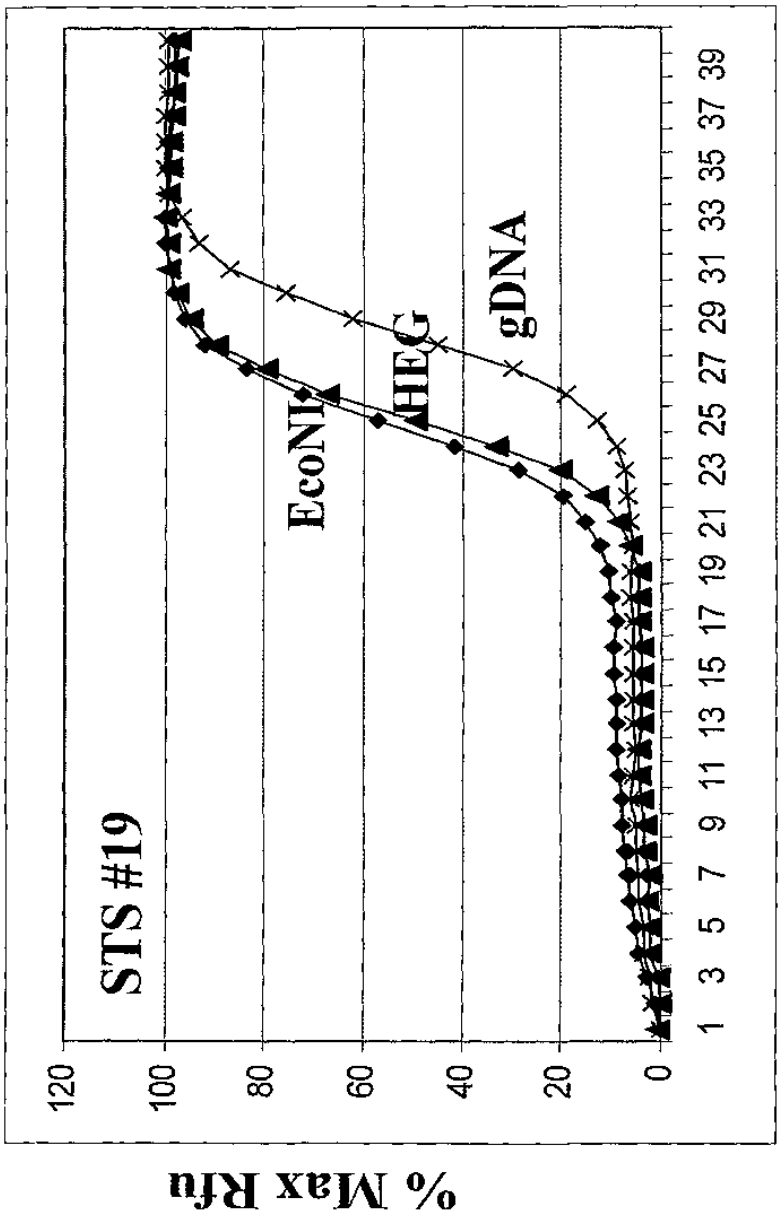

STS #19
EcoNI
HEG
gDNA
% Max Rfu
120
100
80
60
40
20
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
Cycle Number

FIG. 24B

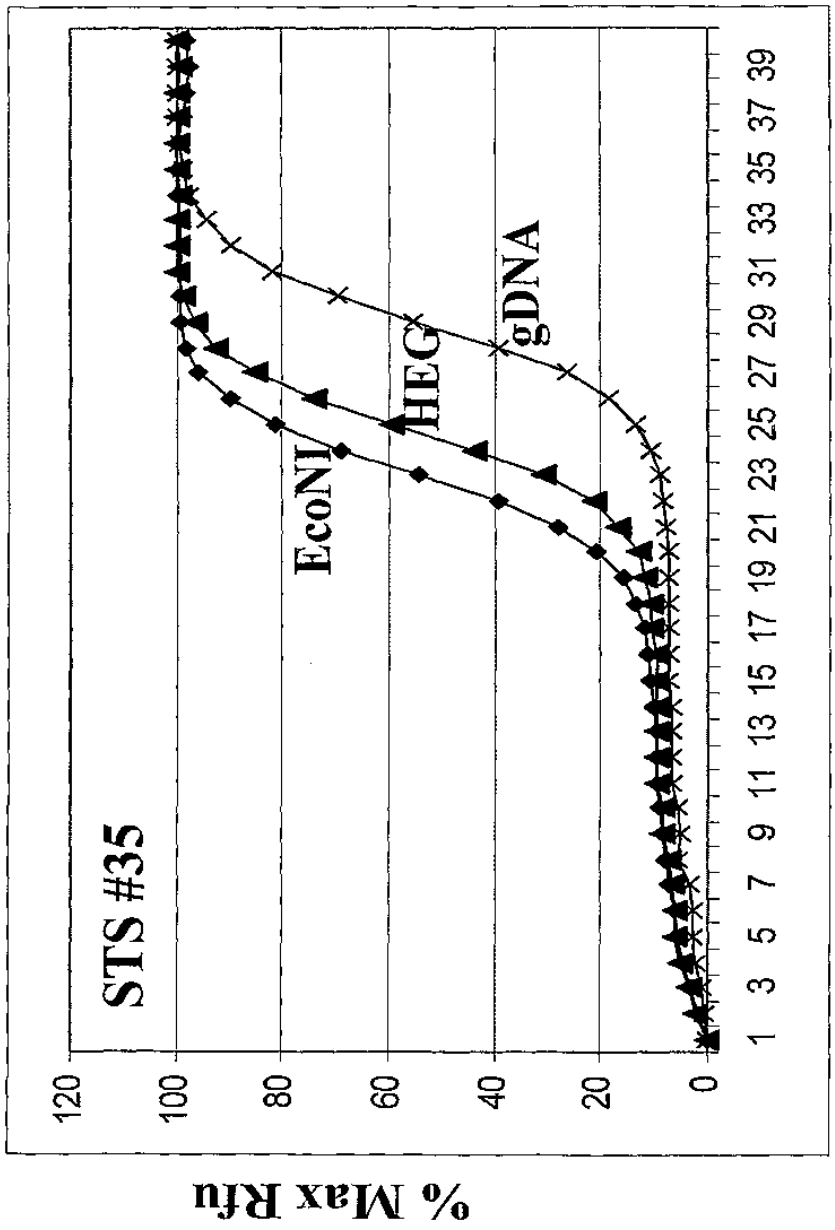

STS #35
EcoNI
HEG
gDNA
% Max Rfu
120
100
80
60
40
20
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
Cycle Number

FIG. 24C

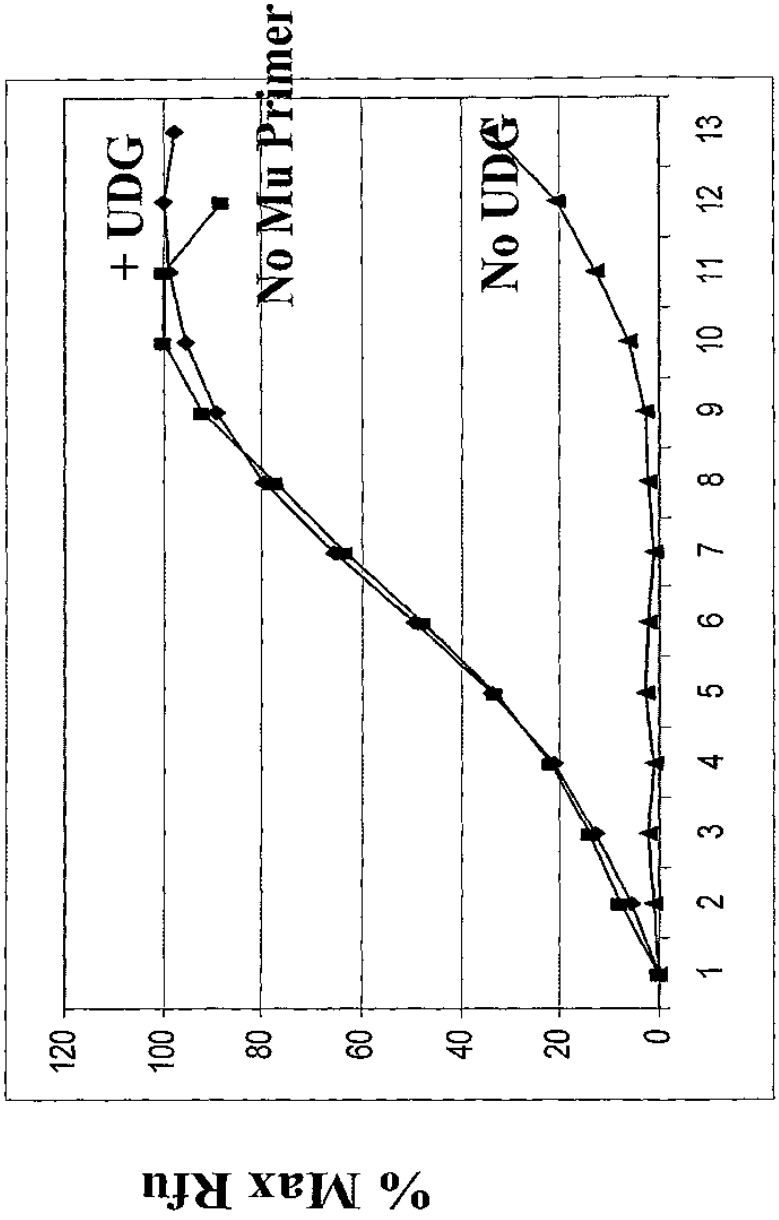

+ UDG
No Mu Primer
No UDG
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
% Max Rfu
Cycle Number

FIG. 25

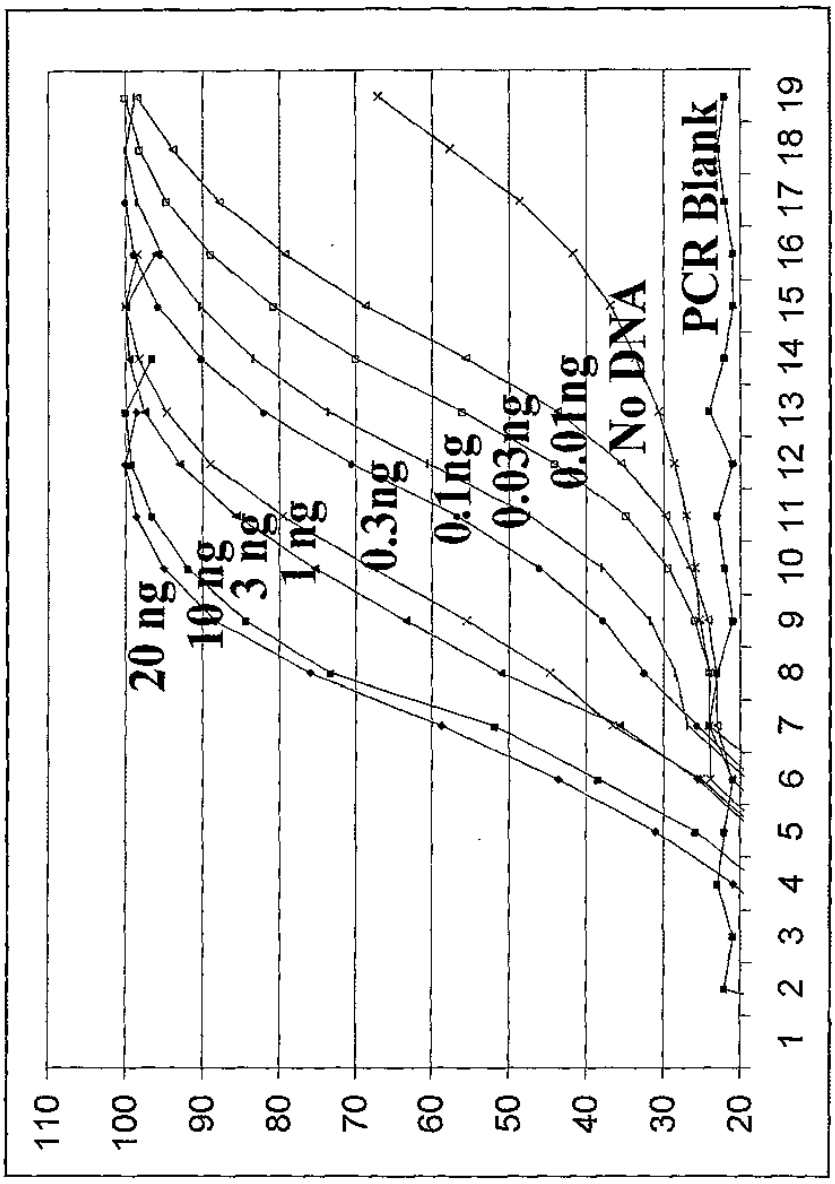
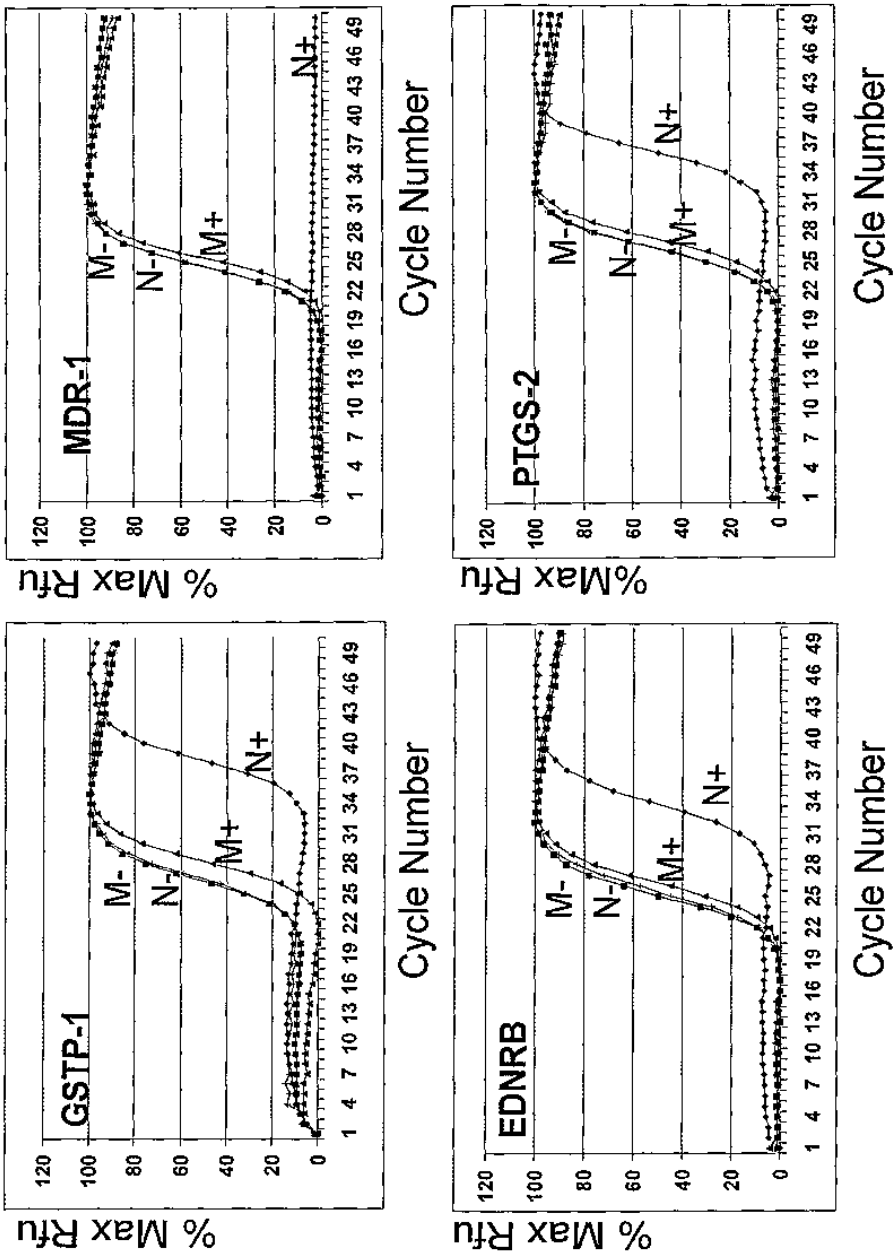

% Max Rfu
110
100
90
80
70
60
50
40
30
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Cycle Number
20 ng
10 ng
3 ng
1 ng
0.3ng
0.1ng
0.03ng
0.01ng
No DNA
PCR Blank

FIG. 26



N+ = Non-methylated Cut, N- = Non-methylated Uncut
M+ = Methylated Cut M- = Methylated Uncut

FIG. 27

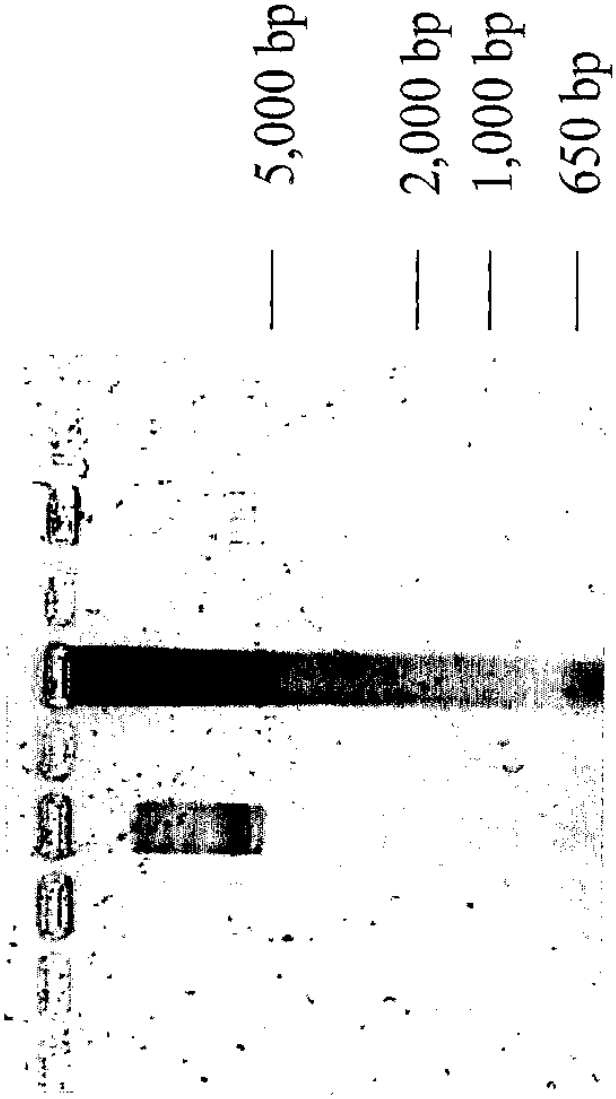

1Kb+ ladder
-T4 Ligase
+T4 Ligase
1Kb+ ladder
5,000 bp
2,000 bp
1,000 bp
650 bp

FIG. 28

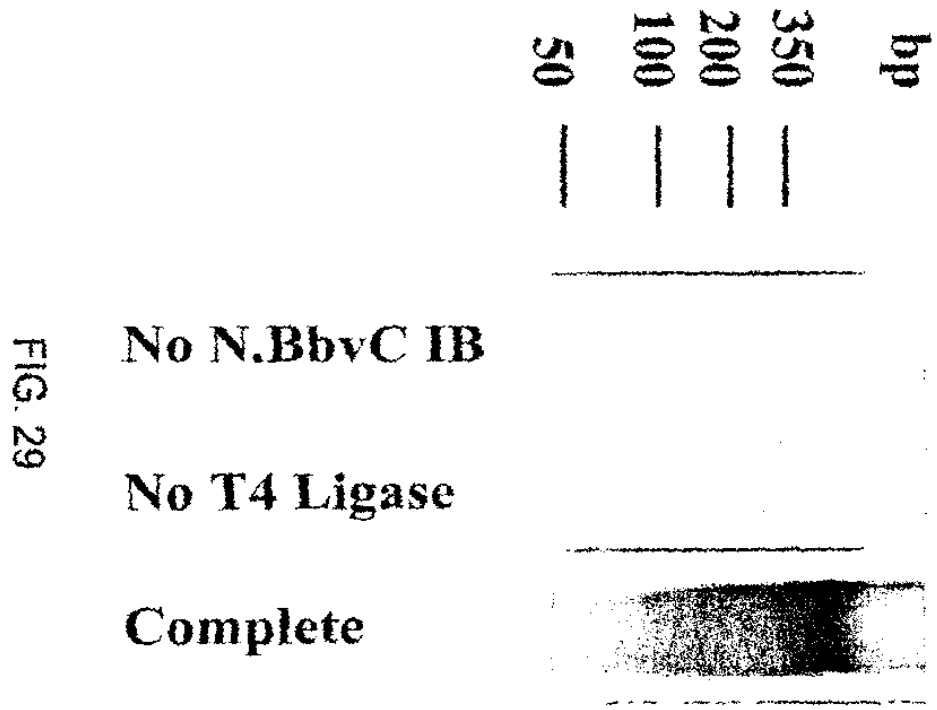


FIG. 29

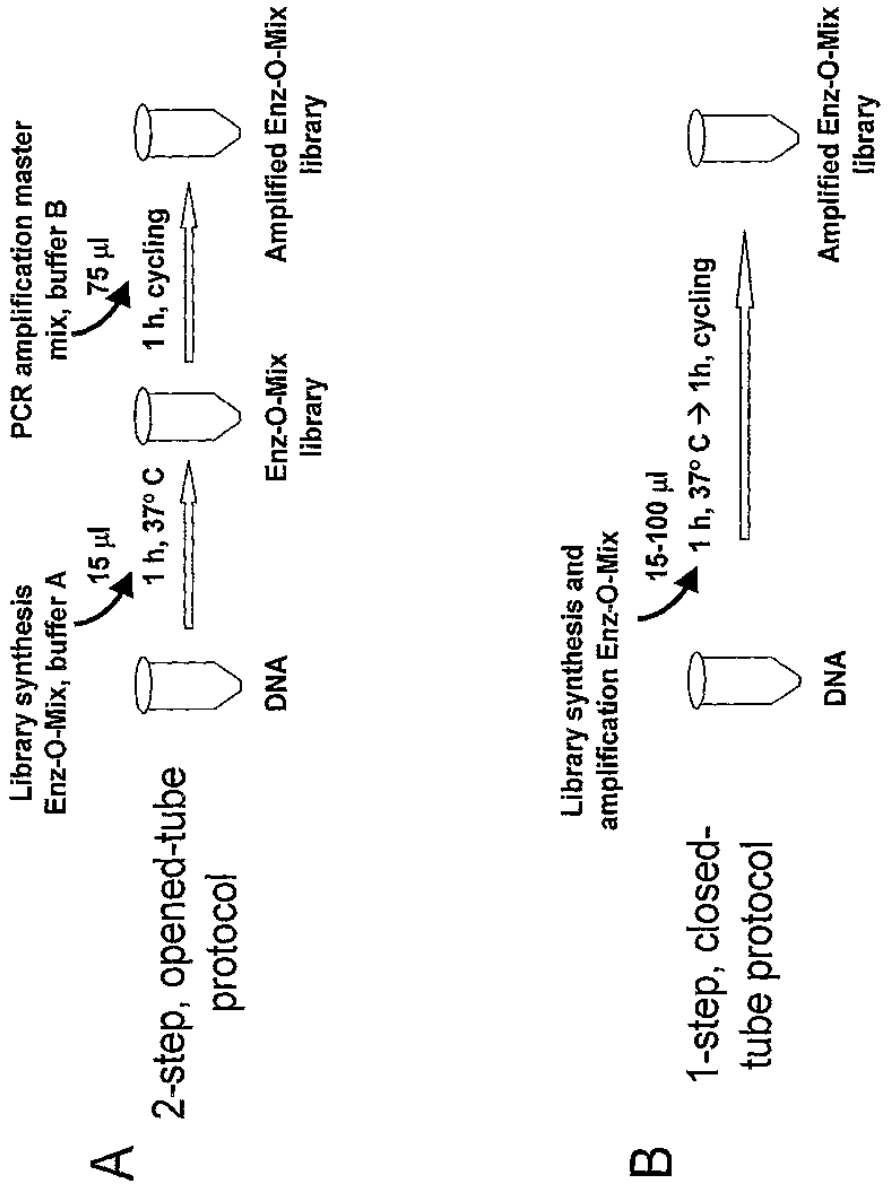

A
2-step, opened-tube protocol
Library synthesis Enz-O-Mix, buffer A
15 µl
1 h, 37° C
PCR amplification master mix, buffer B
75 µl
1 h, cycling
DNA
Enz-O-Mix library
Amplified Enz-O-Mix library
B
1-step, closed-tube protocol
Library synthesis and amplification Enz-O-Mix
15-100 µl
1 h, 37° C → 1h, cycling
DNA
Amplified Enz-O-Mix library

FIG. 30

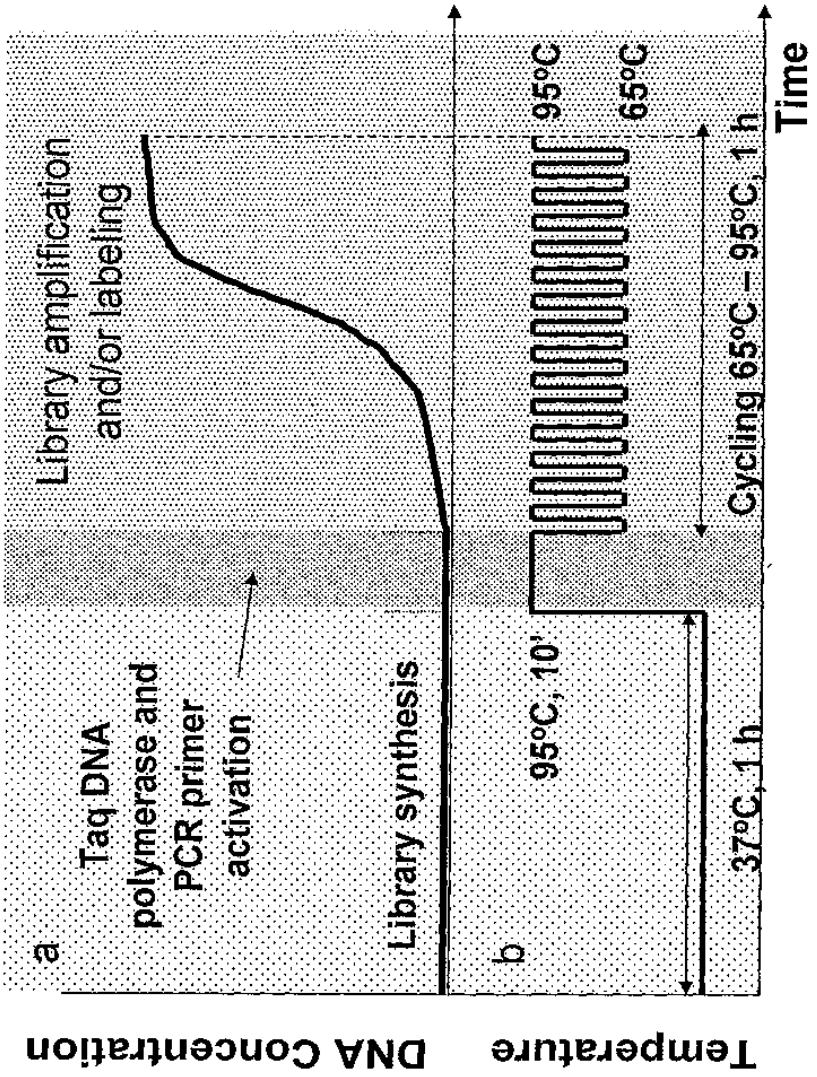

DNA Concentration
Temperature
Time
a
Taq DNA polymerase and PCR primer activation
Library amplification and/or labeling
Library synthesis
b
95°C, 10'
37°C, 1 h
95°C
65°C
Cycling 65°C – 95°C, 1 h

FIG. 31

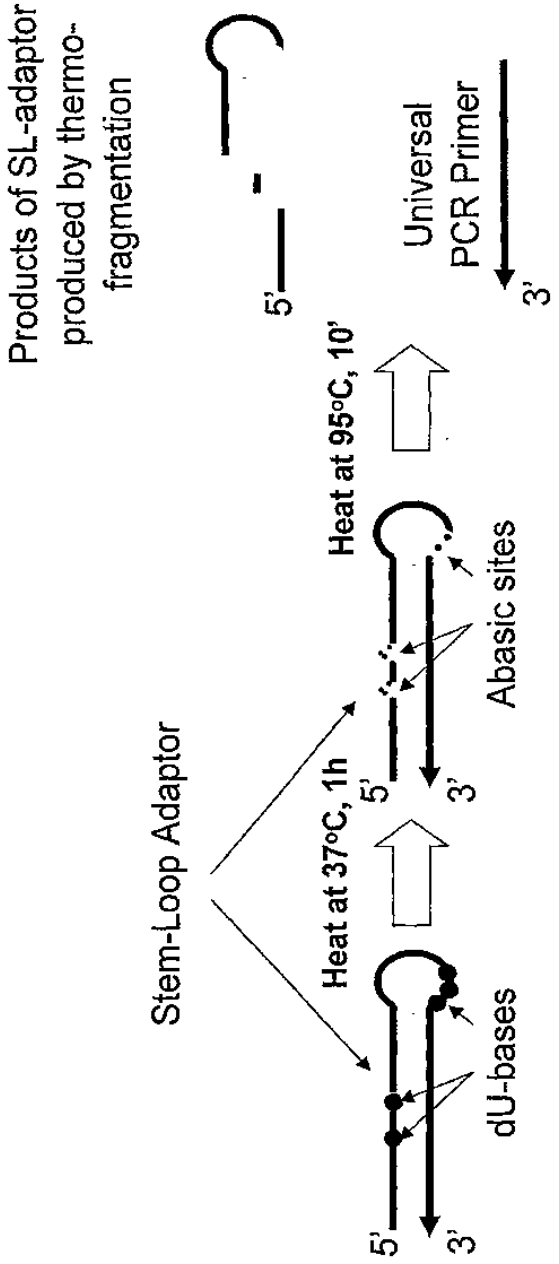

Products of SL-adaptor produced by thermo-fragmentation
5'
Universal PCR Primer
3'
Heat at 95°C, 10'
Abasic sites
Heat at 37°C, 1h
Stem-Loop Adaptor
dU-bases
5'
3'
5'
3'

FIG. 32

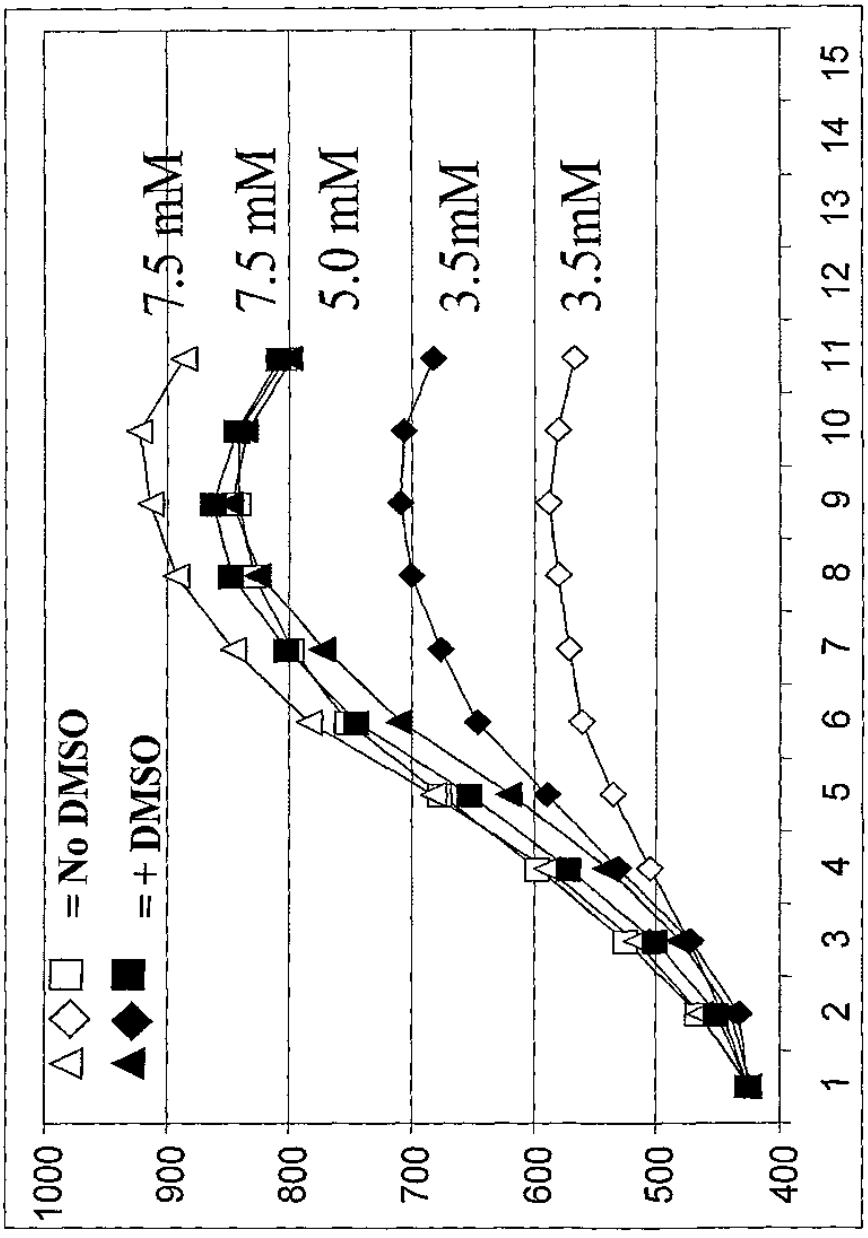

7.5 mM
7.5 mM
5.0 mM
3.5mM
3.5mM
= No DMSO
= + DMSO
1000
900
800
700
600
500
400
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

FIG. 33

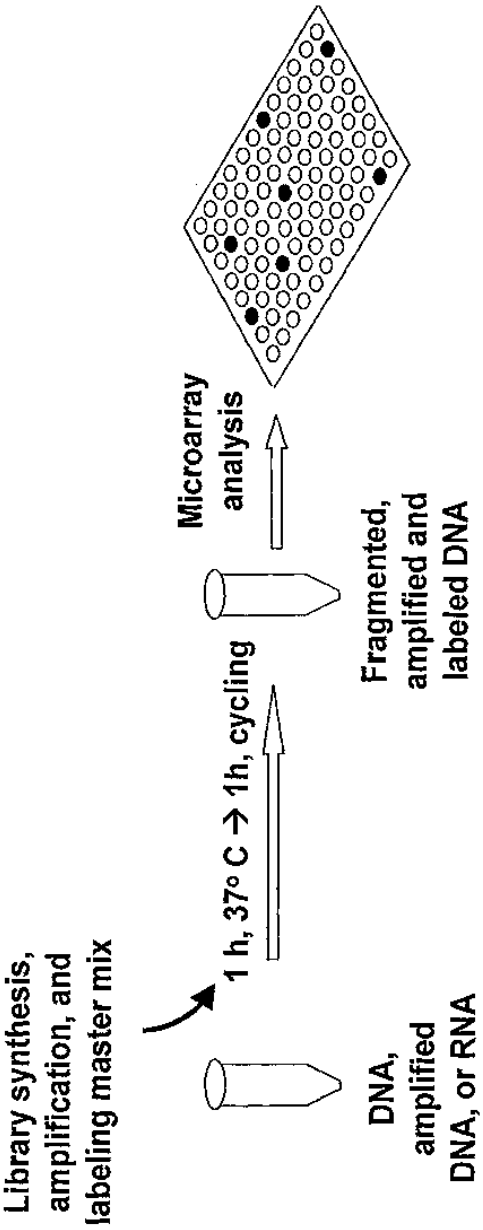

Library synthesis, amplification, and labeling master mix
1 h, 37° C → 1h, cycling
DNA, amplified DNA, or RNA
Fragmented, amplified and labeled DNA
Microarray analysis

FIG. 34

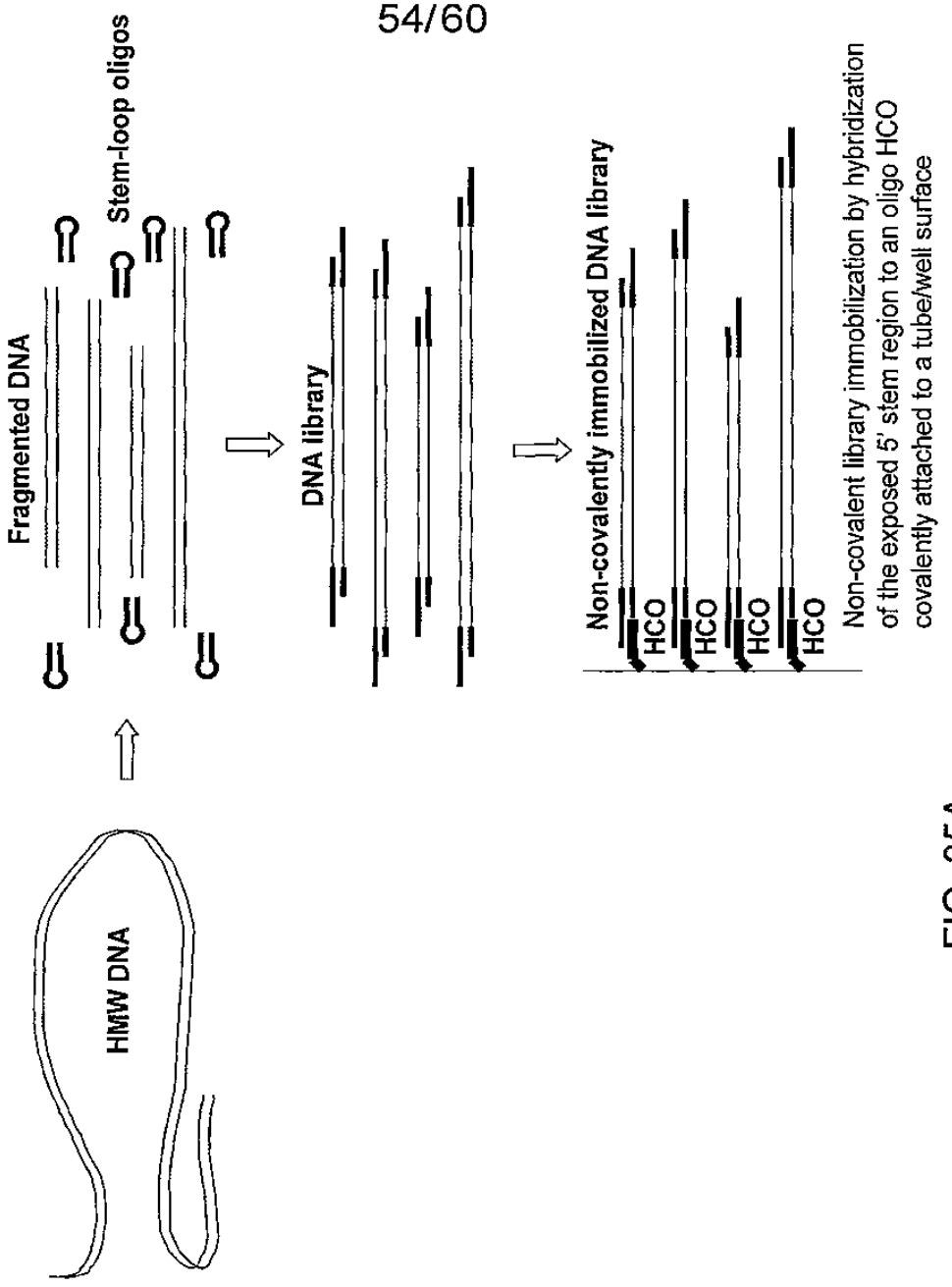

HMW DNA
Fragmented DNA
Stem-loop oligos
DNA library
Non-covalently immobilized DNA library
HCO
HCO
HCO
HCO
Non-covalent library immobilization by hybridization of the exposed 5' stem region to an oligo HCO covalently attached to a tube/well surface

FIG. 35A

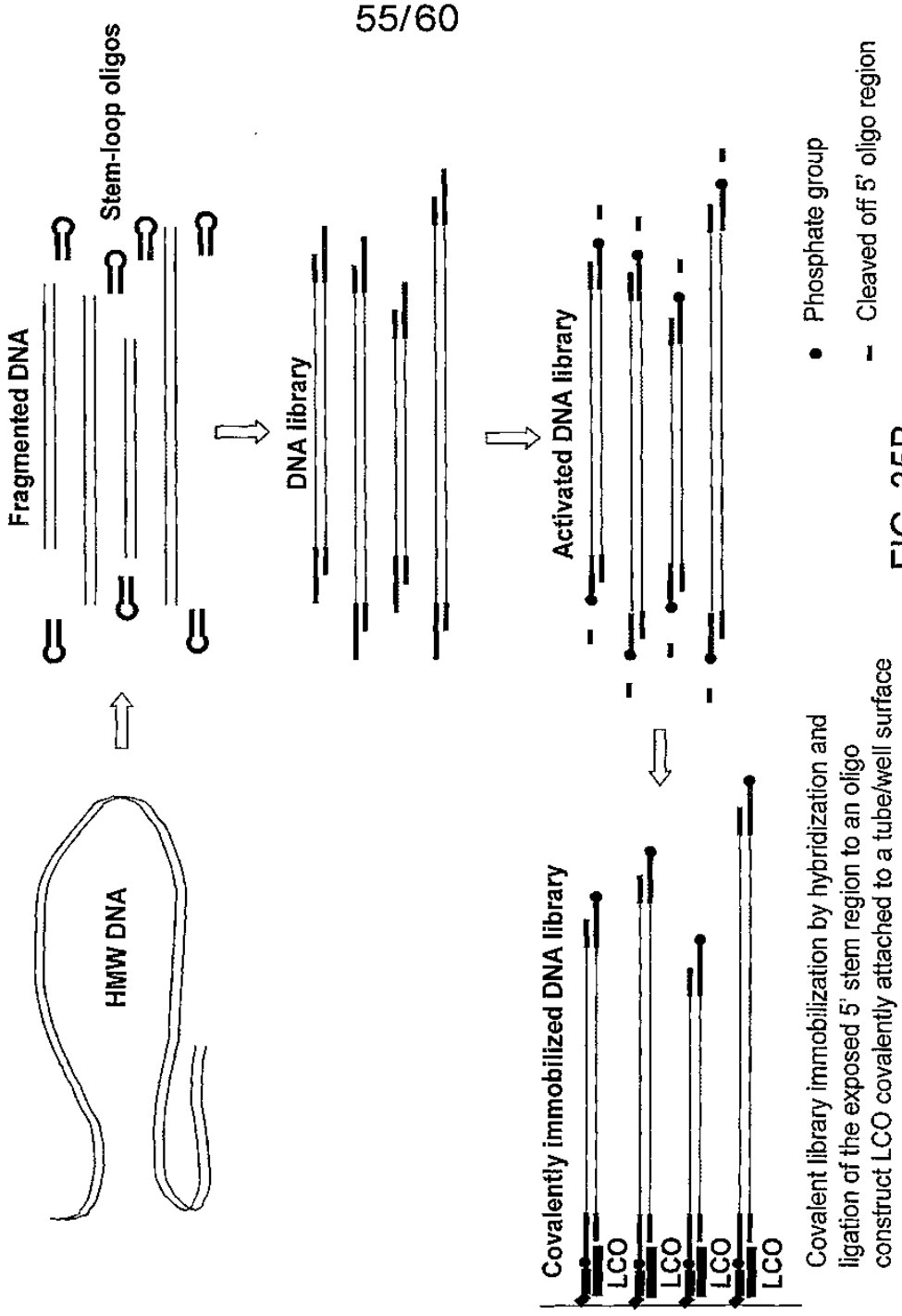

Fragmented DNA
Stem-loop oligos
DNA library
Activated DNA library
Phosphate group
Cleaved off 5' oligo region
HMW DNA
Covalently immobilized DNA library
LCO
LCO
LCO
LCO
Covalent library immobilization by hybridization and ligation of the exposed 5' stem region to an oligo construct LCO covalently attached to a tube/well surface

FIG. 35B

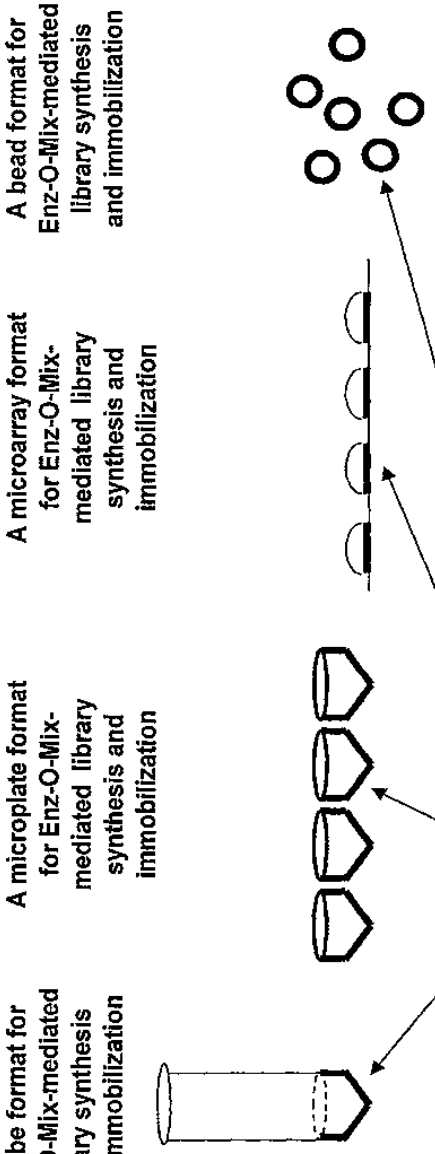

A tube format for Enz-O-Mix-mediated library synthesis and immobilization
A microplate format for Enz-O-Mix-mediated library synthesis and immobilization
A microarray format for Enz-O-Mix-mediated library synthesis and immobilization
A bead format for Enz-O-Mix-mediated library synthesis and immobilization
The solid black lines show the area of a capture oligonucleotide attachment

FIG. 36

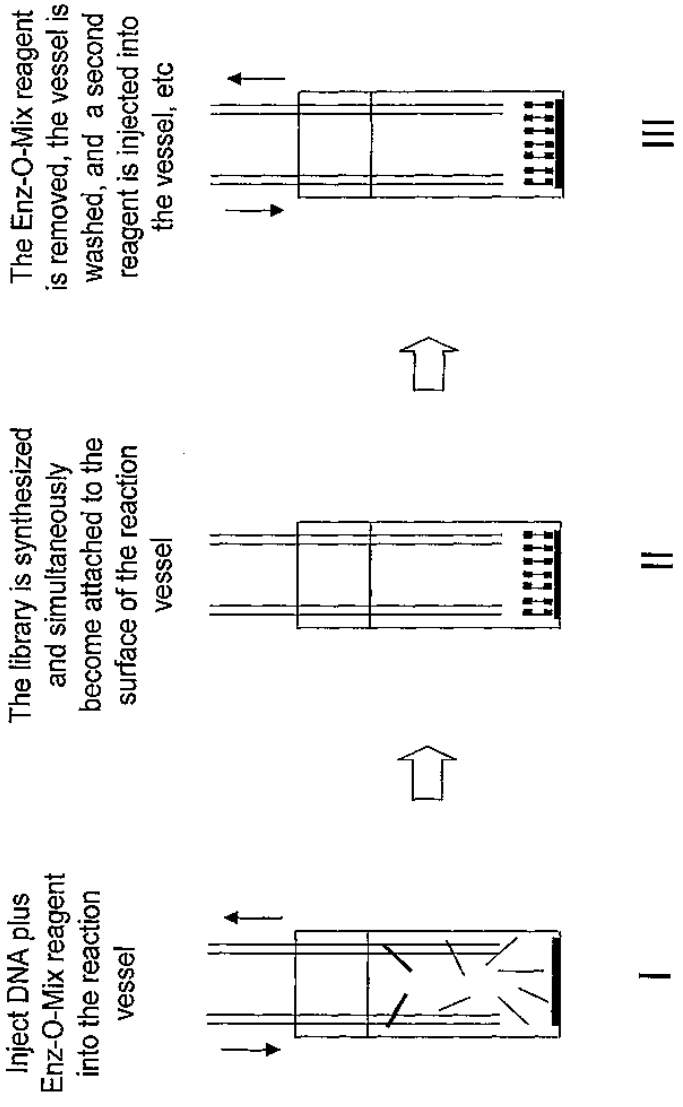

Inject DNA plus Enz-O-Mix reagent into the reaction vessel
I
The library is synthesized and simultaneously become attached to the surface of the reaction vessel
II
The Enz-O-Mix reagent is removed, the vessel is washed, and a second reagent is injected into the vessel, etc
III

FIG. 37

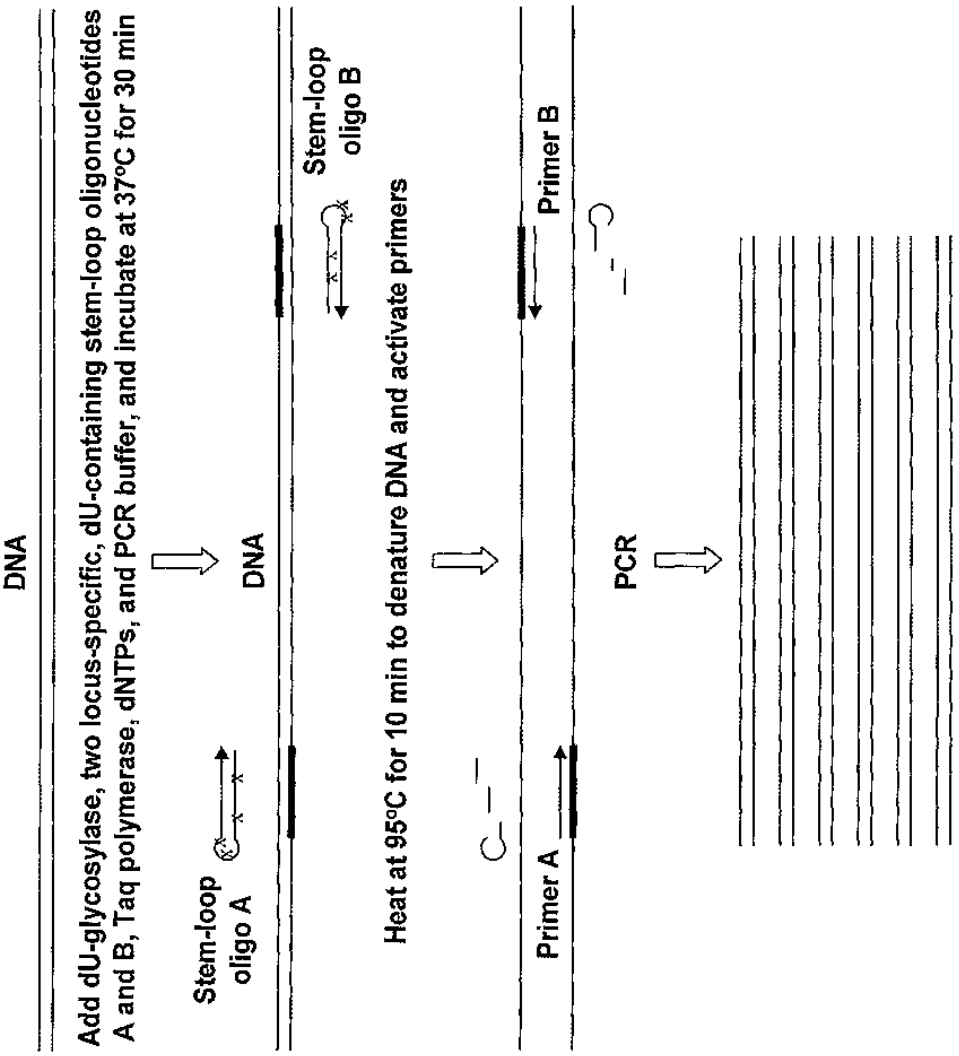

DNA
Add dU-glycosylase, two locus-specific, dU-containing stem-loop oligonucleotides A and B, Taq polymerase, dNTPs, and PCR buffer, and incubate at 37°C for 30 min
Stem-loop oligo A
DNA
Stem-loop oligo B
Heat at 95°C for 10 min to denature DNA and activate primers
Primer A
Primer B
PCR

FIG. 38

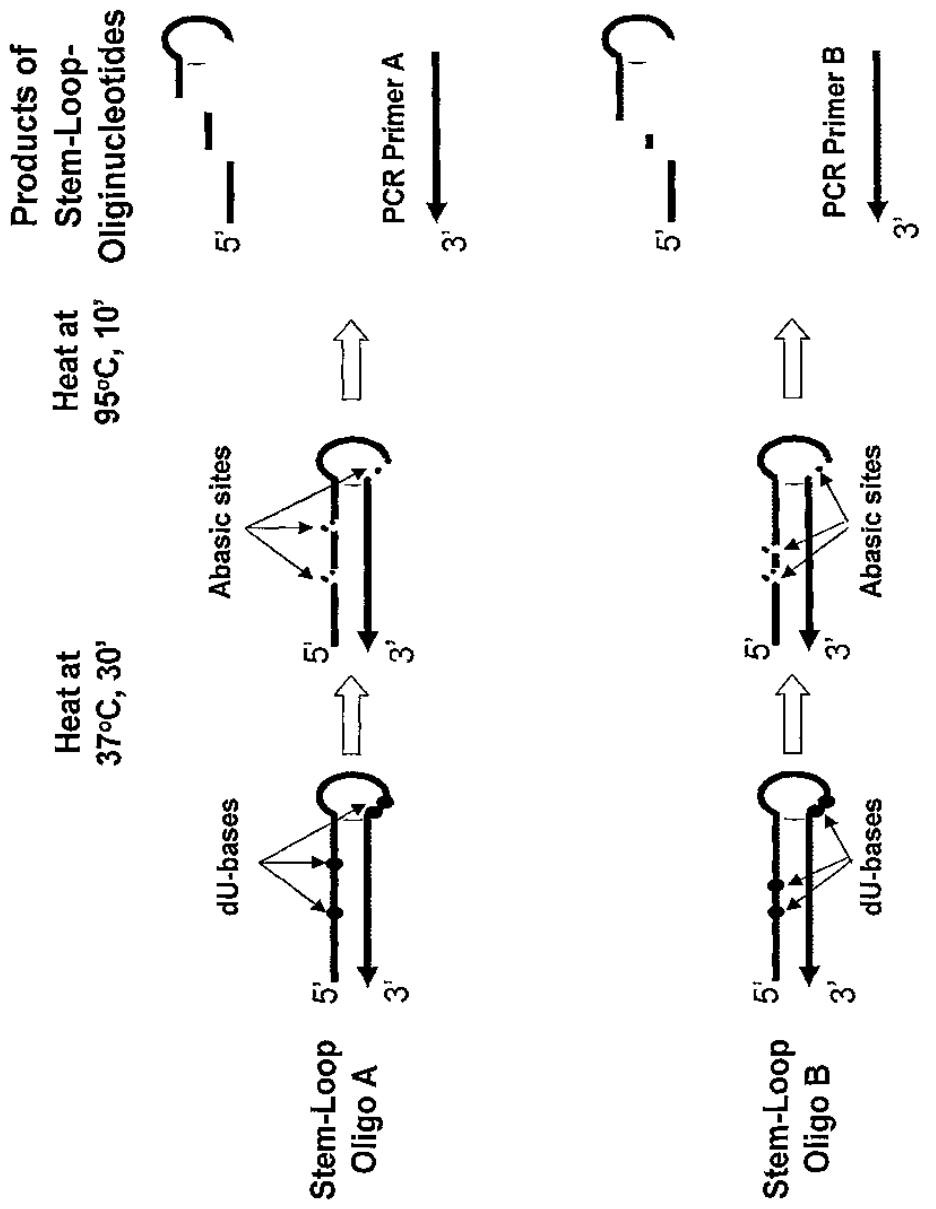

Products of Stem-Loop-Oliginucleotides
Heat at 95°C, 10'
Heat at 37°C, 30'
Stem-Loop Oligo A
Stem-Loop Oligo B
dU-bases
Abasic sites
PCR Primer A
PCR Primer B
5'
3'

FIG. 39

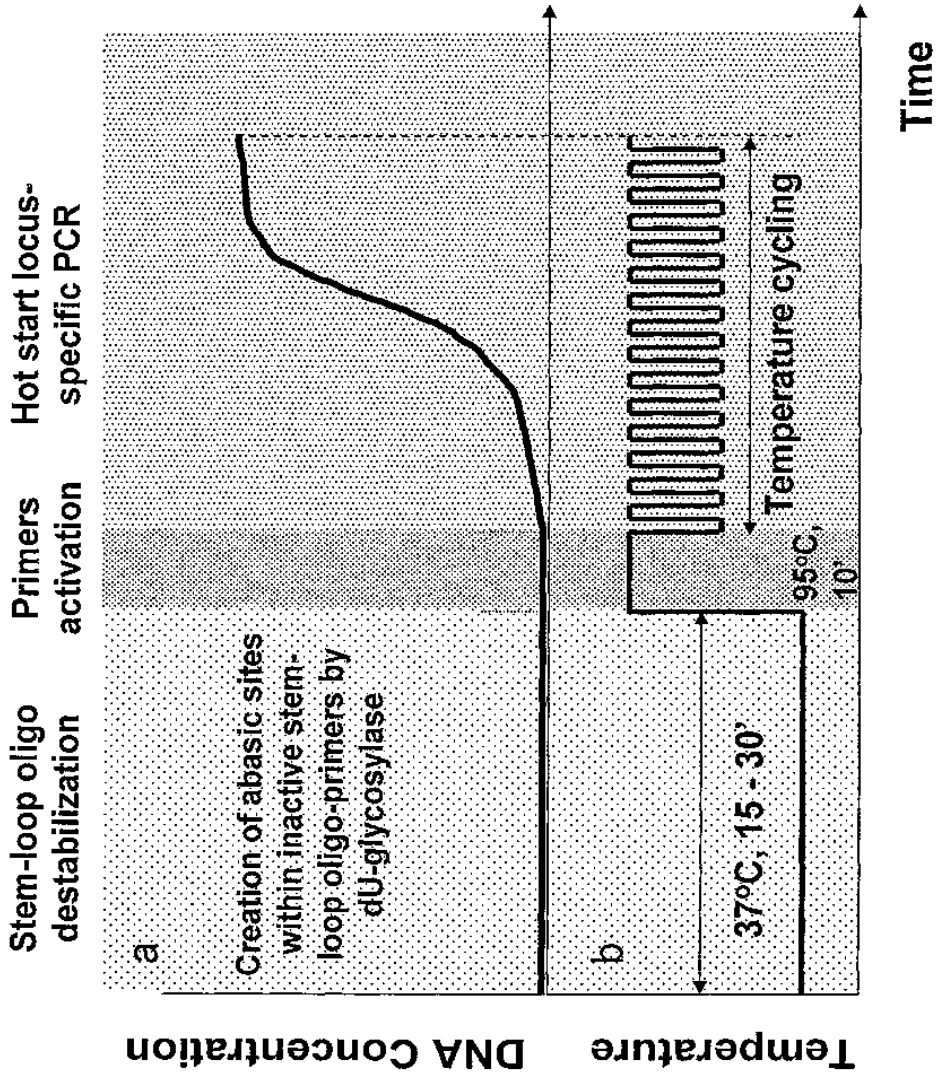

DNA Concentration
Temperature
Time
a
Creation of abasic sites within inactive stem-loop oligo-primers by dU-glycosylase
b
37°C, 15 - 30'
95°C, 10'
Temperature cycling
Stem-loop oligo destabilization
Primers activation
Hot start locus-specific PCR

FIG. 40

RUBC026WO.APP.txt
SEQUENCE LISTING

110> MAKAROV, VLADIMIR L.
KAMBEROV, EMMANUEL
TARRIER, BRENDAN

120> COMPOSITIONS AND METHODS FOR PROCESSING AND AMPLIFICATION OF DNA, INCLUDING USING MULTIPLE ENZYMES IN A SINGLE REACTION

130> RUBC:026WO

140> UNKNOWN
141> 2006-03-02

150> 60/704,932
151> 2005-08-02

160> 20

170> PatentIn Ver. 2.1

210> 1
211> 32
212> DNA
213> Artificial Sequence

220>
223> Description of Artificial Sequence: Synthetic Primer

400> 1
aaacacac ccaacacacc taaaaaaggt gt 32

210> 2
211> 18
212> DNA
213> Artificial Sequence

220>
223> Description of Artificial Sequence: Synthetic Primer

400> 2
tgttgggt gtgtttgg 18

210> 3
211> 40
212> DNA
213> Artificial Sequence

220>
223> Description of Artificial Sequence: Synthetic Primer

400> 3
gagagagg gaaggaagaa aacttccttc cctctctctt 40

210> 4
211> 18

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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

<400> 4
cttccttccc tctctctt 18

<210> 5
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

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ccaaacacac ccaacacaaa aagtgttg 28

<210> 6
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

<400> 6
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<210> 7
<211> 40
<212> DNA
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<220>
<221> modified_base
<222> (10)..(24)
<223> U = a, c, g or t

<220>
<223> Description of Artificial Sequence: Synthetic Primer

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<210> 8
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

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IPR2023-00955
Page 00386

<400> 8
ccacacacac ccaacaca 18

<210> 9
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

<400> 9
ggaggagtct ttcgagttca a 21

<210> 10
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

<400> 10
cgggaggaat acagacacgt ctt 23

<210> 11
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

<400> 11
ggaaagaggg aaaggcttc 19

<210> 12
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

<400> 12
ccccagtgct gagtcacgg 19

<210> 13
<211> 20
<212> DNA
<213> Artificial Sequence

<220>

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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<223> Description of Artificial Sequence: Synthetic Primer

<400> 13
gggtgggagg aagcatcgtc 20

<210> 14
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

<400> 14
ggtctccagc atctccacga a 21

<210> 15
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

<400> 15
agaactggct ctcggaagcg 20

<210> 16
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

<400> 16
gggagcagag ggggtagtc 19

<210> 17
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic Primer

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/007485

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

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)
C12Q

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/57248 A (SOLEXA LTD) 9 August 2001 (2001-08-09) page 6 - page 7	1-5,7, 12-14, 16,17, 21,52-54
X	KABOEV O K ET AL: "PCR hot start using primers with the structure of molecular beacons (hairpin-like structure)" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 28, no. 21, 1 November 2000 (2000-11-01), page E94, XP002244959 ISSN: 0305-1048 cited in the application the whole document	1-3, 52-54

-/--

[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

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 September 2006	12/10/2006

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

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/007485

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 692 915 B1 (NALLUR GIRISH N) 17 February 2004 (2004-02-17) page 23, line 10 - line 14	1-3
A	US 6 261 774 B1 (PAGRATIS NIKOS ET AL) 17 July 2001 (2001-07-17) page 18, line 44 - line 49; figures 41A,B	1,4-11, 14,15,20
A	LIU CHIH LONG ET AL: "Development and validation of a T7 based linear amplification for genomic DNA." BMC GENOMICS, vol. 4, no. 19 Cited June 13, 2003, 9 May 2003 (2003-05-09), XP002394174 ISSN: 1471-2164	15,34
A	KAUR MANJIT ET AL: "Novel amplification of DNA in a hairpin structure: towards a radical elimination of PCR errors from amplified DNA." NUCLEIC ACIDS RESEARCH. 15 MAR 2003, vol. 31, no. 6, 15 March 2003 (2003-03-15), page e26, XP002394810 ISSN: 1362-4962 figures 1,3	
A	WO 98/39485 A (THE REGENTS OF THE UNIVERSITY OF MICHIGAN; MAKAROV, VLADIMIR L; LANGMO) 11 September 1998 (1998-09-11) page 49 - page 50	
A	PIRKER C ET AL: "WHOLE GENOMIC AMPLIFICATION FOR CGH ANALYSIS: LINKER-ADAPTER PCR AS THE METHOD OF CHOICE FOR DIFFICULT AND LIMITED SAMPLES" CYTOMETRY, ALAN LISS, NEW YORK, US, vol. 61A, no. 1, September 2004 (2004-09), pages 26-34, XP008068045 ISSN: 0196-4763	

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

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2006/007485

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [X] table(s) related to the sequence listing

   b. format of material

   [X] on paper

   [X] in electronic form

   c. time of filing/furnishing

   [X] contained in the international application as filed

   [ ] filed together with the international application in electronic form

   [X] furnished subsequently to this Authority for the purpose of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2006/007485

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0157248 A	09-08-2001	AU 3038401 A	14-08-2001
		CA 2398168 A1	09-08-2001
		EP 1252332 A2	30-10-2002
		IS 6489 A	31-07-2002
		JP 2003521693 T	15-07-2003
US 6692915 B1	17-02-2004	NONE	
US 6261774 B1	17-07-2001	US 5637459 A	10-06-1997
		US 5723289 A	03-03-1998
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		US 5496938 A	05-03-1996
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WO 9839485 A	11-09-1998	AU 6544598 A	22-09-1998
		US 6537757 B1	25-03-2003
		US 6117634 A	12-09-2000

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International application published by the World Intellectual Property Organisation under number:

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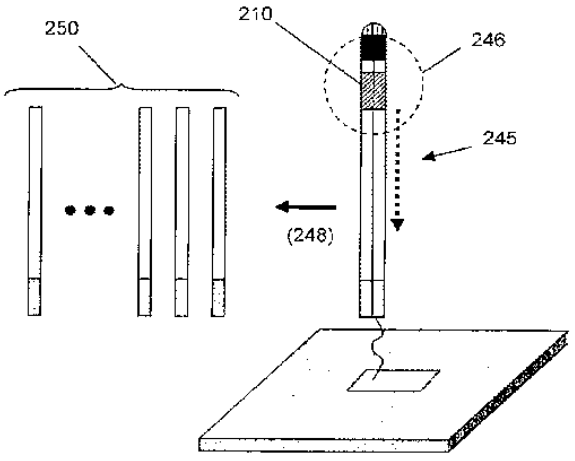
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC,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

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— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ISOTHERMAL DNA AMPLIFICATION



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

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ISOTHERMAL DNA AMPLIFICATION

Field of the Invention

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

BACKGROUND

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

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

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

SUMMARY OF THE INVENTION

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

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

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

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

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

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

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

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BRIEF DESCRIPTION OF THE FIGURES

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

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

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

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

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

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

DEFINITIONS

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

DETAILED DESCRIPTION OF THE INVENTION

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

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

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

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

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

Example 1

Solid Phase Capture and Amplification

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Example 2

Amplification with a Single RNA Polymerase

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

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

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

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

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We claim:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

generating a complementary DNA from the RNA transcript;

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

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

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

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

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

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

5′-GCTAATACGACTCACTATAGGGATTCTATCCCAATTAACCCTCACTAAAGGGAGACCATGGTCTTGCTAGCCTTGAACCGTCGATTGGCTT
CGATTATGCTGAGTGATATCCCTAAGATAGGGTTAATTGGGAGTGATTTCCCTCTGGTACCAGAACGATCGGAACTTGGCAGCTAACCGAA

T7 promoter Stem-Loop T3 promoter NcoI NheI Tag Cap

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

5′-GGGAUUCUAUCCCAAUUAACCCUCACUAAAGGGAGACCAUGGUCUUGCUAGCCUUGAACCGUCGAUUGGCUU

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

105

3′-CCCTAAGATAGGGTTAATTGGGAGTGATTTCCCTCTGGTACCAGAACGATCGGAACTTGGCAGCTAACCGAA

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

G AATCCCAATTAACCCTCACTAAAGGGAGACCATGGTCTTGCTAGCCTTGAACCGTCGATTGGCTT-3′
ATAGGGTTAATTGGGAGTGATTTCCCTCTGGTACCAGAACGATCGGAACTTGGCAGCTAACCGAA-5′

Fig. 1

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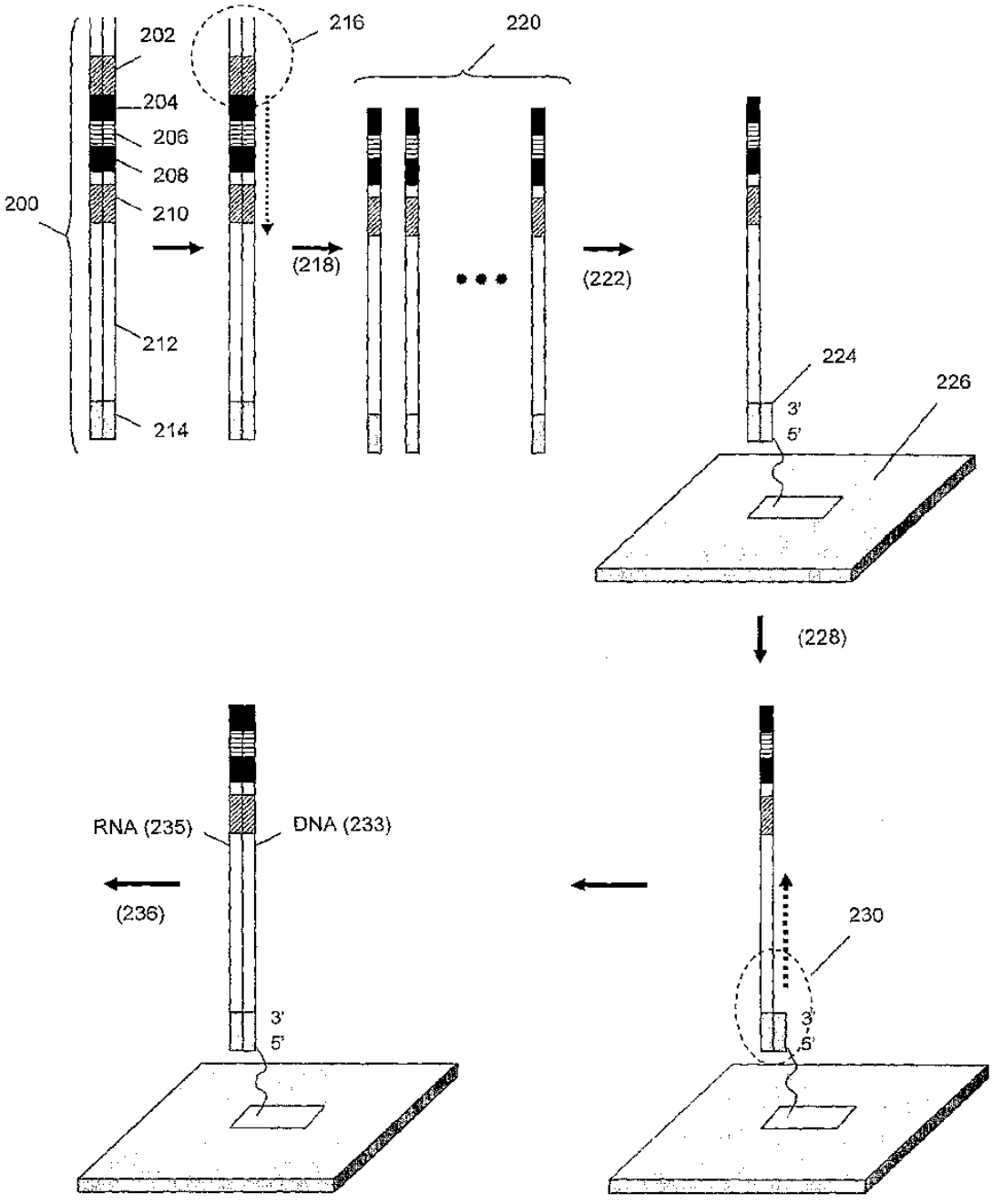

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226
3'
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230
3'
5'
(236)
RNA (235)
DNA (233)
3'
5'

Fig. 2A

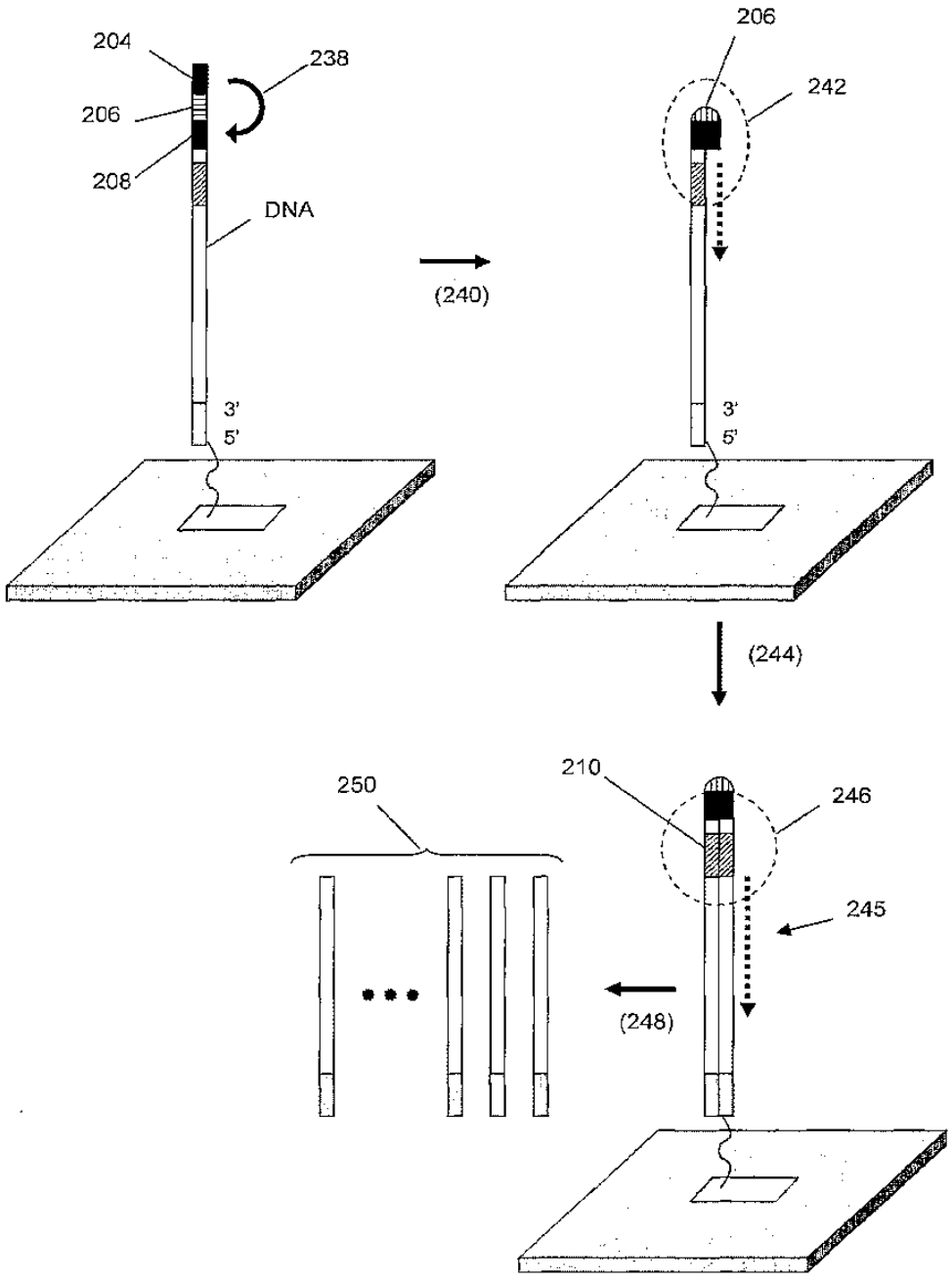


Fig. 2B

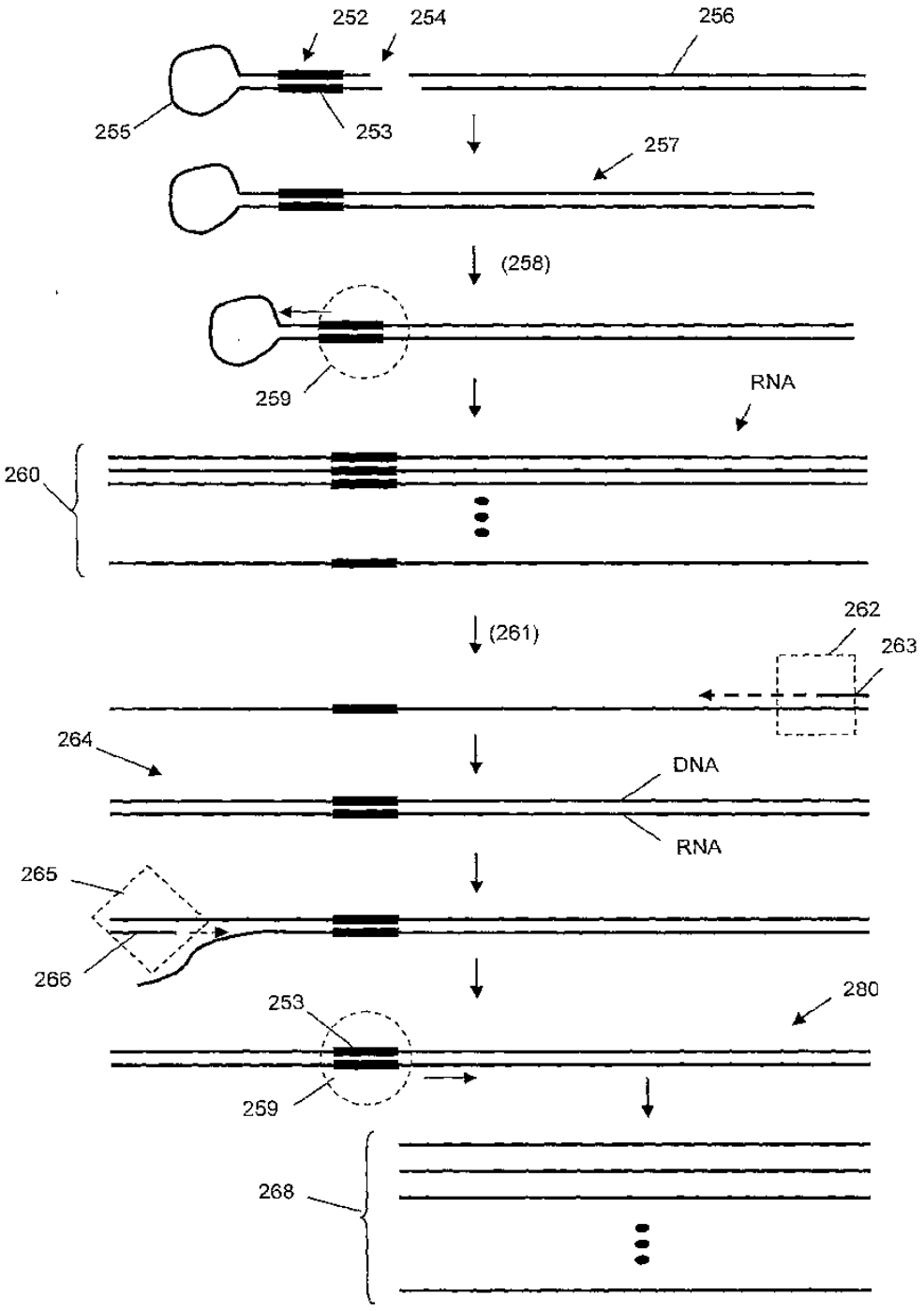

252
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253
257
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RNA
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(261)
262
263
264
DNA
RNA
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266
253
280
259
268

Fig. 2C

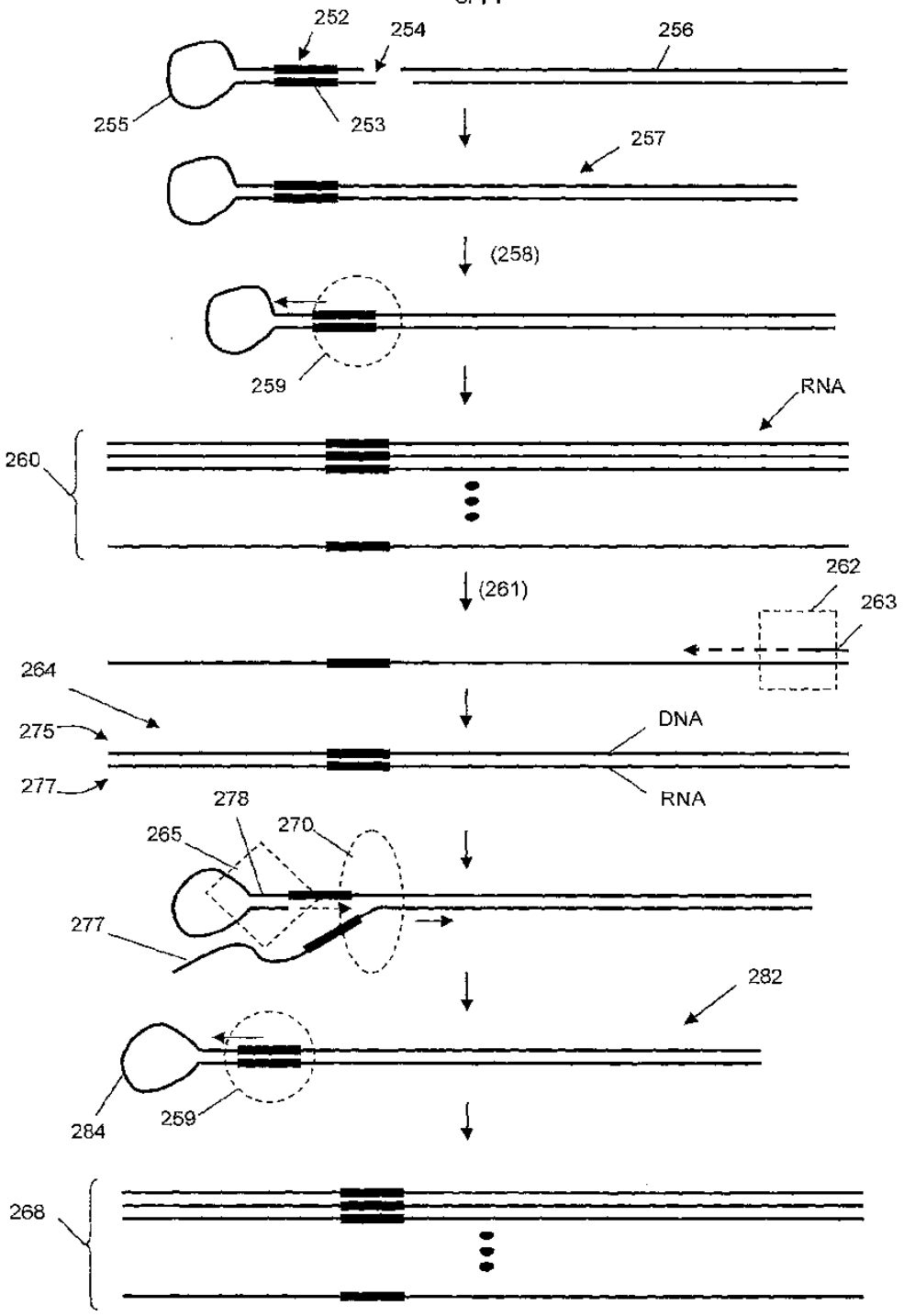

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(258)
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(261)
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259
268

Fig. 2D

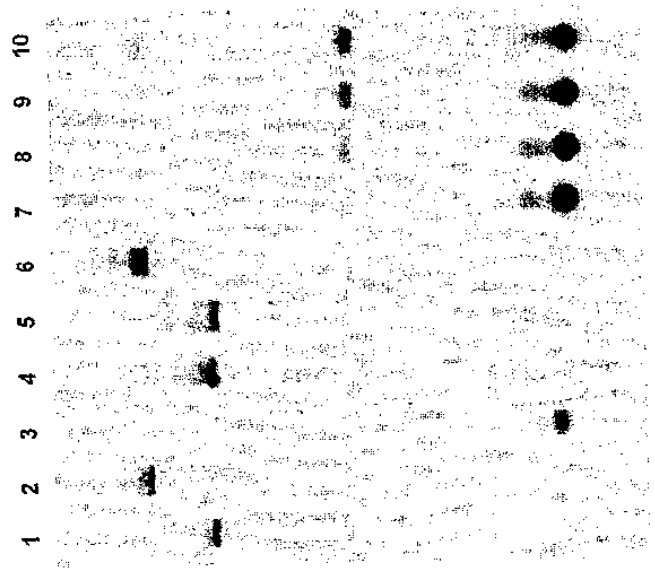


Fig. 3

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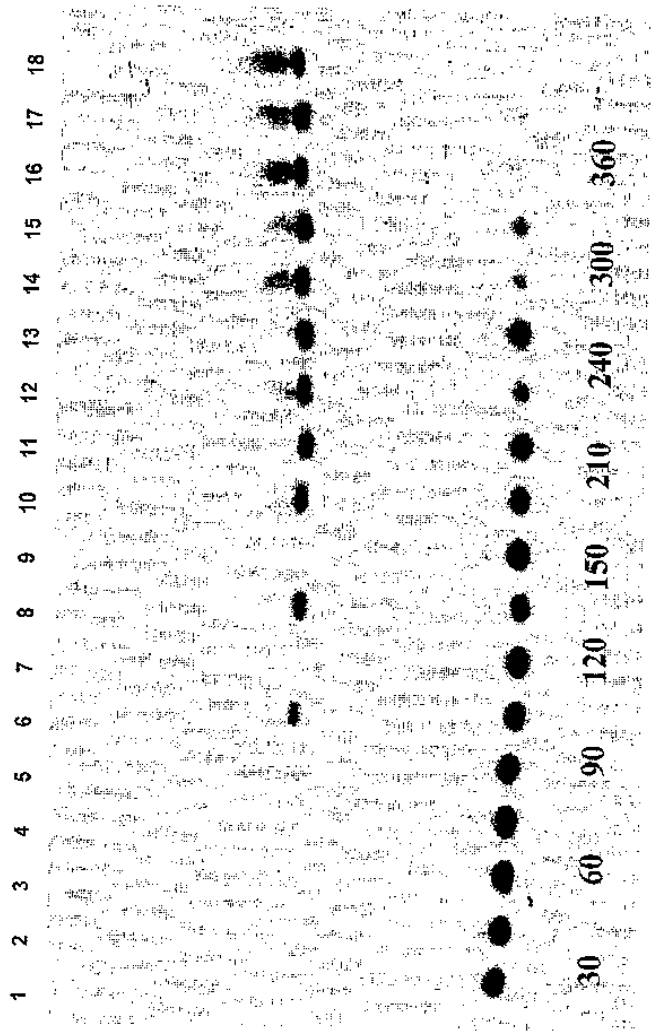

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Fig. 4A

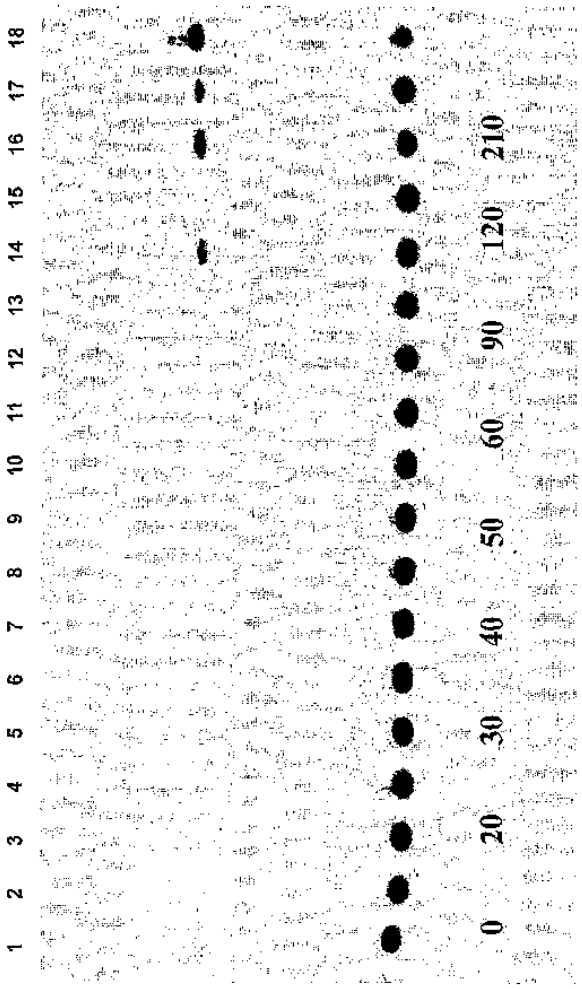


Fig. 4B

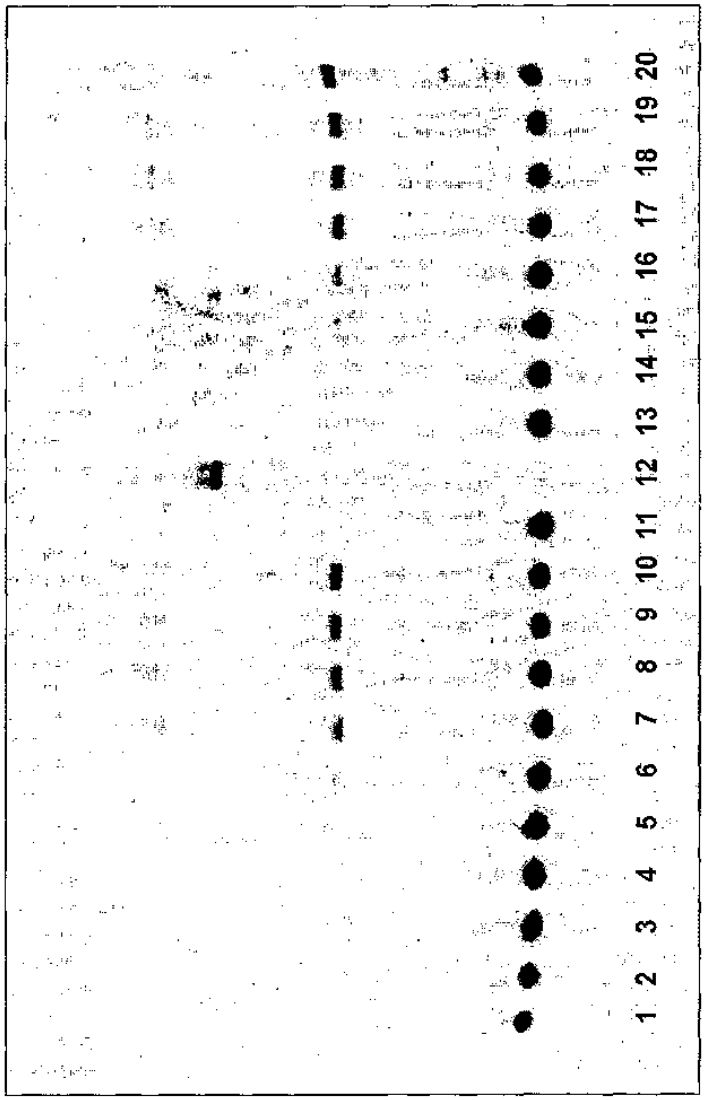


Fig. 5

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

Table 1

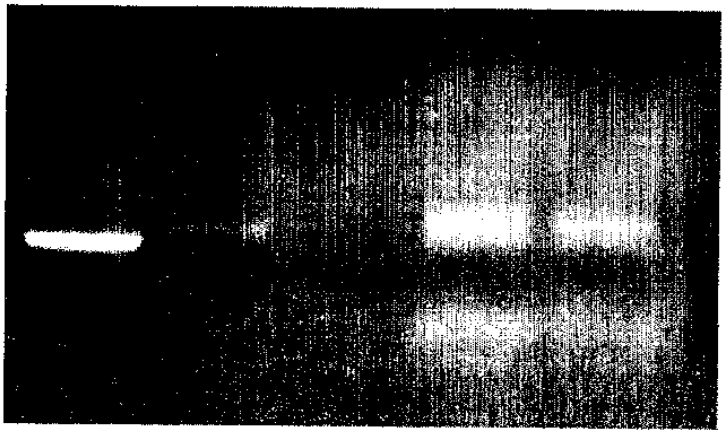


Fig. 6

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SEQUENCE LISTING

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Brenner, Sydney
Mikawa, Gi

<120> Isothermal DNA Amplification

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<150> US 60/647,064
<151> 2005-01-25

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ggatagaatc cc 72

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gtcgtattaa gcaagg 76

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PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

To:
Scherer, David C.
BOZICEVIC, FIELD & FRANCIS LLP
1900 University Avenue Suite 200
East Palo Alto CA 94303
ETATS-UNIS D'AMERIQUE

Date of mailing *(day/month/year)*: 9 February 2011 (09-02-2011)

Applicant's or agent's file reference: OGLO-020WO

FOR FURTHER ACTION See paragraphs 1 and 4 below

International application No.: PCT/IB2010/002243

International filing date *(day/month/year)*: 13 August 2010 (13-08-2010)

Applicant: POPULATION GENETICS TECHNOLOGIES LTD

1 [X] The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the International Search Report.

Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 338.82.70

For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 - 9.011

2 [ ] The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3 [ ] **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4 **Reminders**

The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. Following the expiration of 30 months from the priority date, these comments will also be made available to the public.

Shortly after the expiration of **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before completion of the technical preparations for international publication (Rules 90*bis*.1 and 90*bis*.3).

Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months.

For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

Name and mailing address of the International Searching Authority:
European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040
Fax: (+31-70) 340-3016

Authorized officer:
SCHULZ, Magdalena
Tel: +49 (0)89 2399-6315

Form PCT/ISA/220 (July 2010)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
CGLC-020WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/IB2010/002243	13/08/2010	20/08/2009
Applicant		
POPULATION GENETICS TECHNOLOGIES LTD		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      [X] the international application in the language in which it was filed
      [ ] a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. [ ] This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. [ ] With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. [ ] **Certain claims were found unsearchable** (See Box No. II)

3. [ ] **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   [X] ✓ the text is approved as submitted by the applicant
   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   [X] ✓ the text is approved as submitted by the applicant
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. 1
      [X] as suggested by the applicant
      [ ] as selected by this Authority, because the applicant failed to suggest a figure
      [ ] as selected by this Authority, because this figure better characterizes the invention
   b. [ ] none of the figures is to be published with the abstract

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2010/002243

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/10 C12N15/66 C12Q1/68
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 C12Q

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/061083 A2 (AMGEN INC [US]) 22 July 2004 (2004-07-22) paragraphs [0022], [0141]; claims; figure 7	1-11, 16-20
X	EP 1 841 879 A2 (COMPASS GENETICS LLC [US] POPULATION GENETICS TECHNOLOGI [GB]) 10 October 2007 (2007-10-10) page 12 - page 14; claims; figure 2; examples	1-11, 16-20
X Y	US 2007/238113 A1 (KANDA HIDETOSHI [JP] ET AL) 11 October 2007 (2007-10-11) page 2 - page 19; claims; figures 3-5 paragraph [0024]	1-11, 16-20 12-15

-/--

[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
27 January 2011	09/02/2011

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	Madruga, Jaime

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

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 672 064 A1 (RIKEN [JP]) 21 June 2006 (2006-06-21) paragraphs [0010] - [017,]; figure 5	1-11, 16-20
X	WO 2007/018601 A1 (RUBICON GENOMICS INC [US]; MAKAROV VLADIMIR L [US]; KAMBEROV EMMANUEL) 15 February 2007 (2007-02-15) claims; figure 3	1-11, 16-20
Y	US 2007/259357 A1 (BRENNER SYDNEY [GB]) 8 November 2007 (2007-11-08) cited in the application the whole document	12
Y	YAN PU ET AL: "Rapid one-step construction of hairpin RNA.", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 12 JUN 2009 LNKD-PUBMED:19371727, vol. 383, no. 4, 12 June 2009 (2009-06-12), pages 464-468, XP002618912, ISSN: 1090-2104 the whole document	13-15

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2010/002243

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004061083 A2	22-07-2004	AU 2003299970 A1	29-07-2004
		CA 2509636 A1	22-07-2004
		EP 1575980 A2	21-09-2005
		US 2007111228 A1	17-05-2007
		US 2004248299 A1	09-12-2004
EP 1841879 A2	10-10-2007	WO 2006081222 A2	03-08-2006
US 2007238113 A1	11-10-2007	NONE	
EP 1672064 A1	21-06-2006	WO 2005028646 A1	31-03-2005
		JP 4353945 B2	28-10-2009
		US 2009004739 A1	01-01-2009
WO 2007018601 A1	15-02-2007	EP 1924704 A1	28-05-2008
US 2007259357 A1	08-11-2007	EP 1987162 A2	05-11-2008
		US 2010216125 A1	26-08-2010
		WO 2007087310 A2	02-08-2007

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PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY
(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/IB2010/002243	13.08.2010	20.08.2009

International Patent Classification (IPC) or both national classification and IPC
INV. C12N15/10 C12N15/66 C12Q1/68

Applicant
POPULATION GENETICS TECHNOLOGIES LTD

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA:

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0
Fax: +49 89 2399 - 4465

Date of completion of this opinion
see form PCT/ISA/210

Authorized Officer
Madruga, Jaime
Telephone No. +49 89 2399-4739

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Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed

   ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☐ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	6, 7, 12-16
	No:	Claims	1-5, 8-11, 17-20
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-20
Industrial applicability (IA)	Yes:	Claims	1-20
	No:	Claims	

2. Citations and explanations

see separate sheet

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Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

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Re Item V.

1 **CITATIONS**

1.1 Reference is made to the following documents:

D1 WO 2004/061083 A2 (AMGEN INC [US]) 22 July 2004 (2004-07-22)

D2 EP 1 841 879 A2 (COMPASS GENETICS LLC [US] POPULATION GENETICS TECHNOLOGI [GB]) 10 October 2007 (2007-10-10)

D3 US 2007/238113 A1 (KANDA HIDETOSHI [JP] ET AL) 11 October 2007 (2007-10-11)

D4 EP 1 672 064 A1 (RIKEN [JP]) 21 June 2006 (2006-06-21)

D5 WO 2007/018601 A1 (RUBICON GENOMICS INC [US]; MAKAROV VLADIMIR L [US]; KAMBEROV EMMANUEL) 15 February 2007 (2007-02-15)

D6 US 2007/259357 A1 (BRENNER SYDNEY [GB]) 8 November 2007 (2007-11-08)cited in the application

D7 YAN PU ET AL: "Rapid one-step construction of hairpin RNA.", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 12 JUN 2009 LNKD- PUBMED:19371727, vol. 383, no. 4, 12 June 2009 (2009-06-12), pages 464-468, XP002618912, ISSN: 1090-2104

1.2 **THE APPLICATION**

The present application concerns a method to move a domain (not necessarily a protein domain, can be any sequence, especially polymorphic regions in the genome) in a single stranded nucleic acid to a different position by using a sequence, termed "reflex sequence" (which can be any sequence as far as it is not present anymore in the polynucleotide) and its complement, which are annealed forming a loop or hairpin (comprising the sequence of interest); the complement of the reflex sequence is then extended (primer extension) to have the complement of the domain. The domain and the reflex sequence are preferably removed; the strand is copied to get the reverse

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polarity, whereby the region of interest in the loop has now the reverse orientation and is at a different position in relation to the domain as in the original nucleic acid strand.

2 NOVELTY (Art. 33(2) PCT)

2.1 **D1** discloses methods for RNA interference. In some embodiments (e.g. Fig. 7), D1 discloses a nucleic acid comprising in the 5' to 3' orientation: a first domain(T7 promoter), a reflex sequence (one part of the closing stem), a polynucleotide of interest (loop) and the complement the reflex sequence at the 3' end of the strand. The extended molecule is captured by streptavidin and amplified by PCR. D1 includes a method where the "reflex sequence" is annealed to its complement (closing stem) and there is a 3' extension from the 3' end of the complement of the reflex sequence. In some embodiments, the reflex sequences comprises an RNA polymerase site (D1, [0012]). According to the application, a multiplex identifier (MID) can be any sequence as far as it is associated with the polynucleotide claimed and can be used to differentiate polynucleotides in a sample. Thus, the subject-matter of claims **1-5, 8-11, 17-20** lacks novelty over D1.

2.2 **D2** discloses a method of self-priming for amplifying a nucleic acid (D2, fig 2B and 2D; claim 1, pages 12-13). The nucleic acid in figure 2B comprises in the 5' to 3' orientation: a first domain (e.g. 210), a reflex sequence (one part of the closing stem, 208), a polynucleotide of interest (loop. 206) and the complement the reflex sequence at the 3' end of the strand, 204). The moleule is attached to a solid support by its 5' end and after annealing, the nucleic acid is extended and later amplified by PCR. D1 includes a method where the "reflex sequence" is annealed to its complement (closing stem) and there is a 3' extension from the 3' end of the complement of the reflex sequence. The sequence of the stem has a site for RNA polymerase (see figure 2C (259) and pages 12-14). Thus, the molecule and the method in D2 (fig 2B) have the same features of claim 1 and claim 20. The subject-matter of claims **1-5, 8-11, 17-20** lacks novelty over D2.

2.3 **D3** discloses methods for detecting mutations and polymorphisms. The method relies partly on intramolecularly anneal in a single strand of DNA, which is self priming (loop primer) and comprises all the elements of claims 1 and 20 (D1, figs. 3-5). The nucleic acid in Fig. 5 comprises in the 5' to 3' orientation: a first domain, a reflex sequence (one part of the closing stem, F1c), a polynucleotide of interest (loop, F2) and the complement the reflex

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sequence at the 3' end of the strand (F1). The nucleic acid in Fig. 4, e.g. feature (11), comprises in the 5' to 3' orientation: a first domain (e.g. F1), a reflex sequence (R1c), a polynucleotide of interest (R2c) and the complement the reflex sequence (R1), which is extended from the 3' end. Said molecules are also to be amplified, e.g. by PCR. (See D3, Figures 3-5, pages 2-19). The subject-matter of claims **1-4, 8-11, 17-20** lacks novelty over D3.

2.4 **D4** discloses a method for preparing a chimera gene having an inverted repeat sequence of a target sequence. The method includes steps in which a loop (hairpin) is formed and extended, thereby moving a domain in a nucleic acid based on intramolecular annealing of a sequence (reflex sequence in the application, inverted repeat in D4) to its complementary sequence followed by extension from the 3' end and followed by replication by PCR (e.g. D4, fig 5). This is identical to the method disclosed in claim 1 and the product in claim 20 . The subject-matter of claims **1-4, 8-11, 17-20** lacks novelty over D4.

2.5 **D5** discloses processing and amplification of DNA; in some embodiments D5 discloses a method using nucleic acid hairpin molecules where their 3' end is extended (D5, e.g. fig 3). This falls into the scope of claims 1 and 20. The subject-matter of claims **1-4, 8-11, 17-20** lacks novelty over D5.

2.6 The present application does not satisfy the criterion set forth in Art. 33(2) PCT because the subject-matter of claims **1-5, 8-11, 17-20** is not new in respect of prior art as defined in the regulations (Rule 64(1)-(3) PCT).

3 INVENTIVE STEP (Art. 33(3) PCT)

3.1 Document D3 can be considered to represent the closest prior art (see above).

3.2 The subject-matter of dependent claims **6-7**, differs in that the first domain and the reflex sequence from the 5' end of the double stranded region are removed. A skilled person who wanted to obtain a molecule only with the reflex sequence and the inverted region of interest, would remove said first domain and the reflex sequence from the 5' end without the need of any inventive step.The use of an exonuclease would be one of the preferred options, and T7 is one of the best known for the skilled person, thus, the subject-matter of claims 6-7 does not seem to be inventive.

3.3 The subject-matter of dependent claim **12** differs in that in the method of claim 1, the sample comprises multiple polynucleotide strands, each polynucleotide strand comprising a unique MID in the first domain. The **problem** to be solved

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may be regarded as the provision of a method to move a domain in a nucleic acid sequence in a plurality of nucleic acid strands, wherein the resulting strand with a moved domain can be identified. The **solution** would be a method as in claim 1, wherein each strand in the sample comprises a unique MID in the first domain. This solution is not considered as involving an inventive step since the tagging of polynucleotides allowing detection and selection of a particular strand in a nucleic acid sample comprising multiple polynucleotide strands, was a well known technique in the prior art. For instance, D6, cited in application as US20070656746, discloses the use of tags for nucleic acids and their use in the detection of polymorphisms. The method in D6 includes tagging a plurality of populations of polynucleotides each with a unique sequence token by generating a plurality of unique sequence token tags to provide a plurality of populations of polynucleotides each tagged with a unique sequence token tag, wherein each unique sequence token tag is used to tag only one population of polynucleotides (D6, e.g. [0006], claims). The subject-matter of claim **12** is not inventive over a combination of the teachings of D3 and D6.

3.4 The subject-matter of claim 13 differs from that of claim 1 in that two primers are used. in particular, the main difference is the step of incorporating in a polynucleotide strand the region corresponding to the complement of the "reflex sequence" by extension using a primer which is complementary to the polynucleotide of interest or the second domain. This is a method step which would not represent any inventive activity for the skilled person in the field of in molecular biology. For instance, D7 discloses methods to introduce a complementary sequence to a "reflex" sequence (stem sequence) in a polynucleotide of interest by using a primer complementary to a region in the polynucleotide of interest and the sequence of the stem so that this is incorporated in the polynucleotide by annealing and extension of the polynucleotide to render a product comprising the inverted repeats. The subject-matter of claims **13-15** is not inventive over a combination of the teachings of D3 and D7.

3.5 The subject-matter of dependent claim 16 seems to represent a mere alternative for further amplifications and priming to that disclosed in many prior art documents (D1-D5) and does not appear to involve any inventive step over such prior art and the general knowledge of the skilled person.

3.6 Thus, the present application does therefore not satisfy the criterion set forth in Art. 33(3) PCT and the subject-matter of claims 6-7 and 12-16 does not involve an inventive step (Rule 65(1)(2) PCT).

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Re Item VIII

4 **CLARITY**

4.1 The application does not meet the requirements of Art. 6 PCT because the last part of claim 13 has many possible interpretation and is not considered to be clear.

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	Application Number	
	Filing Date	
	First Named Inventor	Sydney Brenner
	Art Unit	
	Examiner Name	
	Attorney Docket Number	CGLC-020

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	2	2007/018601	WO	A1	2007-02-15	RUBICON GENOMICS INC		☐

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Sydney Brenner
	Art Unit	
	Examiner Name	
	Attorney Docket Number	CGLC-020

	3	1841879	EP	A2	2007-10-10	POPULATION GENETICS TECHNOLOGI		☐
	4	1672064	EP	A1	2006-06-21	RIKEN		☐

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	1	YAN, et al., "Rapid one-step construction of hairpin RNA", Biochemical and Biophysical Research Communications, 2009, 383:464-8.	☐
	2	PCT/IB2010/002243, International Search Report and Written Opinion, mailed February 9, 2011, 13pgs.	☐

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Sydney Brenner
	Art Unit	
	Examiner Name	
	Attorney Docket Number	CGLC-020

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Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

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A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/James S. Keddie, Reg. No. 48,920/	Date (YYYY-MM-DD)	2012-01-26
Name/Print	James S. Keddie	Registration Number	48920

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Electronic Patent Application Fee Transmittal	
Application Number:	
Filing Date:	
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Filer:	James Samuel Keddie/David Castro
Attorney Docket Number:	CGLC-020
Filed as Small Entity	

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Basic National Stage Fee	2631	1	190	190
Natl Stage Search Fee - Report provided	2642	1	245	245
Natl Stage Exam Fee - all other cases	2633	1	125	125
Pages:				
Claims:				
Miscellaneous-Filing:				
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Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		560

Electronic Acknowledgement Receipt	
EFS ID:	11932163
Application Number:	13387343
International Application Number:	PCT/IB10/02243
Confirmation Number:	3537
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	24353
Filer:	James Samuel Keddie
Filer Authorized By:	
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APPLICATION DATA SHEET

APPLICATION INFORMATION

Application Type::	Utility
Title::	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
Attorney Docket Number::	CGLC-020
Request for Non-Publication?::	
Assignee for Publication::	
Total Drawing Sheets::	13
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Sequence Submission?::	No
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APPLICATION DATA SHEET

CONTINUITY INFORMATION

This application is a::	371 National Stage Application of
> Application One::	PCT/IB2010/002243
Filing Date::	August 13, 2010
Which claims priority to::	US Provisional
> Application Two::	61/235,595
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>> Application Three::	61/288,792
Filing Date::	December 21, 2009

PRIOR FOREIGN APPLICATIONS

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(54) Title: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT



Fig. 1

(57) Abstract: Aspects of the present invention are drawn to processes for moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method". In certain embodiments, the reflex method results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer sites and/or MID). Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

WO 2011/021102 A2

COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

BACKGROUND

We have previously described methods that enable tagging each of a population of fragmented genomes and then combining them together to create a 'population library' that can be processed and eventually sequenced as a mixture. The population tags enable analysis software to parse the sequence reads into files that can be attributed to a particular genome in the population. One limitation of the overall process stems from limitations of existing DNA sequencing technologies. In particular, if fragments in the regions of interest of the genome are longer than the lengths that can be sequenced by a particular technology, then such fragments will not be fully analyzed (since sequencing proceeds from an end of a fragment inward). Furthermore, a disadvantage of any sequencing technology dependent on fragmentation is that sequence changes in one part of a particular genomic region may not be able to be linked to sequence changes in other parts of the same genome (e.g., the same chromosome) because the sequence changes reside on different fragments. (See Figure 5 and its description below).

The present invention removes the limitations imposed by current sequencing technologies as well as being useful in a number of other nucleic acid analyses.

SUMMARY OF THE INVENTION

Aspects of the present invention are drawn to processes for moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method" (or reflex process, reflex sequence process, reflex reaction, and the like). In certain embodiments, the reflex method results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer sites and/or MID). Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to scale. Indeed, the

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dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures:

Figure 1: Panel A is a schematic diagram illustrating moving a first domain from one site to another in a nucleic acid molecule using a reflex sequence. Panel B is a schematic diagram depicting the relative position of primer pairs (A_n-B_n primers) that find use in aspects of the reflex process described herein.

Figure 2 shows an exemplary embodiment of using binding partner pairs (biotin/streptavidin) to isolate single stranded polynucleotides of interest.

Figure 3 is a schematic diagram illustrating an exemplary embodiment for moving a primer site and a MID to a specific location in a nucleic acid of interest.

Figure 4 shows a schematic diagram illustrating an exemplary use of the reflex process for generating a sample enriched for fragments having a region of interest (e.g., from a population of randomly fragmented and asymmetrically tagged polynucleotides).

Figure 5 shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information.

Figure 6: Panel A is a schematic showing expected structures and sizes of nucleic acid species in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

Figure 7: Panel A is a schematic showing the structure of the nucleic acid and competitor used in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

Figure 8 shows a flow chart of a reflex process (left) in which the T7 exonuclease step is optional. The gel on the right shows the resultant product of the reflex process either without the T7 exonuclease step (lane 1) or with the T7 exonuclease step (lane 2).

Figure 9 shows an exemplary reflex process workflow with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt beads to remove primer oligos).

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Figure 10 shows the starting material (left panel) and the resultant product generated (right panel) using a reflex process without using a T7 exonuclease step (as described in Example II). The reflex site in the starting material is a sequence normally present in the polynucleotide being processed (also called a "non-artificial" reflex site). This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in transferring an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

Figure 11 shows a schematic and results of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate five different products ("daughter" products) each having a different region of interest (i.e., daughter products are produced having either region 1, 2, 3, 4 or 5).

Figure 12 shows a schematic and results of experiments performed to determine the prevalence of intramolecular rearrangement during the reflex process (as desired) vs. intermolecular rearrangement (MID switching).

Figure 13 shows a diagram of exemplary workflows for preparing material for and performing the reflex process.

DEFINITIONS

Unless otherwise defined, 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. Still, certain elements are defined for the sake of clarity and ease of reference.

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

"Amplicon" means the product of a polynucleotide amplification reaction. That is, it is a population of polynucleotides, usually double stranded, that are replicated from one or more starting sequences. The one or more starting sequences may be one or more copies of the same sequence, or it may be a mixture of different sequences. Amplicons

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

The term "assessing" includes any form of measurement, and includes determining if an element is present or not. The terms "determining", "measuring", "evaluating", "assessing" and "assaying" are used interchangeably and includes quantitative and qualitative determinations. Assessing may be relative or absolute. "Assessing the presence of" includes determining the amount of something present, and/or determining whether it is present or absent. As used herein, the terms "determining," "measuring," and "assessing," and "assaying" are used interchangeably and include both quantitative and qualitative determinations.

Polynucleotides that are "asymmetrically tagged" have left and right adapter domains that are not identical. This process is referred to generically as attaching adapters asymmetrically or asymmetrically tagging a polynucleotide, e.g., a polynucleotide

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fragment. Production of polynucleotides having asymmetric adapter termini may be achieved in any convenient manner. Exemplary asymmetric adapters are described in: U.S. Patents 5,712,126 and 6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters employed are those described in U.S. Patent Application Ser. No. 12/432,080, filed on April 29, 2009, incorporated herein by reference in its entirety.

As one example, a user of the subject invention may use an asymmetric adapter to tag polynucleotides. An "asymmetric adapter" is one that, when ligated to both ends of a double stranded nucleic acid fragment, will lead to the production of primer extension or amplification products that have non-identical sequences flanking the genomic insert of interest. The ligation is usually followed by subsequent processing steps so as to generate the non-identical terminal adapter sequences. For example, replication of an asymmetric adapter attached fragment(s) results in polynucleotide products in which there is at least one nucleic acid sequence difference, or nucleotide/nucleoside modification, between the terminal adapter sequences. Attaching adapters asymmetrically to polynucleotides (e.g., polynucleotide fragments) results in polynucleotides that have one or more adapter sequences on one end (e.g., one or more region or domain, e.g., a primer site) that are either not present or have a different nucleic acid sequence as compared to the adapter sequence on the other end. It is noted that an adapter that is termed an "asymmetric adapter" is not necessarily itself structurally asymmetric, nor does the mere act of attaching an asymmetric adapter to a polynucleotide fragment render it immediately asymmetric. Rather, an asymmetric adapter-attached polynucleotide, which has an identical asymmetric adapter at each end, produces replication products (or isolated single stranded polynucleotides) that are asymmetric with respect to the adapter sequences on opposite ends (e.g., after at least one round of amplification/primer extension).

Any convenient asymmetric adapter, or process for attaching adapters asymmetrically, may be employed in practicing the present invention. Exemplary asymmetric adapters are described in: U.S. Patents 5,712,126 and 6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters employed are those described in U.S. Patent Application Ser. No. 12/432,080, filed on April 29, 2009, incorporated herein by reference in its entirety.

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

"Duplex" means at least two oligonucleotides and/or polynucleotides that are fully or partially complementary undergo Watson-Crick type base pairing among all or most of their nucleotides so that a stable complex is formed. The terms "annealing" and "hybridization" are used interchangeably to mean the formation of a stable duplex. "Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one another such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. A stable duplex can include Watson-Crick base pairing and/or non-Watson-Crick base pairing between the strands of the duplex (where base pairing means the forming hydrogen bonds). In certain embodiments, a non-Watson-Crick base pair includes a nucleoside analog, such as deoxyinosine, 2, 6-diaminopurine, PNAs, LNA's and the like. In certain embodiments, a non-Watson-Crick base pair includes a "wobble base", such as deoxyinosine, 8-oxo-dA, 8-oxo-dG and the like, where by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand (wobble bases are described in further detail below). A "mismatch" in a duplex between two oligonucleotides or polynucleotides means that a pair of nucleotides in the duplex fails to undergo Watson-Crick bonding.

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"Genetic locus," "locus," or "locus of interest" in reference to a genome or target polynucleotide, means a contiguous sub-region or segment of the genome or target polynucleotide. As used herein, genetic locus, locus, or locus of interest may refer to the position of a nucleotide, a gene or a portion of a gene in a genome, including mitochondrial DNA or other non-chromosomal DNA (e.g., bacterial plasmid), or it may refer to any contiguous portion of genomic sequence whether or not it is within, or associated with, a gene. A genetic locus, locus, or locus of interest can be from a single nucleotide to a segment of a few hundred or a few thousand nucleotides in length or more. In general, a locus of interest will have a reference sequence associated with it (see description of "reference sequence" below).

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

"Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, e.g. oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon of a terminal nucleotide of one oligonucleotide with 3' carbon of another oligonucleotide. A variety of template-driven ligation reactions are described in the following references, which are incorporated by reference: Whiteley et al, U.S. patent 4,883,750; Letsinger et al, U.S. patent 5,476,930; Fung et al, U.S. patent 5,593,826; Kool, U.S. patent 5,426,180; Landegren et al, U.S. patent 5,871,921; Xu and Kool, Nucleic Acids Research, 27: 875-881 (1999); Higgins et al, Methods in Enzymology, 68: 50-71 (1979); Engler et al, The Enzymes, 15: 3-29 (1982); and Namsaraev, U.S. patent publication 2004/0110213.

"Multiplex Identifier" (MID) as used herein refers to a tag or combination of tags associated with a polynucleotide whose identity (e.g., the tag DNA sequence)

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can be used to differentiate polynucleotides in a sample. In certain embodiments, the MID on a polynucleotide is used to identify the source from which the polynucleotide is derived. For example, a nucleic acid sample may be a pool of polynucleotides derived from different sources, (e.g., polynucleotides derived from different individuals, different tissues or cells, or polynucleotides isolated at different times points), where the polynucleotides from each different source are tagged with a unique MID. As such, a MID provides a correlation between a polynucleotide and its source. In certain embodiments, MIDs are employed to uniquely tag each individual polynucleotide in a sample. Identification of the number of unique MIDs in a sample can provide a readout of how many individual polynucleotides are present in the sample (or from how many original polynucleotides a manipulated polynucleotide sample was derived; see, e.g., U.S. Patent No. 7,537,897, issued on May 26, 2009, incorporated herein by reference in its entirety). MIDs can range in length from 2 to 100 nucleotide bases or more and may include multiple subunits, where each different MID has a distinct identity and/or order of subunits. Exemplary nucleic acid tags that find use as MIDs are described in U.S. Patent 7,544,473, issued on June 6, 2009, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Patent 7,393,665, issued on July 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., Tm, length, base composition, etc.), as the methods described herein can accommodate a wide variety of unique MID sets. It is emphasized here that MIDs need only be unique within a given experiment. Thus, the same MID may be used to tag a different sample being processed in a different experiment. In addition, in certain experiments, a user may use the same MID to tag a subset of different samples within the same experiment. For example, all samples derived from individuals having a specific phenotype may be tagged with the same MID, e.g., all samples derived from control (or wildtype) subjects can be tagged with a first MID while subjects having a disease condition can be tagged with a second MID (different than the first MID). As another example, it may be desirable to tag different samples derived from the same source with different MIDs (e.g., samples derived over time or derived from different sites within a tissue). Further, MIDs can be generated in a

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variety of different ways, e.g., by a combinatorial tagging approach in which one MID is attached by ligation and a second MID is attached by primer extension. Thus, MIDs can be designed and implemented in a variety of different ways to track polynucleotide fragments during processing and analysis, and thus no limitation in this regard is intended.

"Nucleoside" as used herein includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543-584 (1990), or the like, with the proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like. Polynucleotides comprising analogs with enhanced hybridization or nuclease resistance properties are described in Uhlman and Peyman (cited above); Crooke et al, Exp. Opin. Ther. Patents, 6: 855-870 (1996); Mesmaeker et al, Current Opinion in Structual Biology, 5: 343-355 (1995); and the like. Exemplary types of polynucleotides that are capable of enhancing duplex stability include oligonucleotide N3'→P5' phosphoramidates (referred to herein as "amidates"), peptide nucleic acids (referred to herein as "PNAs"), oligo-2'-O-alkylribonucleotides, polynucleotides containing C-5 propynylpyrimidines, locked nucleic acids ("LNAs"), and like compounds. Such oligonucleotides are either available commercially or may be synthesized using methods described in the literature.

"Polymerase chain reaction," or "PCR," means a reaction for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. In other words, PCR is a reaction for making multiple copies or replicates of a target nucleic acid flanked by primer sites, such reaction comprising one or more repetitions of the following steps: (i) denaturing the target nucleic acid, (ii) annealing primers to the primer sites, and (iii) extending the primers by a nucleic acid polymerase in the presence of nucleoside triphosphates. Usually, the reaction is cycled through different temperatures optimized for each step in a thermal cycler instrument. Particular temperatures, durations at each step, and rates of change between steps depend on many factors well-known to those of ordinary skill in the art, e.g. exemplified by the references: McPherson et al, editors, PCR: A Practical Approach and PCR2: A Practical Approach (IRL Press, Oxford, 1991 and 1995, respectively). For example, in a conventional

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PCR using Taq DNA polymerase, a double stranded target nucleic acid may be denatured at a temperature >90°C, primers annealed at a temperature in the range 50-75°C, and primers extended at a temperature in the range 72-78°C. The term "PCR" encompasses derivative forms of the reaction, including but not limited to, RT-PCR, real-time PCR, nested PCR, quantitative PCR, multiplexed PCR, and the like. Reaction volumes range from a few hundred nanoliters, e.g. 200 nL, to a few hundred μL, e.g. 200 μL. "Reverse transcription PCR," or "RT-PCR," means a PCR that is preceded by a reverse transcription reaction that converts a target RNA to a complementary single stranded DNA, which is then amplified, e.g. Tecott et al, U.S. patent 5,168,038, which patent is incorporated herein by reference. "Real-time PCR" means a PCR for which the amount of reaction product, i.e. amplicon, is monitored as the reaction proceeds. There are many forms of real-time PCR that differ mainly in the detection chemistries used for monitoring the reaction product, e.g. Gelfand et al, U.S. patent 5,210,015 ("TAQMAN™"); Wittwer et al, U.S. patents 6,174,670 and 6,569,627 (intercalating dyes); Tyagi et al, U.S. patent 5,925,517 (molecular beacons); which patents are incorporated herein by reference. Detection chemistries for real-time PCR are reviewed in Mackay et al, Nucleic Acids Research, 30: 1292-1305 (2002), which is also incorporated herein by reference. "Nested PCR" means a two-stage PCR wherein the amplicon of a first PCR becomes the sample for a second PCR using a new set of primers, at least one of which binds to an interior location of the first amplicon. As used herein, "initial primers" in reference to a nested amplification reaction mean the primers used to generate a first amplicon, and "secondary primers" mean the one or more primers used to generate a second, or nested, amplicon. "Multiplexed PCR" means a PCR wherein multiple target sequences (or a single target sequence and one or more reference sequences) are simultaneously carried out in the same reaction mixture, e.g. Bernard et al, Anal. Biochem., 273: 221-228 (1999)(two-color real-time PCR). Usually, distinct sets of primers are employed for each sequence being amplified.

"Quantitative PCR" means a PCR designed to measure the abundance of one or more specific target sequences in a sample or specimen. Quantitative PCR includes both absolute quantitation and relative quantitation of such target sequences. Quantitative measurements are made using one or more reference sequences that may be assayed separately or together with a target sequence. The reference sequence may be endogenous or exogenous to a sample or specimen, and in the latter case, may comprise one or more competitor templates. Typical endogenous reference sequences include segments of transcripts of the following genes: β-actin, GAPDH, β_2-microglobulin, ribosomal RNA, and the like. Techniques for

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quantitative PCR are well-known to those of ordinary skill in the art, as exemplified in the following references that are incorporated by reference: Freeman et al, Biotechniques, 26: 112-126 (1999); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9447 (1989); Zimmerman et al, Biotechniques, 21: 268-279 (1996); Diviacco et al, Gene, 122: 3013-3020 (1992); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9446 (1989); and the like.

"Polynucleotide" or "oligonucleotide" is used interchangeably and each means a linear polymer of nucleotide monomers. Monomers making up polynucleotides and oligonucleotides are capable of specifically binding to a natural polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, wobble base pairing, or the like. As described in detail below, by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand. Such monomers and their internucleosidic linkages may be naturally occurring or may be analogs thereof, e.g. naturally occurring or non-naturally occurring analogs. Non-naturally occurring analogs may include peptide nucleic acids (PNAs, e.g., as described in U.S. Patent 5,539,082, incorporated herein by reference), locked nucleic acids (LNAs, e.g., as described in U.S. Patent 6,670,461, incorporated herein by reference), phosphorothioate internucleosidic linkages, bases containing linking groups permitting the attachment of labels, such as fluorophores, or haptens, and the like. Whenever the use of an oligonucleotide or polynucleotide requires enzymatic processing, such as extension by a polymerase, ligation by a ligase, or the like, one of ordinary skill would understand that oligonucleotides or polynucleotides in those instances would not contain certain analogs of internucleosidic linkages, sugar moieties, or bases at any or some positions. Polynucleotides typically range in size from a few monomeric units, e.g. 5-40, when they are usually referred to as "oligonucleotides," to several thousand monomeric units. Whenever a polynucleotide or oligonucleotide is represented by a sequence of letters (upper or lower case), such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, "I" denotes deoxyinosine, "U" denotes uridine, unless otherwise indicated or obvious from context. Unless otherwise noted the terminology and atom numbering conventions will follow those disclosed in Strachan and Read, Human Molecular Genetics 2 (Wiley-Liss, New York,

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1999). Usually polynucleotides comprise the four natural nucleosides (e.g. deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine for DNA or their ribose counterparts for RNA) linked by phosphodiester linkages; however, they may also comprise non-natural nucleotide analogs, e.g. including modified bases, sugars, or internucleosidic linkages. It is clear to those skilled in the art that where an enzyme has specific oligonucleotide or polynucleotide substrate requirements for activity, e.g. single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for the oligonucleotide or polynucleotide substrates is well within the knowledge of one of ordinary skill, especially with guidance from treatises, such as Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references.

"Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Usually primers are extended by a DNA polymerase. Primers are generally of a length compatible with their use in synthesis of primer extension products, and are usually are in the range of between 8 to 100 nucleotides in length, such as 10 to 75, 15 to 60, 15 to 40, 18 to 30, 20 to 40, 21 to 50, 22 to 45, 25 to 40, and so on, more typically in the range of between 18-40, 20-35, 21-30 nucleotides long, and any length between the stated ranges. Typical primers can be in the range of between 10-50 nucleotides long, such as 15-45, 18-40, 20-30, 21-25 and so on, and any length between the stated ranges. In some embodiments, the primers are usually not more than about 10, 12, 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, or 70 nucleotides in length.

Primers are usually single-stranded for maximum efficiency in amplification, but may alternatively be double-stranded. If double-stranded, the primer is usually first treated to separate its strands before being used to prepare extension products. This denaturation step is typically affected by heat, but may alternatively be carried out using alkali, followed by neutralization. Thus, a "primer" is complementary to a template, and complexes by hydrogen bonding or hybridization with the template to give a primer/template complex for initiation of synthesis by a polymerase, which is extended by the addition of covalently bonded bases linked at its 3' end complementary to the template in the process of DNA synthesis.

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A "primer pair" as used herein refers to first and second primers having nucleic acid sequence suitable for nucleic acid-based amplification of a target nucleic acid. Such primer pairs generally include a first primer having a sequence that is the same or similar to that of a first portion of a target nucleic acid, and a second primer having a sequence that is complementary to a second portion of a target nucleic acid to provide for amplification of the target nucleic acid or a fragment thereof. Reference to "first" and "second" primers herein is arbitrary, unless specifically indicated otherwise. For example, the first primer can be designed as a "forward primer" (which initiates nucleic acid synthesis from a 5' end of the target nucleic acid) or as a "reverse primer" (which initiates nucleic acid synthesis from a 5' end of the extension product produced from synthesis initiated from the forward primer). Likewise, the second primer can be designed as a forward primer or a reverse primer.

"Primer site" (e.g., a sequencing primer site, and amplification primer site, etc.) as used herein refers to a domain in a polynucleotide that includes the sequence of a primer (e.g., a sequencing primer) and/or the complementary sequence of a primer. When present in single stranded form (e.g., in a single stranded polynucleotide), a primer site can be either the identical sequence of a primer or the complementary sequence of a primer. When present in double stranded form, a primer site contains the sequence of a primer hybridized to the complementary sequence of the primer. Thus, a primer site is a region of a polynucleotide that is either identical to or complementary to the sequence of a primer (when in a single stranded form) or a double stranded region formed between a primer sequence and its complement. Primer sites may be present in an adapter attached to a polynucleotide. The specific orientation of a primer site can be inferred by those of ordinary skill in the art from the structural features of the relevant polynucleotide and/or context in which it is used.

"Readout" means a parameter, or parameters, which are measured and/or detected that can be converted to a number or value. In some contexts, readout may refer to an actual numerical representation of such collected or recorded data. For example, a readout of fluorescent intensity signals from a microarray is the address and fluorescence intensity of a signal being generated at each hybridization site of the microarray; thus, such a readout may be registered or stored in various ways, for example, as an image of the microarray, as a table of numbers, or the like.

"Reflex site", "reflex sequence" and equivalents are used to indicate sequences in a polynucleotide that are employed to move a domain intramolecularly from its initial location

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to a different location in the polynucleotide. The sequence of a reflex site can be added to a polynucleotide of interest (e.g., present in an adapter ligated to the polynucleotide), be based on a sequence naturally present within the polynucleotide of interest (e.g., a genomic sequence in the polynucleotide), or a combination of both. The reflex sequence is chosen so as to be distinct from other sequences in the polynucleotide (i.e., with little sequence homology to other sequences likely to be present in the polynucleotide, e.g., genomic or sub-genomic sequences to be processed). As such, a reflex sequence should be selected so as to not hybridize to any sequence except its complement under the conditions employed in the reflex processes herein described. As described later in this application, the complement to the reflex sequence is inserted on the same strand of the polynucleotide (e.g., the same strand of a double-stranded polynucleotide or on the same single stranded polynucleotide) in a particular location so as to facilitate an intramolecular binding event on such particular strand. Reflex sequences employed in the reflex process described herein can thus have a wide range of lengths and sequences. Reflex sequences may range from 5 to 200 nucleotide bases in length.

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

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

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

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature (e.g., as measured in °C) at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are known in the art (see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985). Other references (e.g., Allawi, H.T. & SantaLucia, J., Jr., Biochemistry 36, 10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m.

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

The terms "upstream" and "downstream" in describing nucleic acid molecule orientation and/or polymerization are used herein as understood by one of skill in the art. As such, "downstream" generally means proceeding in the 5' to 3' direction, i.e., the direction in which a nucleotide polymerase normally extends a sequence, and "upstream" generally

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means the converse. For example, a first primer that hybridizes "upstream" of a second primer on the same target nucleic acid molecule is located on the 5' side of the second primer (and thus nucleic acid polymerization from the first primer proceeds towards the second primer).

It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or the use of a "negative" limitation.

DETAILED DESCRIPTION OF THE INVENTION

The invention is drawn to compositions and methods for intramolecular nucleic acid rearrangement that find use in various applications of genetic analysis, including sequencing, as well as general molecular biological manipulations of polynucleotide structures.

Before the present invention is described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

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. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in

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connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" includes a plurality of such nucleic acids and reference to "the compound" includes reference to one or more compounds and equivalents thereof known to those skilled in the art, and so forth.

The practice of the present invention may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such conventional techniques include polymer array synthesis, hybridization, ligation, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the example herein below. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV), *Using Antibodies: A Laboratory Manual, Cells: A Laboratory Manual, PCR Primer: A Laboratory Manual, and Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), Stryer, L. (1995) *Biochemistry* (4th Ed.) Freeman, New York, Gait, "*Oligonucleotide Synthesis: A Practical Approach*" 1984, IRL Press, London, Nelson and Cox (2000), Lehninger, A., *Principles of Biochemistry* 3rd Ed., W. H. Freeman Pub., New York, N.Y. and Berg et al. (2002) *Biochemistry*, 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

As summarized above, aspects of the present invention are drawn to the use of a 'reflex' sequence present in a polynucleotide (e.g., in an adapter structure of the polynucleotide, in a genomic region of the polynucleotide, or a combination of both) to move a domain of the polynucleotide intra-molecularly from a first location to a second location. The reflex process described herein finds use in any number of applications, e.g.,

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placing functional elements of a polynucleotide (e.g., sequencing primer sites and/or MID tags) into proximity to a desired sub-region of interest.

Nucleic Acids

The reflex process (as described in detail below) can be employed for the manipulation and analysis of nucleic acid sequences of interest from virtually any nucleic acid source, including but not limited to genomic DNA, complementary DNA (cDNA), RNA (e.g., messenger RNA, ribosomal RNA, short interfering RNA, microRNA, etc.), plasmid DNA, mitochondrial DNA, synthetic DNA, etc. Furthermore, any organism, organic material or nucleic acid-containing substance can be used as a source of nucleic acids to be processed in accordance with the present invention including, but not limited to, plants, animals (e.g., reptiles, mammals, insects, worms, fish, etc.), tissue samples, bacteria, fungi (e.g., yeast), phage, viruses, cadaveric tissue, archaeological/ancient samples, etc. In certain embodiments, the nucleic acids in the nucleic acid sample are derived from a mammal, where in certain embodiments the mammal is a human.

In certain embodiments, the nucleic acid sequences are enriched prior to the reflex sequence process. By enriched is meant that the nucleic acid is subjected to a process that reduces the complexity of the nucleic acids, generally by increasing the relative concentration of particular nucleic acid species in the sample (e.g., having a specific locus of interest, including a specific nucleic acid sequence, lacking a locus or sequence, being within a specific size range, etc.). There are a wide variety of ways to enrich nucleic acids having a specific characteristic(s) or sequence, and as such any convenient method to accomplish this may be employed. The enrichment (or complexity reduction) can take place at any of a number of steps in the process, and will be determined by the desires of the user. For example, enrichment can take place in individual parental samples (e.g., untagged nucleic acids prior to adaptor ligation) or in multiplexed samples (e.g., nucleic acids tagged with primer sites, MID and/or reflex sequences and pooled; MID are described in further detail below).

In certain embodiments, nucleic acids in the nucleic acid sample are amplified prior to analysis. In certain of these embodiments, the amplification reaction also serves to enrich a starting nucleic acid sample for a sequence or locus of interest. For example, a starting nucleic acid sample can be subjected to a polymerase chain reaction (PCR) that amplifies one or more region of interest. In certain embodiments, the amplification reaction is an exponential amplification reaction, whereas in certain other embodiments, the amplification reaction is a linear amplification reaction. Any convenient method for performing

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amplification reactions on a starting nucleic acid sample can be used in practicing the subject invention. In certain embodiments, the nucleic acid polymerase employed in the amplification reaction is a polymerase that has proofreading capability (e.g., phi29 DNA Polymerase, Thermococcus litoralis DNA polymerase, Pyrococcus furiosus DNA polymerase, etc.).

In certain embodiments, the nucleic acid sample being analyzed is derived from a single source (e.g., a single organism, virus, tissue, cell, subject, etc.), whereas in other embodiments, the nucleic acid sample is a pool of nucleic acids extracted from a plurality of sources (e.g., a pool of nucleic acids from a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. As such, in certain embodiments, a nucleic acid sample can contain nucleic acids from 2 or more sources, 3 or more sources, 5 or more sources, 10 or more sources, 50 or more sources, 100 or more sources, 500 or more sources, 1000 or more sources, 5000 or more sources, up to and including about 10,000 or more sources.

In certain embodiments, nucleic acid fragments that are to be pooled with nucleic acid fragments derived from a plurality of sources (e.g., a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. In such embodiments, the nucleic acids derived from each source includes a multiplex identifier (MID) such that the source from which the each tagged nucleic acid fragment was derived can be determined. In such embodiments, each nucleic acid sample source is correlated with a unique MID, where by unique MID is meant that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID. Any type of MID can be used, including but not limited to those described in co-pending U.S. Patent Application Serial Number 11/656,746, filed on January 22, 2007, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Patent 7,393,665, issued on July 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., T_m, length, base composition, etc.), as the asymmetric tagging methods (and many tag readout methods, including but not limited to sequencing of the tag or measuring the length of the tag) can accommodate a wide variety of unique MID sets.

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In certain embodiments, each individual polynucleotide (e.g., double-stranded or single-stranded, as appropriate to the methodological details employed) in a sample to be analyzed is tagged with a unique MID so that the fate of each polynucleotide can be tracked in subsequent processes (where, as noted above, unique MID is meant to indicate that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID). For example (and as described below), having each nucleic acid tagged with a unique MID allows analysis of the sequence of each individual nucleic acid using the reflex sequence methods described herein. This allows the linkage of sequence information for large nucleic acid fragments that cannot be sequenced in a single sequencing run.

Reflex Sequence Process

As summarized above, aspects of the present invention include methods and compositions for moving a domain in a polynucleotide from a first location to a second location in the polynucleotide. An exemplary embodiment is shown in Figure 1A.

Figure 1A shows a single stranded polynucleotide 100 comprising, in a 5' to 3' orientation, a first domain (102; the domain to be moved); a reflex sequence 104; a nucleic acid sequence 106 having a site distal to the first domain (Site A), and a complement of the reflex sequence 108 (positioned at the 3' terminus of the polynucleotide). The steps of the reflex method described below will move the first domain into closer proximity to Site A. It is noted here that the prime designation in Figure 1A denotes a complementary sequence of a domain. For example, First Domain' is the complement of the First Domain.

In Step 1, the reflex sequence and its complement in the polynucleotide are annealed intramolecularly to form polynucleotide structure 112, with the polynucleotide folding back on itself and hybridizing to form a region of complementarity (i.e., double stranded reflex/reflex' region). In this configuration, the 3' end of the complement of the reflex sequence can serve as a nucleic acid synthesis priming site. Nucleic acid synthesis from this site is then performed in extension Step 2 producing a complement of the first domain at the 3' end of the nucleic acid extension (shown in polynucleotide 114; extension is indicated with dotted arrow labeled "extend").

Denaturation of polynucleotide 114 (e.g., by heat) generates linear single stranded polynucleotide 116. As shown in Figure 1, resultant polynucleotide 116 contains a complement of the first domain at a position proximal to Site A (i.e., separated by only the complement of the reflex sequence). This resultant polynucleotide may be used for any

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subsequent analysis or processing steps as desired by the user (e.g., sequencing, as a template for amplification (linear, PCR, etc.), sequence specific extraction, etc.).

In alternative embodiments, the first domain and reflex sequence are removed from the 5' end of the double-stranded region of polynucleotide 114 (shown in polynucleotide 118; removal is shown in the dotted arrow labeled "remove"). Removal of this region may be accomplished by any convenient method, including, but not limited to, treatment (under appropriate incubation conditions) of polynucleotide structure 114 with T7 exonuclease or by treatment with Lambda exonuclease; the Lambda exonuclease can be employed so long as the 5' end of the polynucleotide is phosphorylated. If the region is removed enzymatically, resultant polynucleotide 118 is used in place of polynucleotide 116 in subsequent steps (e.g., copying to reverse polarity).

In certain embodiments, polynucleotide 116 or 118 is used as a template to produce a double stranded polynucleotide, for example by performing a nucleic acid synthesis reaction with a primer that primes in the complement of the first domain. This step is sometimes referred to as copying to reverse polarity of a single stranded polynucleotide, and in some instances, the double-stranded intermediate product of this copying is not shown (see, e.g., Figure 3). For example, copying to reverse the polarity of polynucleotide 116 results in single-stranded polynucleotide 120 having, in a 5' to 3' orientation, the first domain (122); the reflex sequence (124); the complement of polynucleotide 106 (oriented with the complement of Site A (Site A'; 126) proximal to the reflex sequence); the complement of the reflex sequence (128); and the complement of the first domain (130).

In certain embodiments, the first domain in the polynucleotide comprises one or more elements that find use in one or more subsequent processing or analysis steps. Such sequences include, but are not limited to, restriction enzyme sites, PCR primer sites, linear amplification primer sites, reverse transcription primer sites, RNA polymerase promoter sites (such as for T7, T3 or SP6 RNA polymerase), MID tags, sequencing primer sites, etc. Any convenient element can be included in the first domain and, in certain embodiments, is determined by the desires of the user of the methods described herein.

As an exemplary embodiment, suppose we want to sequence a specific polynucleotide region from multiple genomes in a pooled sample where the polynucleotide region is too long to sequence in a single reaction. For example, sequencing a polynucleotide region that is 2 kilobases or more in length using Roche 454 (Branford, CT) technology, in which the length of a single sequencing run is about 400 bases. In this scenario, we can design a set of left hand primers (A_n) and right hand primers (B_n) specific

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for the polynucleotide region that are positioned in such a way that we can obtain direct sequences of all parts of the insert, as shown in Figure 1B. Note that the polynucleotide shown in Figure 1B (140) has a domain (142) containing a primer site and an MID denoting from which original sample(s) the polynucleotide is derived. Site 142 thus represents an example of a First Domain site such identified as 122 in the Figure 1A. The polynucleotide also includes a reflex site (144), which can be part of the polynucleotide region itself (e.g., a genomic sequence), added in a ligated adapter domain along with the primer site and the MID (an artificial sequence), or a combination of both (a sequence spanning the adapter/polynucleotide junction).

It is noted here that polynucleotide 140 can be categorized as a precursor to polynucleotide 100 in Figure 1A, as it does not include a 3' reflex sequence complementary to the reflex site (domain 108 in Figure 1A). As detailed below, polynucleotide 140 can be converted to a polynucleotide having the structural configuration of polynucleotide 100, a polynucleotide suitable as a substrate for the reflex process described herein (e.g., by primer extension using a B_n primer and reversal of polarity).

In an exemplary embodiment, each A_n-B_n primer pair defines a nucleic acid region that is approximately 400 bases in length or less. This size range is within the single-sequencing run read length of the current Roche 454 sequencing platform; a different size range for the defined nucleic acid region may be utilized for a different sequencing platform. Thus, each product from each reflex process can be sequenced in a single run. It is noted here that primer pairs as shown in Figure 1B can be used to define regions 1 to 5 shown in Figure 3 (described in further detail below).

In certain embodiments, to obtain the first part of the sequence of the polynucleotide region (i.e., in the original structure, that part of the polynucleotide closest to the first domain), we only need a right hand primer (e.g., B_0) and we do not need to transfer the MID as it is within reach of this sequencing primer (i.e., the MID is within 400 bases of sequencing primer B_0). All other B_n primers have the reflex sequence added to their 5' ends ("R" element shown on B primers) so that they read 5' reflex-B_n. However, in certain embodiments, the B_0 primer does include the reflex sequence and is used in the reflex process (along with a corresponding A_0 primer) as detailed below.

As described above, we obtain a single stranded polynucleotide having, in the 5' to 3' orientation, a primer site (e.g., for Roche 454 sequencing), an MID, a reflex sequence and the polynucleotide to be sequenced. Numerous methods for obtaining single-stranded polynucleotides of interest have been described and are known in the art, including in U.S.

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Patent 7,217,522, issued on May 15, 2007; U.S. Patent Application 11/377,462, filed on March 16, 2006; and U.S. Patent Application 12/432,080, filed on April 29, 2009; each of which is incorporated by reference herein in their entirety. For example, a single stranded product can be produced using linear amplification with a primer specific for the primer site of the template. In certain embodiments, the primer includes a binding moiety to facilitate isolation of the single stranded nucleic acid of interest, e.g., to immobilize the top strand on a binding partner of the binding moiety immobilized on a solid support. Removal of a hybridized, non-biotinylated strand by denaturation using heat or high pH (or any other convenient method) serves to isolate the biotinylated strand. Binding moieties and their corresponding binding partners are sometimes referred to herein as binding partner pairs. Any convenient binding partner pairs may be used, including but not limited to biotin/avidin (or streptavidin), antigen/antibody pairs, etc.

It is noted here that while the figures and description of the reflex process provided herein depict manipulations with regard to a single stranded polynucleotide, it is not necessarily required that the single stranded polynucleotide described or depicted in the figures be present in the sample in an isolated form (i.e., isolated from its complementary strand). In other words, double stranded polynucleotides may be used where only one strand is described/depicted, which will generally be determined by the user.

The implementation of a single strand isolation step using the methods described above or variations thereof (or any other convenient single strand isolation step) will generally be based on the desires of the user. One example of isolating single stranded polynucleotides is shown in Figure 2. In this Figure, a starting double stranded template (with 5' to 3' orientation shown as an arrow) is denatured and primed with a biotinylated synthesis primer specific for the primer site. After extension of the primer (i.e., nucleic acid synthesis), the sample is contacted with a solid support having streptavidin bound to it. The biotin moiety (i.e., the binding partner of streptavidin) on the extended strands will bind to the solid-phase streptavidin. Denaturation and washing is then performed to remove all non-biotinylated polynucleotide strands. If desired, the bound polynucleotide, which can be used in subsequent reflex process steps (e.g., as a template for B_n primer extension reactions), may be eluted from the streptavidin support. Alternatively, the bound polynucleotide may be employed in subsequent steps of the desired process while still bound to the solid support (e.g., in solid phase extension reactions using B_n primers). This process, with minor variations depending on the template being used and the identity of the desired single stranded polynucleotide, may be employed at any of a number of steps in which a single

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stranded product is to be isolated. It is noted that in certain embodiments, substrate bound biotinylated polynucleotide can be used to produce and isolate non-biotinylated single stranded products (i.e., by eluting the non-biotinylated products while leaving the biotinylated templates bound to the streptavidin on the solid support). Thus, the specifics of how binding partners are used to isolate single stranded polynucleotides of interest will vary depending on experimental design parameters.

Additional single-stranded isolation/production methods include asymmetric PCR, strand-specific enzymatic degradation, and the use of in-vitro transcription followed by reverse transcriptase (IVT-RT) with subsequent destruction of the RNA strand. As noted above, any convenient single stranded production/isolation method may be employed.

To the single stranded polynucleotide shown in Figure 1B we anneal one of the B_n primers having the appended reflex sequence, denoted with a capital "R" (e.g., B_1) and extend the primer under nucleic acid synthesis conditions to produce a copy of the polynucleotide that has a reflex sequence at its 5' end. A single stranded copy of this polynucleotide is then produced to reverse polarity using a primer specific for the primer site in the first domain' (complement of the first domain 102). The resulting nucleic acid has structure 100 shown in Figure 1A, where the first domain 102 includes the primer site and the MID. Site A (110) in Figure 1 is determined by the specificity of the 5' reflex-B_n primer used.

The reflex process (e.g., as shown in Figure 1) is then performed to produce a product in which the primer site and the MID are now in close proximity to the desired site (or region of interest (ROI)) within the original polynucleotide (i.e., the site defined by the primer used, e.g., B_1). The resulting polynucleotide can be used in subsequent analyses as desired by the user (e.g., Roche 454 sequencing technology).

It is noted here that, while not shown in Figure 1A and 1B, any convenient method for adding adapters to a polynucleotide to be processed as described herein may be used in the practice of the reflex process (adapters containing, e.g., primer sites, polymerase sites, MIDs, restriction enzyme sites, and reflex sequences). For example, adapters can be added at a particular position by ligation. For double stranded polynucleotides, an adapter can be configured to be ligated to a particular restriction enzyme cut site. Where a single stranded polynucleotide is employed, a double stranded adapter construct that possesses an overhang configured to bind to the end of the single-stranded polynucleotide can be used. For example, in the latter case, the end of a single stranded polynucleotide can be modified to include specific nucleotide bases that are complementary to the overhang in the double

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stranded adaptor using terminal transferase and specific nucleotides. In other embodiments, PCR or linear amplification methods using adapter-conjugated primers is employed to add an adapter at a site of interest. Again, any convenient method for producing a starting polynucleotide may be employed in practicing the methods of the subject invention.

In certain embodiments, the nucleic acid may be sequenced directly using a sequencing primer specific for the primer site. This sequencing reaction will read through the MID and desired site in the insert.

In certain embodiments, the polynucleotide may be isolated (or fractionated) using an appropriate A_n primer (e.g., when using B_1 as the first primer, primer A_1 can be used). In certain embodiments, the A_n primed polynucleotide is subjected to nucleic acid synthesis conditions to produce a copy of the fragment produced in the reflex process. In certain of these embodiments, the A_n primer has appended on its 5' end a primer site that can be used in subsequent steps, including sequencing reactions. Providing a primer site in the A_n primer allows amplifying and/or sequencing from both ends of the resultant fragment: from the primer site in the first domain 102 and the primer site in the A_n primer (not shown in Figure 1B). Because of the position of the primer sites and their distance apart (i.e., less than one sequencing run apart), sequencing from both ends will usually capture the sequence of the desired site (or ROI) and the sequence of the MID, which can be used for subsequent bioinformatic analyses, e.g., to positively identify the sample of origin. It is noted here that while sequencing in both directions is possible, it is not necessary, as sequencing from either primer site alone will capture the sequence of the ROI as well as its corresponding MID sequence.

Note that in certain embodiments, the first fragment obtained by amplification/extension from primer B_0 directly, the polarity of the ROI in the resulting fragment is reversed as compared to the ROI in fragments obtained by primers B_1-B_n. This is because the B_0-generated fragment, unlike the B_1-B_n generated fragments, has not been subjected to a reflex process which reverses the orientation of the ROI sequence with respect to the first domain/reflex sequence (as described above). Therefore, the B_0 primer may have appended to it a primer site (e.g., at its 5' end) that can be used for subsequent amplification and/or sequencing reactions (e.g., in Roche 454 sequencing system) rather than a reflex sequence as with primers B_1-B_n. However, in certain embodiments, as noted above, the reflex process may be used with a corresponding B_0-A_0 primer pair as described above, i.e., using a B_0 primer having a 5' reflex sequence and a corresponding A_0 primer with its corresponding 5' adapter domain (e.g., a primer site).

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It is noted here that because the particular sections of sequence to be analyzed are defined by the A_n-B_n primer pairs (as shown and described above), a much higher sequence specificity is achieved as compared to using previous extraction methods that employ only a single oligo binding event (e.g., using probes on a microarray).

Figure 3 provides a detailed flow chart for an exemplary embodiment that employs reflex sequences for use in sequencing multiple specific regions in a polynucleotide (i.e., regions 1, 2, 3, 4 and 5 in an 11kb region of lambda DNA).

A single parent DNA fragment 202 is generated that includes adapter domains (i.e., a Roche 454 sequencing primer site, a single MID, and a reflex sequence) and the sequence of interest. In the example shown, the sequence of interest is from lambda DNA and the reflex sequence is present on the top strand (with its complement shown in the bottom strand). Any convenient method for producing this parent DNA fragment may be used, including amplification with a primer that includes the adapter domains (e.g., using PCR), cloning the fragment into a vector that includes the adapter domains (e.g., a vector with the adapter domains adjacent to a cloning site), or by attaching adapters to polynucleotide fragments (e.g., fragment made by random fragmentation, by sequence-specific restriction enzyme digestion, or combinations thereof). While only a single fragment with a single MID is shown, the steps in Figure 3 are applicable to samples having multiple different fragments each with a different MID, e.g., a sample having a population of homologous fragments from any number of different sources (e.g., different individuals). Figure 3 describes the subsequent enzymatic steps involved in creating the five daughter fragments in which regions 1, 2, 3, 4 and 5 (shown in polynucleotide 204) are rearranged to be placed within a functional distance of the adapter domains (i.e., close enough to the adapter domains to be sequenced in a single Roche 454 sequencing reaction). Note that certain steps are shown for region 4 only (206).

In step 1, the five regions of interest are defined within the parent fragment (labeled 1 to 5 in polynucleotide 204) and corresponding primer pairs are designed for each. The distance of each region of interest from the reflex sequence is shown below polynucleotide 204. The primer pairs are designed as described and shown in Figure 1B (i.e., the A_n-B_n primer pairs). For clarity, only primer sites for region 4 are shown in Figure 3 ("primer sites" surrounding region 4). In step 2, sequence specific primer extensions are performed (only region 4 is shown) with corresponding B_n primers to produce single stranded polynucleotides having structure 208 (i.e., having the reflex sequence on the 5' terminus). As shown, the B_n primer for region 4 will include a sequence specific primer site that primes

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at the 3'-most primer site noted for region 4 (where "3'-most" refers to the template strand, which in Figure 3 is the top strand). This polynucleotide is copied back to produce polynucleotide 210 having reversed polarity (e.g., copied using a primer that hybridizes to the 454A' domain). Polynucleotide 210 has structure similar to polynucleotide 100 shown at the top of Figure 1. Step 4 depicts the result of the intramolecular priming between the reflex sequence and its complement followed by extension to produce the MID' and 454A' structures at the 3' end (polynucleotide 212). In the embodiments shown in Figure 3, polynucleotide 212 is treated with T7 exonuclease to remove double stranded DNA from the 5' end (as indicated above, this step is optional). The polynucleotide formed for region 4 is shown as 216 with polynucleotides for the other regions also shown (214).

It is noted here that the formation of each of the polynucleotides 214 may be accomplished either in separate reactions (i.e., structure with region 1 in proximity to the adapter domains is in a first sample, the structure with region 2 in proximity to the adapter region is in a second sample, etc.) or in one or more combined sample.

In step 6 the polynucleotides 214 are copied to reverse polarity to form polynucleotides 218. In step 7, each of these products are then primed with the second primer of the specific primer pair (see A_3 primers as shown in Figure 1B) each having a second Roche 454 primer site (454B) attached at the 5' end, and extended to form products 220. Steps 6 and 7 may be combined (e.g., in a single PCR or other amplification reaction).

In summary, Figure 3 shows how the reflex process can be employed to produce five daughter fragments 220 of similar length (e.g., ~500 bp) each of which contain DNA sequences that differ in their distance from the reflex sequence in the starting structure 202 while maintaining the original MID.

Figure 4 shows another exemplary use of the reflex process as described herein. In the embodiment shown in Figure 4, a target sequence (i.e., containing region of interest "E") is enriched from a pool of adapter-attached fragments. In certain embodiments, the fragments are randomly sheared, selected for a certain size range (e.g., DNA having a length from 100 to 5000 base pairs), and tagged with adapters (e.g., asymmetric adapters, e.g., as described in US Patent Application Ser. No. 12/432,080, filed on April 29, 2009). The asymmetric adaptor employed in Figure 4 contains a sequencing primer site (454A, as used in the Roche 454 sequencing platform), an MID, an X sequence, and an internal stem region (ISR), which denotes the region of complementarity for the asymmetric adapter that is adjacent to the adapter attachment site (see, e.g., the description in US Application Ser. No. 12/432,080, filed on April 29, 2009, incorporated herein by reference in its entirety). The X

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sequence can be any sequence that can serve as a binding site for a polynucleotide containing the complement of the X sequence (similar to a primer site). As described below, the X sequence allows for the annealing of an oligonucleotide having a 5' overhang that can serve as a template for extension of the 3' end of the adaptor oligonucleotide. The sequencing direction of the sequencing primer site (454A primer site in structure 401 of Figure 4) is oriented such that amplification of the adapter ligated fragment using the sequencing primer site proceeds away from the ligated genomic insert. This has the effect of making the initial asymmetric adapter ligated library 'inert' to amplification using this primer, e.g., in a PCR reaction.

To extract a region of interest (the "E" region), the library is mixed with an oligonucleotide (403) containing a 3' X' sequence and a target specific priming sequence (the 1' sequence) under hybridization/annealing conditions. The target specific sequence 1' is designed to flank one side of the region of interest (the 1' sequence adjacent to E in the genomic insert; note that only the E-containing polynucleotide fragment is shown in Figure 4), much like a PCR primer. After annealing primer 403, the hybridized complex is extended, whereby all of the adaptor tagged fragments will obtain the complement of the target specific sequence (i.e., the 1 sequence) on the 3' end (see structure 405; arrows denote the direction of extension).

Extended products 405 are then denatured and the 1/1' regions allowed to hybridize intramolecularly in a reflex process priming event, after which nucleic acid extension is performed to form structure 407 (extension is from the 1 priming site; shown with an arrow). This reflex reaction creates a product (407) that, unlike its parent structure (405), has a sequencing primer site (454A) that is oriented such the extension using this primer sequence proceeds towards the region of interest. Thus, in the absence of a priming and extension reflex reaction, extension with a sequencing primer will not generate a product containing the region of interest (the E region). In other words, only E-region containing target polynucleotides will have a 454A sequence that can amplify genomic material (structure 407).

After completing the reflex process (using 1/1' as the reflex sequences), a PCR amplification reaction is performed to amplify the region of interest (with associated adapter domains). However, before performing the PCR reaction, the fragment sample is "inactivated" from further extension using terminal transferase and ddNTPs. This inactivation prevents non-target adaptor tagged molecules from performing primer extension from the 3' primer 1 site. Once inactivated, a PCR reaction is performed using a sequencing

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primer (i.e., 454A primer 409) and a second primer that primes and extends from the opposite side of the region of interest (i.e., primer 411, which includes a 5' 454B sequencing primer site and a 3' "2" region that primes on the opposite end of E from the 1 region). Only fragments that have undergone the reflex process and contain the E region will be suitable templates for the PCR reaction and produce the desired product (413).

Thus, the process exemplified in Figure 4 allows for the movement of an adapter domain (e.g., containing functional elements and/or MID) into proximity to a desired region of interest.

The reflex process described herein can be used to perform powerful linkage analysis by combining it with nucleic acid counting methods. Any convenient method for tagging and /or counting individual nucleic acid molecules with unique tags may be employed (see, e.g., U.S. Patent 7,537,897, issued on May 26, 2009; U.S. Patent 7,217,522, issued on May 15, 2007; U.S. Patent Application 11/377,462, filed on March 16, 2006; and U.S. Patent Application 12/432,080, filed on April 29, 2009; each of which is incorporated by reference herein in their). All of this can be conducted in parallel thus saving on the cost of labor, time and materials.

In one exemplary embodiment, a large collection of sequences is tagged with MID such that each polynucleotide molecule in the sample has a unique MID. In other words, each polynucleotide in the sample (e.g., each individual double stranded or single stranded polynucleotide) is tagged with a MID that is different from every other MID on every other polynucleotide in the sample. In general, to accomplish such molecular tagging the number of distinct MID tags to be used should be many times greater than the actual number of molecules to be analyzed. This will result in the majority of individual nucleic acid molecules being labeled with a unique ID tag (see, e.g., Brenner et al., Proc. Natl. Acad. Sci. USA. 2000 97(4):1665-70). Any sequences that then result from the reflex process on that particular molecule (e.g., as described above) will thus be labeled with the same unique MID tag and thus inherently linked. Note that once all molecules in a sample are individually tagged, they can be manipulated and amplified as much as needed for processing so long as the MID tag is maintained in the products generated.

For example, we might want to sequence one thousand viral genomes (or a specific genomic region) or one thousand copies of a gene present in somatic cells. After tagging each polynucleotide in the sample with a sequencing primer site, MID and reflex sequence (as shown in the figures and described above), we use the reflex process to break each polynucleotide into lengths appropriate to the sequencing procedure being used, transferring

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the sequencing primer site and MID to each fragment (as described above). Obtaining sequence information from all of the reflex-processed samples can be used to determine the sequence of each individual polynucleotide in the starting sample, using the MID sequence to defining linkage relationships between sequences from different regions in the polynucleotide being sequenced. Using a sequencing platform with longer read lengths can minimize the number of primers to be used (and reflex fragments generated).

The advantages noted above are shown in Figure 5. This figure shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information. It is noted that not all domain structures and steps are shown in the reflex process for simplicity.

Kits and Systems

Also provided by the subject invention are kits and systems for practicing the subject methods, as described above, such vectors configured to add reflex sequences to nucleic acid inserts of interest and regents for performing any steps in the cloning or reflex process described herein (e.g., restriction enzymes, nucleotides, polymerases, primers, exonucleases, etc.). The various components of the kits may be present in separate containers or certain compatible components may be precombined into a single container, as desired.

The subject systems and kits may also include one or more other reagents for preparing or processing a nucleic acid sample according to the subject methods. The reagents may include one or more matrices, solvents, sample preparation reagents, buffers, desalting reagents, enzymatic reagents, denaturing reagents, where calibration standards such as positive and negative controls may be provided as well. As such, the kits may include one or more containers such as vials or bottles, with each container containing a separate component for carrying out a sample processing or preparing step and/or for carrying out one or more steps of a nucleic acid variant isolation assay according to the present invention.

In addition to above-mentioned components, the subject kits typically further include instructions for using the components of the kit to practice the subject methods, e.g., to prepare nucleic acid samples for perform the reflex process according to aspects of the

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subject methods. The instructions for practicing the subject methods are generally recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or sub-packaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g. CD-ROM, diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

In addition to the subject database, programming and instructions, the kits may also include one or more control samples and reagents, e.g., two or more control samples for use in testing the kit.

Utility

The reflex process described herein provides significant advantages in numerous applications, a few of which are noted below (as well as described above).

For example, as described above, certain aspects of the reflex process define the particular sections of sequence to be analyzed by a primer pair, as in PCR (e.g., the two oligos shown as A_n-B_n in Figure 1B). This results in higher sequence specificity as compared to other extraction methods (e.g., using probes on a microarray) that only use a single oligo sequence. The separation of the probes defines a length that can be relatively uniform (hence making subsequent handling including amplification more uniform) and can also be tailored to the particular sequencing platform being employed.

Further, as described above, aspects of the present invention can be used to analyze homologous genomic locations in a multiplexed sample (i.e., a sample having polynucleotides from different genomic samples) in which the polynucleotides are tagged with the MID. This is possible because the reflex process, which operates intramolecularly, maintains the MID thus linking any particular fragment to the sample from which it originates.

Finally, as the reflex processes described herein function intramolecularly, one can determine the genetic linkage between different regions on the same large fragment that are too far apart to be sequenced in one sequence read. Such a determination of linkage may be of great value in plant or animal genetics (e.g., to decide if a particular set of variations are

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linked together on the same stretch of chromosome) or in viral studies (e.g., to determine if particular variations are linked together on the same stretch of a viral genome/transcripts, e.g., HIV, hepatitis virus, etc.).

EXAMPLES

Example 1

Figures 6 and 7 provide experimental data and validation of the reflex process described herein using synthetic polynucleotide substrates.

METHODS

Substrate:

The 100 base oligonucleotide substrate (as shown diagrammatically in Figure 6A) was synthesized with internal fluorescein-dT positioned between the REFLEX and REFLEX' sequences. This label provides convenient and sensitive method of detection of oligonucleotide species using polyacrylamide gel electrophoresis.

Extension reactions:

Reactions were prepared which contained 1 µM of the 100 base oligonucleotide substrate, 200 µM dNTPs, presence or absence of 1 µM competitor oligonucleotide, 0.5 µl of each DNA polymerase ("DNAP"): Vent (NEB, 2 units/µl), Taq (Qiagen HotStarTaq 5 units/µl) and Herculase (Stratagene), and made up to 50 µl with the appropriate commercial buffers for each polymerase and dH_20. For Taq titrations 0.5 µl, 1 µl, 2 µl, and 3 µl enzyme was used in the same 50 µl volume. Reactions were heated in a Biometra thermocycler to 95 °C for 15 minutes (Taq) or 5 minutes (Herculase, Vent), followed by 55 °C or 50 °C for 30 seconds, and a final incubation at 72 °C for 10 minutes.

T7 exonuclease digestions:

Reactions were prepared with 10 µl extension reactions above, 0.5 µl T7 exonuclease (NEB, 10 units/µl), and made up to 50 µl using NEB Buffer 4 and dH_20. Reactions were incubated at 25 °C for 30 minutes.

Gel Electrophoresis analysis:

An 8% denaturing polyacrylamide gel was used to analyze reaction species. 0.4 µl of extension reactions, and 2 µl of digestion reactions were loaded and run at 800V for ~1.5 hours. Gels were analyzed for fluorescein using an Amersham/General Electric Typhoon imager.

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RESULTS

Figure 6A shows the structure of each stage of reflex sequence processing with the expected nucleic acid size shown on the left. The initial single stranded nucleic acid having a sequencing primer site (the Roche 454 sequencing primer A site; listed as 454A); an MID; a reflex sequence; the insert; and a complement of the reflex sequence is 100 nucleotides in length. After self-annealing and extension, the product is expected to be 130 nucleotides in length. After removal of the double stranded region from the 5' end, the nucleic acid is expected to be 82 bases in length.

Figure 6B shows the results of three experiments using three different nucleic acid polymerases (Vent, Herculase and Taq, indicated at the top of the lanes). The temperature at which the annealing was carried out is shown at the top of each lane (either 50°C or 55°C). The sizes of the three nucleic acids as noted above are indicated on the left and right side of the gel.

As shown in figure 6B, extension appears to be most efficient under the conditions used with Herculase (Herculase is a mixture of two enzymes: modified Pfu DNAP and Archaemax (dUTPase)). Most (or all) of the initial 100 base pair nucleic acid are converted to the 130 base pair product (see lanes 6 and 7). However, after T7 exonuclease digestion the 3'-5' exonuclease activity of Herculase results in partial digestion of the desired 82 base product (note bands at and below the 82 base pairs in lanes 8 and 9).

Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product after T7 exonuclease digestion (see lane 13).

Figure 7 shows the effect on the reflex process of increasing amounts of Taq polymerase as well as the use of a reflex sequence competitor (schematically shown in Figure 7A).

As shown in lanes 2 to 5, increased Taq concentration improves extension to ~90% conversion of the starting nucleic acid (see lane 5). Lanes 7 to 8 show that T7 exonuclease digestion does not leave a perfect 82 base product. This may be due to collapse of dsDNA when T7 exonuclease has nearly completed its digestion from the 5' end in the double stranded region of the fold-back structure. It is noted that in many embodiments, the removal of a few additional bases from the 5' end of the polynucleotide will not interfere with subsequent analyses, as nucleotide bases at the 5' end are often removed during subsequent steps.

As shown in Lanes 11-14, addition of a competitor (which can interfere with annealing of the reflex sequences to form the fold-back structure) results in only a small

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decrease (~5-10%) of fully extended product. Thus, as expected, the intramolecular reaction is heavily favored. Although not shown, we have observed that the competitor oligonucleotide also gets extended by the same amount (~5-10%).

The concentration of the competitor, the concentration of the reflex substrate, and the overall genetic complexity, will all likely affect specific results. The experiments shown in Figures 6 and 7 demonstrate that the core parts of the reflex processes as described herein is functional and can be implemented.

Example II

Figure 8 shows the reflex workflow (diagram at left) and exemplary results of the workflow (gel at right) for a specific region of interest (ROI). The starting material is a double stranded nucleic acid molecule (700) that contains a 454A primer site, an MID, a reflex site, and a polynucleotide of interest having three ROIs (2, 3 and 4) at different locations therein. This starting material was subjected to reflex processes (as described in above) specific for ROI 2 as shown in the diagram at the left of the figure, both with and without the use of a T7 exonuclease step (the T7 exonuclease step is shown in the diagram is indicated as "Optional").

Completion of all steps shown in the reflex process should result in a double stranded polynucleotide of 488 base pairs (702) with or without the T7 exonuclease step.

As shown in the gel on the right of Figure 8, the 488 base pair product was produced in reflex processes with and without the T7 exonuclease step.

Figure 9 shows an exemplary protocol for a reflex process based on the results discussed above. The diagram shows specific reflex process steps with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt SPRI beads to remove primer oligos). One reason for performing such purification steps is to reduce the potential for generating side products in a reaction (e.g., undesirable amplicons). While Figure 9 indicates three purification steps, fewer or additional purification steps may be employed depending on the desires of the user. It is noted that the steps of reversing polarity, reflex priming and extension, and "stretch out" (or denaturation) / second reversing polarity step can be performed without intervening purification steps.

The protocol shown in Figure 9 includes the following steps:

annealing a first primer containing a 5' reflex sequence (or reflex tail, as noted in the figure) specific for the 3' primer site for the R' region to the starting polynucleotide and extending (the primer anneals to the top strand at the primer site at the right of R in

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polynucleotide 902, indicated with a *; this step represents the first denature, anneal and extend process indicated on the right);

after purification, adding a 454A primer and performing three cycles of denaturing, annealing and extending: the first cycle results in the copy-back from the 454A primer to reverse the polarity of the strand just synthesized; the second cycle breaks apart the double stranded structure produced, allows the reflex structure to form and then extend; the third cycle results in another copy-back using the same 454A primer originally added;

after purification, adding a second primer specific for the second primer site for the R' region having a 5' 454B tail (this primer anneals to the primer site 3' of the R' region in polynucleotide 904, indicated with a *) and denaturing, annealing and extending resulting in a polynucleotide product having 454A and 454B sites surrounding the MID, the reflex sequence, and R'. Note that the first primer specific for the R' region and the second primer specific for the R' region define its boundaries, as described above and depicted in Figure 1B);

after another purification, adding 454A and 454B primers and performing a PCR amplification reaction.

Example III

As described above, a reflex sequence can be an "artificial" sequence added to a polynucleotide as part of an adapter or can be based on a sequence present in the polynucleotide of interest being analyzed, e.g., a genomic sequence (or "non-artificial").

The data shown in prior Examples used "artificial" reflex sites. In this Example, the reflex site is a genomic sequence present in the polynucleotide being analyzed.

The starting material is a double stranded DNA containing a 454A site, an MID and a polynucleotide to be analyzed. The 454A and MID were added by adapter ligation to parent polynucleotide fragments followed by enrichment of the polynucleotide to be analyzed by a hybridization-based pull-out reaction and subsequent secondary PCR amplification (see Route 1 in Figure 13). Thus, the reflex site employed in this example is a sequence normally present at the 5' end of the subject polynucleotide (a genomic sequence). The polynucleotide being analyzed includes a region of interest distal to the 454A and MID sequences that is 354 base pairs in length.

This starting double stranded nucleic acid is 755 base pairs in length. Based on the length of each of the relevant domains in this starting nucleic acid, the reflex process should result in a product of 461 base pairs.

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Figure 10 shows the starting material for the reflex process (left panel) and the resultant product generated using the reflex process (right panel; reflex process was performed as described in Example II, without using a T7 exonuclease step). A size ladder is included in the left hand lane of each gel to allow estimation of the size of the test material. This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in moving an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

Example IV

Figure 11 shows a schematic of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate 5 different products ("daughter" products) each having a different region of interest (i.e., daughter products are produced having either region 1, 2, 3, 4 or 5). The schematic in Figure 11 shows the starting fragment (11,060 base pairs) and resulting products (each 488 base pairs) generated from each of the different region of interest-specific reflex reactions (reflex reactions are performed as described above). The panel (gel) on the bottom of Figure 11 shows the larger starting fragment (Lane 1) and the resulting daughter products for each region-specific reflex reaction (lanes 2 to 6, with the region of interest noted in each in the box), where the starting and daughter fragments have the expected lengths. Sequencing of the products confirmed the identity of the region of interest in each of the reflex products shown in the gel. These results demonstrate that multiple different reflex products can be generated from a single, asymmetrically tagged parent fragment while maintaining the adapter domains (e.g., the primer sites and MID).

Example V

Figure 12 details experiments performed to determine the prevalence of intramolecular rearrangement (as desired in the reflex process) vs. intermolecular rearrangement. Intermolecular rearrangement is undesirable because it can lead to the transfer of an MID from one fragment to another (also called MID switching). MID switching can occur if a reflex sequence in a first fragment hybridizes to its complement in a second fragment during the reflex process, leading to appending the MID from the second fragment to the first fragment. Thus, intermolecular rearrangement, or MID switching, should be minimized to prevent the transfer of an MID from one fragment in the sample to another, which could lead to a misrepresentation of the source of a fragment.

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To measure the prevalence of MID switching under different reflex conditions, fragments having different sizes were generated that included two different MIDs, as shown in the top panel of Figure 12. The common sequence on these fragments serves as the priming site for the first extension reaction to add the second reflex sequence (see, e.g., step 2 of Figure 3). Three exemplary fragments are shown in Figure 12 for each different fragment size (i.e., 800 base pairs with an MIDB and MIDA combination; 1900 base pairs with MIDC and MIDA combination; and 3000 base pairs with MIDD and MIDA combination). For each MID family (A, B, C and D), there are 10 different members (i.e., MIDA had 10 different members, MIDB has 10 different members, etc.). A set of 10 dual MID fragments for each different size fragment (i.e., 800, 1900 and 3000 base pairs) were generated, where the MID pairs (i.e., MIDA/MIDB, MIDA/MIDC, and MIDA/MIDD) were designated as 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10. All 10 fragments of the same size were then mixed together and a reflex protocol was performed.

Due to the domain structure of the fragments, a successful reflex process results in the two MIDs for each fragment being moved to within close enough proximity to be sequenced in a single read using the Roche 454 sequencing platform (see the reflex products shown in the schematic of Figure 12). The reflex reactions for each fragment size were performed at four different fragment concentrations to determine the effect of this parameter, as well as fragment length, in the prevalence of MID switching. The reflex products from each reaction performed were subjected to 454 sequencing to determine the identity of both MIDs on each fragment, and thereby the proportion of MID switching that occurred.

The panel on the bottom left of Figure 12 shows the rate of MID switching (Y axis, shown in % incorrect (or switched) MID pair) for each different length fragment at each different concentration (X axis; 300, 30, 3 and 0.3 nM). As shown in this panel, the MID switch rate decreases with lower concentrations, as would be expected, because intermolecular, as opposed to intramolecular, binding events are concentration dependent (i.e., lower concentrations lead to reduced intermolecular hybridization/binding). In addition, the MID switch rate decreases slightly with length. This is somewhat unexpected as the ends of longer DNA fragments are effectively at a lower concentration with respect to one another. The reasons for why we do not see this is probably because the production of reflex priming intermediates continues during the final PCR, which means that reflex priming reactions are happening continuously which contributes to MID switching. It is probably the case that the shorter reflex products are able to undergo a higher rate of 'background' reflexing, and therefore increase the overall MID switch rate a little.

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These results demonstrate that MID switching can be minimized (e.g., to below 2%, below 1% or even to nearly undetectable levels) by altering certain parameters of the reaction, e.g., by reducing fragment concentration and/or fragment length.

The panel on the bottom right of Figure 12 shows the frequency of MID switching in the reflex process for the 800 base pair fragments (i.e., MIDA/MIDB containing fragments). In this figure, the area of each circle is proportional to the number of reads containing the corresponding MIDA and MIDB species (e.g., MIDA1/MIDB1; MIDA1/MIDB2; etc.). Thus, a circle representing 200 reads will be 40 times larger in terms of area than a circle representing 5 reads.

As noted above, the MIDA/MIDB combinations having the same number (shown on the X and Y axis, respectively) represent the MIDA/MIDB combinations present in the sample prior to the reflex process being performed (i.e., MIDA/MIDB combinations 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10 were present in the starting sample). All other MIDA/MIDB combinations identified by Roche 454 sequencing were the result of MID switching.

This figure shows that the MID switching that occurs during the reflex process is random, i.e., that MID switching is not skewed based on the identity of the MIDs in the reaction).

Exemplary Reflex Protocols

Figure 13 shows a diagram of exemplary protocols for performing the reflex process on pools of nucleic acids, for example, pools of nucleic acids from different individuals, each of which are labeled with a unique MID. In Route 3, a pooled and tagged extended library is subjected directly to a reflex process. In Route 2, the pooled library is enriched by target-specific hybridization followed by performing the reflex process. In Route 1 employs enrichment by PCR amplification. As shown in Figure 13, PCR enrichment can be performed directly on the pooled tagged extended library or in a secondary PCR reaction after a hybridization-based enrichment step has been performed (as in Route 2) to generate an amplicon substrate that is suitable for the reflex process. Additional routes for preparing a polynucleotide sample for performing a reflex process can be implemented (e.g., having additional amplification, purification, and/or enrichment steps), which will generally be dependent on the desires of the user.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes

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and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

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CLAIMS

That which is claimed is:

1. A method of moving a domain in a nucleic acid, the method comprising:

a) annealing a reflex sequence in a polynucleotide strand in a sample with a complement of the reflex sequence present in the polynucleotide strand, wherein the polynucleotide strand comprises the following in a 5' to 3' orientation:

a first domain;

the reflex sequence;

a polynucleotide of interest; and

the complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand; and

b) extending from the 3' end of the complement of the reflex sequence through the first domain to produce a double stranded region;

whereby a complement of the first domain is present at the 3' end of the extended polynucleotide strand, thereby moving the first domain in the polynucleotide strand.

2. The method of claim 1, wherein the first domain comprises a multiplex identifier (MID).

3. The method of claim 1, wherein the first domain comprises a primer site.

4. The method of claim 1, wherein the reflex sequence is from 10 to 50 nucleotides in length.

5. The method of claim 1, wherein the reflex sequence comprises an RNA polymerase site.

6. The method of claim 1, the method further comprising removing the first domain and the reflex sequence from the 5' end of the double stranded region.

7. The method of claim 6, wherein the removing step comprises treating the nucleic acid of step b with T7 exonuclease.

8. The method of claim 1, wherein the first domain is present in an adapter region attached to the polynucleotide of interest.

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9. The method of claim 8, wherein the reflex sequence is present in the adapter region.

10. The method of claim 8, wherein the reflex sequence is a sequence present within the 5' region of the polynucleotide of interest.

11. The method of claim 1, wherein the sample is a multiplexed sample, wherein the first domain in each of the polynucleotide strands in the multiplexed sample comprises a MID corresponding to its parental sample.

12. The method of claim 1, wherein the sample comprises multiple polynucleotide strands, and wherein each polynucleotide strand in the sample comprises a unique MID in the first domain.

13. The method of claim 1, wherein the polynucleotide strand is produced by:

annealing a nucleic acid synthesis primer to a parent polynucleotide strand, wherein the parent polynucleotide strand comprises the following in a 5' to 3' orientation:

the first domain;

the reflex sequence; and

a second domain comprising the polynucleotide of interest;

wherein the nucleic acid synthesis primer comprises:

a 3' priming domain that is complementary to a site adjacent to and 3' of the polynucleotide of interest in the second domain; and

a 5' reflex sequence;

extending the nucleic acid synthesis primer to produce an extension product; and

replicating the resultant extension product with a second nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain, thereby producing the polynucleotide strand.

14. The method of claim 13, wherein the length of the second domain is from 30 bases to 30 kilobases.

15. The method of claim 13, wherein the parent polynucleotide strand is enriched prior to the annealing step based on its sequence.

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16. The method of claim 1, further comprising the steps of:

c) replicating the polynucleotide strand of step b with a first nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain to produce a first extension product;

d) annealing a second nucleic acid synthesis primer to the first extension product, wherein the second nucleic acid synthesis primer comprises a 3' priming domain that is complementary to a site within the polynucleotide of interest in the first extension product; and

e) extending the second synthesis primer to produce a second extension product.

17. The method of claim 16, wherein the second synthesis primer comprises a 5' domain that is not complementary to the polynucleotide of interest in the first extension product.

18. The method of claim 17, wherein the first domain comprises a first primer site and the 5' domain of the second synthesis primer comprises a second primer site.

19. The method of claim 18, wherein the length of the polynucleotide of interest in the second extension product is from 50 to 10,000 nucleotides.

20. A polynucleotide strand comprising the following in a 5' to 3' orientation:

a first domain;

a reflex sequence;

a polynucleotide of interest; and

a complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand.

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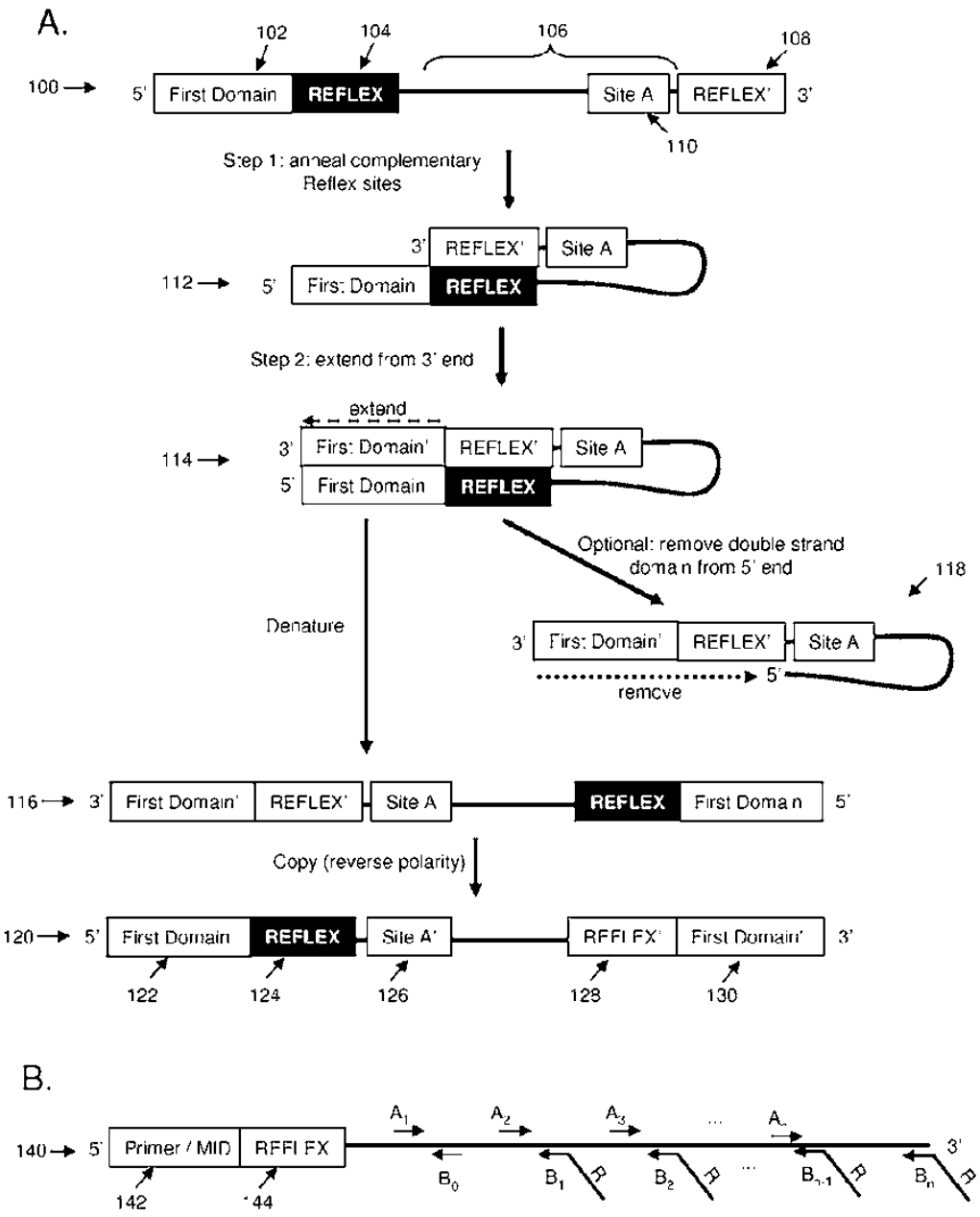

A.
102
104
106
108
100
5'
First Domain
REFLEX
Site A
REFLEX'
3'
110
Step 1: anneal complementary Reflex sites
3'
REFLEX'
Site A
112
5'
First Domain
REFLEX
Step 2: extend from 3' end
extend
114
3'
First Domain'
REFLEX'
Site A
5'
First Domain
REFLEX
Optional: remove double strand domain from 5' end
118
Denature
3'
First Domain'
REFLEX'
Site A
5'
remove
116
3'
First Domain'
REFLEX'
Site A
REFLEX
First Domain
5'
Copy (reverse polarity)
120
5'
First Domain
REFLEX
Site A'
RFFI FX'
First Domain'
3'
122
124
126
128
130
B.
140
5'
Primer / MID
RFFI FX
142
144
A1
A2
A3
...
An
B0
B1
R
B2
R
...
Bn-1
R
Bn
R
3'

Fig. 1

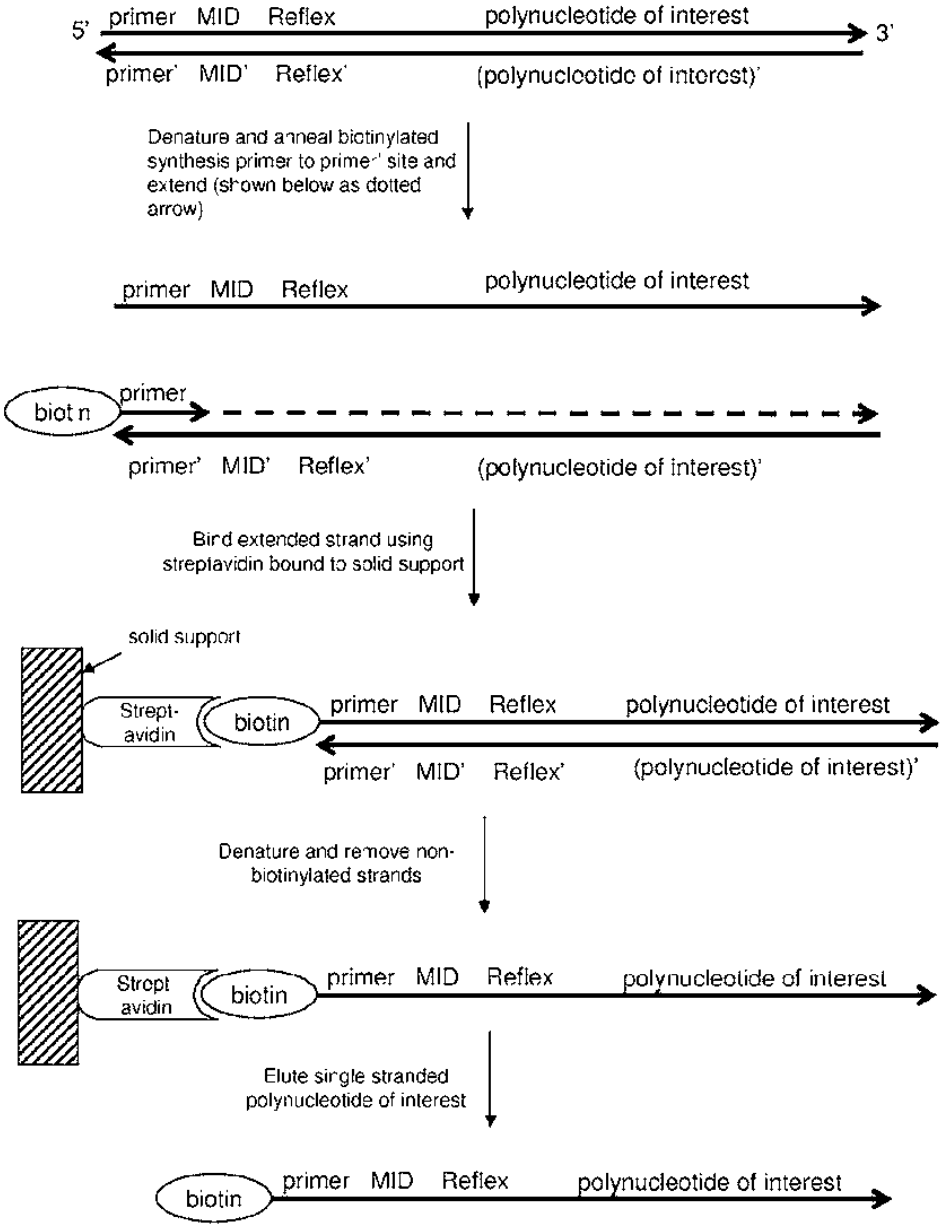
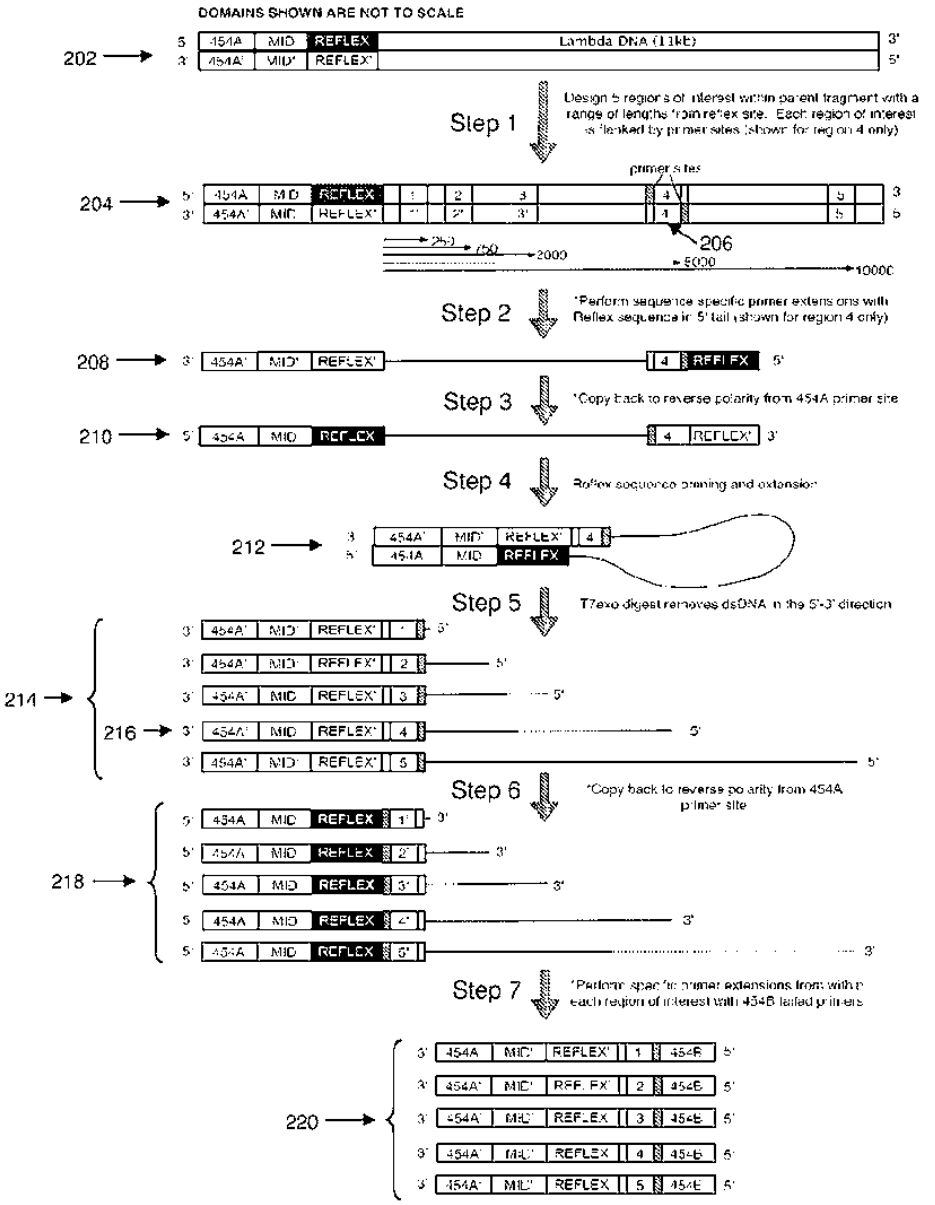

5' primer MID Reflex polynucleotide of interest 3'
primer' MID' Reflex' (polynucleotide of interest)'
Denature and anneal biotinylated synthesis primer to primer' site and extend (shown below as dotted arrow)
primer MID Reflex polynucleotide of interest
biotin
primer
primer' MID' Reflex' (polynucleotide of interest)'
Bind extended strand using streptavidin bound to solid support
solid support
Strept-avidin
biotin
primer MID Reflex polynucleotide of interest
primer' MID' Reflex' (polynucleotide of interest)'
Denature and remove non-biotinylated strands
Strept avidin
biotin
primer MID Reflex polynucleotide of interest
Elute single stranded polynucleotide of interest
biotin
primer MID Reflex polynucleotide of interest

Fig. 2



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g., using primers with binding moieties and/or multiple cycles of extension)

Fig. 3

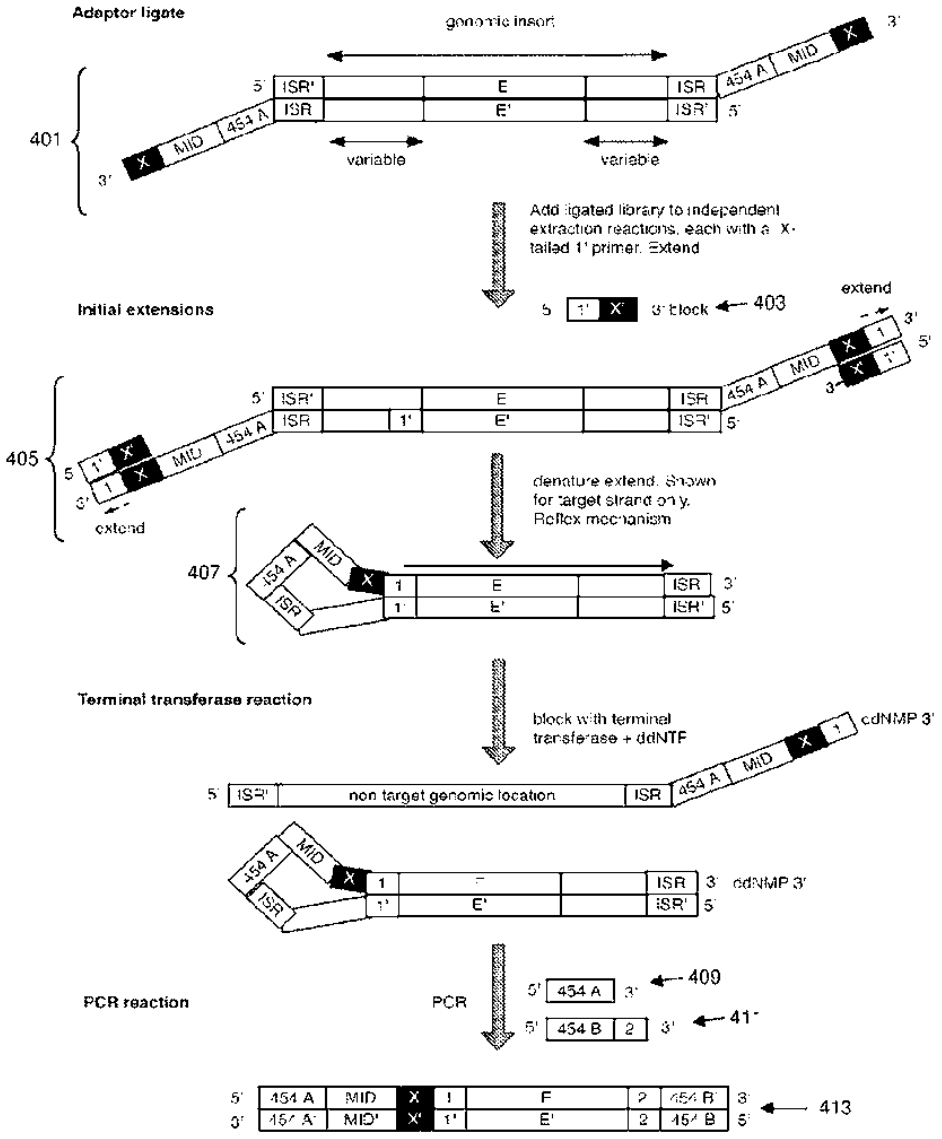

Adaptor ligate
genomic insert
401
5'
ISR'
ISR
E
E'
ISR
ISR'
454 A
MID
X
3'
variable
variable
Add ligated library to independent extraction reactions, each with a X-tailed 1' primer. Extend
Initial extensions
5' 1' X' 3' Block
403
extend
405
1
1'
extend
denature extend. Shown for target strand only. Reflex mechanism
407
Terminal transferase reaction
block with terminal transferase + ddNTP
ddNMP 3'
5' ISR' non target genomic location ISR 454 A MID X 1
PCR reaction
PCR
409
411
454 A
454 B
2
413

Fig. 4

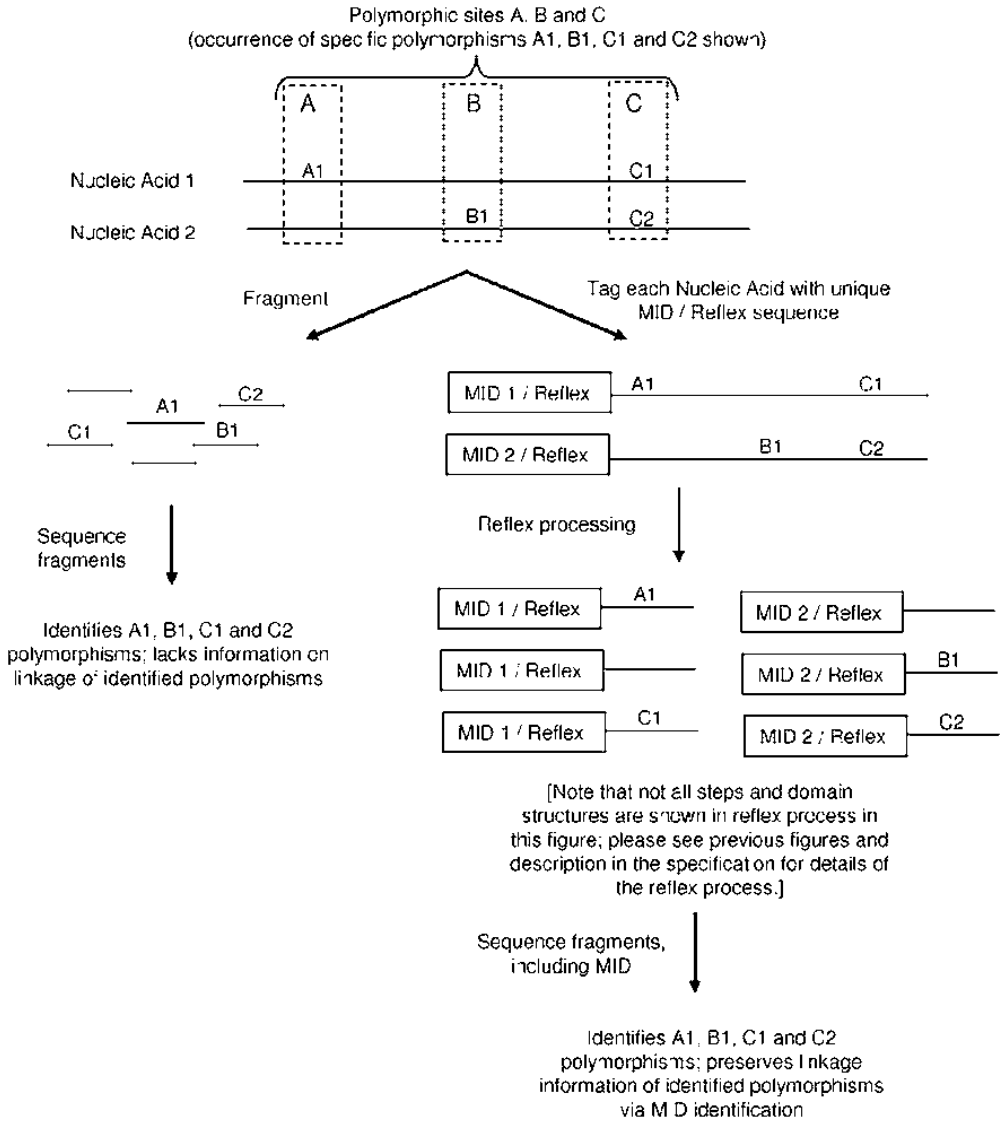
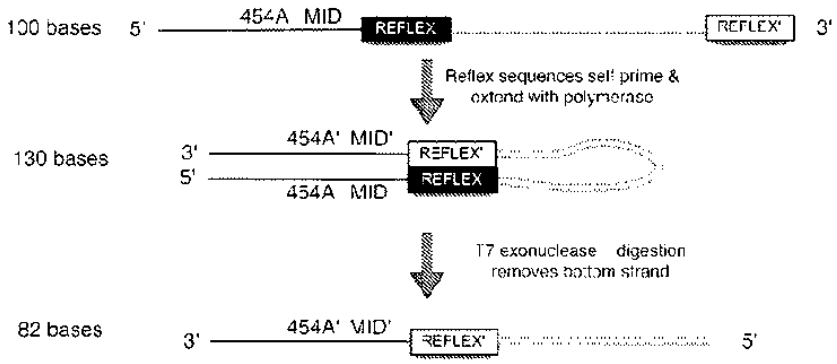

Polymorphic sites A, B and C
(occurrence of specific polymorphisms A1, B1, C1 and C2 shown)
A
B
C
Nucleic Acid 1
A1
C1
Nucleic Acid 2
B1
C2
Fragment
Tag each Nucleic Acid with unique MID / Reflex sequence
A1
C2
C1
B1
Sequence fragments
Identifies A1, B1, C1 and C2 polymorphisms; lacks information on linkage of identified polymorphisms
MID 1 / Reflex
A1
C1
MID 2 / Reflex
B1
C2
Reflex processing
MID 1 / Reflex
A1
MID 2 / Reflex
MID 1 / Reflex
MID 2 / Reflex
B1
MID 1 / Reflex
C1
MID 2 / Reflex
C2
[Note that not all steps and domain structures are shown in reflex process in this figure; please see previous figures and description in the specification for details of the reflex process.]
Sequence fragments, including MID
Identifies A1, B1, C1 and C2 polymorphisms; preserves linkage information of identified polymorphisms via MID identification

Fig. 5

A.


100 bases
5'
454A MID
REFLEX
REFLEX'
3'
Reflex sequences self prime & extend with polymerase
130 bases
3'
454A' MID'
REFLEX'
5'
REFLEX
454A MID
T7 exonuclease digestion removes bottom strand
82 bases
3'
454A' MID'
REFLEX'
5'

B.

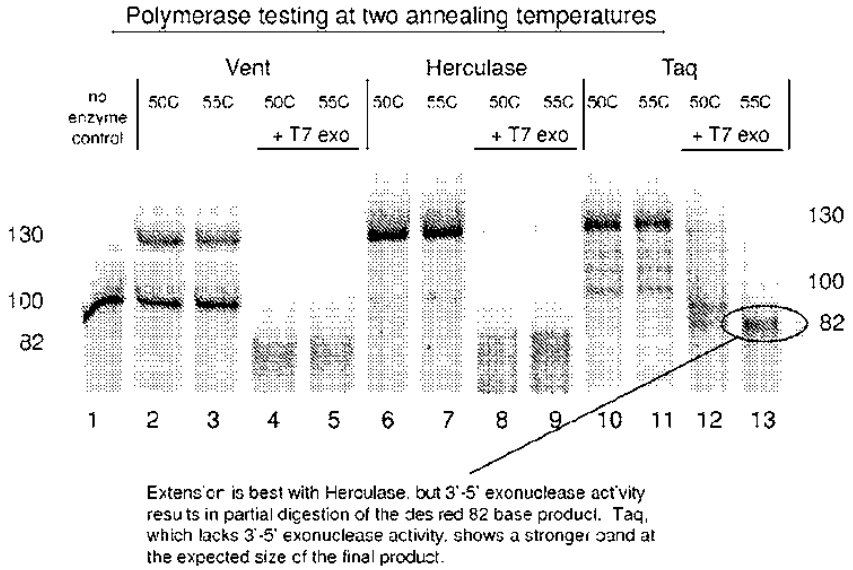

Polymerase testing at two annealing temperatures
Vent
Herculase
Taq
no enzyme control
50C
55C
50C
55C
+ T7 exo
50C
55C
50C
55C
+ T7 exo
50C
55C
50C
55C
+ T7 exo
130
100
82
130
100
82
1
2
3
4
5
6
7
8
9
10
11
12
13
Extension is best with Herculase, but 3'-5' exonuclease activity results in partial digestion of the desired 82 base product. Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product.

Fig. 6

A.


5'
454A MID
REFLEX
REFLEX'
3'
+ Competitor
5'
REFLEX'
3'

B.

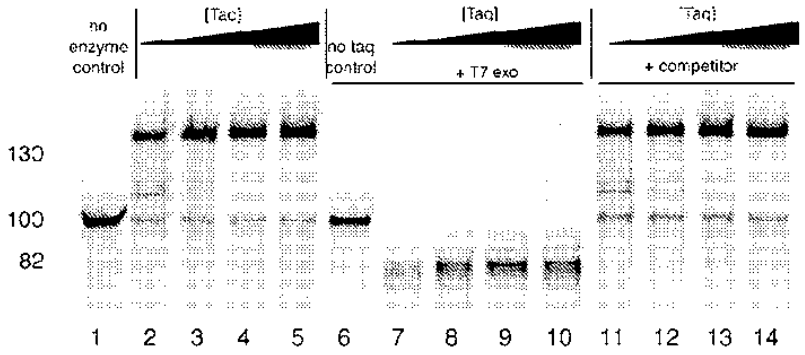
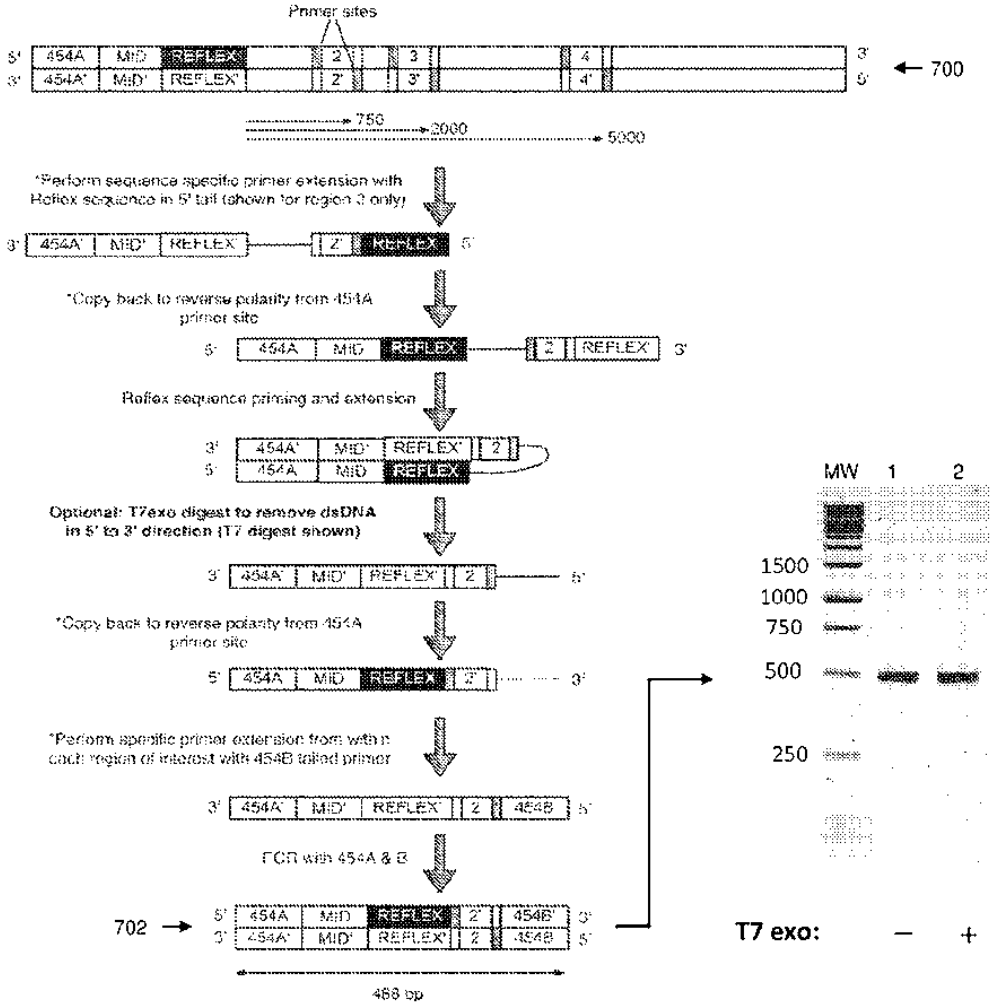

no enzyme control
[Taq]
no taq control
[Taq]
+ T7 exo
[Taq]
+ competitor
130
100
82
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 7



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g., using primers with binding moieties and/or multiple cycles of extension)

Fig. 8

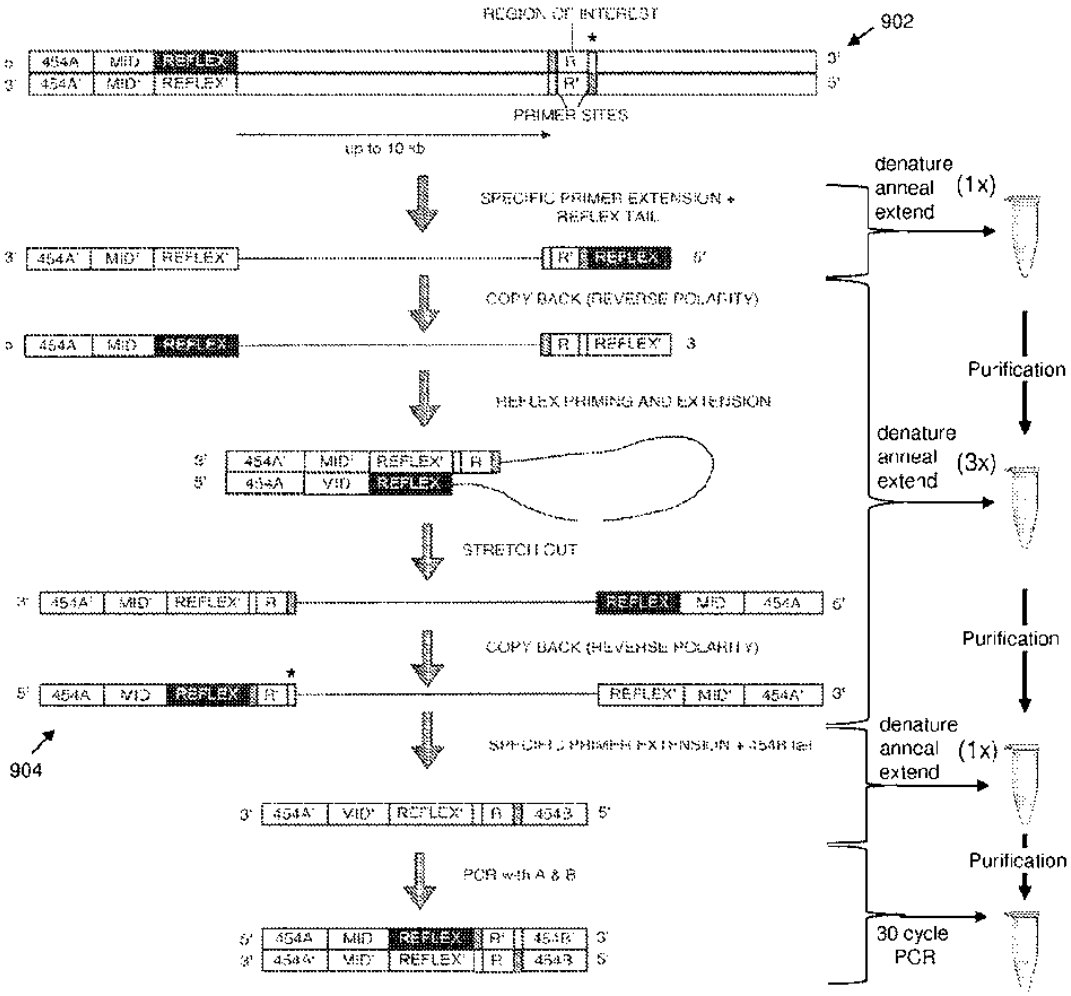

REGION OF INTEREST
902
454A
MID
REFLEX
R
PRIMER SITES
up to 10 kb
SPECIFIC PRIMER EXTENSION + REFLEX TAIL
COPY BACK (REVERSE POLARITY)
REFLEX PRIMING AND EXTENSION
STRETCH OUT
COPY BACK (REVERSE POLARITY)
SPECIFIED PRIMER EXTENSION + 454B tail
PCR with A & B
904
denature anneal extend (1x)
Purification
denature anneal extend (3x)
Purification
denature anneal extend (1x)
Purification
30 cycle PCR

Fig. 9

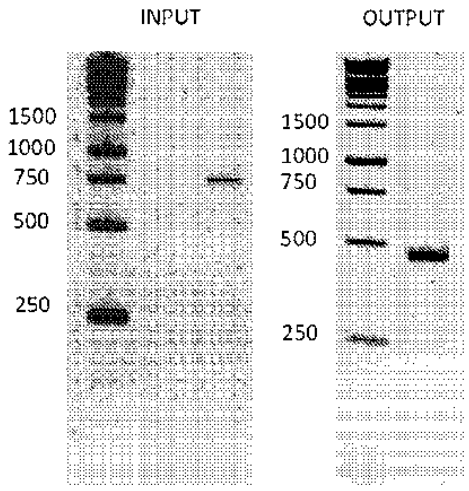
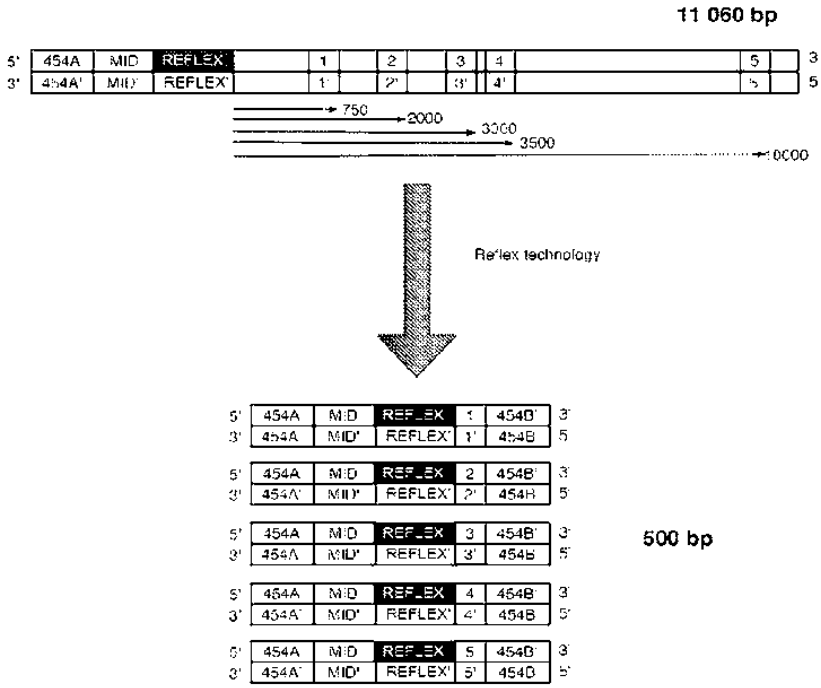


Fig. 10


11 060 bp
5' 454A MID REFLEX 1 2 3 4 5 3'
3' 454A' MID' REFLEX' 1' 2' 3' 4' 5' 5'
750
2000
3000
3500
10000
Reflex technology
454A MID REFLEX 1 454B
454A MID' REFLEX' 1' 454B
454A MID REFLEX 2 454B
454A' MID' REFLEX' 2' 454B
454A MID REFLEX 3 454B
454A MID' REFLEX' 3' 454B
454A MID REFLEX 4 454B
454A' MID' REFLEX' 4' 454B
454A MID REFLEX 5 454B
454A' MID' REFLEX' 5' 454B
500 bp

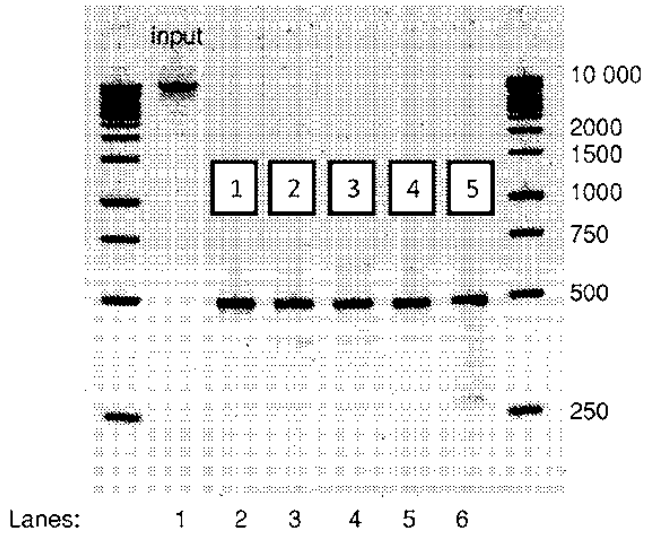
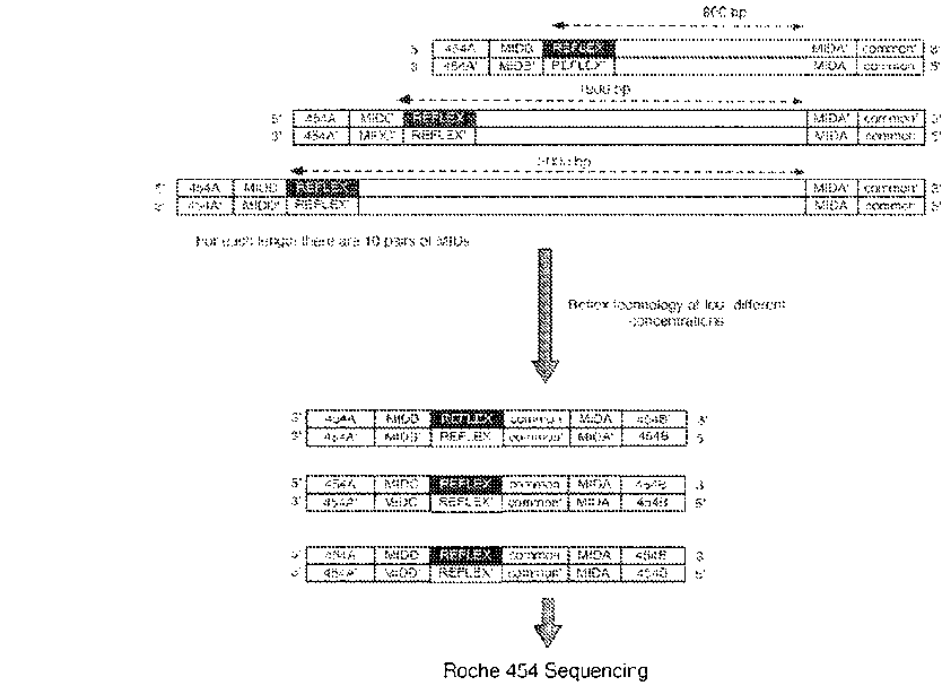

input
1
2
3
4
5
10 000
2000
1500
1000
750
500
250
Lanes: 1 2 3 4 5 6

Fig. 11



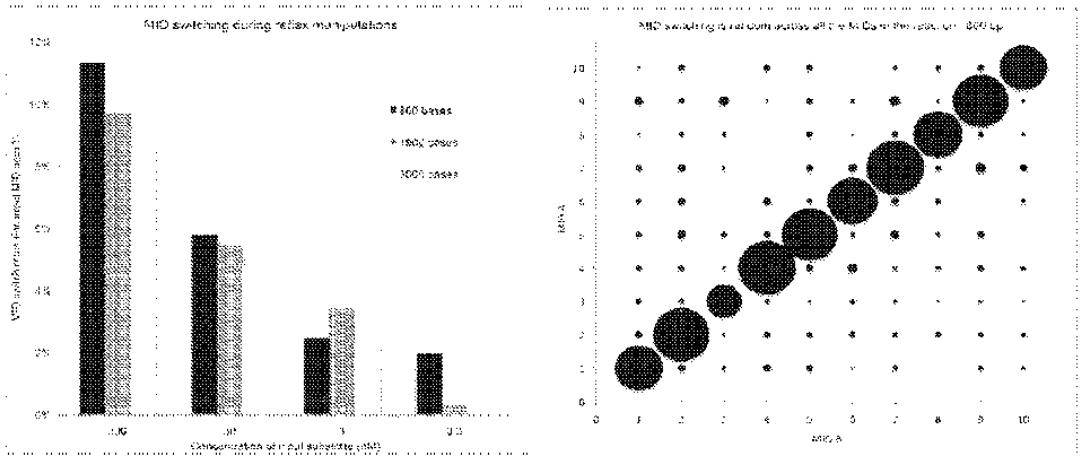


Fig. 12

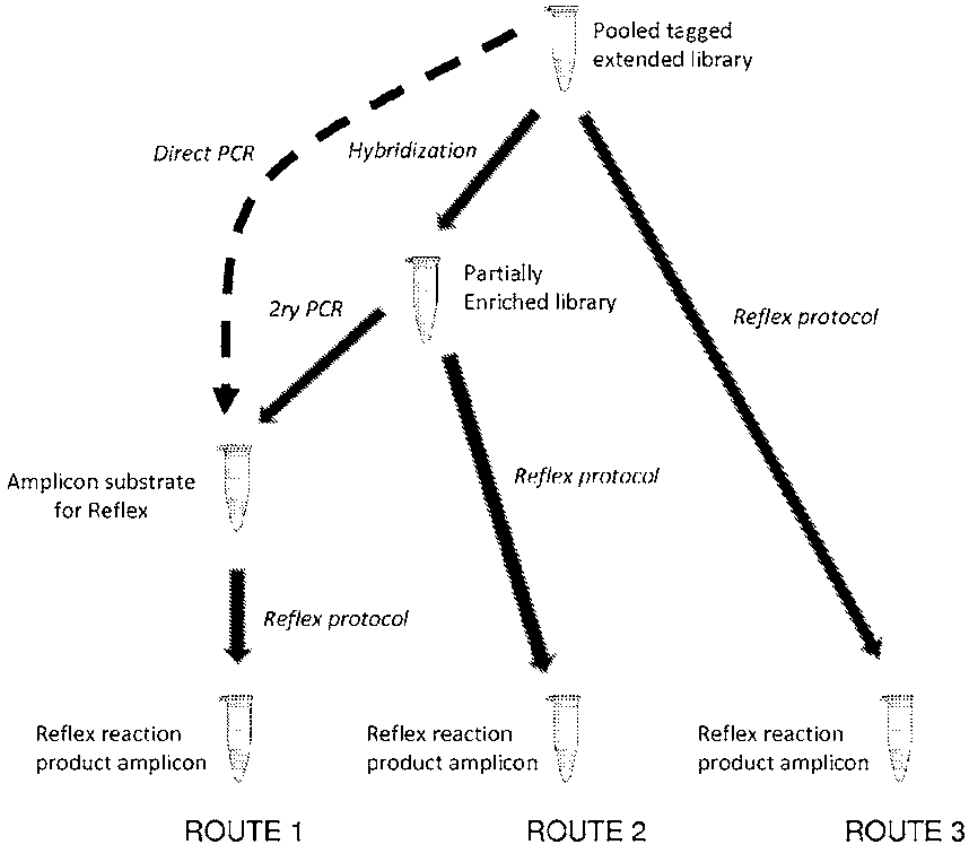

Pooled tagged extended library
Direct PCR
Hybridization
Partially Enriched library
2ry PCR
Reflex protocol
Amplicon substrate for Reflex
Reflex protocol
Reflex protocol
Reflex reaction product amplicon
Reflex reaction product amplicon
Reflex reaction product amplicon
ROUTE 1
ROUTE 2
ROUTE 3

Fig. 13

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A SUBMISSION UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER CGLC-020 U.S. APPLICATION NO. (If known, see 37 CFR 1.5)
INTERNATIONAL APPLICATION NO. IB2010/002243	INTERNATIONAL FILING DATE 8/13/2010	PRIORITY DATE CLAIMED August 20, 2009
TITLE OF INVENTION **Compositions and Methods for Intramolecular Nucleic Acid Rearrangement**		
APPLICANT(S) FOR DO/EO/US **Brenner, Sydney Mikawa, Gi Osborne, Robert Slatter, Andrew**		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a submission under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a submission under 35 U.S.C. 371.

3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.

4. ☐ The US has been elected (Article 31).

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))

   a. ☒ is attached hereto (required only if not communicated by the International Bureau).

   b. ☐ has been communicated by the International Bureau.

   c. ☐ is not required, as the application was filed in the United States Receiving Office(RO/US)

6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).

   a. ☐ is attached hereto.

   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))

   a. ☐ are attached hereto (required only if not communicated by the International Bureau).

   b. ☐ have been communicated by the International Bureau.

   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.

   d. ☒ have not been made and will not be made.

8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☒ A preliminary amendment.

14. ☒ An Application Data Sheet under 37 CFR 1.76.

15. ☐ A substitute specification.

16. ☐ A power of attorney and/or change of address letter.

17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13*ter*.2 and 37 CFR 1.821 - 1.825.

18. ☐ A second copy of the published International Application under 35 U.S.C. 154(d)(4).

19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).

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U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
	IB2010/002243	CGLC-020

20. Other items or information:

	CALCULATIONS	PTO USE ONLY
The following fees have been submitted 21. ☒ Basic national fee (37 CFR 1.492(a)) $190	$ 190	
22. ☒ Examination fee (37 CFR 1.492(c)) If the written opinion prepared by ISA/US or the International preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4) ..... $0 All other situations $125	$ 125	
23. ☒ Search fee (37 CFR 1.492(b)) If the written opinion prepared by ISA/US or the International preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4) ..... $0 Search fee (37 CFR 1.445(a)(2)) has been paid on the international application to the USPTO as an International Searching Authority ..... $60 International Search Report prepared by an ISA other than the US and provided to the Office or previously communicated to the US by the IB ..... $245 All other situations $310	$ 245	
TOTAL OF 21, 22 and 23 =	$ 560	

☐ Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing in compliance with 37 CFR 1.82(c) or (e) or computer program listing in an electronic medium) (37 CFR 1.492(j)).
The fee is $270 for each additional 50 sheets of paper or fraction thereof.

Total Sheets	Extra sheets	Number of each additional 50 or fraction thereof (round up to a whole number)	RATE	
- 100 =	/50 =		X $270	$

Surcharge of $130.00 for furnishing any of the search fee, examination fee, or the oath or declaration after the date of commencement of the national stage (37 CFR 1.492(h)).	$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	- 20 =		x $52	$	
Independent Claims	- 3 =		x $220	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $390	$	
			TOTAL OF ABOVE CALCULATIONS =	$	

☒ Applicant claims small entity status. See 37 CFR 1.27. Fees above are reduced by ½.		
SUBTOTAL =	$ 560	
Processing fee of $130.00 for furnishing the English translation later than 30 months from the earliest claimed priority date (37 CFR 1.492(i)). +	$	
TOTAL NATIONAL FEE =	$ 560	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +	$	
TOTAL FEES ENCLOSED =	$ 560	
	Amount to be refunded:	$
	Amount to be charged:	$560

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a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 50-0815 in the amount of $_____ to cover the above fees.
A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees (other than excess claim fees unless indicated above) which may be required, or credit any overpayment to Deposit Account No. 50-0815. A duplicate copy of this sheet is enclosed.

d. ☒ **Fees are to be charged to a credit card. WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.
The PTO-2038 should only be mailed or faxed to the USPTO. However, when paying the basic national fee, the PTO-2038 may NOT be faxed to the UPSTO.

ADVISORY: If filing by EFS-Web, do NOT attach the PTO-2038 form as a PDF along with your EFS-Web submission. Please be advised that this is not recommended and by doing so your credit card information may be displayed via PAIR. To protect your information, it is recommended paying fees online by using the electronic payment method.

NOTE: Where an appropriate time limit under 37 CFR 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the International Application to pending status.

SEND ALL CORRESPONDENCE TO:

Bozicevic, Field & Francis, LLP
1900 University Avenue, Suite 200
East Palo Alto
California
94303

/James S. Keddie, Ph.D., Reg. No. 48,920/
SIGNATURE

James S. Keddie, Ph.D.
NAME

48,920
REGISTRATION NUMBER

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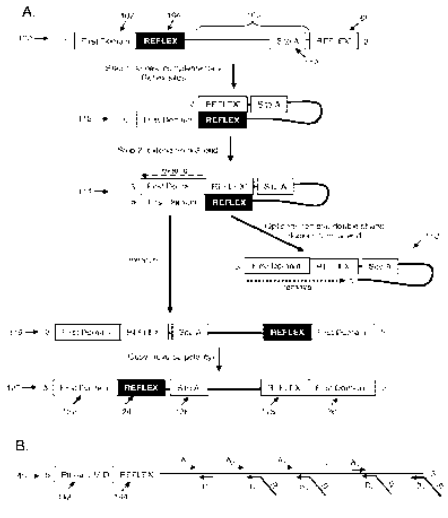


Fig. 1

(57) Abstract: Aspects of the present invention are drawn to processes for moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method". In certain embodiments, the reflex method results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer sites and/or MID). Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

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COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

BACKGROUND

We have previously described methods that enable tagging each of a population of fragmented genomes and then combining them together to create a 'population library' that can be processed and eventually sequenced as a mixture. The population tags enable analysis software to parse the sequence reads into files that can be attributed to a particular genome in the population. One limitation of the overall process stems from limitations of existing DNA sequencing technologies. In particular, if fragments in the regions of interest of the genome are longer than the lengths that can be sequenced by a particular technology, then such fragments will not be fully analyzed (since sequencing proceeds from an end of a fragment inward). Furthermore, a disadvantage of any sequencing technology dependent on fragmentation is that sequence changes in one part of a particular genomic region may not be able to be linked to sequence changes in other parts of the same genome (e.g., the same chromosome) because the sequence changes reside on different fragments. (See Figure 5 and its description below).

The present invention removes the limitations imposed by current sequencing technologies as well as being useful in a number of other nucleic acid analyses.

SUMMARY OF THE INVENTION

Aspects of the present invention are drawn to processes for moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method" (or reflex process, reflex sequence process, reflex reaction, and the like). In certain embodiments, the reflex method results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer sites and/or MID). Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to scale. Indeed, the

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dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures:

Figure 1: Panel A is a schematic diagram illustrating moving a first domain from one site to another in a nucleic acid molecule using a reflex sequence. Panel B is a schematic diagram depicting the relative position of primer pairs (A_n-B_n primers) that find use in aspects of the reflex process described herein.

Figure 2 shows an exemplary embodiment of using binding partner pairs (biotin/streptavidin) to isolate single stranded polynucleotides of interest.

Figure 3 is a schematic diagram illustrating an exemplary embodiment for moving a primer site and a MID to a specific location in a nucleic acid of interest.

Figure 4 shows a schematic diagram illustrating an exemplary use of the reflex process for generating a sample enriched for fragments having a region of interest (e.g., from a population of randomly fragmented and asymmetrically tagged polynucleotides).

Figure 5 shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information.

Figure 6: Panel A is a schematic showing expected structures and sizes of nucleic acid species in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

Figure 7: Panel A is a schematic showing the structure of the nucleic acid and competitor used in the reflex process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

Figure 8 shows a flow chart of a reflex process (left) in which the T7 exonuclease step is optional. The gel on the right shows the resultant product of the reflex process either without the T7 exonuclease step (lane 1) or with the T7 exonuclease step (lane 2).

Figure 9 shows an exemplary reflex process workflow with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt beads to remove primer oligos).

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Figure 10 shows the starting material (left panel) and the resultant product generated (right panel) using a reflex process without using a T7 exonuclease step (as described in Example II). The reflex site in the starting material is a sequence normally present in the polynucleotide being processed (also called a "non-artificial" reflex site). This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in transferring an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

Figure 11 shows a schematic and results of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate five different products ("daughter" products) each having a different region of interest (i.e., daughter products are produced having either region 1, 2, 3, 4 or 5).

Figure 12 shows a schematic and results of experiments performed to determine the prevalence of intramolecular rearrangement during the reflex process (as desired) vs. intermolecular rearrangement (MID switching).

Figure 13 shows a diagram of exemplary workflows for preparing material for and performing the reflex process.

DEFINITIONS

Unless otherwise defined, 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. Still, certain elements are defined for the sake of clarity and ease of reference.

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

"Amplicon" means the product of a polynucleotide amplification reaction. That is, it is a population of polynucleotides, usually double stranded, that are replicated from one or more starting sequences. The one or more starting sequences may be one or more copies of the same sequence, or it may be a mixture of different sequences. Amplicons

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

The term "assessing" includes any form of measurement, and includes determining if an element is present or not. The terms "determining", "measuring", "evaluating", "assessing" and "assaying" are used interchangeably and includes quantitative and qualitative determinations. Assessing may be relative or absolute. "Assessing the presence of" includes determining the amount of something present, and/or determining whether it is present or absent. As used herein, the terms "determining," "measuring," and "assessing," and "assaying" are used interchangeably and include both quantitative and qualitative determinations.

Polynucleotides that are "asymmetrically tagged" have left and right adapter domains that are not identical. This process is referred to generically as attaching adapters asymmetrically or asymmetrically tagging a polynucleotide, e.g., a polynucleotide

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fragment. Production of polynucleotides having asymmetric adapter termini may be achieved in any convenient manner. Exemplary asymmetric adapters are described in: U.S. Patents 5,712,126 and 6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters employed are those described in U.S. Patent Application Ser. No. 12/432,080, filed on April 29, 2009, incorporated herein by reference in its entirety.

As one example, a user of the subject invention may use an asymmetric adapter to tag polynucleotides. An "asymmetric adapter" is one that, when ligated to both ends of a double stranded nucleic acid fragment, will lead to the production of primer extension or amplification products that have non-identical sequences flanking the genomic insert of interest. The ligation is usually followed by subsequent processing steps so as to generate the non-identical terminal adapter sequences. For example, replication of an asymmetric adapter attached fragment(s) results in polynucleotide products in which there is at least one nucleic acid sequence difference, or nucleotide/nucleoside modification, between the terminal adapter sequences. Attaching adapters asymmetrically to polynucleotides (e.g., polynucleotide fragments) results in polynucleotides that have one or more adapter sequences on one end (e.g., one or more region or domain, e.g., a primer site) that are either not present or have a different nucleic acid sequence as compared to the adapter sequence on the other end. It is noted that an adapter that is termed an "asymmetric adapter" is not necessarily itself structurally asymmetric, nor does the mere act of attaching an asymmetric adapter to a polynucleotide fragment render it immediately asymmetric. Rather, an asymmetric adapter-attached polynucleotide, which has an identical asymmetric adapter at each end, produces replication products (or isolated single stranded polynucleotides) that are asymmetric with respect to the adapter sequences on opposite ends (e.g., after at least one round of amplification/primer extension).

Any convenient asymmetric adapter, or process for attaching adapters asymmetrically, may be employed in practicing the present invention. Exemplary asymmetric adapters are described in: U.S. Patents 5,712,126 and 6,372,434; U.S. Patent Publications 2007/0128624 and 2007/0172839; and PCT publication WO/2009/032167; all of which are incorporated by reference herein in their entirety. In certain embodiments, the asymmetric adapters employed are those described in U.S. Patent Application Ser. No. 12/432,080, filed on April 29, 2009, incorporated herein by reference in its entirety.

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

"Duplex" means at least two oligonucleotides and/or polynucleotides that are fully or partially complementary undergo Watson-Crick type base pairing among all or most of their nucleotides so that a stable complex is formed. The terms "annealing" and "hybridization" are used interchangeably to mean the formation of a stable duplex. "Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one another such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. A stable duplex can include Watson-Crick base pairing and/or non-Watson-Crick base pairing between the strands of the duplex (where base pairing means the forming hydrogen bonds). In certain embodiments, a non-Watson-Crick base pair includes a nucleoside analog, such as deoxyinosine, 2, 6-diaminopurine, PNAs, LNA's and the like. In certain embodiments, a non-Watson-Crick base pair includes a "wobble base", such as deoxyinosine, 8-oxo-dA, 8-oxo-dG and the like, where by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand (wobble bases are described in further detail below). A "mismatch" in a duplex between two oligonucleotides or polynucleotides means that a pair of nucleotides in the duplex fails to undergo Watson-Crick bonding.

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"Genetic locus," "locus," or "locus of interest" in reference to a genome or target polynucleotide, means a contiguous sub-region or segment of the genome or target polynucleotide. As used herein, genetic locus, locus, or locus of interest may refer to the position of a nucleotide, a gene or a portion of a gene in a genome, including mitochondrial DNA or other non-chromosomal DNA (e.g., bacterial plasmid), or it may refer to any contiguous portion of genomic sequence whether or not it is within, or associated with, a gene. A genetic locus, locus, or locus of interest can be from a single nucleotide to a segment of a few hundred or a few thousand nucleotides in length or more. In general, a locus of interest will have a reference sequence associated with it (see description of "reference sequence" below).

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

"Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, e.g. oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon of a terminal nucleotide of one oligonucleotide with 3' carbon of another oligonucleotide. A variety of template-driven ligation reactions are described in the following references, which are incorporated by reference: Whiteley et al, U.S. patent 4,883,750; Letsinger et al, U.S. patent 5,476,930; Fung et al, U.S. patent 5,593,826; Kool, U.S. patent 5,426,180; Landegren et al, U.S. patent 5,871,921; Xu and Kool, Nucleic Acids Research, 27: 875-881 (1999); Higgins et al, Methods in Enzymology, 68: 50-71 (1979); Engler et al, The Enzymes, 15: 3-29 (1982); and Namsaraev, U.S. patent publication 2004/0110213.

"Multiplex Identifier" (MID) as used herein refers to a tag or combination of tags associated with a polynucleotide whose identity (e.g., the tag DNA sequence)

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can be used to differentiate polynucleotides in a sample. In certain embodiments, the MID on a polynucleotide is used to identify the source from which the polynucleotide is derived. For example, a nucleic acid sample may be a pool of polynucleotides derived from different sources, (e.g., polynucleotides derived from different individuals, different tissues or cells, or polynucleotides isolated at different times points), where the polynucleotides from each different source are tagged with a unique MID. As such, a MID provides a correlation between a polynucleotide and its source. In certain embodiments, MIDs are employed to uniquely tag each individual polynucleotide in a sample. Identification of the number of unique MIDs in a sample can provide a readout of how many individual polynucleotides are present in the sample (or from how many original polynucleotides a manipulated polynucleotide sample was derived; see, e.g., U.S. Patent No. 7,537,897, issued on May 26, 2009, incorporated herein by reference in its entirety). MIDs can range in length from 2 to 100 nucleotide bases or more and may include multiple subunits, where each different MID has a distinct identity and/or order of subunits. Exemplary nucleic acid tags that find use as MIDs are described in U.S. Patent 7,544,473, issued on June 6, 2009, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Patent 7,393,665, issued on July 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., Tm, length, base composition, etc.), as the methods described herein can accommodate a wide variety of unique MID sets. It is emphasized here that MIDs need only be unique within a given experiment. Thus, the same MID may be used to tag a different sample being processed in a different experiment. In addition, in certain experiments, a user may use the same MID to tag a subset of different samples within the same experiment. For example, all samples derived from individuals having a specific phenotype may be tagged with the same MID, e.g., all samples derived from control (or wildtype) subjects can be tagged with a first MID while subjects having a disease condition can be tagged with a second MID (different than the first MID). As another example, it may be desirable to tag different samples derived from the same source with different MIDs (e.g., samples derived over time or derived from different sites within a tissue). Further, MIDs can be generated in a

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variety of different ways, e.g., by a combinatorial tagging approach in which one MID is attached by ligation and a second MID is attached by primer extension. Thus, MIDs can be designed and implemented in a variety of different ways to track polynucleotide fragments during processing and analysis, and thus no limitation in this regard is intended.

"Nucleoside" as used herein includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543-584 (1990), or the like, with the proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like. Polynucleotides comprising analogs with enhanced hybridization or nuclease resistance properties are described in Uhlman and Peyman (cited above); Crooke et al, Exp. Opin. Ther. Patents, 6: 855-870 (1996); Mesmaeker et al, Current Opinion in Structual Biology, 5: 343-355 (1995); and the like. Exemplary types of polynucleotides that are capable of enhancing duplex stability include oligonucleotide N3'→P5' phosphoramidates (referred to herein as "amidates"), peptide nucleic acids (referred to herein as "PNAs"), oligo-2'-O-alkylribonucleotides, polynucleotides containing C-5 propynylpyrimidines, locked nucleic acids ("LNAs"), and like compounds. Such oligonucleotides are either available commercially or may be synthesized using methods described in the literature.

"Polymerase chain reaction," or "PCR," means a reaction for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. In other words, PCR is a reaction for making multiple copies or replicates of a target nucleic acid flanked by primer sites, such reaction comprising one or more repetitions of the following steps: (i) denaturing the target nucleic acid, (ii) annealing primers to the primer sites, and (iii) extending the primers by a nucleic acid polymerase in the presence of nucleoside triphosphates. Usually, the reaction is cycled through different temperatures optimized for each step in a thermal cycler instrument. Particular temperatures, durations at each step, and rates of change between steps depend on many factors well-known to those of ordinary skill in the art, e.g. exemplified by the references: McPherson et al, editors, PCR: A Practical Approach and PCR2: A Practical Approach (IRL Press, Oxford, 1991 and 1995, respectively). For example, in a conventional

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PCR using Taq DNA polymerase, a double stranded target nucleic acid may be denatured at a temperature >90°C, primers annealed at a temperature in the range 50-75°C, and primers extended at a temperature in the range 72-78°C. The term "PCR" encompasses derivative forms of the reaction, including but not limited to, RT-PCR, real-time PCR, nested PCR, quantitative PCR, multiplexed PCR, and the like. Reaction volumes range from a few hundred nanoliters, e.g. 200 nL, to a few hundred μL, e.g. 200 μL. "Reverse transcription PCR," or "RT-PCR," means a PCR that is preceded by a reverse transcription reaction that converts a target RNA to a complementary single stranded DNA, which is then amplified, e.g. Tecott et al, U.S. patent 5,168,038, which patent is incorporated herein by reference. "Real-time PCR" means a PCR for which the amount of reaction product, i.e. amplicon, is monitored as the reaction proceeds. There are many forms of real-time PCR that differ mainly in the detection chemistries used for monitoring the reaction product, e.g. Gelfand et al, U.S. patent 5,210,015 ("TAQMAN™"); Wittwer et al, U.S. patents 6,174,670 and 6,569,627 (intercalating dyes); Tyagi et al, U.S. patent 5,925,517 (molecular beacons); which patents are incorporated herein by reference. Detection chemistries for real-time PCR are reviewed in Mackay et al, Nucleic Acids Research, 30: 1292-1305 (2002), which is also incorporated herein by reference. "Nested PCR" means a two-stage PCR wherein the amplicon of a first PCR becomes the sample for a second PCR using a new set of primers, at least one of which binds to an interior location of the first amplicon. As used herein, "initial primers" in reference to a nested amplification reaction mean the primers used to generate a first amplicon, and "secondary primers" mean the one or more primers used to generate a second, or nested, amplicon. "Multiplexed PCR" means a PCR wherein multiple target sequences (or a single target sequence and one or more reference sequences) are simultaneously carried out in the same reaction mixture, e.g. Bernard et al, Anal. Biochem., 273: 221-228 (1999)(two-color real-time PCR). Usually, distinct sets of primers are employed for each sequence being amplified.

"Quantitative PCR" means a PCR designed to measure the abundance of one or more specific target sequences in a sample or specimen. Quantitative PCR includes both absolute quantitation and relative quantitation of such target sequences. Quantitative measurements are made using one or more reference sequences that may be assayed separately or together with a target sequence. The reference sequence may be endogenous or exogenous to a sample or specimen, and in the latter case, may comprise one or more competitor templates. Typical endogenous reference sequences include segments of transcripts of the following genes: β-actin, GAPDH, β_2-microglobulin, ribosomal RNA, and the like. Techniques for

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quantitative PCR are well-known to those of ordinary skill in the art, as exemplified in the following references that are incorporated by reference: Freeman et al, Biotechniques, 26: 112-126 (1999); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9447 (1989); Zimmerman et al, Biotechniques, 21: 268-279 (1996); Diviacco et al, Gene, 122: 3013-3020 (1992); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9446 (1989); and the like.

"Polynucleotide" or "oligonucleotide" is used interchangeably and each means a linear polymer of nucleotide monomers. Monomers making up polynucleotides and oligonucleotides are capable of specifically binding to a natural polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, wobble base pairing, or the like. As described in detail below, by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand. Such monomers and their internucleosidic linkages may be naturally occurring or may be analogs thereof, e.g. naturally occurring or non-naturally occurring analogs. Non-naturally occurring analogs may include peptide nucleic acids (PNAs, e.g., as described in U.S. Patent 5,539,082, incorporated herein by reference), locked nucleic acids (LNAs, e.g., as described in U.S. Patent 6,670,461, incorporated herein by reference), phosphorothioate internucleosidic linkages, bases containing linking groups permitting the attachment of labels, such as fluorophores, or haptens, and the like. Whenever the use of an oligonucleotide or polynucleotide requires enzymatic processing, such as extension by a polymerase, ligation by a ligase, or the like, one of ordinary skill would understand that oligonucleotides or polynucleotides in those instances would not contain certain analogs of internucleosidic linkages, sugar moieties, or bases at any or some positions. Polynucleotides typically range in size from a few monomeric units, e.g. 5-40, when they are usually referred to as "oligonucleotides," to several thousand monomeric units. Whenever a polynucleotide or oligonucleotide is represented by a sequence of letters (upper or lower case), such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, "I" denotes deoxyinosine, "U" denotes uridine, unless otherwise indicated or obvious from context. Unless otherwise noted the terminology and atom numbering conventions will follow those disclosed in Strachan and Read, Human Molecular Genetics 2 (Wiley-Liss, New York,

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1999). Usually polynucleotides comprise the four natural nucleosides (e.g. deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine for DNA or their ribose counterparts for RNA) linked by phosphodiester linkages; however, they may also comprise non-natural nucleotide analogs, e.g. including modified bases, sugars, or internucleosidic linkages. It is clear to those skilled in the art that where an enzyme has specific oligonucleotide or polynucleotide substrate requirements for activity, e.g. single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for the oligonucleotide or polynucleotide substrates is well within the knowledge of one of ordinary skill, especially with guidance from treatises, such as Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references.

"Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Usually primers are extended by a DNA polymerase. Primers are generally of a length compatible with their use in synthesis of primer extension products, and are usually are in the range of between 8 to 100 nucleotides in length, such as 10 to 75, 15 to 60, 15 to 40, 18 to 30, 20 to 40, 21 to 50, 22 to 45, 25 to 40, and so on, more typically in the range of between 18-40, 20-35, 21-30 nucleotides long, and any length between the stated ranges. Typical primers can be in the range of between 10-50 nucleotides long, such as 15-45, 18-40, 20-30, 21-25 and so on, and any length between the stated ranges. In some embodiments, the primers are usually not more than about 10, 12, 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, or 70 nucleotides in length.

Primers are usually single-stranded for maximum efficiency in amplification, but may alternatively be double-stranded. If double-stranded, the primer is usually first treated to separate its strands before being used to prepare extension products. This denaturation step is typically affected by heat, but may alternatively be carried out using alkali, followed by neutralization. Thus, a "primer" is complementary to a template, and complexes by hydrogen bonding or hybridization with the template to give a primer/template complex for initiation of synthesis by a polymerase, which is extended by the addition of covalently bonded bases linked at its 3' end complementary to the template in the process of DNA synthesis.

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A "primer pair" as used herein refers to first and second primers having nucleic acid sequence suitable for nucleic acid-based amplification of a target nucleic acid. Such primer pairs generally include a first primer having a sequence that is the same or similar to that of a first portion of a target nucleic acid, and a second primer having a sequence that is complementary to a second portion of a target nucleic acid to provide for amplification of the target nucleic acid or a fragment thereof. Reference to "first" and "second" primers herein is arbitrary, unless specifically indicated otherwise. For example, the first primer can be designed as a "forward primer" (which initiates nucleic acid synthesis from a 5' end of the target nucleic acid) or as a "reverse primer" (which initiates nucleic acid synthesis from a 5' end of the extension product produced from synthesis initiated from the forward primer). Likewise, the second primer can be designed as a forward primer or a reverse primer.

"Primer site" (e.g., a sequencing primer site, and amplification primer site, etc.) as used herein refers to a domain in a polynucleotide that includes the sequence of a primer (e.g., a sequencing primer) and/or the complementary sequence of a primer. When present in single stranded form (e.g., in a single stranded polynucleotide), a primer site can be either the identical sequence of a primer or the complementary sequence of a primer. When present in double stranded form, a primer site contains the sequence of a primer hybridized to the complementary sequence of the primer. Thus, a primer site is a region of a polynucleotide that is either identical to or complementary to the sequence of a primer (when in a single stranded form) or a double stranded region formed between a primer sequence and its complement. Primer sites may be present in an adapter attached to a polynucleotide. The specific orientation of a primer site can be inferred by those of ordinary skill in the art from the structural features of the relevant polynucleotide and/or context in which it is used.

"Readout" means a parameter, or parameters, which are measured and/or detected that can be converted to a number or value. In some contexts, readout may refer to an actual numerical representation of such collected or recorded data. For example, a readout of fluorescent intensity signals from a microarray is the address and fluorescence intensity of a signal being generated at each hybridization site of the microarray; thus, such a readout may be registered or stored in various ways, for example, as an image of the microarray, as a table of numbers, or the like.

"Reflex site", "reflex sequence" and equivalents are used to indicate sequences in a polynucleotide that are employed to move a domain intramolecularly from its initial location

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to a different location in the polynucleotide. The sequence of a reflex site can be added to a polynucleotide of interest (e.g., present in an adapter ligated to the polynucleotide), be based on a sequence naturally present within the polynucleotide of interest (e.g., a genomic sequence in the polynucleotide), or a combination of both. The reflex sequence is chosen so as to be distinct from other sequences in the polynucleotide (i.e., with little sequence homology to other sequences likely to be present in the polynucleotide, e.g., genomic or sub-genomic sequences to be processed). As such, a reflex sequence should be selected so as to not hybridize to any sequence except its complement under the conditions employed in the reflex processes herein described. As described later in this application, the complement to the reflex sequence is inserted on the same strand of the polynucleotide (e.g., the same strand of a double-stranded polynucleotide or on the same single stranded polynucleotide) in a particular location so as to facilitate an intramolecular binding event on such particular strand. Reflex sequences employed in the reflex process described herein can thus have a wide range of lengths and sequences. Reflex sequences may range from 5 to 200 nucleotide bases in length.

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

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

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

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature (e.g., as measured in °C) at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are known in the art (see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985). Other references (e.g., Allawi, H.T. & SantaLucia, J., Jr., Biochemistry 36, 10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m.

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

The terms "upstream" and "downstream" in describing nucleic acid molecule orientation and/or polymerization are used herein as understood by one of skill in the art. As such, "downstream" generally means proceeding in the 5' to 3' direction, i.e., the direction in which a nucleotide polymerase normally extends a sequence, and "upstream" generally

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means the converse. For example, a first primer that hybridizes "upstream" of a second primer on the same target nucleic acid molecule is located on the 5' side of the second primer (and thus nucleic acid polymerization from the first primer proceeds towards the second primer).

It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or the use of a "negative" limitation.

DETAILED DESCRIPTION OF THE INVENTION

The invention is drawn to compositions and methods for intramolecular nucleic acid rearrangement that find use in various applications of genetic analysis, including sequencing, as well as general molecular biological manipulations of polynucleotide structures.

Before the present invention is described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

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. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in

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connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" includes a plurality of such nucleic acids and reference to "the compound" includes reference to one or more compounds and equivalents thereof known to those skilled in the art, and so forth.

The practice of the present invention may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such conventional techniques include polymer array synthesis, hybridization, ligation, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the example herein below. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV), *Using Antibodies: A Laboratory Manual, Cells: A Laboratory Manual, PCR Primer: A Laboratory Manual, and Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), Stryer, L. (1995) *Biochemistry* (4th Ed.) Freeman, New York, Gait, "*Oligonucleotide Synthesis: A Practical Approach*" 1984, IRL Press, London, Nelson and Cox (2000), Lehninger, A., *Principles of Biochemistry* 3rd Ed., W. H. Freeman Pub., New York, N.Y. and Berg et al. (2002) *Biochemistry*, 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

As summarized above, aspects of the present invention are drawn to the use of a 'reflex' sequence present in a polynucleotide (e.g., in an adapter structure of the polynucleotide, in a genomic region of the polynucleotide, or a combination of both) to move a domain of the polynucleotide intra-molecularly from a first location to a second location. The reflex process described herein finds use in any number of applications, e.g.,

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placing functional elements of a polynucleotide (e.g., sequencing primer sites and/or MID tags) into proximity to a desired sub-region of interest.

Nucleic Acids

The reflex process (as described in detail below) can be employed for the manipulation and analysis of nucleic acid sequences of interest from virtually any nucleic acid source, including but not limited to genomic DNA, complementary DNA (cDNA), RNA (e.g., messenger RNA, ribosomal RNA, short interfering RNA, microRNA, etc.), plasmid DNA, mitochondrial DNA, synthetic DNA, etc. Furthermore, any organism, organic material or nucleic acid-containing substance can be used as a source of nucleic acids to be processed in accordance with the present invention including, but not limited to, plants, animals (e.g., reptiles, mammals, insects, worms, fish, etc.), tissue samples, bacteria, fungi (e.g., yeast), phage, viruses, cadaveric tissue, archaeological/ancient samples, etc. In certain embodiments, the nucleic acids in the nucleic acid sample are derived from a mammal, where in certain embodiments the mammal is a human.

In certain embodiments, the nucleic acid sequences are enriched prior to the reflex sequence process. By enriched is meant that the nucleic acid is subjected to a process that reduces the complexity of the nucleic acids, generally by increasing the relative concentration of particular nucleic acid species in the sample (e.g., having a specific locus of interest, including a specific nucleic acid sequence, lacking a locus or sequence, being within a specific size range, etc.). There are a wide variety of ways to enrich nucleic acids having a specific characteristic(s) or sequence, and as such any convenient method to accomplish this may be employed. The enrichment (or complexity reduction) can take place at any of a number of steps in the process, and will be determined by the desires of the user. For example, enrichment can take place in individual parental samples (e.g., untagged nucleic acids prior to adaptor ligation) or in multiplexed samples (e.g., nucleic acids tagged with primer sites, MID and/or reflex sequences and pooled; MID are described in further detail below).

In certain embodiments, nucleic acids in the nucleic acid sample are amplified prior to analysis. In certain of these embodiments, the amplification reaction also serves to enrich a starting nucleic acid sample for a sequence or locus of interest. For example, a starting nucleic acid sample can be subjected to a polymerase chain reaction (PCR) that amplifies one or more region of interest. In certain embodiments, the amplification reaction is an exponential amplification reaction, whereas in certain other embodiments, the amplification reaction is a linear amplification reaction. Any convenient method for performing

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amplification reactions on a starting nucleic acid sample can be used in practicing the subject invention. In certain embodiments, the nucleic acid polymerase employed in the amplification reaction is a polymerase that has proofreading capability (e.g., phi29 DNA Polymerase, Thermococcus litoralis DNA polymerase, Pyrococcus furiosus DNA polymerase, etc.).

In certain embodiments, the nucleic acid sample being analyzed is derived from a single source (e.g., a single organism, virus, tissue, cell, subject, etc.), whereas in other embodiments, the nucleic acid sample is a pool of nucleic acids extracted from a plurality of sources (e.g., a pool of nucleic acids from a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. As such, in certain embodiments, a nucleic acid sample can contain nucleic acids from 2 or more sources, 3 or more sources, 5 or more sources, 10 or more sources, 50 or more sources, 100 or more sources, 500 or more sources, 1000 or more sources, 5000 or more sources, up to and including about 10,000 or more sources.

In certain embodiments, nucleic acid fragments that are to be pooled with nucleic acid fragments derived from a plurality of sources (e.g., a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. In such embodiments, the nucleic acids derived from each source includes a multiplex identifier (MID) such that the source from which the each tagged nucleic acid fragment was derived can be determined. In such embodiments, each nucleic acid sample source is correlated with a unique MID, where by unique MID is meant that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID. Any type of MID can be used, including but not limited to those described in co-pending U.S. Patent Application Serial Number 11/656,746, filed on January 22, 2007, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Patent 7,393,665, issued on July 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., T_m, length, base composition, etc.), as the asymmetric tagging methods (and many tag readout methods, including but not limited to sequencing of the tag or measuring the length of the tag) can accommodate a wide variety of unique MID sets.

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In certain embodiments, each individual polynucleotide (e.g., double-stranded or single-stranded, as appropriate to the methodological details employed) in a sample to be analyzed is tagged with a unique MID so that the fate of each polynucleotide can be tracked in subsequent processes (where, as noted above, unique MID is meant to indicate that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID). For example (and as described below), having each nucleic acid tagged with a unique MID allows analysis of the sequence of each individual nucleic acid using the reflex sequence methods described herein. This allows the linkage of sequence information for large nucleic acid fragments that cannot be sequenced in a single sequencing run.

Reflex Sequence Process

As summarized above, aspects of the present invention include methods and compositions for moving a domain in a polynucleotide from a first location to a second location in the polynucleotide. An exemplary embodiment is shown in Figure 1A.

Figure 1A shows a single stranded polynucleotide 100 comprising, in a 5' to 3' orientation, a first domain (102; the domain to be moved); a reflex sequence 104; a nucleic acid sequence 106 having a site distal to the first domain (Site A), and a complement of the reflex sequence 108 (positioned at the 3' terminus of the polynucleotide). The steps of the reflex method described below will move the first domain into closer proximity to Site A. It is noted here that the prime designation in Figure 1A denotes a complementary sequence of a domain. For example, First Domain' is the complement of the First Domain.

In Step 1, the reflex sequence and its complement in the polynucleotide are annealed intramolecularly to form polynucleotide structure 112, with the polynucleotide folding back on itself and hybridizing to form a region of complementarity (i.e., double stranded reflex/reflex' region). In this configuration, the 3' end of the complement of the reflex sequence can serve as a nucleic acid synthesis priming site. Nucleic acid synthesis from this site is then performed in extension Step 2 producing a complement of the first domain at the 3' end of the nucleic acid extension (shown in polynucleotide 114; extension is indicated with dotted arrow labeled "extend").

Denaturation of polynucleotide 114 (e.g., by heat) generates linear single stranded polynucleotide 116. As shown in Figure 1, resultant polynucleotide 116 contains a complement of the first domain at a position proximal to Site A (i.e., separated by only the complement of the reflex sequence). This resultant polynucleotide may be used for any

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subsequent analysis or processing steps as desired by the user (e.g., sequencing, as a template for amplification (linear, PCR, etc.), sequence specific extraction, etc.).

In alternative embodiments, the first domain and reflex sequence are removed from the 5' end of the double-stranded region of polynucleotide 114 (shown in polynucleotide 118; removal is shown in the dotted arrow labeled "remove"). Removal of this region may be accomplished by any convenient method, including, but not limited to, treatment (under appropriate incubation conditions) of polynucleotide structure 114 with T7 exonuclease or by treatment with Lambda exonuclease; the Lambda exonuclease can be employed so long as the 5' end of the polynucleotide is phosphorylated. If the region is removed enzymatically, resultant polynucleotide 118 is used in place of polynucleotide 116 in subsequent steps (e.g., copying to reverse polarity).

In certain embodiments, polynucleotide 116 or 118 is used as a template to produce a double stranded polynucleotide, for example by performing a nucleic acid synthesis reaction with a primer that primes in the complement of the first domain. This step is sometimes referred to as copying to reverse polarity of a single stranded polynucleotide, and in some instances, the double-stranded intermediate product of this copying is not shown (see, e.g., Figure 3). For example, copying to reverse the polarity of polynucleotide 116 results in single-stranded polynucleotide 120 having, in a 5' to 3' orientation, the first domain (122); the reflex sequence (124); the complement of polynucleotide 106 (oriented with the complement of Site A (Site A'; 126) proximal to the reflex sequence); the complement of the reflex sequence (128); and the complement of the first domain (130).

In certain embodiments, the first domain in the polynucleotide comprises one or more elements that find use in one or more subsequent processing or analysis steps. Such sequences include, but are not limited to, restriction enzyme sites, PCR primer sites, linear amplification primer sites, reverse transcription primer sites, RNA polymerase promoter sites (such as for T7, T3 or SP6 RNA polymerase), MID tags, sequencing primer sites, etc. Any convenient element can be included in the first domain and, in certain embodiments, is determined by the desires of the user of the methods described herein.

As an exemplary embodiment, suppose we want to sequence a specific polynucleotide region from multiple genomes in a pooled sample where the polynucleotide region is too long to sequence in a single reaction. For example, sequencing a polynucleotide region that is 2 kilobases or more in length using Roche 454 (Branford, CT) technology, in which the length of a single sequencing run is about 400 bases. In this scenario, we can design a set of left hand primers (A_n) and right hand primers (B_n) specific

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for the polynucleotide region that are positioned in such a way that we can obtain direct sequences of all parts of the insert, as shown in Figure 1B. Note that the polynucleotide shown in Figure 1B (140) has a domain (142) containing a primer site and an MID denoting from which original sample(s) the polynucleotide is derived. Site 142 thus represents an example of a First Domain site such identified as 122 in the Figure 1A. The polynucleotide also includes a reflex site (144), which can be part of the polynucleotide region itself (e.g., a genomic sequence), added in a ligated adapter domain along with the primer site and the MID (an artificial sequence), or a combination of both (a sequence spanning the adapter/polynucleotide junction).

It is noted here that polynucleotide 140 can be categorized as a precursor to polynucleotide 100 in Figure 1A, as it does not include a 3' reflex sequence complementary to the reflex site (domain 108 in Figure 1A). As detailed below, polynucleotide 140 can be converted to a polynucleotide having the structural configuration of polynucleotide 100, a polynucleotide suitable as a substrate for the reflex process described herein (e.g., by primer extension using a B_n primer and reversal of polarity).

In an exemplary embodiment, each A_n-B_n primer pair defines a nucleic acid region that is approximately 400 bases in length or less. This size range is within the single-sequencing run read length of the current Roche 454 sequencing platform; a different size range for the defined nucleic acid region may be utilized for a different sequencing platform. Thus, each product from each reflex process can be sequenced in a single run. It is noted here that primer pairs as shown in Figure 1B can be used to define regions 1 to 5 shown in Figure 3 (described in further detail below).

In certain embodiments, to obtain the first part of the sequence of the polynucleotide region (i.e., in the original structure, that part of the polynucleotide closest to the first domain), we only need a right hand primer (e.g., B_0) and we do not need to transfer the MID as it is within reach of this sequencing primer (i.e., the MID is within 400 bases of sequencing primer B_0). All other B_n primers have the reflex sequence added to their 5' ends ("R" element shown on B primers) so that they read 5' reflex-B_n. However, in certain embodiments, the B_0 primer does include the reflex sequence and is used in the reflex process (along with a corresponding A_0 primer) as detailed below.

As described above, we obtain a single stranded polynucleotide having, in the 5' to 3' orientation, a primer site (e.g., for Roche 454 sequencing), an MID, a reflex sequence and the polynucleotide to be sequenced. Numerous methods for obtaining single-stranded polynucleotides of interest have been described and are known in the art, including in U.S.

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Patent 7,217,522, issued on May 15, 2007; U.S. Patent Application 11/377,462, filed on March 16, 2006; and U.S. Patent Application 12/432,080, filed on April 29, 2009; each of which is incorporated by reference herein in their entirety. For example, a single stranded product can be produced using linear amplification with a primer specific for the primer site of the template. In certain embodiments, the primer includes a binding moiety to facilitate isolation of the single stranded nucleic acid of interest, e.g., to immobilize the top strand on a binding partner of the binding moiety immobilized on a solid support. Removal of a hybridized, non-biotinylated strand by denaturation using heat or high pH (or any other convenient method) serves to isolate the biotinylated strand. Binding moieties and their corresponding binding partners are sometimes referred to herein as binding partner pairs. Any convenient binding partner pairs may be used, including but not limited to biotin/avidin (or streptavidin), antigen/antibody pairs, etc.

It is noted here that while the figures and description of the reflex process provided herein depict manipulations with regard to a single stranded polynucleotide, it is not necessarily required that the single stranded polynucleotide described or depicted in the figures be present in the sample in an isolated form (i.e., isolated from its complementary strand). In other words, double stranded polynucleotides may be used where only one strand is described/depicted, which will generally be determined by the user.

The implementation of a single strand isolation step using the methods described above or variations thereof (or any other convenient single strand isolation step) will generally be based on the desires of the user. One example of isolating single stranded polynucleotides is shown in Figure 2. In this Figure, a starting double stranded template (with 5' to 3' orientation shown as an arrow) is denatured and primed with a biotinylated synthesis primer specific for the primer site. After extension of the primer (i.e., nucleic acid synthesis), the sample is contacted with a solid support having streptavidin bound to it. The biotin moiety (i.e., the binding partner of streptavidin) on the extended strands will bind to the solid-phase streptavidin. Denaturation and washing is then performed to remove all non-biotinylated polynucleotide strands. If desired, the bound polynucleotide, which can be used in subsequent reflex process steps (e.g., as a template for B_n primer extension reactions), may be eluted from the streptavidin support. Alternatively, the bound polynucleotide may be employed in subsequent steps of the desired process while still bound to the solid support (e.g., in solid phase extension reactions using B_n primers). This process, with minor variations depending on the template being used and the identity of the desired single stranded polynucleotide, may be employed at any of a number of steps in which a single

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stranded product is to be isolated. It is noted that in certain embodiments, substrate bound biotinylated polynucleotide can be used to produce and isolate non-biotinylated single stranded products (i.e., by eluting the non-biotinylated products while leaving the biotinylated templates bound to the streptavidin on the solid support). Thus, the specifics of how binding partners are used to isolate single stranded polynucleotides of interest will vary depending on experimental design parameters.

Additional single-stranded isolation/production methods include asymmetric PCR, strand-specific enzymatic degradation, and the use of in-vitro transcription followed by reverse transcriptase (IVT-RT) with subsequent destruction of the RNA strand. As noted above, any convenient single stranded production/isolation method may be employed.

To the single stranded polynucleotide shown in Figure 1B we anneal one of the B_n primers having the appended reflex sequence, denoted with a capital "R" (e.g., B_1) and extend the primer under nucleic acid synthesis conditions to produce a copy of the polynucleotide that has a reflex sequence at its 5` end. A single stranded copy of this polynucleotide is then produced to reverse polarity using a primer specific for the primer site in the first domain' (complement of the first domain 102). The resulting nucleic acid has structure 100 shown in Figure 1A, where the first domain 102 includes the primer site and the MID. Site A (110) in Figure 1 is determined by the specificity of the 5' reflex-B_n primer used.

The reflex process (e.g., as shown in Figure 1) is then performed to produce a product in which the primer site and the MID are now in close proximity to the desired site (or region of interest (ROI)) within the original polynucleotide (i.e., the site defined by the primer used, e.g., B_1). The resulting polynucleotide can be used in subsequent analyses as desired by the user (e.g., Roche 454 sequencing technology).

It is noted here that, while not shown in Figure 1A and 1B, any convenient method for adding adapters to a polynucleotide to be processed as described herein may be used in the practice of the reflex process (adapters containing, e.g., primer sites, polymerase sites, MIDs, restriction enzyme sites, and reflex sequences). For example, adapters can be added at a particular position by ligation. For double stranded polynucleotides, an adapter can be configured to be ligated to a particular restriction enzyme cut site. Where a single stranded polynucleotide is employed, a double stranded adapter construct that possesses an overhang configured to bind to the end of the single-stranded polynucleotide can be used. For example, in the latter case, the end of a single stranded polynucleotide can be modified to include specific nucleotide bases that are complementary to the overhang in the double

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stranded adaptor using terminal transferase and specific nucleotides. In other embodiments, PCR or linear amplification methods using adapter-conjugated primers is employed to add an adapter at a site of interest. Again, any convenient method for producing a starting polynucleotide may be employed in practicing the methods of the subject invention.

In certain embodiments, the nucleic acid may be sequenced directly using a sequencing primer specific for the primer site. This sequencing reaction will read through the MID and desired site in the insert.

In certain embodiments, the polynucleotide may be isolated (or fractionated) using an appropriate A_n primer (e.g., when using B_1 as the first primer, primer A_1 can be used). In certain embodiments, the A_n primed polynucleotide is subjected to nucleic acid synthesis conditions to produce a copy of the fragment produced in the reflex process. In certain of these embodiments, the A_n primer has appended on its 5' end a primer site that can be used in subsequent steps, including sequencing reactions. Providing a primer site in the A_n primer allows amplifying and/or sequencing from both ends of the resultant fragment: from the primer site in the first domain 102 and the primer site in the A_n primer (not shown in Figure 1B). Because of the position of the primer sites and their distance apart (i.e., less than one sequencing run apart), sequencing from both ends will usually capture the sequence of the desired site (or ROI) and the sequence of the MID, which can be used for subsequent bioinformatic analyses, e.g., to positively identify the sample of origin. It is noted here that while sequencing in both directions is possible, it is not necessary, as sequencing from either primer site alone will capture the sequence of the ROI as well as its corresponding MID sequence.

Note that in certain embodiments, the first fragment obtained by amplification/extension from primer B_0 directly, the polarity of the ROI in the resulting fragment is reversed as compared to the ROI in fragments obtained by primers B_1-B_n. This is because the B_0-generated fragment, unlike the B_1-B_n generated fragments, has not been subjected to a reflex process which reverses the orientation of the ROI sequence with respect to the first domain/reflex sequence (as described above). Therefore, the B_0 primer may have appended to it a primer site (e.g., at its 5' end) that can be used for subsequent amplification and/or sequencing reactions (e.g., in Roche 454 sequencing system) rather than a reflex sequence as with primers B_1-B_n. However, in certain embodiments, as noted above, the reflex process may be used with a corresponding B_0-A_0 primer pair as described above, i.e., using a B_0 primer having a 5' reflex sequence and a corresponding A_0 primer with its corresponding 5' adapter domain (e.g., a primer site).

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It is noted here that because the particular sections of sequence to be analyzed are defined by the A_n-B_n primer pairs (as shown and described above), a much higher sequence specificity is achieved as compared to using previous extraction methods that employ only a single oligo binding event (e.g., using probes on a microarray).

Figure 3 provides a detailed flow chart for an exemplary embodiment that employs reflex sequences for use in sequencing multiple specific regions in a polynucleotide (i.e., regions 1, 2, 3, 4 and 5 in an 11kb region of lambda DNA).

A single parent DNA fragment 202 is generated that includes adapter domains (i.e., a Roche 454 sequencing primer site, a single MID, and a reflex sequence) and the sequence of interest. In the example shown, the sequence of interest is from lambda DNA and the reflex sequence is present on the top strand (with its complement shown in the bottom strand). Any convenient method for producing this parent DNA fragment may be used, including amplification with a primer that includes the adapter domains (e.g., using PCR), cloning the fragment into a vector that includes the adapter domains (e.g., a vector with the adapter domains adjacent to a cloning site), or by attaching adapters to polynucleotide fragments (e.g., fragment made by random fragmentation, by sequence-specific restriction enzyme digestion, or combinations thereof). While only a single fragment with a single MID is shown, the steps in Figure 3 are applicable to samples having multiple different fragments each with a different MID, e.g., a sample having a population of homologous fragments from any number of different sources (e.g., different individuals). Figure 3 describes the subsequent enzymatic steps involved in creating the five daughter fragments in which regions 1, 2, 3, 4 and 5 (shown in polynucleotide 204) are rearranged to be placed within a functional distance of the adapter domains (i.e., close enough to the adapter domains to be sequenced in a single Roche 454 sequencing reaction). Note that certain steps are shown for region 4 only (206).

In step 1, the five regions of interest are defined within the parent fragment (labeled 1 to 5 in polynucleotide 204) and corresponding primer pairs are designed for each. The distance of each region of interest from the reflex sequence is shown below polynucleotide 204. The primer pairs are designed as described and shown in Figure 1B (i.e., the A_n-B_n primer pairs). For clarity, only primer sites for region 4 are shown in Figure 3 ("primer sites" surrounding region 4). In step 2, sequence specific primer extensions are performed (only region 4 is shown) with corresponding B_n primers to produce single stranded polynucleotides having structure 208 (i.e., having the reflex sequence on the 5' terminus). As shown, the B_n primer for region 4 will include a sequence specific primer site that primes

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at the 3'-most primer site noted for region 4 (where "3'-most" refers to the template strand, which in Figure 3 is the top strand). This polynucleotide is copied back to produce polynucleotide 210 having reversed polarity (e.g., copied using a primer that hybridizes to the 454A' domain). Polynucleotide 210 has structure similar to polynucleotide 100 shown at the top of Figure 1. Step 4 depicts the result of the intramolecular priming between the reflex sequence and its complement followed by extension to produce the MID' and 454A' structures at the 3' end (polynucleotide 212). In the embodiments shown in Figure 3, polynucleotide 212 is treated with T7 exonuclease to remove double stranded DNA from the 5' end (as indicated above, this step is optional). The polynucleotide formed for region 4 is shown as 216 with polynucleotides for the other regions also shown (214).

It is noted here that the formation of each of the polynucleotides 214 may be accomplished either in separate reactions (i.e., structure with region 1 in proximity to the adapter domains is in a first sample, the structure with region 2 in proximity to the adapter region is in a second sample, etc.) or in one or more combined sample.

In step 6 the polynucleotides 214 are copied to reverse polarity to form polynucleotides 218. In step 7, each of these products are then primed with the second primer of the specific primer pair (see A_n primers as shown in Figure 1B) each having a second Roche 454 primer site (454B) attached at the 5' end, and extended to form products 220. Steps 6 and 7 may be combined (e.g., in a single PCR or other amplification reaction).

In summary, Figure 3 shows how the reflex process can be employed to produce five daughter fragments 220 of similar length (e.g., ~500 bp) each of which contain DNA sequences that differ in their distance from the reflex sequence in the starting structure 202 while maintaining the original MID.

Figure 4 shows another exemplary use of the reflex process as described herein. In the embodiment shown in Figure 4, a target sequence (i.e., containing region of interest "E") is enriched from a pool of adapter-attached fragments. In certain embodiments, the fragments are randomly sheared, selected for a certain size range (e.g., DNA having a length from 100 to 5000 base pairs), and tagged with adapters (e.g., asymmetric adapters, e.g., as described in US Patent Application Ser. No. 12/432,080, filed on April 29, 2009). The asymmetric adaptor employed in Figure 4 contains a sequencing primer site (454A, as used in the Roche 454 sequencing platform), an MID, an X sequence, and an internal stem region (ISR), which denotes the region of complementarity for the asymmetric adapter that is adjacent to the adapter attachment site (see, e.g., the description in US Application Ser. No. 12/432,080, filed on April 29, 2009, incorporated herein by reference in its entirety). The X

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sequence can be any sequence that can serve as a binding site for a polynucleotide containing the complement of the X sequence (similar to a primer site). As described below, the X sequence allows for the annealing of an oligonucleotide having a 5' overhang that can serve as a template for extension of the 3' end of the adaptor oligonucleotide. The sequencing direction of the sequencing primer site (454A primer site in structure 401 of Figure 4) is oriented such that amplification of the adapter ligated fragment using the sequencing primer site proceeds away from the ligated genomic insert. This has the effect of making the initial asymmetric adapter ligated library 'inert' to amplification using this primer, e.g., in a PCR reaction.

To extract a region of interest (the "E" region), the library is mixed with an oligonucleotide (403) containing a 3' X' sequence and a target specific priming sequence (the 1' sequence) under hybridization/annealing conditions. The target specific sequence 1' is designed to flank one side of the region of interest (the 1' sequence adjacent to E in the genomic insert; note that only the E-containing polynucleotide fragment is shown in Figure 4), much like a PCR primer. After annealing primer 403, the hybridized complex is extended, whereby all of the adaptor tagged fragments will obtain the complement of the target specific sequence (i.e., the 1 sequence) on the 3' end (see structure 405; arrows denote the direction of extension).

Extended products 405 are then denatured and the 1/1' regions allowed to hybridize intramolecularly in a reflex process priming event, after which nucleic acid extension is performed to form structure 407 (extension is from the 1 priming site; shown with an arrow). This reflex reaction creates a product (407) that, unlike its parent structure (405), has a sequencing primer site (454A) that is oriented such the extension using this primer sequence proceeds towards the region of interest. Thus, in the absence of a priming and extension reflex reaction, extension with a sequencing primer will not generate a product containing the region of interest (the E region). In other words, only E-region containing target polynucleotides will have a 454A sequence that can amplify genomic material (structure 407).

After completing the reflex process (using 1/1' as the reflex sequences), a PCR amplification reaction is performed to amplify the region of interest (with associated adapter domains). However, before performing the PCR reaction, the fragment sample is "inactivated" from further extension using terminal transferase and ddNTPs. This inactivation prevents non-target adaptor tagged molecules from performing primer extension from the 3' primer 1 site. Once inactivated, a PCR reaction is performed using a sequencing

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primer (i.e., 454A primer 409) and a second primer that primes and extends from the opposite side of the region of interest (i.e., primer 411, which includes a 5' 454B sequencing primer site and a 3' "2" region that primes on the opposite end of E from the 1 region). Only fragments that have undergone the reflex process and contain the E region will be suitable templates for the PCR reaction and produce the desired product (413).

Thus, the process exemplified in Figure 4 allows for the movement of an adapter domain (e.g., containing functional elements and/or MID) into proximity to a desired region of interest.

The reflex process described herein can be used to perform powerful linkage analysis by combining it with nucleic acid counting methods. Any convenient method for tagging and /or counting individual nucleic acid molecules with unique tags may be employed (see, e.g., U.S. Patent 7,537,897, issued on May 26, 2009; U.S. Patent 7,217,522, issued on May 15, 2007; U.S. Patent Application 11/377,462, filed on March 16, 2006; and U.S. Patent Application 12/432,080, filed on April 29, 2009; each of which is incorporated by reference herein in their). All of this can be conducted in parallel thus saving on the cost of labor, time and materials.

In one exemplary embodiment, a large collection of sequences is tagged with MID such that each polynucleotide molecule in the sample has a unique MID. In other words, each polynucleotide in the sample (e.g., each individual double stranded or single stranded polynucleotide) is tagged with a MID that is different from every other MID on every other polynucleotide in the sample. In general, to accomplish such molecular tagging the number of distinct MID tags to be used should be many times greater than the actual number of molecules to be analyzed. This will result in the majority of individual nucleic acid molecules being labeled with a unique ID tag (see, e.g., Brenner et al., Proc. Natl. Acad. Sci. USA. 2000 97(4):1665-70). Any sequences that then result from the reflex process on that particular molecule (e.g., as described above) will thus be labeled with the same unique MID tag and thus inherently linked. Note that once all molecules in a sample are individually tagged, they can be manipulated and amplified as much as needed for processing so long as the MID tag is maintained in the products generated.

For example, we might want to sequence one thousand viral genomes (or a specific genomic region) or one thousand copies of a gene present in somatic cells. After tagging each polynucleotide in the sample with a sequencing primer site, MID and reflex sequence (as shown in the figures and described above), we use the reflex process to break each polynucleotide into lengths appropriate to the sequencing procedure being used, transferring

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the sequencing primer site and MID to each fragment (as described above). Obtaining sequence information from all of the reflex-processed samples can be used to determine the sequence of each individual polynucleotide in the starting sample, using the MID sequence to defining linkage relationships between sequences from different regions in the polynucleotide being sequenced. Using a sequencing platform with longer read lengths can minimize the number of primers to be used (and reflex fragments generated).

The advantages noted above are shown in Figure 5. This figure shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information. It is noted that not all domain structures and steps are shown in the reflex process for simplicity.

Kits and Systems

Also provided by the subject invention are kits and systems for practicing the subject methods, as described above, such vectors configured to add reflex sequences to nucleic acid inserts of interest and regents for performing any steps in the cloning or reflex process described herein (e.g., restriction enzymes, nucleotides, polymerases, primers, exonucleases, etc.). The various components of the kits may be present in separate containers or certain compatible components may be precombined into a single container, as desired.

The subject systems and kits may also include one or more other reagents for preparing or processing a nucleic acid sample according to the subject methods. The reagents may include one or more matrices, solvents, sample preparation reagents, buffers, desalting reagents, enzymatic reagents, denaturing reagents, where calibration standards such as positive and negative controls may be provided as well. As such, the kits may include one or more containers such as vials or bottles, with each container containing a separate component for carrying out a sample processing or preparing step and/or for carrying out one or more steps of a nucleic acid variant isolation assay according to the present invention.

In addition to above-mentioned components, the subject kits typically further include instructions for using the components of the kit to practice the subject methods, e.g., to prepare nucleic acid samples for perform the reflex process according to aspects of the

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subject methods. The instructions for practicing the subject methods are generally recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or sub-packaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g. CD-ROM, diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

In addition to the subject database, programming and instructions, the kits may also include one or more control samples and reagents, e.g., two or more control samples for use in testing the kit.

Utility

The reflex process described herein provides significant advantages in numerous applications, a few of which are noted below (as well as described above).

For example, as described above, certain aspects of the reflex process define the particular sections of sequence to be analyzed by a primer pair, as in PCR (e.g., the two oligos shown as A_n-B_n in Figure 1B). This results in higher sequence specificity as compared to other extraction methods (e.g., using probes on a microarray) that only use a single oligo sequence. The separation of the probes defines a length that can be relatively uniform (hence making subsequent handling including amplification more uniform) and can also be tailored to the particular sequencing platform being employed.

Further, as described above, aspects of the present invention can be used to analyze homologous genomic locations in a multiplexed sample (i.e., a sample having polynucleotides from different genomic samples) in which the polynucleotides are tagged with the MID. This is possible because the reflex process, which operates intramolecularly, maintains the MID thus linking any particular fragment to the sample from which it originates.

Finally, as the reflex processes described herein function intramolecularly, one can determine the genetic linkage between different regions on the same large fragment that are too far apart to be sequenced in one sequence read. Such a determination of linkage may be of great value in plant or animal genetics (e.g., to decide if a particular set of variations are

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linked together on the same stretch of chromosome) or in viral studies (e.g., to determine if particular variations are linked together on the same stretch of a viral genome/transcripts, e.g., HIV, hepatitis virus, etc.).

EXAMPLES

Example I

Figures 6 and 7 provide experimental data and validation of the reflex process described herein using synthetic polynucleotide substrates.

METHODS

Substrate:

The 100 base oligonucleotide substrate (as shown diagrammatically in Figure 6A) was synthesized with internal fluorescein-dT positioned between the REFLEX and REFLEX' sequences. This label provides convenient and sensitive method of detection of oligonucleotide species using polyacrylamide gel electrophoresis.

Extension reactions:

Reactions were prepared which contained 1 μM of the 100 base oligonucleotide substrate, 200 μM dNTPs, presence or absence of 1 μM competitor oligonucleotide, 0.5 μl of each DNA polymerase ("DNAP"): Vent (NEB, 2 units/μl), Taq (Qiagen HotStarTaq 5 units/μl) and Herculase (Stratagene), and made up to 50 μl with the appropriate commercial buffers for each polymerase and dH_20. For Taq titrations 0.5 μl, 1 μl, 2 μl, and 3 μl enzyme was used in the same 50 μl volume. Reactions were heated in a Biometra thermocycler to 95 °C for 15 minutes (Taq) or 5 minutes (Herculase, Vent), followed by 55 °C or 50 °C for 30 seconds, and a final incubation at 72 °C for 10 minutes.

T7 exonuclease digestions:

Reactions were prepared with 10 μl extension reactions above, 0.5 μl T7 exonuclease (NEB, 10 units/μl), and made up to 50 μl using NEB Buffer 4 and dH_20. Reactions were incubated at 25 °C for 30 minutes.

Gel Electrophoresis analysis:

An 8% denaturing polyacrylamide gel was used to analyze reaction species. 0.4 μl of extension reactions, and 2 μl of digestion reactions were loaded and ran at 800V for ~1.5 hours. Gels were analyzed for fluorescein using an Amersham/General Electric Typhoon imager.

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RESULTS

Figure 6A shows the structure of each stage of reflex sequence processing with the expected nucleic acid size shown on the left. The initial single stranded nucleic acid having a sequencing primer site (the Roche 454 sequencing primer A site; listed as 454A); an MID; a reflex sequence; the insert; and a complement of the reflex sequence is 100 nucleotides in length. After self-annealing and extension, the product is expected to be 130 nucleotides in length. After removal of the double stranded region from the 5' end, the nucleic acid is expected to be 82 bases in length.

Figure 6B shows the results of three experiments using three different nucleic acid polymerases (Vent, Herculase and Taq, indicated at the top of the lanes). The temperature at which the annealing was carried out is shown at the top of each lane (either 50°C or 55°C). The sizes of the three nucleic acids as noted above are indicated on the left and right side of the gel.

As shown in figure 6B, extension appears to be most efficient under the conditions used with Herculase (Herculase is a mixture of two enzymes: modified Pfu DNAP and Archaemax (dUTPase)). Most (or all) of the initial 100 base pair nucleic acid are converted to the 130 base pair product (see lanes 6 and 7). However, after T7 exonuclease digestion the 3'-5' exonuclease activity of Herculase results in partial digestion of the desired 82 base product (note bands at and below the 82 base pairs in lanes 8 and 9).

Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product after T7 exonuclease digestion (see lane 13).

Figure 7 shows the effect on the reflex process of increasing amounts of Taq polymerase as well as the use of a reflex sequence competitor (schematically shown in Figure 7A).

As shown in lanes 2 to 5, increased Taq concentration improves extension to ~90% conversion of the starting nucleic acid (see lane 5). Lanes 7 to 8 show that T7 exonuclease digestion does not leave a perfect 82 base product. This may be due to collapse of dsDNA when T7 exonuclease has nearly completed its digestion from the 5' end in the double stranded region of the fold-back structure. It is noted that in many embodiments, the removal of a few additional bases from the 5' end of the polynucleotide will not interfere with subsequent analyses, as nucleotide bases at the 5' end are often removed during subsequent steps.

As shown in Lanes 11-14, addition of a competitor (which can interfere with annealing of the reflex sequences to form the fold-back structure) results in only a small

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decrease (~5-10%) of fully extended product. Thus, as expected, the intramolecular reaction is heavily favored. Although not shown, we have observed that the competitor oligonucleotide also gets extended by the same amount (~5-10%).

The concentration of the competitor, the concentration of the reflex substrate, and the overall genetic complexity, will all likely affect specific results. The experiments shown in Figures 6 and 7 demonstrate that the core parts of the reflex processes as described herein is functional and can be implemented.

Example II

Figure 8 shows the reflex workflow (diagram at left) and exemplary results of the workflow (gel at right) for a specific region of interest (ROI). The starting material is a double stranded nucleic acid molecule (700) that contains a 454A primer site, an MID, a reflex site, and a polynucleotide of interest having three ROIs (2, 3 and 4) at different locations therein. This starting material was subjected to reflex processes (as described in above) specific for ROI 2 as shown in the diagram at the left of the figure, both with and without the use of a T7 exonuclease step (the T7 exonuclease step is shown in the diagram is indicated as "Optional").

Completion of all steps shown in the reflex process should result in a double stranded polynucleotide of 488 base pairs (702) with or without the T7 exonuclease step.

As shown in the gel on the right of Figure 8, the 488 base pair product was produced in reflex processes with and without the T7 exonuclease step.

Figure 9 shows an exemplary protocol for a reflex process based on the results discussed above. The diagram shows specific reflex process steps with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt SPRI beads to remove primer oligos). One reason for performing such purification steps is to reduce the potential for generating side products in a reaction (e.g., undesirable amplicons). While Figure 9 indicates three purification steps, fewer or additional purification steps may be employed depending on the desires of the user. It is noted that the steps of reversing polarity, reflex priming and extension, and "stretch out" (or denaturation) / second reversing polarity step can be performed without intervening purification steps.

The protocol shown in Figure 9 includes the following steps:

annealing a first primer containing a 5' reflex sequence (or reflex tail, as noted in the figure) specific for the 3' primer site for the R' region to the starting polynucleotide and extending (the primer anneals to the top strand at the primer site at the right of R in

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polynucleotide 902, indicated with a *; this step represents the first denature, anneal and extend process indicated on the right);

after purification, adding a 454A primer and performing three cycles of denaturing, annealing and extending: the first cycle results in the copy-back from the 454A primer to reverse the polarity of the strand just synthesized; the second cycle breaks apart the double stranded structure produced, allows the reflex structure to form and then extend; the third cycle results in another copy-back using the same 454A primer originally added;

after purification, adding a second primer specific for the second primer site for the R' region having a 5' 454B tail (this primer anneals to the primer site 3' of the R' region in polynucleotide 904, indicated with a *) and denaturing, annealing and extending resulting in a polynucleotide product having 454A and 454B sites surrounding the MID, the reflex sequence, and R'. Note that the first primer specific for the R' region and the second primer specific for the R' region define its boundaries, as described above and depicted in Figure 1B);

after another purification, adding 454A and 454B primers and performing a PCR amplification reaction.

Example III

As described above, a reflex sequence can be an "artificial" sequence added to a polynucleotide as part of an adapter or can be based on a sequence present in the polynucleotide of interest being analyzed, e.g., a genomic sequence (or "non-artificial").

The data shown in prior Examples used "artificial" reflex sites. In this Example, the reflex site is a genomic sequence present in the polynucleotide being analyzed.

The starting material is a double stranded DNA containing a 454A site, an MID and a polynucleotide to be analyzed. The 454A and MID were added by adapter ligation to parent polynucleotide fragments followed by enrichment of the polynucleotide to be analyzed by a hybridization-based pull-out reaction and subsequent secondary PCR amplification (see Route 1 in Figure 13). Thus, the reflex site employed in this example is a sequence normally present at the 5' end of the subject polynucleotide (a genomic sequence). The polynucleotide being analyzed includes a region of interest distal to the 454A and MID sequences that is 354 base pairs in length.

This starting double stranded nucleic acid is 755 base pairs in length. Based on the length of each of the relevant domains in this starting nucleic acid, the reflex process should result in a product of 461 base pairs.

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Figure 10 shows the starting material for the reflex process (left panel) and the resultant product generated using the reflex process (right panel; reflex process was performed as described in Example II, without using a T7 exonuclease step). A size ladder is included in the left hand lane of each gel to allow estimation of the size of the test material. This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in moving an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

Example IV

Figure 11 shows a schematic of an experiment in which the reflex process is performed on a single large initial template (a "parent" fragment) to generate 5 different products ("daughter" products) each having a different region of interest (i.e., daughter products are produced having either region 1, 2, 3, 4 or 5). The schematic in Figure 11 shows the starting fragment (11,060 base pairs) and resulting products (each 488 base pairs) generated from each of the different region of interest-specific reflex reactions (reflex reactions are performed as described above). The panel (gel) on the bottom of Figure 11 shows the larger starting fragment (Lane 1) and the resulting daughter products for each region-specific reflex reaction (lanes 2 to 6, with the region of interest noted in each in the box), where the starting and daughter fragments have the expected lengths. Sequencing of the products confirmed the identity of the region of interest in each of the reflex products shown in the gel. These results demonstrate that multiple different reflex products can be generated from a single, asymmetrically tagged parent fragment while maintaining the adapter domains (e.g., the primer sites and MID).

Example V

Figure 12 details experiments performed to determine the prevalence of intramolecular rearrangement (as desired in the reflex process) vs. intermolecular rearrangement. Intermolecular rearrangement is undesirable because it can lead to the transfer of an MID from one fragment to another (also called MID switching). MID switching can occur if a reflex sequence in a first fragment hybridizes to its complement in a second fragment during the reflex process, leading to appending the MID from the second fragment to the first fragment. Thus, intermolecular rearrangement, or MID switching, should be minimized to prevent the transfer of an MID from one fragment in the sample to another, which could lead to a misrepresentation of the source of a fragment.

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To measure the prevalence of MID switching under different reflex conditions, fragments having different sizes were generated that included two different MIDs, as shown in the top panel of Figure 12. The common sequence on these fragments serves as the priming site for the first extension reaction to add the second reflex sequence (see, e.g., step 2 of Figure 3). Three exemplary fragments are shown in Figure 12 for each different fragment size (i.e., 800 base pairs with an MIDB and MIDA combination; 1900 base pairs with MIDC and MIDA combination; and 3000 base pairs with MIDD and MIDA combination). For each MID family (A, B, C and D), there are 10 different members (i.e., MIDA had 10 different members, MIDB has 10 different members, etc.). A set of 10 dual MID fragments for each different size fragment (i.e., 800, 1900 and 3000 base pairs) were generated, where the MID pairs (i.e., MIDA/MIDB, MIDA/MIDC, and MIDA/MIDD) were designated as 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10. All 10 fragments of the same size were then mixed together and a reflex protocol was performed.

Due to the domain structure of the fragments, a successful reflex process results in the two MIDs for each fragment being moved to within close enough proximity to be sequenced in a single read using the Roche 454 sequencing platform (see the reflex products shown in the schematic of Figure 12). The reflex reactions for each fragment size were performed at four different fragment concentrations to determine the effect of this parameter, as well as fragment length, in the prevalence of MID switching. The reflex products from each reaction performed were subjected to 454 sequencing to determine the identity of both MIDs on each fragment, and thereby the proportion of MID switching that occurred.

The panel on the bottom left of Figure 12 shows the rate of MID switching (Y axis, shown in % incorrect (or switched) MID pair) for each different length fragment at each different concentration (X axis; 300, 30, 3 and 0.3 nM). As shown in this panel, the MID switch rate decreases with lower concentrations, as would be expected, because intermolecular, as opposed to intramolecular, binding events are concentration dependent (i.e., lower concentrations lead to reduced intermolecular hybridization/binding). In addition, the MID switch rate decreases slightly with length. This is somewhat unexpected as the ends of longer DNA fragments are effectively at a lower concentration with respect to one another. The reasons for why we do not see this is probably because the production of reflex priming intermediates continues during the final PCR, which means that reflex priming reactions are happening continuously which contributes to MID switching. It is probably the case that the shorter reflex products are able to undergo a higher rate of 'background' reflexing, and therefore increase the overall MID switch rate a little.

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These results demonstrate that MID switching can be minimized (e.g., to below 2%, below 1% or even to nearly undetectable levels) by altering certain parameters of the reaction, e.g., by reducing fragment concentration and/or fragment length.

The panel on the bottom right of Figure 12 shows the frequency of MID switching in the reflex process for the 800 base pair fragments (i.e., MIDA/MIDB containing fragments). In this figure, the area of each circle is proportional to the number of reads containing the corresponding MIDA and MIDB species (e.g., MIDA1/MIDB1; MIDA1/MIDB2; etc.). Thus, a circle representing 200 reads will be 40 times larger in terms of area than a circle representing 5 reads.

As noted above, the MIDA/MIDB combinations having the same number (shown on the X and Y axis, respectively) represent the MIDA/MIDB combinations present in the sample prior to the reflex process being performed (i.e., MIDA/MIDB combinations 1/1, 2/2, 3/3, 4/4, 5/5, 6/6, 7/7, 8/8, 9/9, and 10/10 were present in the starting sample). All other MIDA/MIDB combinations identified by Roche 454 sequencing were the result of MID switching.

This figure shows that the MID switching that occurs during the reflex process is random, i.e., that MID switching is not skewed based on the identity of the MIDs in the reaction).

Exemplary Reflex Protocols

Figure 13 shows a diagram of exemplary protocols for performing the reflex process on pools of nucleic acids, for example, pools of nucleic acids from different individuals, each of which are labeled with a unique MID. In Route 3, a pooled and tagged extended library is subjected directly to a reflex process. In Route 2, the pooled library is enriched by target-specific hybridization followed by performing the reflex process. In Route 1 employs enrichment by PCR amplification. As shown in Figure 13, PCR enrichment can be performed directly on the pooled tagged extended library or in a secondary PCR reaction after a hybridization-based enrichment step has been performed (as in Route 2) to generate an amplicon substrate that is suitable for the reflex process. Additional routes for preparing a polynucleotide sample for performing a reflex process can be implemented (e.g., having additional amplification, purification, and/or enrichment steps), which will generally be dependent on the desires of the user.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes

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and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

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CLAIMS

That which is claimed is:

1. A method of moving a domain in a nucleic acid, the method comprising:

a) annealing a reflex sequence in a polynucleotide strand in a sample with a complement of the reflex sequence present in the polynucleotide strand, wherein the polynucleotide strand comprises the following in a 5' to 3' orientation:

a first domain;

the reflex sequence;

a polynucleotide of interest; and

the complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand; and

b) extending from the 3' end of the complement of the reflex sequence through the first domain to produce a double stranded region;

whereby a complement of the first domain is present at the 3' end of the extended polynucleotide strand, thereby moving the first domain in the polynucleotide strand.

2. The method of claim 1, wherein the first domain comprises a multiplex identifier (MID).

3. The method of claim 1, wherein the first domain comprises a primer site.

4. The method of claim 1, wherein the reflex sequence is from 10 to 50 nucleotides in length.

5. The method of claim 1, wherein the reflex sequence comprises an RNA polymerase site.

6. The method of claim 1, the method further comprising removing the first domain and the reflex sequence from the 5' end of the double stranded region.

7. The method of claim 6, wherein the removing step comprises treating the nucleic acid of step b with T7 exonuclease.

8. The method of claim 1, wherein the first domain is present in an adapter region attached to the polynucleotide of interest.

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9. The method of claim 8, wherein the reflex sequence is present in the adapter region.

10. The method of claim 8, wherein the reflex sequence is a sequence present within the 5' region of the polynucleotide of interest.

11. The method of claim 1, wherein the sample is a multiplexed sample, wherein the first domain in each of the polynucleotide strands in the multiplexed sample comprises a MID corresponding to its parental sample.

12. The method of claim 1, wherein the sample comprises multiple polynucleotide strands, and wherein each polynucleotide strand in the sample comprises a unique MID in the first domain.

13. The method of claim 1, wherein the polynucleotide strand is produced by:

annealing a nucleic acid synthesis primer to a parent polynucleotide strand, wherein the parent polynucleotide strand comprises the following in a 5' to 3' orientation:

the first domain;

the reflex sequence; and

a second domain comprising the polynucleotide of interest;

wherein the nucleic acid synthesis primer comprises:

a 3' priming domain that is complementary to a site adjacent to and 3' of the polynucleotide of interest in the second domain; and

a 5' reflex sequence;

extending the nucleic acid synthesis primer to produce an extension product; and

replicating the resultant extension product with a second nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain, thereby producing the polynucleotide strand.

14. The method of claim 13, wherein the length of the second domain is from 30 bases to 30 kilobases.

15. The method of claim 13, wherein the parent polynucleotide strand is enriched prior to the annealing step based on its sequence.

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16. The method of claim 1, further comprising the steps of:

c) replicating the polynucleotide strand of step b with a first nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain to produce a first extension product;

d) annealing a second nucleic acid synthesis primer to the first extension product, wherein the second nucleic acid synthesis primer comprises a 3' priming domain that is complementary to a site within the polynucleotide of interest in the first extension product; and

e) extending the second synthesis primer to produce a second extension product.

17. The method of claim 16, wherein the second synthesis primer comprises a 5' domain that is not complementary to the polynucleotide of interest in the first extension product.

18. The method of claim 17, wherein the first domain comprises a first primer site and the 5' domain of the second synthesis primer comprises a second primer site.

19. The method of claim 18, wherein the length of the polynucleotide of interest in the second extension product is from 50 to 10,000 nucleotides.

20. A polynucleotide strand comprising the following in a 5' to 3' orientation:

a first domain;

a reflex sequence;

a polynucleotide of interest; and

a complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand.

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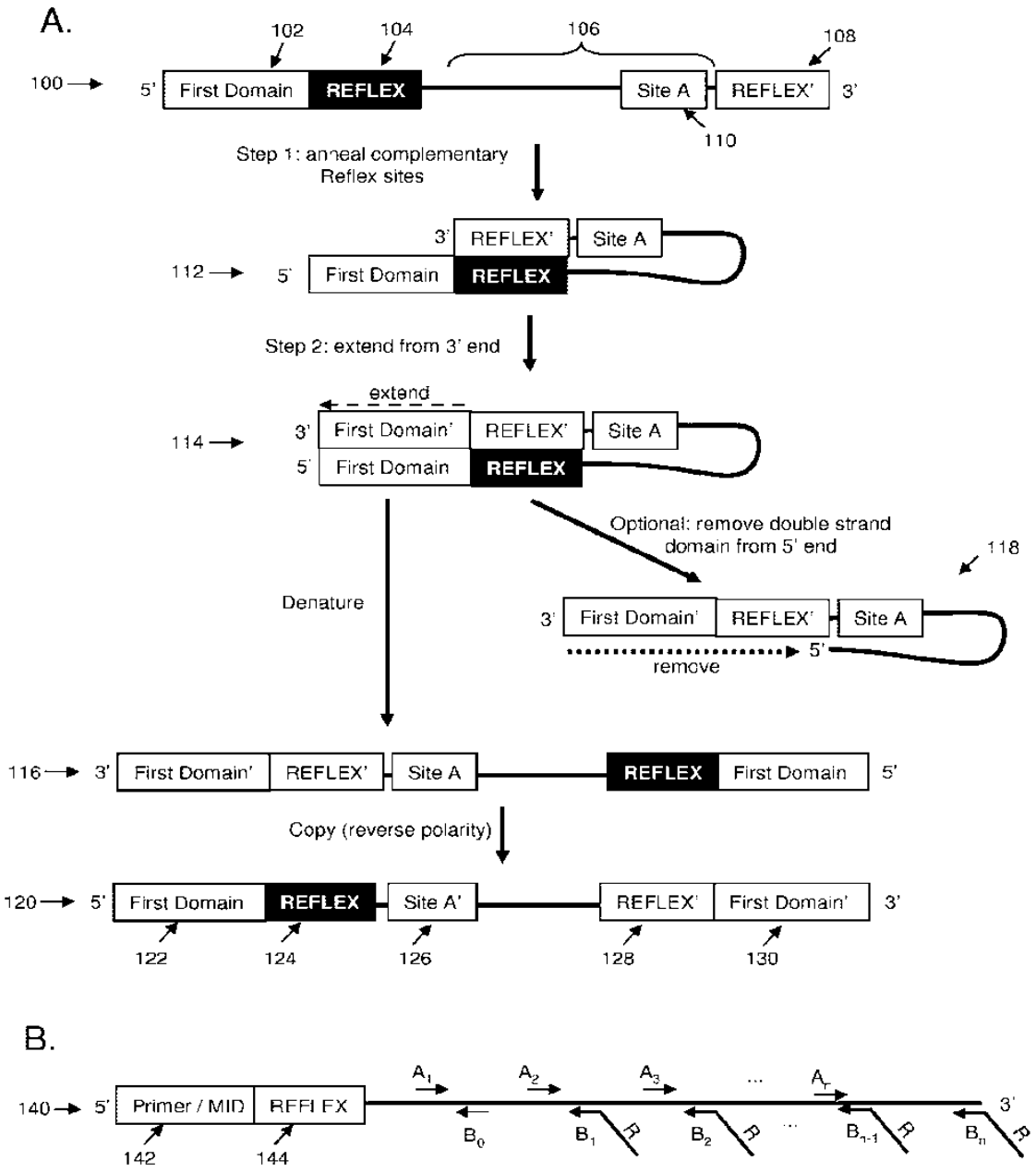

A.
100
5'
First Domain
REFLEX
102
104
106
108
Site A
REFLEX'
3'
110
Step 1: anneal complementary Reflex sites
112
3'
REFLEX'
Site A
5'
First Domain
REFLEX
Step 2: extend from 3' end
extend
114
3'
First Domain'
REFLEX'
Site A
5'
First Domain
REFLEX
Optional: remove double strand domain from 5' end
118
Denature
3'
First Domain'
REFLEX'
Site A
5'
remove
116
3'
First Domain'
REFLEX'
Site A
REFLEX
First Domain
5'
Copy (reverse polarity)
120
5'
First Domain
REFLEX
Site A'
REFLEX'
First Domain'
3'
122
124
126
128
130
B.
140
5'
Primer / MID
REFLEX
142
144
A1
A2
A3
...
Ar
3'
B0
B1
R
B2
R
...
Bn-1
R
Bn
R

Fig. 1

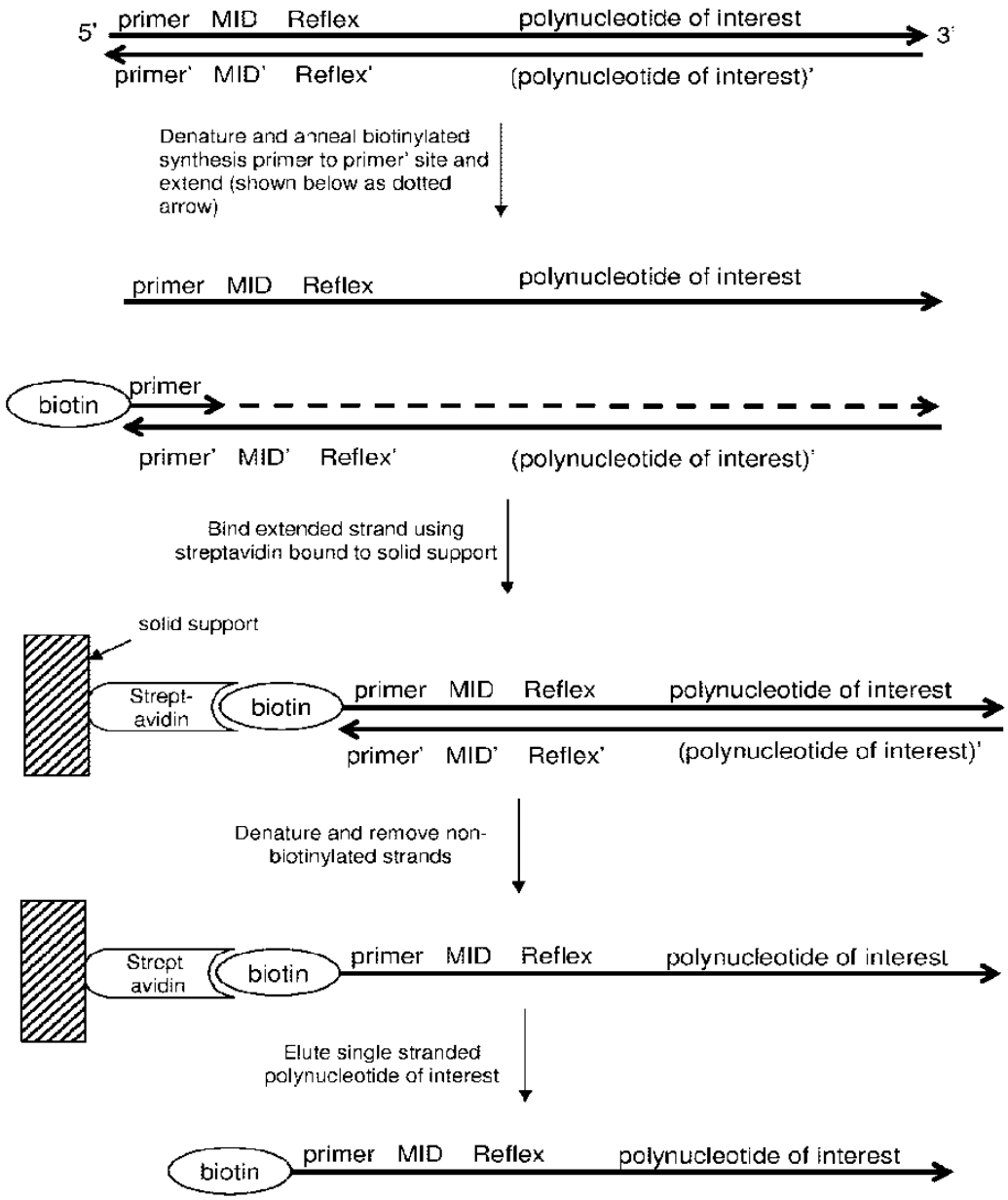
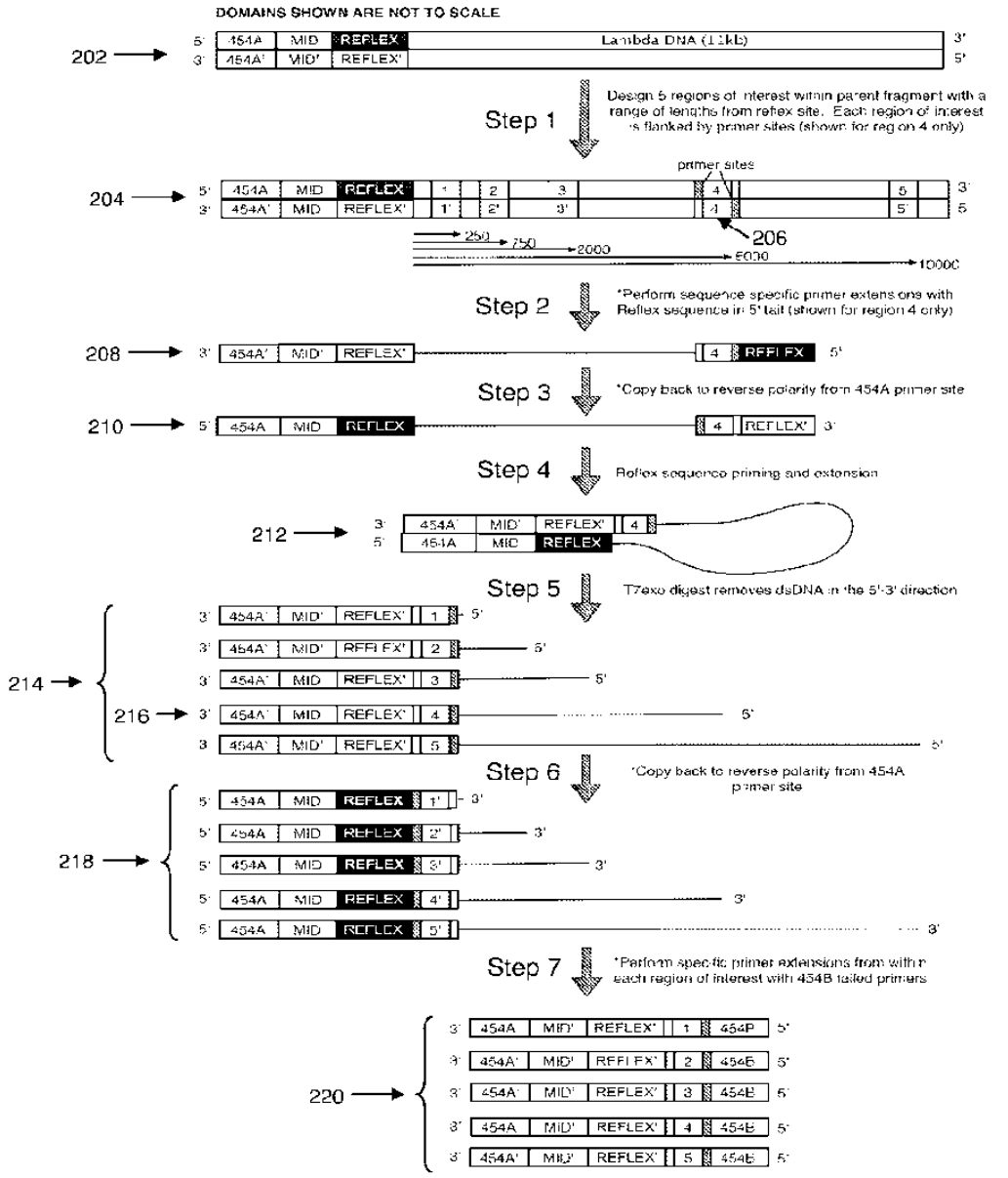

5'
primer MID Reflex
polynucleotide of interest
3'
primer' MID' Reflex'
(polynucleotide of interest)'
Denature and anneal biotinylated synthesis primer to primer' site and extend (shown below as dotted arrow)
primer MID Reflex
polynucleotide of interest
biotin
primer
primer' MID' Reflex'
(polynucleotide of interest)'
Bind extended strand using streptavidin bound to solid support
solid support
Strept-avidin
biotin
primer MID Reflex
polynucleotide of interest
primer' MID' Reflex'
(polynucleotide of interest)'
Denature and remove non-biotinylated strands
Strept avidin
biotin
primer MID Reflex
polynucleotide of interest
Elute single stranded polynucleotide of interest
biotin
primer MID Reflex
polynucleotide of interest

Fig. 2



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g. using primers with binding moieties and/or multiple cycles of extension)

Fig. 3

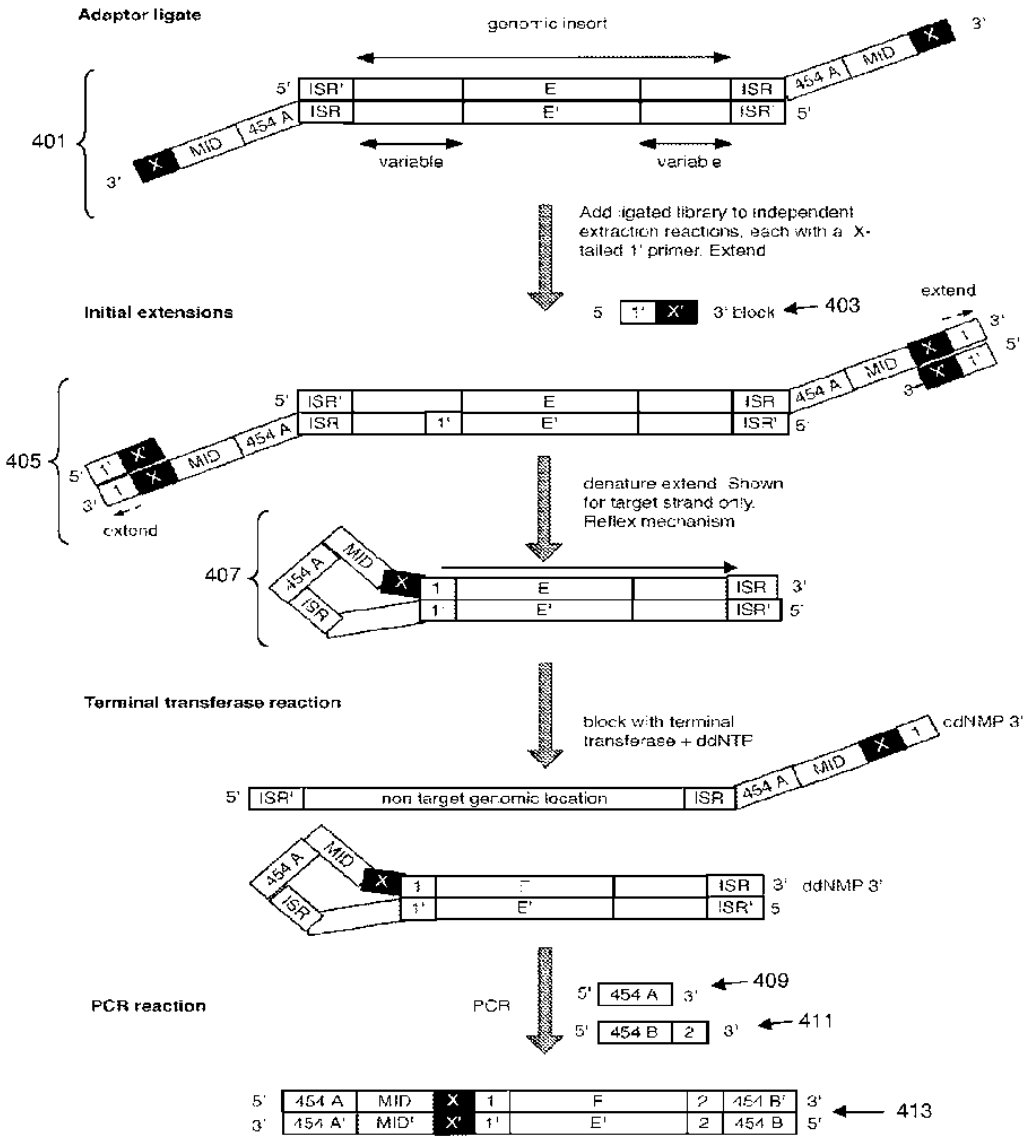

Adaptor ligate
genomic insert
401
ISR'
ISR
454 A
MID
X
E
E'
variable
Add ligated library to independent extraction reactions, each with a X-tailed 1' primer. Extend
Initial extensions
403
extend
405
1'
denature extend Shown for target strand only. Reflex mechanism
407
Terminal transferase reaction
block with terminal transferase + ddNTP
ddNMP 3'
non target genomic location
PCR reaction
PCR
409
411
413

Fig. 4

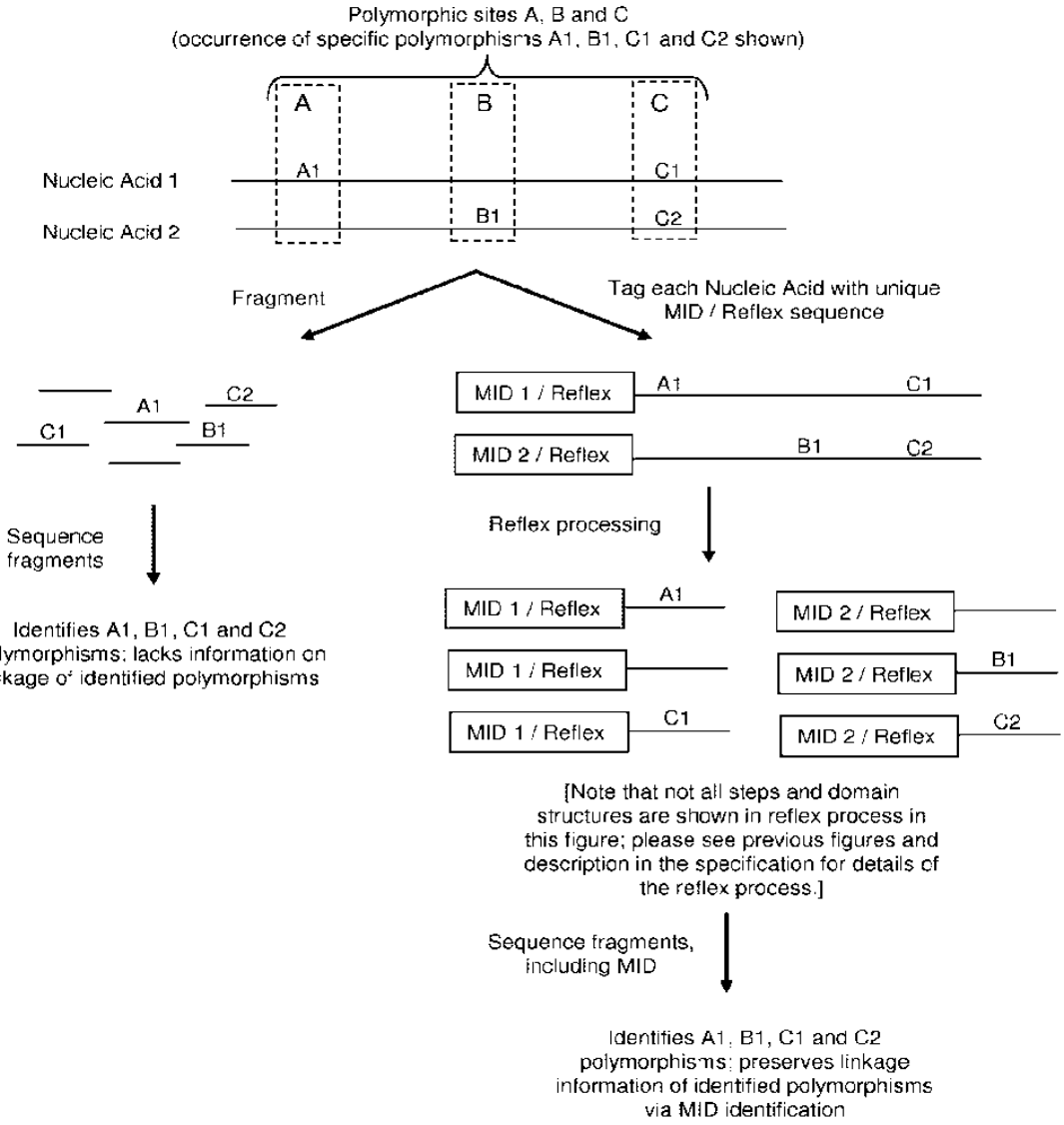
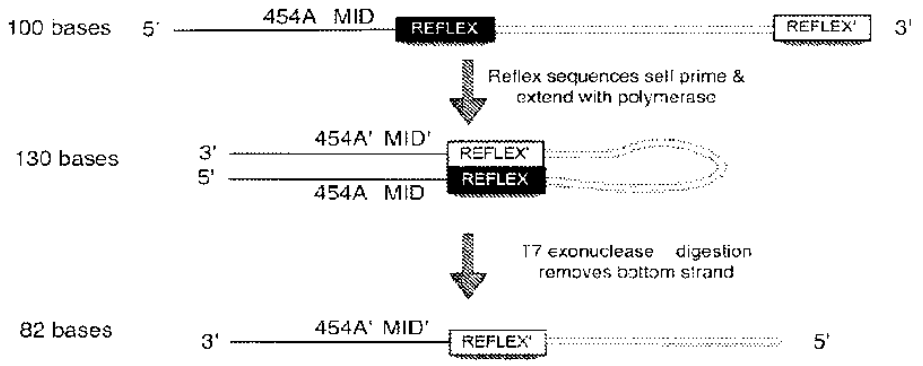

Polymorphic sites A, B and C
(occurrence of specific polymorphisms A1, B1, C1 and C2 shown)
A
B
C
Nucleic Acid 1
A1
C1
Nucleic Acid 2
B1
C2
Fragment
Tag each Nucleic Acid with unique MID / Reflex sequence
A1
C2
C1
B1
Sequence fragments
Identifies A1, B1, C1 and C2 polymorphisms; lacks information on linkage of identified polymorphisms
MID 1 / Reflex
A1
C1
MID 2 / Reflex
B1
C2
Reflex processing
MID 1 / Reflex
A1
MID 2 / Reflex
MID 1 / Reflex
MID 2 / Reflex
B1
MID 1 / Reflex
C1
MID 2 / Reflex
C2
[Note that not all steps and domain structures are shown in reflex process in this figure; please see previous figures and description in the specification for details of the reflex process.]
Sequence fragments, including MID
Identifies A1, B1, C1 and C2 polymorphisms; preserves linkage information of identified polymorphisms via MID identification

Fig. 5

A.


100 bases
5'
454A MID
REFLEX
REFLEX'
3'
Reflex sequences self prime & extend with polymerase
130 bases
3'
454A' MID'
REFLEX'
5'
REFLEX
454A MID
T7 exonuclease digestion removes bottom strand
82 bases
3'
454A' MID'
REFLEX'
5'

B.

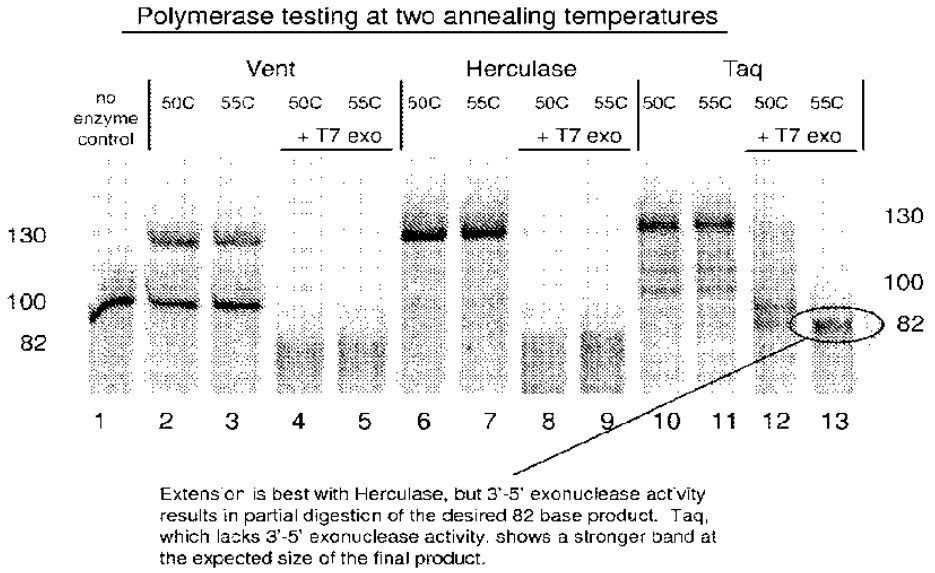

Polymerase testing at two annealing temperatures
Vent
Herculase
Taq
no enzyme control
50C
55C
50C
55C
+ T7 exo
50C
55C
50C
55C
+ T7 exo
50C
55C
50C
55C
+ T7 exo
130
100
82
130
100
82
1
2
3
4
5
6
7
8
9
10
11
12
13
Extension is best with Herculase, but 3'-5' exonuclease activity results in partial digestion of the desired 82 base product. Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product.

Fig. 6

A.


5'
454A MID
REFLEX
REFLEX'
3'
+ Competitor
5'
REFLEX'
3'

B.

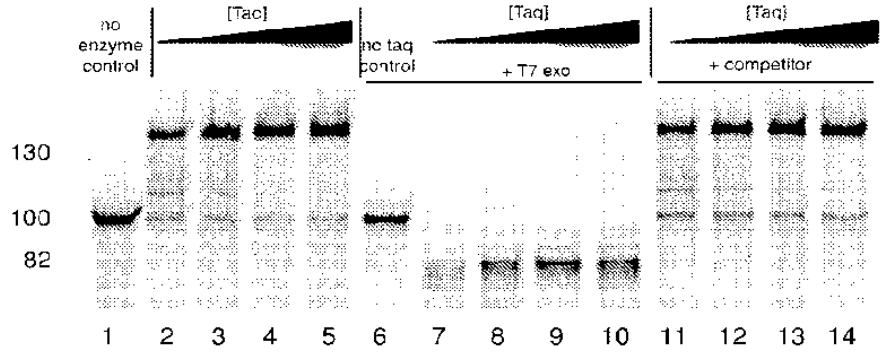
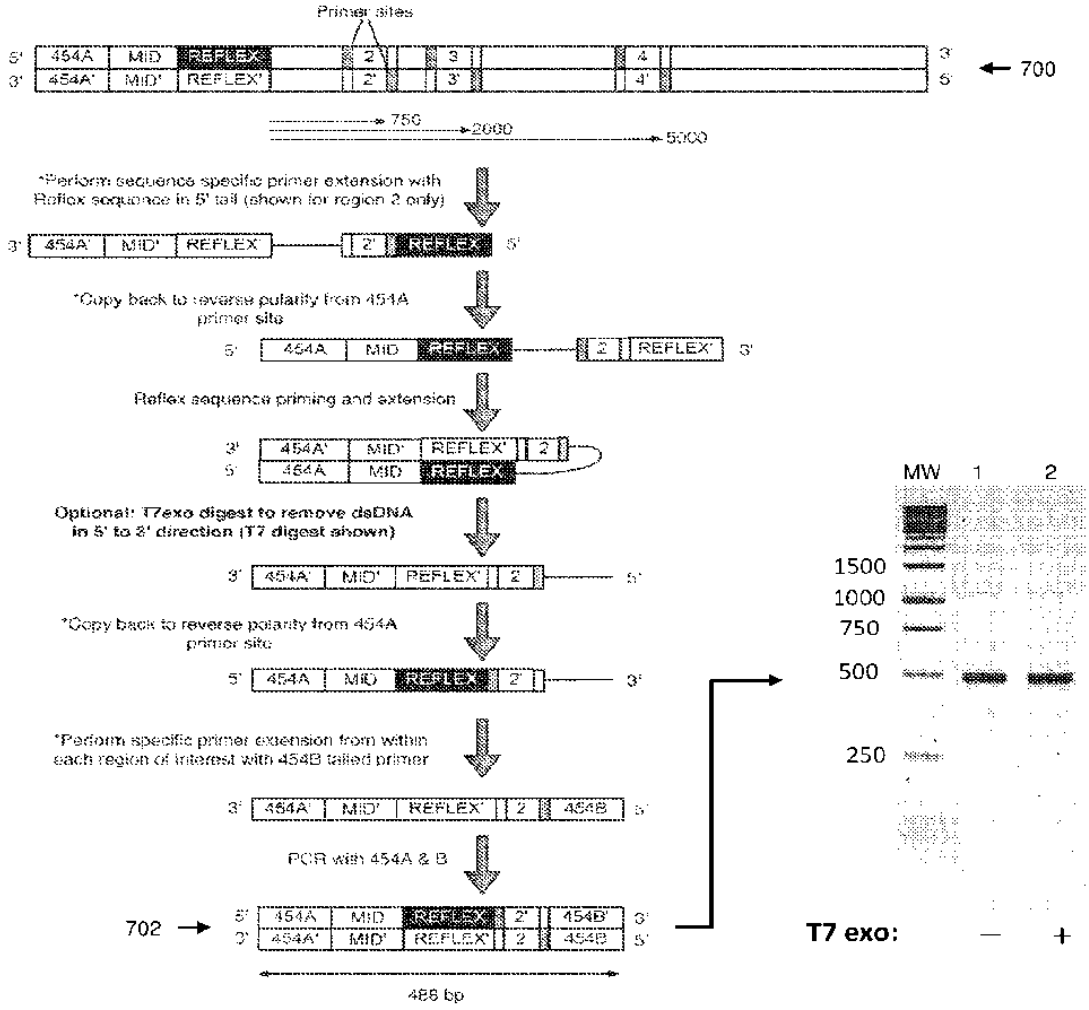

no enzyme control
[Taq]
no taq control
[Taq]
+ T7 exo
[Taq]
+ competitor
130
100
82
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 7



Primer extension reactions with * may be performed such that isolation of single strand species is facilitated (e.g., using primers with binding moieties and/or multiple cycles of extension)

Fig. 8

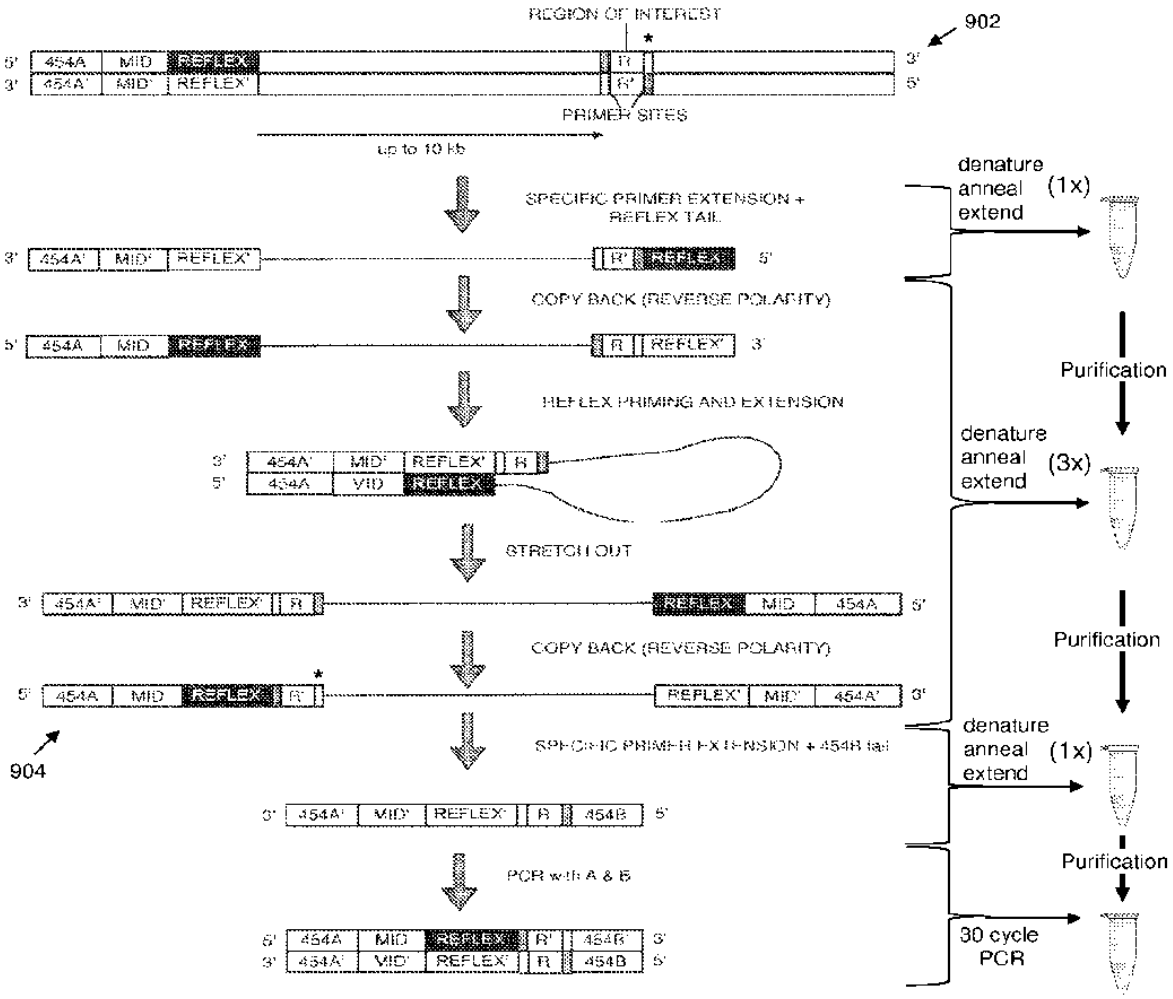

REGION OF INTEREST
902
454A
MID
REFLEX
R
PRIMER SITES
up to 10 kb
SPECIFIC PRIMER EXTENSION + REFLEX TAIL
COPY BACK (REVERSE POLARITY)
REFLEX PRIMING AND EXTENSION
STRETCH OUT
COPY BACK (REVERSE POLARITY)
SPECIFIC PRIMER EXTENSION + 454B tail
PCR with A & B
904
denature anneal extend (1x)
Purification
denature anneal extend (3x)
Purification
denature anneal extend (1x)
Purification
30 cycle PCR

Fig. 9

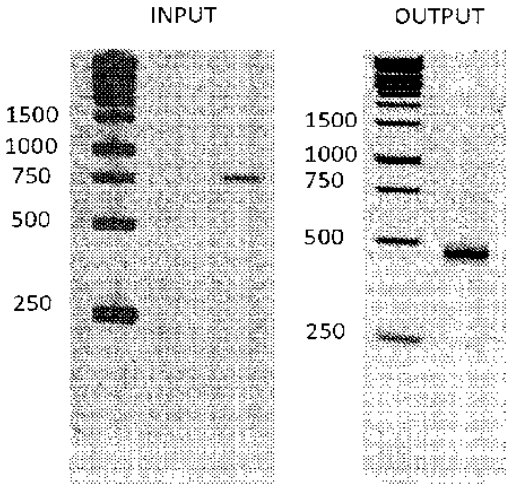
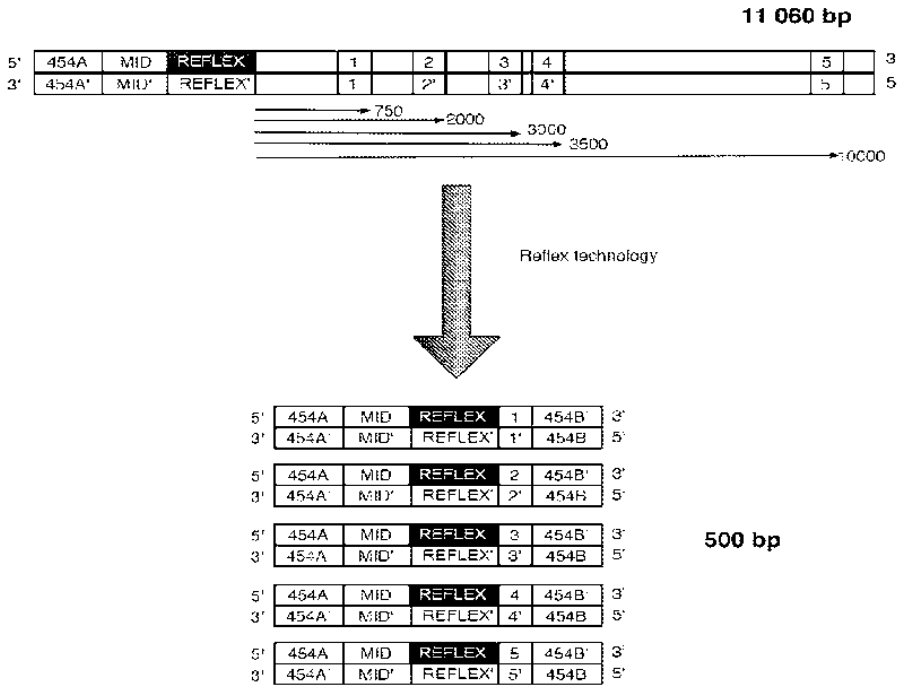


Fig. 10


11 060 bp
5' 454A MID REFLEX 1 2 3 4 5 3'
3' 454A' MID' REFLEX' 1' 2' 3' 4' 5' 5'
750
2000
3000
3500
10000
Reflex technology
500 bp

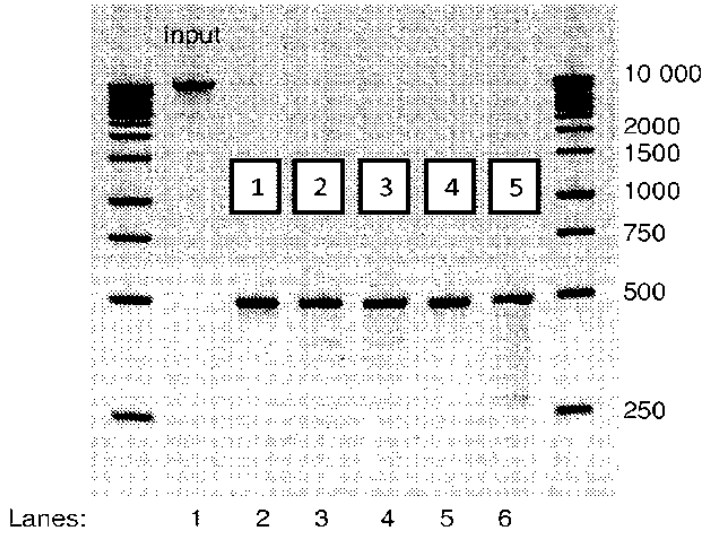
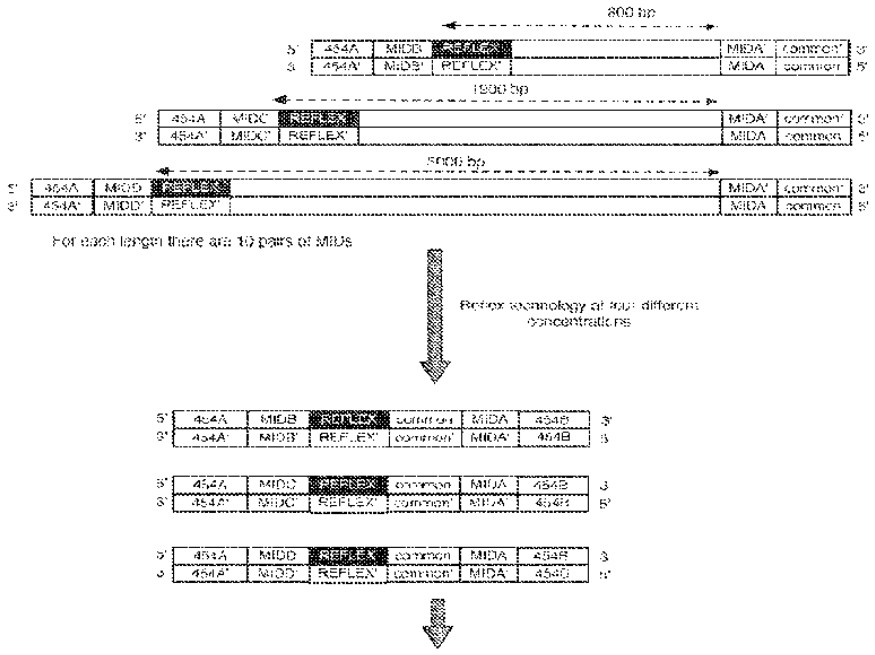

Input
10 000
2000
1500
1000
750
500
250
1
2
3
4
5
Lanes: 1 2 3 4 5 6

Fig. 11


800 bp
1500 bp
5000 bp
For each length there are 10 pairs of MIDs
Roche 454 Sequencing

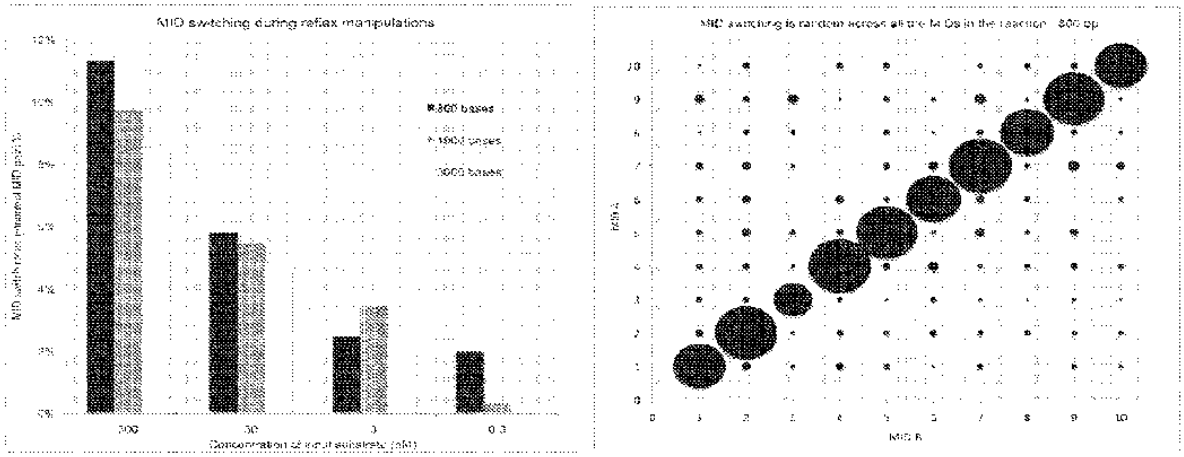

MID switching during reflex manipulations
MID switching is random across all the MIDs in the reaction - 800 bp

Fig. 12

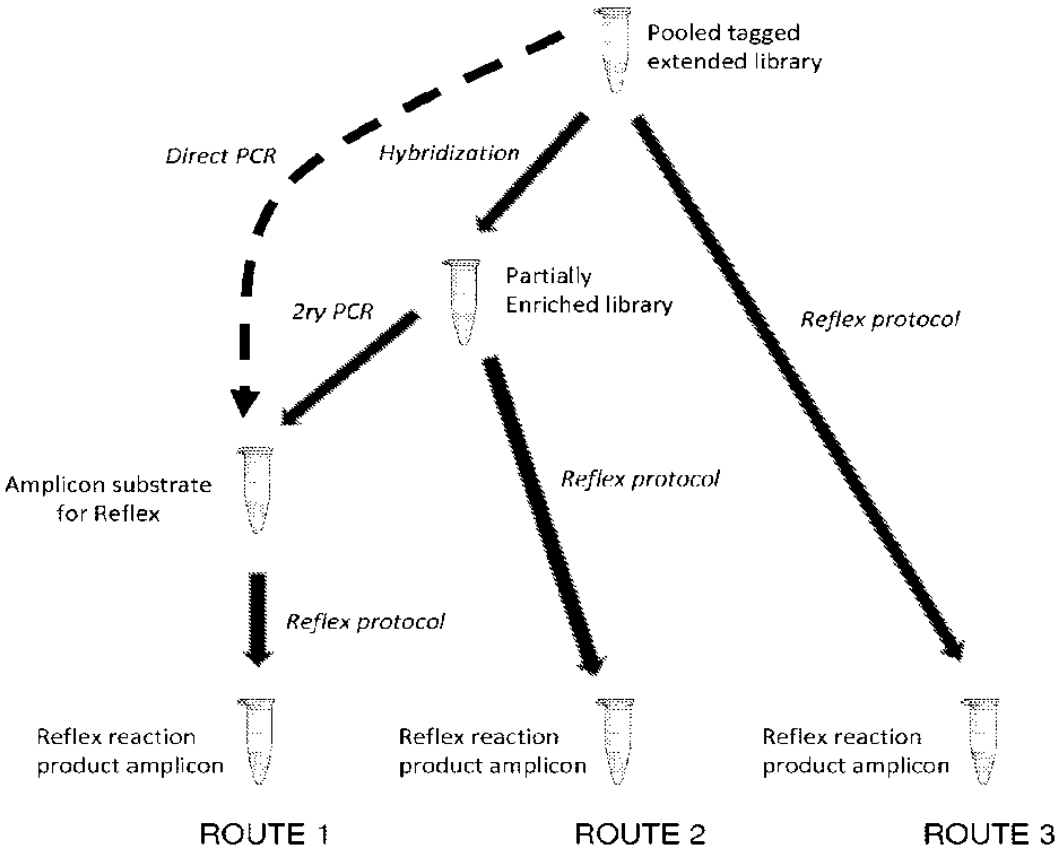

Pooled tagged extended library
Direct PCR
Hybridization
Partially Enriched library
2ry PCR
Reflex protocol
Amplicon substrate for Reflex
Reflex protocol
Reflex protocol
Reflex reaction product amplicon
Reflex reaction product amplicon
Reflex reaction product amplicon
ROUTE 1
ROUTE 2
ROUTE 3

Fig. 13

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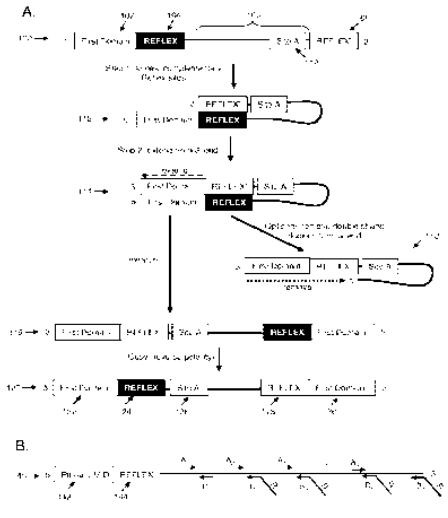


Fig. 1

(57) Abstract: Aspects of the present invention are drawn to processes for moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method". In certain embodiments, the reflex method results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer sites and/or MID). Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

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COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

BACKGROUND

We have previously described methods that enable tagging each of a population of fragmented genomes and then combining them together to create a 'population library' that can be processed and eventually sequenced as a mixture. The population tags enable analysis software to parse the sequence reads into files that can be attributed to a particular genome in the population. One limitation of the overall process stems from limitations of existing DNA sequencing technologies. In particular, if fragments in the regions of interest of the genome are longer than the lengths that can be sequenced by a particular technology, then such fragments will not be fully analyzed (since sequencing proceeds from an end of a fragment inward). Furthermore, a disadvantage of any sequencing technology dependent on fragmentation is that one can never relate sequence changes in one part of a particular genomic region to the sequence changes of other parts of the same genome that may be on the same chromosome but on a different fragment. (See Figure 5 and its description below).

The present invention removes the limitations imposed by current sequencing technologies as well as being useful in a number of other nucleic acid analyses.

SUMMARY OF THE INVENTION

Aspects of the present invention are drawn to the use of a specifically designed 'reflex' sequence present in a nucleic acid (e.g., as part of an adapter structure which is ligated onto the ends of a nucleic acid to be analyzed) that can be used to move a desired region of the nucleic acid to be analyzed to a position into functional proximity to elements present elsewhere in the nucleic acid (e.g., sequencing primer binding sites, MID, etc.). The reflex method thus allows one to transform a long nucleic acid fragment that is too long to sequence completely into a set of shorter fragments, each one of which can be sequenced within the limits of a particular sequencing platform. Because the reflex process functions on an intramolecular basis, the identity of each nucleic acid can be maintained in downstream processes (e.g., by including a multiplex identifier domain in the starting nucleic acid at a position that will be carried through in the reflex process).

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Aspects of the present invention include methods of moving a domain in a nucleic acid, the method comprising:

a) annealing a reflex sequence in a single stranded nucleic acid in a sample with a complement of the reflex sequence in the single stranded nucleic acid, wherein the single stranded nucleic acid comprises the following in a 5' to 3' orientation:

a first domain;

the reflex sequence;

a nucleic acid sequence of interest; and

the complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the single stranded nucleic acid;

b) extending from the 3' end of the complement of the reflex sequence through the first domain to produce a double stranded region; and

c) removing the first domain and the reflex sequence from the 5' end of the double stranded region, whereby a complement of the first domain is present at the 3' end of the single stranded nucleic acid fragment, thereby moving the first domain in the single stranded nucleic acid.

In certain embodiments, the first domain comprises a multiplex identifier (MID).

In certain embodiments, the first domain comprises a primer binding site.

In certain embodiments, the first reflex sequence is from 10 to 50 nucleotides in length.

In certain embodiments, the first reflex sequence comprises an RNA polymerase binding site.

In certain embodiments, the removing step comprises treating the nucleic acid of step b with T7 exonuclease.

In certain embodiments, the sample is a multiplexed sample, wherein the first domain in each of the single stranded nucleic acids in the multiplexed sample comprises a MID corresponding to its parental sample.

In certain embodiments, the sample comprises multiple single stranded nucleic acids, and wherein each single stranded nucleic acid in the sample comprises a unique MID in the first domain.

In certain embodiments, the single stranded nucleic acid is produced by:

annealing a nucleic acid synthesis primer to a parent nucleic acid strand, wherein the parent nucleic acid strand comprises the following in a 5' to 3' orientation:

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the first domain;

the reflex sequence; and

a second domain comprising the nucleic acid sequence of interest;

wherein the nucleic acid synthesis primer comprises:

a 3' priming domain that is complementary to a site adjacent to and 3' of the nucleic acid sequence of interest in the second domain; and

a 5' reflex sequence;

extending the nucleic acid synthesis primer to produce an extension product; and

replicating the resultant extension product with a second nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain, thereby producing the single stranded nucleic acid.

In certain embodiments, the length of the second domain is from 0.1 to 30 kilobases.

In certain embodiments, the parent nucleic acid strand is enriched prior to the annealing step based on a sequence in the nucleic acid sequence of interest.

In certain embodiments, the method further comprises the steps of:

d) replicating the single stranded nucleic acid of step c with a first nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain to produce a first extension product;

e) annealing a second nucleic acid synthesis primer to the first extension product, wherein the second nucleic acid synthesis primer comprises a 3' priming domain that is complementary to a site within the sequence of interest in the first extension product; and

f) extending the second synthesis primer to produce a second extension product.

In certain embodiments, the second synthesis primer comprises a 5' domain that is not complementary to the sequence of interest in the first extension product.

In certain embodiments, the first domain comprises a first primer binding site and the 5' domain of the second synthesis primer comprises a second primer binding site.

In certain embodiments, the length of the sequence of interest in the second extension product is from 50 to 10,000 nucleotides.

Aspects of the present invention include a polynucleotide comprising the following in a 5' to 3' orientation:

a first domain;

a first reflex sequence;

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a nucleic acid sequence of interest; and

a complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the single stranded nucleic acid.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to-scale. Indeed, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures:

Figure 1: Panel A is a schematic diagram illustrating moving a first domain from one site to another in a nucleic acid molecule using a reflex sequence. Panel B is a schematic diagram depicting the relative position of primer pairs (A_n-B_n primers) that find us in aspects of the reflex process described herein.

Figure 2 is a schematic diagram illustrating an exemplary embodiment for moving a primer binding site and a MID to a specific location in a nucleic acid of interest.

Figure 3: Panel A is a schematic showing expected structures and sizes of nucleic acid species in the reflex site process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

Figure 4: Panel A is a schematic showing the structure of the nucleic acid and competitor used in the reflex sequence process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

Figure 5 shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information.

Figure 6 shows an exemplary embodiment of using binding partner pairs (biotin/avidin) to isolate single stranded polynucleotides of interest.

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DEFINITIONS

Unless otherwise defined, 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. Still, certain elements are defined for the sake of clarity and ease of reference.

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

"Amplicon" means the product of a polynucleotide amplification reaction. That is, it is a population of polynucleotides, usually double stranded, that are replicated from one or more starting sequences. The one or more starting sequences may be one or more copies of the same sequence, or it may be a mixture of different sequences. Amplicons may be produced by a variety of amplification reactions whose products are multiple replicates of one or more target nucleic acids. Generally, amplification reactions producing amplicons are "template-driven" in that base pairing of reactants, either nucleotides or oligonucleotides, have complements in a template polynucleotide that are required for the creation of reaction products. In one aspect, template-driven reactions are primer extensions with a nucleic acid polymerase or oligonucleotide ligations with a nucleic acid ligase. Such reactions include, but are not limited to, polymerase chain reactions (PCRs), linear polymerase reactions, nucleic acid sequence-based amplification (NASBAs), rolling circle amplifications, and the like, disclosed in the following references that are incorporated herein by reference: Mullis et al, U.S. patents 4,683,195; 4,965,188; 4,683,202; 4,800,159 (PCR); Gelfand et al, U.S. patent 5,210,015 (real-time PCR with "TAQMAN™" probes); Wittwer et al, U.S. patent 6,174,670; Kacian et al, U.S. patent 5,399,491 ("NASBA"); Lizardi, U.S. patent 5,854,033; Aono et al, Japanese patent publ. JP 4-262799 (rolling circle amplification); and the like. In one aspect, amplicons of the invention are produced by PCRs. An amplification reaction may

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be a "real-time" amplification if a detection chemistry is available that permits a reaction product to be measured as the amplification reaction progresses, e.g. "real-time PCR" described below, or "real-time NASBA" as described in Leone et al, Nucleic Acids Research, 26: 2150-2155 (1998), and like references. As used herein, the term "amplifying" means performing an amplification reaction. A "reaction mixture" means a solution containing all the necessary reactants for performing a reaction, which may include, but not be limited to, buffering agents to maintain pH at a selected level during a reaction, salts, co-factors, scavengers, and the like.

The term "assessing" includes any form of measurement, and includes determining if an element is present or not. The terms "determining", "measuring", "evaluating", "assessing" and "assaying" are used interchangeably and includes quantitative and qualitative determinations. Assessing may be relative or absolute. "Assessing the presence of" includes determining the amount of something present, and/or determining whether it is present or absent. As used herein, the terms "determining," "measuring," and "assessing," and "assaying" are used interchangeably and include both quantitative and qualitative determinations.

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

"Duplex" means at least two oligonucleotides and/or polynucleotides that are fully or partially complementary undergo Watson-Crick type base pairing among all or

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most of their nucleotides so that a stable complex is formed. The terms "annealing" and "hybridization" are used interchangeably to mean the formation of a stable duplex. "Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one another such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. A stable duplex can include Watson-Crick base pairing and/or non-Watson-Crick base pairing between the strands of the duplex (where base pairing means the forming hydrogen bonds). In certain embodiments, a non-Watson-Crick base pair includes a nucleoside analog, such as deoxyinosine, 2, 6-diaminopurine, PNAs, LNA's and the like. In certain embodiments, a non-Watson-Crick base pair includes a "wobble base", such as deoxyinosine, 8-oxo-dA, 8-oxo-dG and the like, where by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand (wobble bases are described in further detail below). A "mismatch" in a duplex between two oligonucleotides or polynucleotides means that a pair of nucleotides in the duplex fails to undergo Watson-Crick bonding.

"Genetic locus," "locus," or "locus of interest" in reference to a genome or target polynucleotide, means a contiguous sub-region or segment of the genome or target polynucleotide. As used herein, genetic locus, locus, or locus of interest may refer to the position of a nucleotide, a gene or a portion of a gene in a genome, including mitochondrial DNA or other non-chromosomal DNA (e.g., bacterial plasmid), or it may refer to any contiguous portion of genomic sequence whether or not it is within, or associated with, a gene. A genetic locus, locus, or locus of interest can be from a single nucleotide to a segment of a few hundred or a few thousand nucleotides in length or more. In general, a locus of interest will have a reference sequence associated with it (see description of "reference sequence" below).

"Kit" refers to any delivery system for delivering materials or reagents for carrying out a method of the invention. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., probes, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one

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location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. Such contents may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains probes.

"Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, e.g. oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon of a terminal nucleotide of one oligonucleotide with 3' carbon of another oligonucleotide. A variety of template-driven ligation reactions are described in the following references, which are incorporated by reference: Whiteley et al, U.S. patent 4,883,750; Letsinger et al, U.S. patent 5,476,930; Fung et al, U.S. patent 5,593,826; Kool, U.S. patent 5,426,180; Landegren et al, U.S. patent 5,871,921; Xu and Kool, Nucleic Acids Research, 27: 875-881 (1999); Higgins et al, Methods in Enzymology, 68: 50-71 (1979); Engler et al, The Enzymes, 15: 3-29 (1982); and Namsaraev, U.S. patent publication 2004/0110213.

"Multiplex Identifer" (MID) as used herein refers to a tag associated with a nucleic acid whose identity (e.g., sequence) can be used to differentiate nucleic acids in a sample. In certain embodiments, the MID on a nucleic acid is used to identify the source from which a nucleic acid is derived. For example, a nucleic acid sample may be a pool of nucleic acids derived from different sources, (e.g., nucleic acids derived from different individuals, different tissues or cells, or nucleic acids isolated at different times points), where the nucleic acids from each different source is tagged with a unique MID. Identification of the MID on a nucleic acid thus allows one to determine from which source the nucleic acid is derived. In other words, the MID provides a correlation between a nucleic acid and its source. In certain embodiments, MIDs are employed to uniquely tag each individual nucleic acid in a sample. Identification of the number of unique MIDs in a sample will provide a readout of how many individual nucleic acids are present in the sample (or from how many original nucleic acids a manipulated nucleic acid sample was derived; see, e.g., U.S. Patent No. 7,537,897, issued on May 26, 2009, incorporated herein by reference in its entirety). MIDs can range in

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length from 2 to 100 nucleotide bases or more. Exemplary nucleic acid tags that find use as MIDs are described in U.S. Patent 7,544,473, issued on June 6, 2009, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Patent 7,393,665, issued on July 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides.

"Nucleoside" as used herein includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543-584 (1990), or the like, with the proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like. Polynucleotides comprising analogs with enhanced hybridization or nuclease resistance properties are described in Uhlman and Peyman (cited above); Crooke et al, Exp. Opin. Ther. Patents, 6: 855-870 (1996); Mesmaeker et al, Current Opinion in Structual Biology, 5: 343-355 (1995); and the like. Exemplary types of polynucleotides that are capable of enhancing duplex stability include oligonucleotide N3'→P5' phosphoramidates (referred to herein as "amidates"), peptide nucleic acids (referred to herein as "PNAs"), oligo-2'-O-alkylribonucleotides, polynucleotides containing C-5 propynylpyrimidines, locked nucleic acids ("LNAs"), and like compounds. Such oligonucleotides are either available commercially or may be synthesized using methods described in the literature.

"Polymerase chain reaction," or "PCR," means a reaction for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. In other words, PCR is a reaction for making multiple copies or replicates of a target nucleic acid flanked by primer binding sites, such reaction comprising one or more repetitions of the following steps: (i) denaturing the target nucleic acid, (ii) annealing primers to the primer binding sites, and (iii) extending the primers by a nucleic acid polymerase in the presence of nucleoside triphosphates. Usually, the reaction is cycled through different temperatures optimized for each step in a

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thermal cycler instrument. Particular temperatures, durations at each step, and rates of change between steps depend on many factors well-known to those of ordinary skill in the art, e.g. exemplified by the references: McPherson et al, editors, PCR: A Practical Approach and PCR2: A Practical Approach (IRL Press, Oxford, 1991 and 1995, respectively). For example, in a conventional PCR using Taq DNA polymerase, a double stranded target nucleic acid may be denatured at a temperature >90°C, primers annealed at a temperature in the range 50-75°C, and primers extended at a temperature in the range 72-78°C. The term "PCR" encompasses derivative forms of the reaction, including but not limited to, RT-PCR, real-time PCR, nested PCR, quantitative PCR, multiplexed PCR, and the like. Reaction volumes range from a few hundred nanoliters, e.g. 200 nL, to a few hundred μL, e.g. 200 μL. "Reverse transcription PCR," or "RT-PCR," means a PCR that is preceded by a reverse transcription reaction that converts a target RNA to a complementary single stranded DNA, which is then amplified, e.g. Tecott et al, U.S. patent 5,168,038, which patent is incorporated herein by reference. "Real-time PCR" means a PCR for which the amount of reaction product, i.e. amplicon, is monitored as the reaction proceeds. There are many forms of real-time PCR that differ mainly in the detection chemistries used for monitoring the reaction product, e.g. Gelfand et al, U.S. patent 5,210,015 ("TAQMAN™"); Wittwer et al, U.S. patents 6,174,670 and 6,569,627 (intercalating dyes); Tyagi et al, U.S. patent 5,925,517 (molecular beacons); which patents are incorporated herein by reference. Detection chemistries for real-time PCR are reviewed in Mackay et al, Nucleic Acids Research, 30: 1292-1305 (2002), which is also incorporated herein by reference. "Nested PCR" means a two-stage PCR wherein the amplicon of a first PCR becomes the sample for a second PCR using a new set of primers, at least one of which binds to an interior location of the first amplicon. As used herein, "initial primers" in reference to a nested amplification reaction mean the primers used to generate a first amplicon, and "secondary primers" mean the one or more primers used to generate a second, or nested, amplicon. "Multiplexed PCR" means a PCR wherein multiple target sequences (or a single target sequence and one or more reference sequences) are simultaneously carried out in the same reaction mixture, e.g. Bernard et al, Anal. Biochem., 273: 221-228 (1999)(two-color real-time PCR). Usually, distinct sets of primers are employed for each sequence being amplified.

"Quantitative PCR" means a PCR designed to measure the abundance of one or more specific target sequences in a sample or specimen. Quantitative PCR includes both

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absolute quantitation and relative quantitation of such target sequences. Quantitative measurements are made using one or more reference sequences that may be assayed separately or together with a target sequence. The reference sequence may be endogenous or exogenous to a sample or specimen, and in the latter case, may comprise one or more competitor templates. Typical endogenous reference sequences include segments of transcripts of the following genes: β-actin, GAPDH, β_2-microglobulin, ribosomal RNA, and the like. Techniques for quantitative PCR are well-known to those of ordinary skill in the art, as exemplified in the following references that are incorporated by reference: Freeman et al, Biotechniques, 26: 112-126 (1999); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9447 (1989); Zimmerman et al, Biotechniques, 21: 268-279 (1996); Diviacco et al, Gene, 122: 3013-3020 (1992); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9446 (1989); and the like.

"Polynucleotide" or "oligonucleotide" is used interchangeably and each means a linear polymer of nucleotide monomers. Monomers making up polynucleotides and oligonucleotides are capable of specifically binding to a natural polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, wobble base pairing, or the like. As described in detail below, by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand. Such monomers and their internucleosidic linkages may be naturally occurring or may be analogs thereof, e.g. naturally occurring or non-naturally occurring analogs. Non-naturally occurring analogs may include peptide nucleic acids (PNAs, e.g., as described in U.S. Patent 5,539,082, incorporated herein by reference), locked nucleic acids (LNAs, e.g., as described in U.S. Patent 6,670,461, incorporated herein by reference), phosphorothioate internucleosidic linkages, bases containing linking groups permitting the attachment of labels, such as fluorophores, or haptens, and the like. Whenever the use of an oligonucleotide or polynucleotide requires enzymatic processing, such as extension by a polymerase, ligation by a ligase, or the like, one of ordinary skill would understand that oligonucleotides or polynucleotides in those instances would not contain certain analogs of internucleosidic linkages, sugar moieties, or bases at any or some positions. Polynucleotides typically range in size from a few

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

"Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension process is determined by the sequence of the template polynucleotide. Usually primers are extended by a DNA polymerase. Primers are generally of a length compatible with its use in synthesis of primer extension products, and are usually are in the range of between 8 to 100 nucleotides in length, such as 10 to 75, 15 to 60, 15 to 40, 18 to 30, 20 to 40, 21 to 50, 22 to 45, 25 to 40, and so on, more typically in the range of between 18-40, 20-35, 21-30 nucleotides long, and any length between the stated ranges. Typical primers can be in the range of between 10-50 nucleotides long, such as 15-45, 18-40, 20-30, 21-25 and so on, and any length between the stated ranges. In some embodiments, the primers are usually not more than about 10, 12, 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, or 70 nucleotides in length.

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Primers are usually single-stranded for maximum efficiency in amplification, but may alternatively be double-stranded. If double-stranded, the primer is usually first treated to separate its strands before being used to prepare extension products. This denaturation step is typically affected by heat, but may alternatively be carried out using alkali, followed by neutralization. Thus, a "primer" is complementary to a template, and complexes by hydrogen bonding or hybridization with the template to give a primer/template complex for initiation of synthesis by a polymerase, which is extended by the addition of covalently bonded bases linked at its 3' end complementary to the template in the process of DNA synthesis.

A "primer pair" as used herein refers to first and second primers having nucleic acid sequence suitable for nucleic acid-based amplification of a target nucleic acid. Such primer pairs generally include a first primer having a sequence that is the same or similar to that of a first portion of a target nucleic acid, and a second primer having a sequence that is complementary to a second portion of a target nucleic acid to provide for amplification of the target nucleic acid or a fragment thereof. Reference to "first" and "second" primers herein is arbitrary, unless specifically indicated otherwise. For example, the first primer can be designed as a "forward primer" (which initiates nucleic acid synthesis from a 5' end of the target nucleic acid) or as a "reverse primer" (which initiates nucleic acid synthesis from a 5' end of the extension product produced from synthesis initiated from the forward primer). Likewise, the second primer can be designed as a forward primer or a reverse primer.

"Readout" means a parameter, or parameters, which are measured and/or detected that can be converted to a number or value. In some contexts, readout may refer to an actual numerical representation of such collected or recorded data. For example, a readout of fluorescent intensity signals from a microarray is the address and fluorescence intensity of a signal being generated at each hybridization site of the microarray; thus, such a readout may be registered or stored in various ways, for example, as an image of the microarray, as a table of numbers, or the like.

"Reflex site", "reflex sequence" and equivalents are used to indicate one or more domain structure in a polynucleotide that are employed to move a domain intramolecularly from its initial location to a different location in the polynucleotide. The reflex sequence is chosen so as to be distinct from other sequences in the polynucleotide (i.e., with little sequence homology to other sequences likely to be present in the polynucleotide, e.g.,

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genomic or sub-genomic sequences to be processed). As such, a reflex sequence should be selected so as to not hybridize to any sequence except its complement under the conditions employed in the reflex processes herein described. As described later in this application, the complement to the reflex sequence is inserted on the same strand of the polynucleotide (e.g., the same strand of a double-stranded polynucleotide or on the same single stranded polynucleotide) in a particular location so as to facilitate an intramolecular binding event on such particular strand. Reflex sequences employed in the reflex process described herein can thus have a wide range of lengths and sequences. Reflex sequences may range from 5 to 200 nucleotide bases in length.

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

"Specific" or "specificity" in reference to the binding of one molecule to another molecule, such as a labeled target sequence for a probe, means the recognition, contact, and formation of a stable complex between the two molecules, together with substantially less recognition, contact, or complex formation of that molecule with other molecules. In one aspect, "specific" in reference to the binding of a first molecule to a second molecule means that to the extent the first molecule recognizes and forms a complex with another molecule in a reaction or sample, it forms the largest number of the complexes with the second molecule. Preferably, this largest number is at least fifty percent. Generally, molecules involved in a specific binding event have areas on their surfaces or in cavities giving rise to specific recognition between the molecules binding to each other. Examples of specific binding include antibody-antigen interactions, enzyme-substrate interactions, formation of duplexes or triplexes among polynucleotides and/or oligonucleotides, receptor-ligand interactions, and the like. As used herein, "contact" in reference to specificity or specific binding means two molecules are close enough that weak noncovalent chemical interactions, such as Van der Waal forces, hydrogen bonding, base-

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stacking interactions, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules.

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

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

The terms "upstream" and "downstream" in describing nucleic acid molecule orientation and/or polymerization are used herein as understood by one of skill in the art. As such, "downstream" generally means proceeding in the 5' to 3' direction, i.e., the

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direction in which a nucleotide polymerase normally extends a sequence, and "upstream" generally means the converse. For example, a first primer that hybridizes "upstream" of a second primer on the same target nucleic acid molecule is located on the 5' side of the second primer (and thus nucleic acid polymerization from the first primer proceeds towards the second primer).

It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or the use of a "negative" limitation.

DETAILED DESCRIPTION OF THE INVENTION

The invention is drawn to compositions and methods for intramolecular nucleic acid rearrangement that find use in various applications of genetic analysis, including sequencing, as well as general molecular biological manipulations of polynucleotide structures.

Before the present invention is described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

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

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invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" includes a plurality of such nucleic acids and reference to "the compound" includes reference to one or more compounds and equivalents thereof known to those skilled in the art, and so forth.

The practice of the present invention may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such conventional techniques include polymer array synthesis, hybridization, ligation, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the example herein below. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV), *Using Antibodies: A Laboratory Manual, Cells: A Laboratory Manual, PCR Primer: A Laboratory Manual, and Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), Stryer, L. (1995) *Biochemistry* (4th Ed.) Freeman, New York, Gait, "*Oligonucleotide Synthesis: A Practical Approach*" 1984, IRL Press, London, Nelson and Cox (2000), Lehninger, A., *Principles of Biochemistry* 3rd Ed., W. H. Freeman Pub., New York, N.Y. and Berg et al. (2002) *Biochemistry*, 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue

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of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

As summarized above, aspects of the present invention are drawn to the use of a specifically designed 'reflex' sequence present in a polynucleotide (e.g., as part of an adapter structure which is ligated onto the ends of a polynucleotide to be analyzed) that can be used to move a desired region of the polynucleotide to be analyzed to a position into functional proximity to elements present in the polynucleotide (e.g., sequencing primer binding sites and/or MID tags).

Nucleic Acids

The reflex process (as described in detail below) can be employed for the manipulation and analysis of nucleic acid sequences of interest from virtually any nucleic acid source, including but not limited to genomic DNA, complementary DNA (cDNA), RNA (e.g., messenger RNA, ribosomal RNA, short interfering RNA, microRNA, etc.), plasmid DNA, mitochondrial DNA, etc. Furthermore, as any organism can be used as a source of nucleic acids to be processed in accordance with the present invention, no limitation in that regard is intended. Exemplary organisms include, but are not limited to, plants, animals (e.g., reptiles, mammals, insects, worms, fish, etc.), bacteria, fungi (e.g., yeast), viruses, etc. In certain embodiments, the nucleic acids in the nucleic acid sample are derived from a mammal, where in certain embodiments the mammal is a human.

In certain embodiments, the nucleic acid sequences are enriched prior to the reflex sequence process. By enriched is meant that the nucleic acid is subjected to a process that reduces the complexity of the nucleic acids, generally by increasing the relative concentration of particular nucleic acid species in the sample (e.g., having a specific locus of interest, including a specific nucleic acid sequence, lacking a locus or sequence, being within a specific size range, etc.). There are a wide variety of ways to enrich nucleic acids having a specific characteristic(s) or sequence, and as such any convenient method to accomplish this may be employed. The enrichment (or complexity reduction) can take place at any of a number of steps in the process, and will be determined by the desires of the user. For example, enrichment can take place in individual parental samples (e.g., untagged nucleic acids prior to adaptor ligation) or in multiplexed samples (e.g., nucleic

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acids tagged with primer binding sites, MID and/or reflex sequences and pooled; MID are described in further detail below).

In certain embodiments, nucleic acids in the nucleic acid sample are amplified prior to analysis. In certain of these embodiments, the amplification reaction also serves to enrich a starting nucleic acid sample for a sequence or locus of interest. For example, a starting nucleic acid sample can be subjected to a polymerase chain reaction (PCR) that amplifies one or more region of interest. In certain embodiments, the amplification reaction is an exponential amplification reaction, whereas in certain other embodiments, the amplification reaction is a linear amplification reaction. Any convenient method for performing amplification reactions on a starting nucleic acid sample can be used in practicing the subject invention. In certain embodiments, the nucleic acid polymerase employed in the amplification reaction is a polymerase that has proofreading capability (e.g., phi29 DNA Polymerase, Thermococcus litoralis DNA polymerase, Pyrococcus furiosus DNA polymerase, etc.).

In certain embodiments, the nucleic acid sample being analyzed is derived from a single source (e.g., a single organism, virus, tissue, cell, subject, etc.), whereas in other embodiments, the nucleic acid sample is a pool of nucleic acids extracted from a plurality of sources (e.g., a pool of nucleic acids from a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. As such, in certain embodiments, a nucleic acid sample can contain nucleic acids from 2 or more sources, 3 or more sources, 5 or more sources, 10 or more sources, 50 or more sources, 100 or more sources, 500 or more sources, 1000 or more sources, 5000 or more sources, up to and including about 10,000 or more sources.

In certain embodiments, nucleic acid fragments that are to be pooled with nucleic acid fragments derived from a plurality of sources (e.g., a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. In such embodiments, the nucleic acids derived from each source includes a multiplex identifier (MID) such that the source from which the each tagged nucleic acid fragment was derived can be determined. In such embodiments, each nucleic acid sample source is correlated with a unique MID, where by unique MID is meant that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID. Any type of MID can be used, including but not limited to those described in co-pending U.S. Patent Application Serial Number

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11/656,746, filed on January 22, 2007, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Patent 7,393,665, issued on July 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., T_m, length, base composition, etc.), as the asymmetric tagging methods (and many tag readout methods, including but not limited to sequencing of the tag or measuring the length of the tag) can accommodate a wide variety of unique MID sets.

In certain embodiments, each individual polynucleotide (e.g., double-stranded or single-stranded, as appropriate to the methodological details employed) in a sample to be analyzed is tagged with a unique MID so that the fate of each polynucleotide can be tracked in downstream processes (where, as noted above, unique MID is meant to indicate that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID). For example (and as described below), having each nucleic acid tagged with a unique MID allows analysis of the sequence of each individual nucleic acid using the reflex sequence methods described herein. This allows the linkage of sequence information for large nucleic acid fragments that cannot be sequenced in a single sequencing run.

Reflex Sequence Process

As summarized above, aspects of the present invention include methods and compositions for moving a first domain in a nucleic acid from a first location to a second location in the nucleic acid. An exemplary embodiment is shown in Figure 1A.

Figure 1A shows a single stranded polynucleotide 100 comprising, in a 5' to 3' orientation, a first domain (102; the domain comprising the sequence elements to be moved); a reflex sequence 104; a nucleic acid sequence of interest (or insert) 106 and a complement of the reflex sequence 108 (positioned at the 3' terminus of the nucleic acid). The nucleic acid sequence of interest 106 in this figure includes Site A (110) adjacent to the complement of the reflex sequence. The steps of the reflex method described below will move the first domain into closer proximity to Site A (to the 3' end of the complement of the reflex sequence).

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In Step 1, the reflex sequence and its complement in the polynucleotide are annealed intramolecularly to form structure 112, with the polynucleotide folding back on itself. In this configuration, the 3' end of the complement of the reflex sequence can serve as a nucleic acid synthesis priming site. Nucleic acid synthesis from this site is then performed in extension Step 2 producing a complement of the first domain at the 3' end of the nucleic acid extension (shown in structure114; extension is indicated with dotted arrow labeled "extend"). In Step 3 (after extension), the first domain and reflex sequence are removed from the 5' end of the double-stranded region (shown in structure 116; removal is shown in the dotted arrow labeled "remove"). Removal of this region may be accomplished by any convenient method, including, but not limited to, treatment (under appropriate incubation conditions) of polynucleotide structure 114 with T7 exonuclease or by treatment with Lambda exonuclease; the Lambda exonuclease can be employed so long as the 5' end of the polynucleotide is phosphorylated.

The resultant structure 116 shows that a complement of the first domain has been moved intramolecularly from a position distant from Site A to a position that is separated from Site A by only the complement of the reflex sequence. In certain embodiments, a copy of structure 116 is generated, for example performing a nucleic acid synthesis reaction with a primer that primes in the complement of the first domain. The resultant copy has the structure, in a 5' to 3' orientation, of: the first domain; the reflex sequence; and the nucleic acid sequence of interest (or insert), with Site A in the nucleic acid sequence of interest adjacent to the reflex sequence. This step is shown in more detail in Figure 2 (described below).

In certain embodiments, the first domain in the polynucleotide comprises one or more elements that find use in downstream analyses. Such sequences include, but are not limited to, restriction enzyme sites, PCR primer binding sites, linear amplification primer sites, reverse transcription primer sites, RNA polymerase promoter sites (such as for T7, T3 or SP6 RNA polymerase), MID tags, sequencing primer sites, etc. Any convenient element can be included in the first domain and, in certain embodiments, is determined by the desires of the user of the methods described herein.

As an exemplary embodiment, suppose we want to sequence specific regions of a large nucleic acid (e.g., 2 kilobases or more in length) from multiple genomes in a pooled sample using Roche 454 (Branford, CT) technology, in which the length of a single sequencing run is about 400 bases. We can choose a set of left hand primers (A_n) and

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right hand primers (B_n) that overlap in such a way that we can obtain direct sequences of all parts of the insert, as shown in Figure 1B. Each A_n-B_n primer pair defines a nucleic acid region that is less than approximately 400 bases in length, which is below the sequencing run length of the current Roche 454 sequencing platform. Thus, each product from each reflex process can be sequenced in a single run. It is noted here that primer pairs as shown in Figure 1B can be used to define regions 1 to 5 shown in Figure 2 (described in further detail below).

In certain embodiments, to obtain the first part (i.e., in the original structure that part of the sequence of interest closest to the first domain) of the sequence of the insert, we only need a right hand primer (e.g., B_0) and we do not need to transfer the MID as it is within reach of this sequencing primer (i.e., the MID is within 400 bases of sequencing primer B_0). All other B_n primers have the reflex sequence added to their 5' ends ("R" element shown on B primers) so that they read 5' reflex-B_n. However, in certain embodiments, the B_0 primer does include the reflex sequence and is used in the reflex process (along with a corresponding A_0 primer) as detailed below.

As described above, we obtain a single stranded polynucleotide having, in the 5' to 3' orientation, a primer binding site (e.g., for Roche 454 sequencing), a MID, a reflex sequence and the insert to be sequenced. Numerous methods for obtaining single-stranded polynucleotides of interest have been described and are known in the art, including in U.S. Patent 7,217,522, issued on May 15, 2007; U.S. Patent Application 11/377,462, filed on March 16, 2006; and U.S. Patent Application 12/432,080, filed on April 29, 2009; each of which is incorporated by reference herein in their entirety. For example, a single stranded product can be produced using linear amplification with a primer specific for the primer binding site of the template. In certain embodiments, the primer includes a binding moiety to facilitate isolation of the single stranded nucleic acid of interest, e.g., to immobilize the top strand on a binding partner of the binding moiety immobilized on a solid support. Removal of a hybridized, non-biotinylated strand by denaturation using heat or high pH (or any other convenient method) serves to isolate the biotinylated strand. Binding moieties and their corresponding binding partners are sometimes referred to herein as binding partner pairs. Any convenient binding partner pairs may be used, including but not limited to biotin/avidin (or streptavidin), antigen/antibody pairs, etc.

The implementation of a single strand isolation step using the methods described above or variations thereof (or any other convenient single strand isolation step) will

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generally be based on the desires of the user. One example of isolating single stranded polynucleotides is shown in Figure 6. In this Figure, a starting double stranded template (with 5' to 3' orientation shown as an arrow) is denatured and primed with a biotinylated synthesis primer specific for the primer site. After extension of the primer (i.e., nucleic acid synthesis), the sample is contacted with a solid support having avidin bound to it. The biotin moiety (i.e., the binding partner of avidin) on the extended strands will bind to the solid-phase avidin. Denaturation and washing is then performed to remove all non-biotinylated polynucleotide strands. If desired, the bound polynucleotide, which can be used in subsequent reflex process steps (e.g., as a template for Bn primer extension reactions), may be eluted from the avidin support. Alternatively, the bound polynucleotide may be employed in subsequent steps of the desired process while still bound to the solid support (e.g., in solid phase extension reactions using Bn primers). This process, with minor variations depending on the template being used and the identity of the desired single stranded polynucleotide, may be employed at any of a number of steps in which a single stranded product is to be isolated. It is noted that in certain embodiments, substrate bound biotinylated polynucleotide can be used to produce and isolate non-biotinylated single stranded products (i.e., by eluting the non-biotinylated products while leaving the biotinylated templates bound to the avidin on the solid support). Thus, the specifics of how binding partners are used to isolate single stranded polynucleotides of interest will vary depending on experimental design parameters.

To the single stranded polynucleotide we anneal one of the B_n primers having the appended reflex sequence (e.g., B_1) and extend the primer under nucleic acid synthesis conditions to produce a copy of the polynucleotide that has a reflex sequence at its 5' end. A single stranded copy of this polynucleotide is then produced to reverse polarity using a primer specific for the primer binding site in the domain 118. The resulting nucleic acid has structure 100 shown in Figure 1A, where the first domain 102 includes the primer binding site and the MID. Site A (110) in Figure 1 is determined by the specificity of the 5' reflex-Bn primer used.

Annealing, extending and removal steps are then performed as shown in Figure 1 to produce a product in which the primer binding site and the MID are now in close proximity to the desired site (i.e., the site defined by the primer used, e.g., B1). The resulting nucleic acid can be used in downstream analyses as desired by the user.

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It is noted here that, while not shown in Figure 1A and 1B, any convenient method for adding a reflex sequence (or any other domain or adapter) may be used in the practice of the reflex process. For example, the reflex sequence (or its complement) can be added at a particular position by ligation. For double stranded polynucleotides, an adapter can be configured to be ligated to a particular restriction enzyme cut site. Where a single stranded polynucleotide is employed, a double stranded adapter construct that possesses an overhang configured to bind to the end of the single-stranded polynucleotide can be used. For example, in the latter case, the end of a single stranded polynucleotide can be modified to include specific nucleotide bases that are complementary to the overhang in the double stranded adaptor using terminal transferase and specific nucleotides. Again, any convenient method for producing a starting polynucleotide may be employed in practicing the methods of the subject invention.

In certain embodiments, the nucleic acid may be sequenced directly using a sequencing primer specific for the primer binding site. This sequencing reaction will read through the MID and desired site in the insert.

In certain embodiments, the polynucleotide may be isolated (or fractionated) using an appropriate A_n primer (e.g., when using B_1 as the first primer, primer A_1 can be used). In certain embodiments, the A_n primed polynucleotide is subjected to nucleic acid synthesis conditions to produce a copy of the fragment produced in the reflex process. In certain of these embodiments, the A_n primer has appended on its 5' end a primer binding site that can be used in further downstream steps, including sequencing reactions. Providing a primer binding site in the A_n primer allows amplifying and/or sequencing from both ends of the resultant fragment: from the primer site in the first domain 118 and the primer site in the A_n primer (not shown in Figure 1B). Because of the position of the primer binding sites and their distance apart (i.e., less than one sequencing run apart), sequencing from both ends will capture the sequence of the desired site and the sequence of the MID, which can be used for downstream bioinformatic analyses, e.g., to positively identify the sample of origin. It is noted here that while sequencing in both directions is possible, it is not necessary, as sequencing from either primer site alone will capture the sequence of interest or a polynucleotide as well as its corresponding MID sequence.

Note that in certain embodiments, the first fragment which is obtained by B_0 directly, the polarity of the sequence is reversed as there has been no transfer. In this case B_0 may have appended to it a primer binding site (e.g., at its 5' end) that can be used for

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downstream amplification and/or sequencing reactions (e.g., in Roche 454 sequencing system). However, in certain embodiments, as noted above, the reflex process may be used with a corresponding B_0-A_0 primer pair as described above, i.e., using a B_0 primer having a 5' reflex sequence and a corresponding A_0 primer with its corresponding 5' adapter domain (e.g., a primer binding site).

It is noted here that because the particular sections of sequence to be analyzed are defined by the A_n-B_n primer pairs (as shown and described above), a much higher sequence specificity is achieved as compared to using previous extraction methods that employ only a single primer (e.g., using probes on a microarray). By using primer pairs, the specificity achieved is akin to that of PCR.

Figure 2 provides a detailed flow chart for an exemplary embodiment that employs reflex sequences for use in sequencing multiple specific regions in a nucleic acid sequence (i.e., regions 1, 2, 3, 4 and 5 in an 11kb region of lambda DNA).

A single parent DNA fragment 202 is generated that includes adapter domains (i.e., a Roche 454 sequencing primer site, a single MID, and a reflex sequence) and the sequence of interest. In the example shown, the sequence of interest is from lambda DNA and the reflex sequence is present on the top strand (with its complement shown in the bottom strand). Any convenient method for producing this parent DNA fragment may be used, including amplification with a primer that includes the adapter domains (e.g., using PCR) or by cloning the fragment into a vector that includes the adapter domains (e.g., a vector with the adapter domains adjacent to a cloning site). While only a single fragment with a single MID is shown, the steps in Figure 2 are applicable to samples having multiple different fragments each with a different MID, e.g., a sample having a population of homologous fragments from any number of different sources (e.g., different individuals). Figure 2 describes the subsequent enzymatic steps involved in creating the five daughter fragments in which regions 1, 2, 3, 4 and 5 (shown in structure 204) are rearranged to be placed within a functional distance of the adapter domains (i.e., close enough to the adapter domains to be sequenced in a single Roche 454 sequencing reaction). Note that certain steps are shown for region 4 only (206).

In step 1, the regions of interest are defined within the parent fragment (see 204) and corresponding primer pairs are designed for each (A_n-B_n primers as shown and described in Figure 1B). In step 2, sequence specific primer extensions are performed (only region 4 is shown) with corresponding B_n primers to produce single stranded

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polynucleotides having structure 208 (i.e., having the reflex sequence on the 5' terminus). Note that the B_n primer for region 4 will include a sequence specific primer binding site that primes at the 3'-most end of region 4 (where "3'-most" refers to the template strand). As such, the indication of regions 1 to 5 in Figure 2 is showing the entire sub-region of interest, not merely the corresponding B_n primer binding site. This structure is copied back to produce structure 210 having reversed polarity (e.g., with a primer that lands in the 454A' domain). Polynucleotide 210 has structure similar to structure 100 shown at the top of Figure 1. Steps 4 and 5 are as shown in Figure 1, specifically forming the intramolecular structure 212 and removing double stranded DNA from the 5' end. The structure formed for region 4 is shown as 216 with structures for the other regions also shown (214). Note that the formation of each of the structures 214 are formed in separate reactions (i.e., structure with region 1 in proximity to the adapter domains is in a first sample, the structure with region 2 in proximity to the adapter region is in a second sample, etc.). In step 6 the polynucleootides 214 are copied to reverse polarity to form polynucleotides 218. In step 7, each of these products are then primed with the second primer of the specific primer pair (see A_n primers as shown in Figure 1B) each having a second Roche 454 primer site (454B) and extended to form products 220.

Figure 2 shows that the reflex process produces five daughter fragments 220 of defined length (e.g., ~500 bp) defined by DNA sequences that differ in their distance from the reflex sequence in the starting structure 202 while maintaining the original MID.

The reflex process described herein can be used to perform powerful linkage analysis by combining it with nucleic acid counting methods. Any convenient method for tagging and /or counting individual nucleic acid molecules with unique tags may be employed (see, e.g., U.S. Patent 7,537,897, issued on May 26, 2009; U.S. Patent 7,217,522, issued on May 15, 2007; U.S. Patent Application 11/377,462, filed on March 16, 2006; and U.S. Patent Application 12/432,080, filed on April 29, 2009; each of which is incorporated by reference herein in their). All of this can be conducted in parallel thus saving on the cost of labor, time and materials.

In one exemplary embodiment, a large collection of sequences is tagged with MID such that each polynucleotide molecule in the sample has a unique MID. In other words, each polynucleotide in the sample (e.g., each individual double stranded or single stranded polynucleotide) is tagged with a MID that is different from every other MID on every other polynucleotide in the sample. In general, to accomplish such molecular tagging the

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number of distinct MID tags to be used should be many times greater than the actual number of molecules to be analyzed. This will result in each individual nucleic acid molecule being labeled with a unique ID tag. Any sequences that then result from the reflex process on that particular molecule (e.g., as described above) will thus be labeled with the same unique MID tag and thus inherently linked. Note that once all molecules in a sample are individually tagged, they can be manipulated and amplified as much as needed for processing so long as the MID tag is maintained in the products generated.

For example, we might want to sequence one thousand viral genomes or one thousand copies of a gene present in somatic cells. After tagging each polynucleotide in the sample with a sequencing primer site, MID and reflex sequence (as shown in the figures and described above), we use the reflex process to break each polynucleotide into lengths appropriate to the sequencing procedure being used, transferring the sequencing primer site and MID to each fragment (as described above). Obtaining sequence information from all of the reflex-processed samples can be used to determine the sequence of each individual polynucleotide in the starting sample, using the MID sequence to defining linkage relationships between sequences from different regions in the polynucleotide being sequenced. Using a sequencing platform with longer read lengths can minimize the number of primers to be used (and reflex fragments generated).

The advantages noted above are shown in Figure 5. This figure shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information. It is noted that not all domain structures and steps are shown in the reflex process for simplicity.

Kits and Systems

Also provided by the subject invention are kits and systems for practicing the subject methods, as described above, such vectors configured to add reflex sequences to nucleic acid inserts of interest and regents for performing any steps in the cloning or reflex

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process described herein (e.g., restriction enzymes, nucleotides, polymerases, primers, exonucleases, etc.). The various components of the kits may be present in separate containers or certain compatible components may be precombined into a single container, as desired.

The subject systems and kits may also include one or more other reagents for preparing or processing a nucleic acid sample according to the subject methods. The reagents may include one or more matrices, solvents, sample preparation reagents, buffers, desalting reagents, enzymatic reagents, denaturing reagents, where calibration standards such as positive and negative controls may be provided as well. As such, the kits may include one or more containers such as vials or bottles, with each container containing a separate component for carrying out a sample processing or preparing step and/or for carrying out one or more steps of a nucleic acid variant isolation assay according to the present invention.

In addition to above-mentioned components, the subject kits typically further include instructions for using the components of the kit to practice the subject methods, e.g., to prepare nucleic acid samples for perform the reflex process according to aspects of the subject methods. The instructions for practicing the subject methods are generally recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or sub-packaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g. CD-ROM, diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

In addition to the subject database, programming and instructions, the kits may also include one or more control samples and reagents, e.g., two or more control samples for use in testing the kit.

Utility

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The reflex process described herein provides significant advantages in numerous applications, a few of which are noted below (as well as described above

For example, as described above, certain aspects of the reflex process define the particular sections of sequence to be analyzed by a primer pair, as in PCR (e.g., the two oligos shown as An-Bn in Figure 1B). This results in a higher sequence specificity as compared to other extraction methods (e.g., using probes on a microarray) that only use a single oligo sequence. The separation of the probes defines a length that can be relatively uniform (hence making subsequent handling including amplification more uniform) and can also be tailored to the particular sequencing platform being employed.

Further, as described above, aspects of the present invention can be used to analyze homologous genomic locations in a multiplexed sample (i.e., a sample having polynucleotides from different genomic samples) in which the polynucleotides are tagged with the MID. This is possible because the reflex process, which operates intramolecularly, maintains the MID thus linking any particular fragment to the sample from which it originates.

Finally, as the reflex processes described herein functions intramolecularly, one can determine the genetic linkage between different regions on the same large fragment that are too far apart to be sequenced in one sequence read. Such a determination of linkage may be of great value in plant or animal genetics (e.g., to decide if a particular set of variations are linked together on the same stretch of chromosome) or in viral studies (e.g., to determine if particular variations are linked together on the same stretch of a viral genome/transcripts, e.g., HIV, hepatitis virus, etc.).

EXAMPLES

Example I

Figures 3 and 4 provide experimental data and validation of the reflex process described herein using synthetic polynucleotide substrate.

METHODS

Substrate:

The 100 base oligonucleotide substrate (as shown diagramatically in Figure 3A) was synthesized with internal fluorescein-dT positioned between the reflex sequences. This

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label provides convenient and sensitive method of detection of oligonucleotide species using polyacrylamide gel electrophoresis.

Extension reactions:

Reactions were prepared which contained 1 μM of the 100 base oligonucleotide substrate, 200 μM dNTPs, + or – 1 μM competitor oligonucleotide, 0.5 μl of each polymerase: Vent (NEB), Taq (Qiagen) and Herculase (Stratagene), and made up to 50 μl with the appropriate commercial buffers for each polymerase and dH_20. For Taq titrations 0.5 μl, 1 μl, 2 μl, and 3 μl enzyme was used in the same 50 μl volume. Reactions were heated in a Biometra thermocycler to 95 °C for 15 minutes (Taq) or 5 minutes (Herculase, Vent), followed by 55 °C or 50 °C for 30 seconds, and a final incubation at 72 °C for 10 minutes.

T7 exonuclease digestions:

Reactions were prepared with 10 μl extension reactions above, 0.5 μl T7 exonuclease (NEB), and made up to 50 μl using NEB buffer 4 and dH_20. Reactions were incubated at 25 °C for 30 minutes.

Gel Electrophoresis analysis:

An 8% denaturing polyacrylamide gel was used to analyze reaction species. 0.4 μl of extension reactions, and 2 μl of digestion reactions were loaded and ran at 800V for ~1.5 hours. Gels were analyzed for fluorescein using a Typhoon imager.

RESULTS

Figure 3A shows the structure of each stage of reflex sequence processing with the expected nucleic acid size shown on the left. The initial single stranded nucleic acid having a sequencing primer site (Roche 454 sequencing primer binding site A; listed as 454A); a MID; insert and a complement of the reflex sequence is 100 nucleotides in length. After self annealing and extension, the product is expected to be 130 nucleotides in length. After removal of the double stranded region from the 5' end, the nucleic acid is expected to be 82 bases in length.

Figure 3B shows the results of three experiments using three different nucleic acid polymerases (Vent, Herculase and Taq, indicated at the top of the lanes). The temperature

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at which the reactions were carried out is shown at the top of each lane (either 50°C or 55°C). The sizes of the three nucleic acids as noted above are indicated on the left and right side of the gel.

As shown in figure 3B, extension appears to be most efficient under the conditions used with Herculase (Herculase is a mixture of three enzymes: modified Pfu DNAP, Taq and dUTPase). Most (or all) of the initial 100 base pair nucleic acid are converted to the 130 base pair product (see lanes 6 and 7). However, 3'-5' exonuclease activity of Herculase results in partial digestion of the desired 82 base product (note bands at and below the 82 base pairs in lanes 8 and 9).

Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product (see lane 13).

Figure 4 shows the effect on the reflex process of increasing amounts of Taq polymerase as well as the use of a reflex sequence competitor (schematically shown in Figure 4A).

As shown in lanes 2 to 5, increased Taq concentration improves extension to ~90% conversion of the starting nucleic acid (see lane 5). Lanes 7 to 8 show that T7 exonuclease digestion does not leave a perfect 82 base product. This may be due to collapse of dsDNA when T7 exonuclease has nearly completed its digestion from the 5' end in the double stranded region of the fold-back structure. It is noted that in many embodiments, the removal of a few additional bases from the 5' end of the polynucleotide will not interfere with downstream analyses, as nucleotide bases at the 5' end are often removed during downstream steps.

As shown in Lanes 11-14, addition of a competitor (which can interfere with annealing of the reflex sequences to form the fold-back structure) results in a small decrease (~5-10%) of fully extended product. Although not shown, we have observed that the competitor oligonucleotide also gets extended by the same amount (5-10%). The concentration of the competitor, and the overall genetic complexity, will likely affect this. The important conclusions of the experiments shown in Figures 3 and 4 are that the essential processes (fold-back binding, extension, and chew-back) are all functional and can be implemented.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to

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those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

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CLAIMS

That which is claimed is:

1. A method of moving a domain in a nucleic acid, the method comprising:

a) annealing a reflex sequence in a single stranded nucleic acid in a sample with a complement of the reflex sequence in the single stranded nucleic acid, wherein the single stranded nucleic acid comprises the following in a 5' to 3' orientation:

a first domain;

the reflex sequence;

a nucleic acid sequence of interest; and

the complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the single stranded nucleic acid;

b) extending from the 3' end of the complement of the reflex sequence through the first domain to produce a double stranded region; and

c) removing the first domain and the reflex sequence from the 5' end of the double stranded region,

whereby a complement of the first domain is present at the 3' end of the single stranded nucleic acid fragment, thereby moving the first domain in the single stranded nucleic acid.

2. The method of claim 1, wherein the first domain comprises a multiplex identifier (MID).

3. The method of claim 1, wherein the first domain comprises a primer binding site.

4. The method of claim 1, wherein the first reflex sequence is from 10 to 50 nucleotides in length.

5. The method of claim 1, wherein the first reflex sequence comprises an RNA polymerase binding site.

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6. The method of claim 1, wherein the removing step comprises treating the nucleic acid of step b with T7 exonuclease.

7. The method of claim 1, wherein the sample is a multiplexed sample, wherein the first domain in each of the single stranded nucleic acids in the multiplexed sample comprises a MID corresponding to its parental sample.

8. The method of claim 1, wherein the sample comprises multiple single stranded nucleic acids, and wherein each single stranded nucleic acid in the sample comprises a unique MID in the first domain.

9. The method of claim 1, wherein the single stranded nucleic acid is produced by:

annealing a nucleic acid synthesis primer to a parent nucleic acid strand, wherein the parent nucleic acid strand comprises the following in a 5' to 3' orientation:

the first domain;

the reflex sequence; and

a second domain comprising the nucleic acid sequence of interest;

wherein the nucleic acid synthesis primer comprises:

a 3' priming domain that is complementary to a site adjacent to and 3' of the nucleic acid sequence of interest in the second domain; and

a 5' reflex sequence;

extending the nucleic acid synthesis primer to produce an extension product; and

replicating the resultant extension product with a second nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain, thereby producing the single stranded nucleic acid.

10. The method of claim 9, wherein the length of the second domain is from 0.1 to 30 kilobases.

11. The method of claim 9, wherein the parent nucleic acid strand is enriched prior to the annealing step based on a sequence in the nucleic acid sequence of interest.

12. The method of claim 1, further comprising the steps of:

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d) replicating the single stranded nucleic acid of step c with a first nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain to produce a first extension product;

e) annealing a second nucleic acid synthesis primer to the first extension product, wherein the second nucleic acid synthesis primer comprises a 3' priming domain that is complementary to a site within the sequence of interest in the first extension product; and

f) extending the second synthesis primer to produce a second extension product.

13. The method of claim 12, wherein the second synthesis primer comprises a 5' domain that is not complementary to the sequence of interest in the first extension product.

14. The method of claim 13, wherein the first domain comprises a first primer binding site and the 5' domain of the second synthesis primer comprises a second primer binding site.

15. The method of claim 14, wherein the length of the sequence of interest in the second extension product is from 50 to 10,000 nucleotides.

16. A nucleic acid comprising the following in a 5' to 3' orientation:

a first domain;

a first reflex sequence;

a nucleic acid sequence of interest; and

a complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the single stranded nucleic acid.

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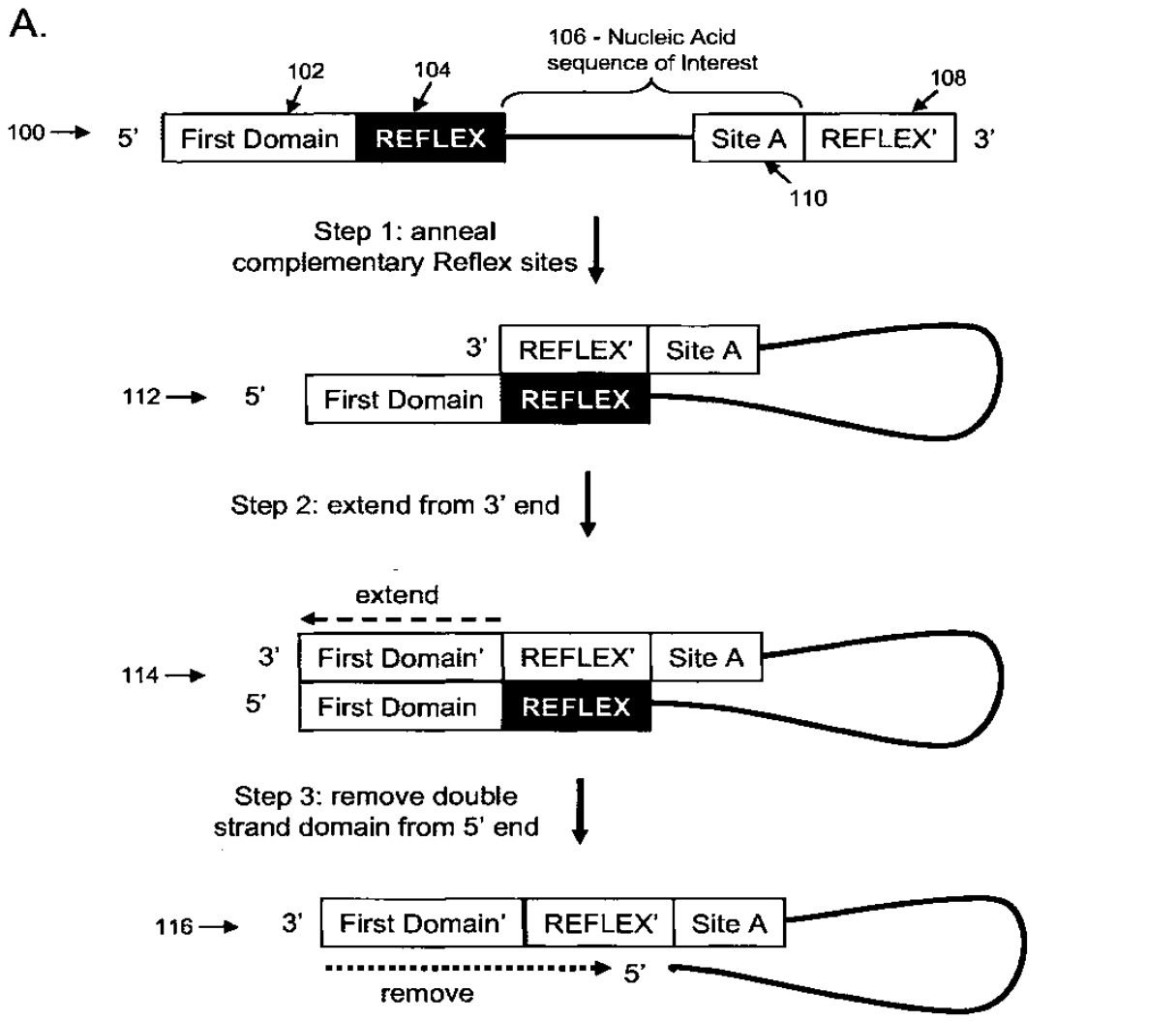
COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

ABSTRACT OF THE DISCLOSURE

Aspects of the present invention are drawn to the use of a specifically designed 'reflex' sequence present in a nucleic acid (e.g., as part of an adapter structure which is ligated onto the ends of a nucleic acid to be analyzed) that can be used to move a desired region of the nucleic acid to be analyzed to a position into functional proximity to elements present elsewhere in the nucleic acid (e.g., primer binding sites and/or MID).

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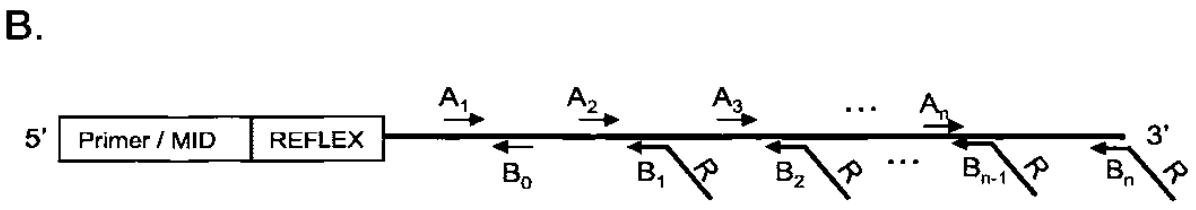
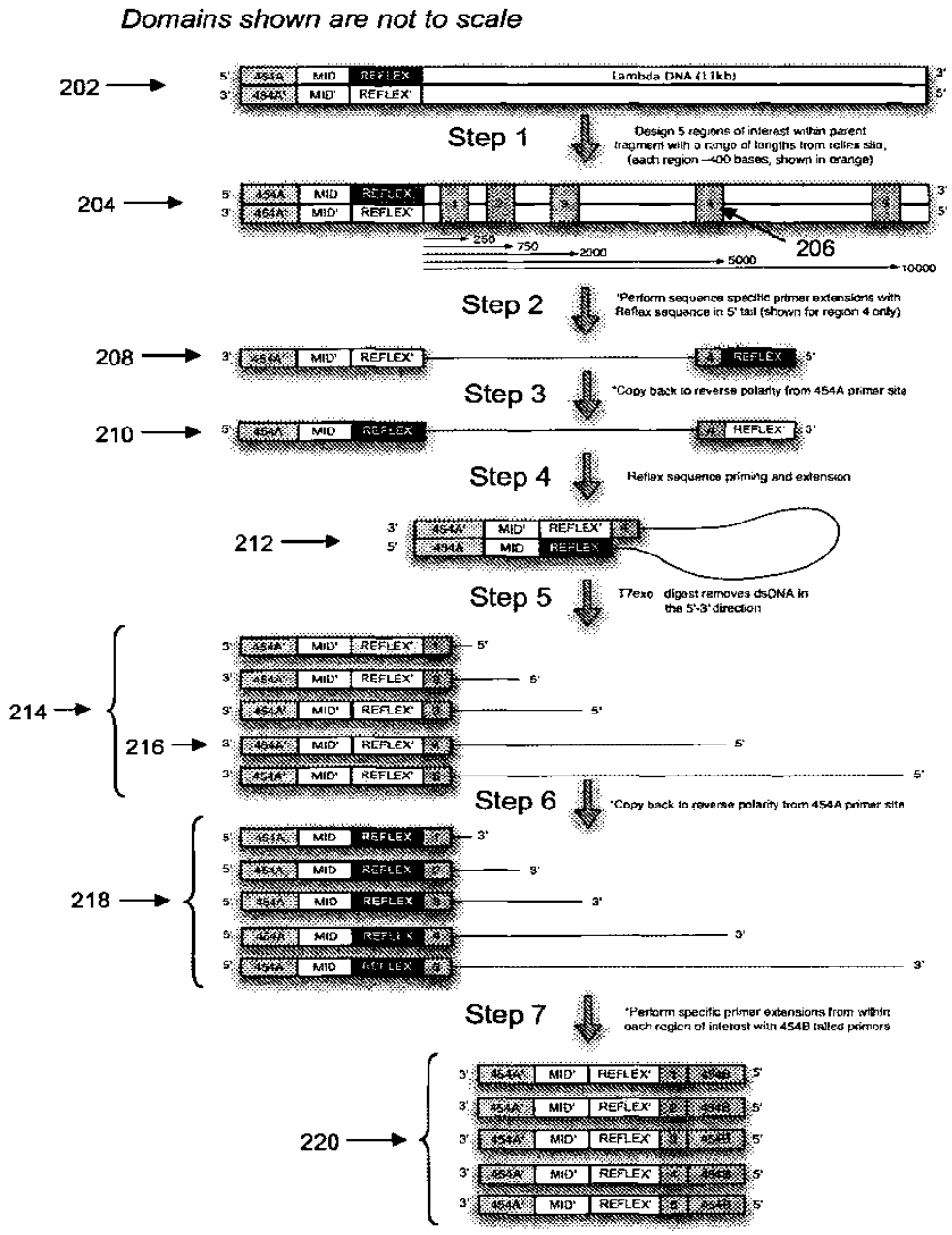


Fig. 1

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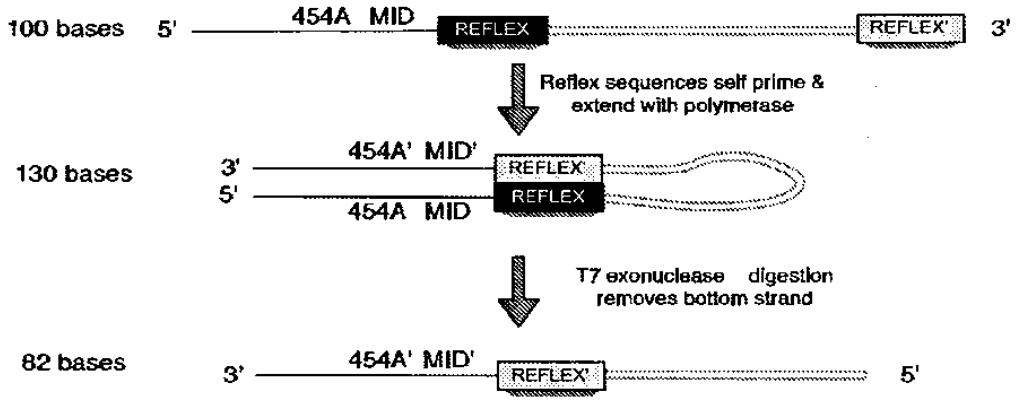


primer extensions with * may be performed such that isolation of single stranded species is facilitated (e.g., using primers with binding moieties and/or linear amplification)

Fig. 2

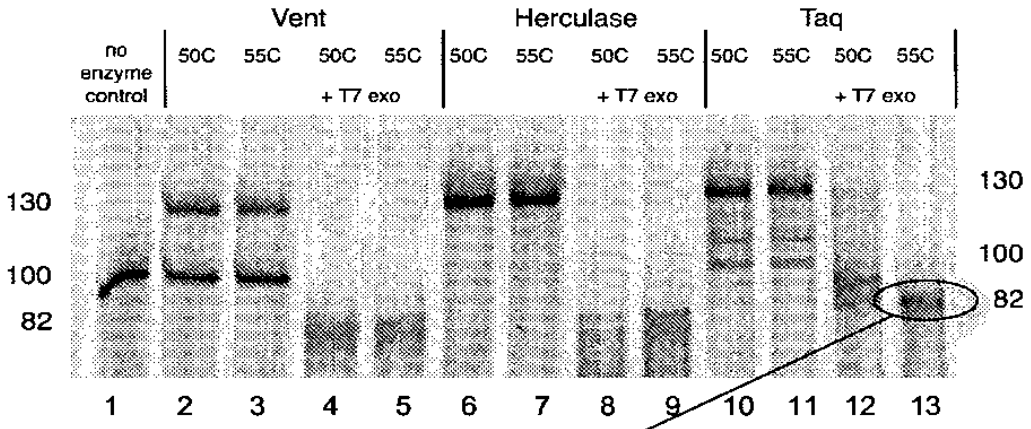
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A.



B.

Polymerase Testing at two annealing temps



Extension is best with herculase, but 3-5' exo activity results in partial digestion of the desired 82 base product. Taq, which lacks 3-5' exo activity shows a stronger band at the expected size of the final product.

Fig. 3

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A.



B.

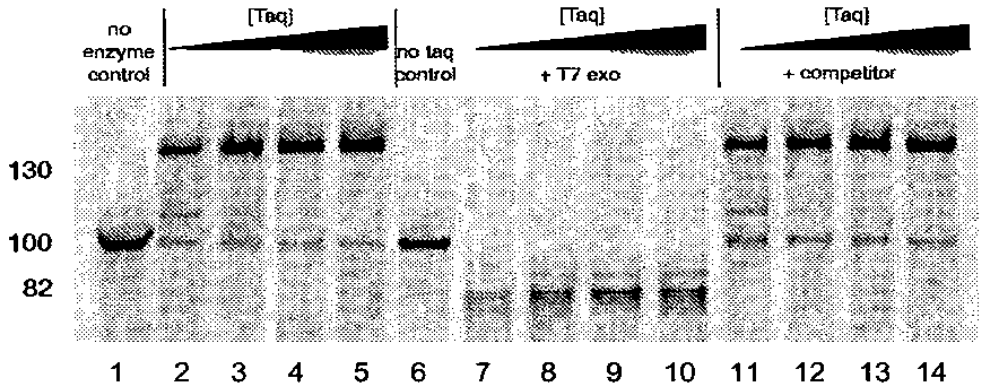


Fig. 4

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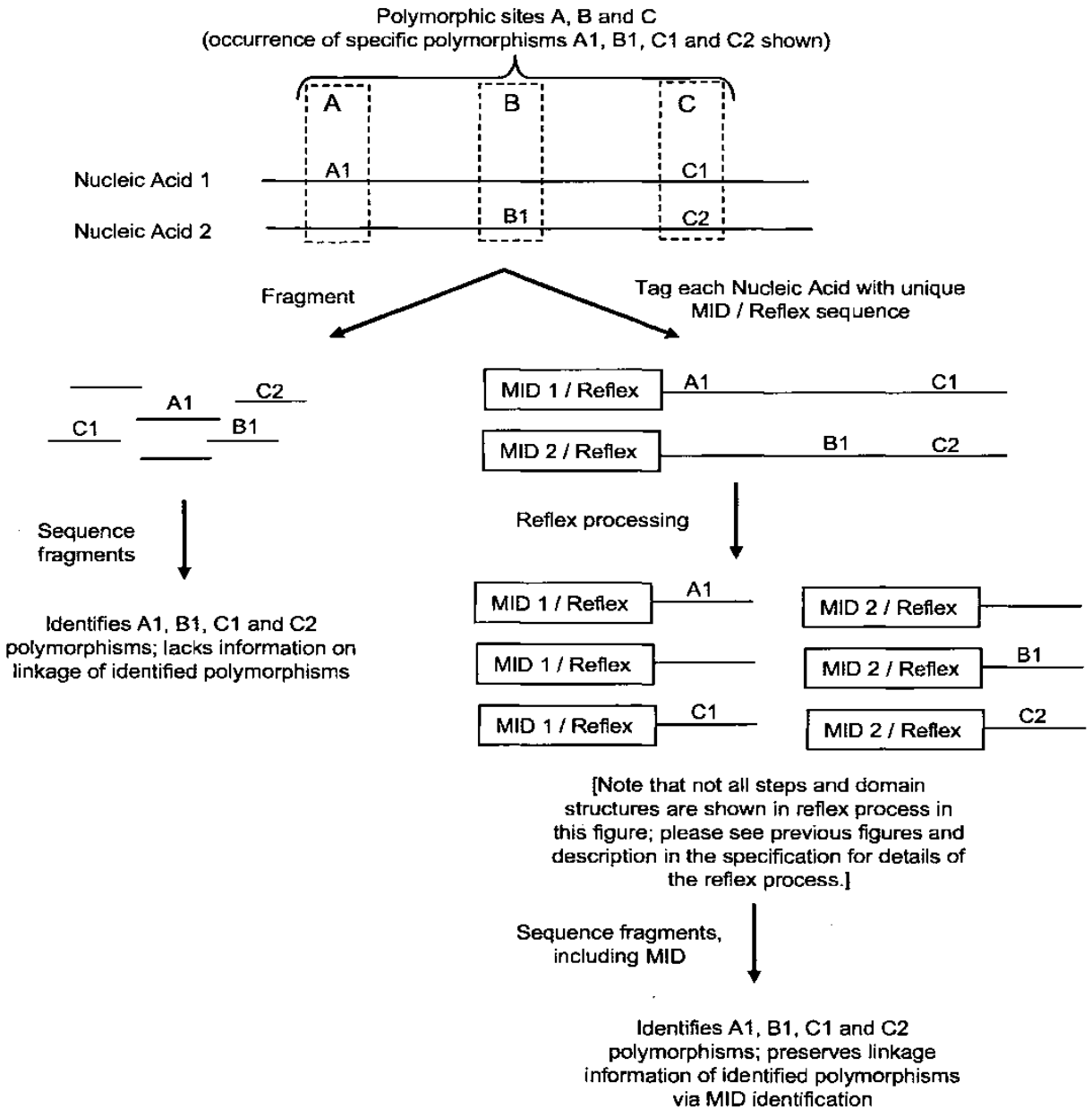


Fig. 5

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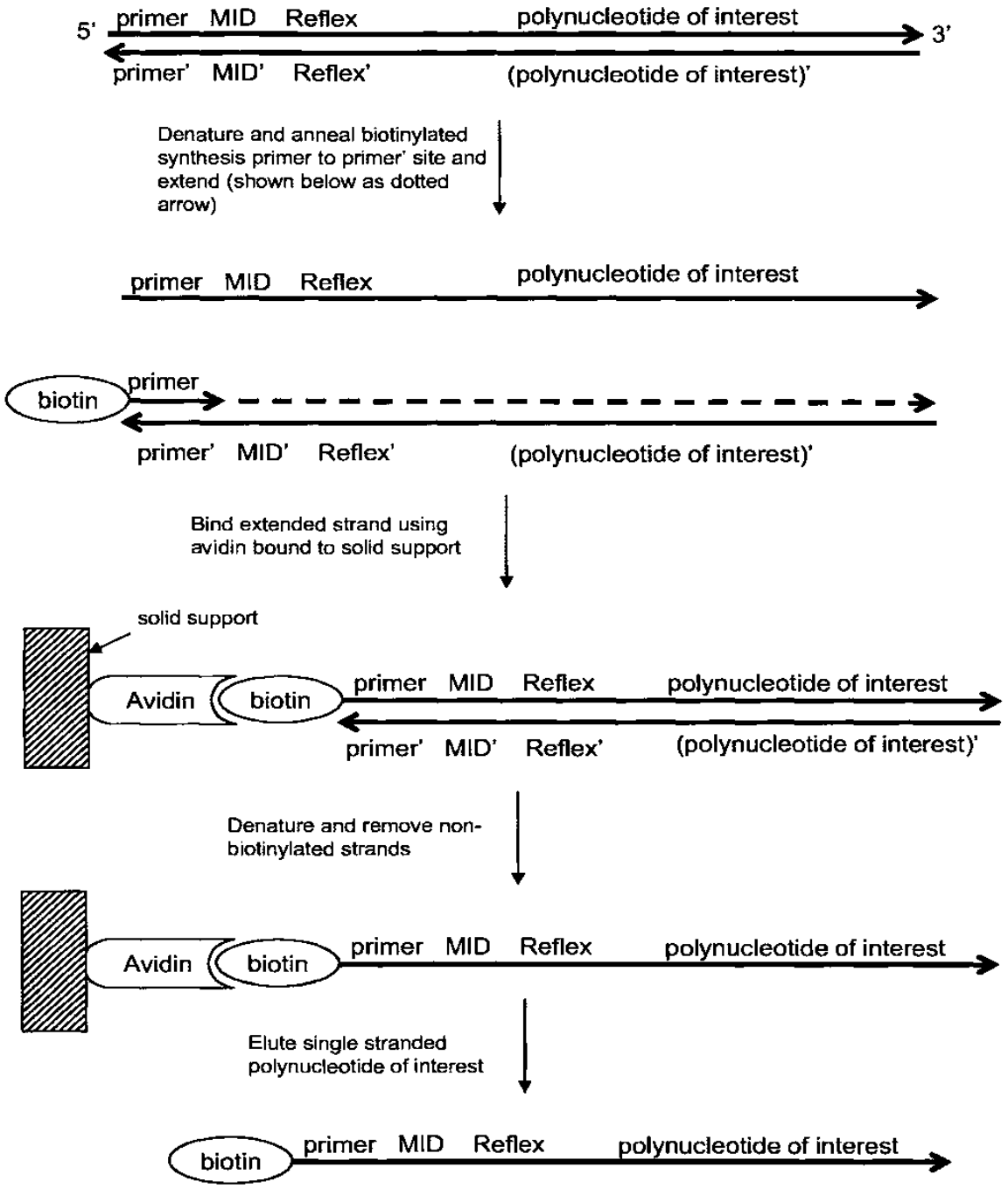


Fig. 6

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APPLICATION DATA SHEET

APPLICATION INFORMATION

Application Type::	Provisional
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Request for Non-Publication?::	
Assignee for Publication::	
Total Drawing Sheets::	6
Small Entity?::	Yes
Sequence Submission?::	No
Computer Readable Form (CRF)?::	

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APPLICATION DATA SHEET

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CONTINUITY INFORMATION

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Warnings:					
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2		CGLC-020PRV2_app.pdf	1400561 c3203d3323f38a6eb009eb67da5e2b1701666439	yes	38
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Specification		1	34	
	Claims		35	37	
	Abstract		38	38	
Warnings:					
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3	Drawings-only black and white line drawings	CGLC-020PRV2_Figures.pdf	510199 18bf192b18b8d422e52f6165dfbd504a4bba7f8a	no	10
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COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

BACKGROUND

We have previously described methods that enable tagging each of a population of fragmented genomes and then combining them together to create a 'population library' that can be processed and eventually sequenced as a mixture. The population tags enable analysis software to parse the sequence reads into files that can be attributed to a particular genome in the population. One limitation of the overall process stems from limitations of existing DNA sequencing technologies. In particular, if fragments in the regions of interest of the genome are longer than the lengths that can be sequenced by a particular technology, then such fragments will not be fully analyzed (since sequencing proceeds from an end of a fragment inward). Furthermore, a disadvantage of any sequencing technology dependent on fragmentation is that one can never relate sequence changes in one part of a particular genomic region to the sequence changes of other parts of the same genome that may be on the same chromosome but on a different fragment. (See Figure 5 and its description below).

The present invention removes the limitations imposed by current sequencing technologies as well as being useful in a number of other nucleic acid analyses.

SUMMARY OF THE INVENTION

Aspects of the present invention are drawn to processes for moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method" (or reflex process, reflex reaction, and the like). In certain embodiments, the reflex method results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer binding sites and/or MID). Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to

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common practice, the various features of the drawings are not to scale. Indeed, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures:

Figure 1: Panel A is a schematic diagram illustrating moving a first domain from one site to another in a nucleic acid molecule using a reflex sequence. Panel B is a schematic diagram depicting the relative position of primer pairs (A_n-B_n primers) that find us in aspects of the reflex process described herein.

Figure 2 is a schematic diagram illustrating an exemplary embodiment for moving a primer binding site and a MID to a specific location in a nucleic acid of interest.

Figure 3: Panel A is a schematic showing expected structures and sizes of nucleic acid species in the reflex site process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

Figure 4: Panel A is a schematic showing the structure of the nucleic acid and competitor used in the reflex sequence process; Panel B is a polyacrylamide gel showing the nucleic acid species produced in the reflex process described in Example 1.

Figure 5 shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information.

Figure 6 shows an exemplary embodiment of using binding partner pairs (biotin/avidin) to isolate single stranded polynucleotides of interest.

Figure 7 shows a flow chart of a reflex process (left) in which the T7 exonuclease step is optional. The gel on the right shows the resultant product of the reflex process either without the T7 exonuclease step (lane 1) or with the T7 exonuclease step (lane 2).

Figure 8 shows an exemplary reflex process workflow with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt beads to remove primer oligos).

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Figure 9 shows the starting material (left panel) and the resultant product generated (right panel) using a reflex process without using a T7 exonuclease step (as described in Example II). The reflex site in the starting material is a sequence normally present in the polynucleotide being processed (also called a "non-artificial" reflex site). This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in transferring an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

Figure 10 shows a diagram of exemplary workflows for preparing material for and performing the reflex process.

DEFINITIONS

Unless otherwise defined, 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. Still, certain elements are defined for the sake of clarity and ease of reference.

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

"Amplicon" means the product of a polynucleotide amplification reaction. That is, it is a population of polynucleotides, usually double stranded, that are replicated from one or more starting sequences. The one or more starting sequences may be one or more copies of the same sequence, or it may be a mixture of different sequences. Amplicons may be produced by a variety of amplification reactions whose products are multiple replicates of one or more target nucleic acids. Generally, amplification reactions producing amplicons are "template-driven" in that base pairing of reactants, either nucleotides or oligonucleotides, have complements in a template polynucleotide that are required for the creation of reaction products. In one aspect, template-driven reactions

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are primer extensions with a nucleic acid polymerase or oligonucleotide ligations with a nucleic acid ligase. Such reactions include, but are not limited to, polymerase chain reactions (PCRs), linear polymerase reactions, nucleic acid sequence-based amplification (NASBAs), rolling circle amplifications, and the like, disclosed in the following references that are incorporated herein by reference: Mullis et al, U.S. patents 4,683,195; 4,965,188; 4,683,202; 4,800,159 (PCR); Gelfand et al, U.S. patent 5,210,015 (real-time PCR with "TAQMAN™" probes); Wittwer et al, U.S. patent 6,174,670; Kacian et al, U.S. patent 5,399,491 ("NASBA"); Lizardi, U.S. patent 5,854,033; Aono et al, Japanese patent publ. JP 4-262799 (rolling circle amplification); and the like. In one aspect, amplicons of the invention are produced by PCRs. An amplification reaction may be a "real-time" amplification if a detection chemistry is available that permits a reaction product to be measured as the amplification reaction progresses, e.g. "real-time PCR" described below, or "real-time NASBA" as described in Leone et al, Nucleic Acids Research, 26: 2150-2155 (1998), and like references. As used herein, the term "amplifying" means performing an amplification reaction. A "reaction mixture" means a solution containing all the necessary reactants for performing a reaction, which may include, but not be limited to, buffering agents to maintain pH at a selected level during a reaction, salts, co-factors, scavengers, and the like.

The term "assessing" includes any form of measurement, and includes determining if an element is present or not. The terms "determining", "measuring", "evaluating", "assessing" and "assaying" are used interchangeably and includes quantitative and qualitative determinations. Assessing may be relative or absolute. "Assessing the presence of" includes determining the amount of something present, and/or determining whether it is present or absent. As used herein, the terms "determining," "measuring," and "assessing," and "assaying" are used interchangeably and include both quantitative and qualitative determinations.

"Complementary" or "substantially complementary" refers to the hybridization or base pairing or the formation of a duplex between nucleotides or nucleic acids, such as, for instance, between the two strands of a double stranded DNA molecule or between an oligonucleotide primer and a primer binding site on a single stranded nucleic acid. Complementary nucleotides are, generally, A and T (or A and U), or C and G. Two single stranded RNA or DNA molecules are said to be substantially complementary when the nucleotides of one strand, optimally aligned and compared and with appropriate

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nucleotide insertions or deletions, pair with at least about 80% of the nucleotides of the other strand, usually at least about 90% to 95%, and more preferably from about 98 to 100%. Alternatively, substantial complementarity exists when an RNA or DNA strand will hybridize under selective hybridization conditions to its complement. Typically, selective hybridization will occur when there is at least about 65% complementary over a stretch of at least 14 to 25 nucleotides, preferably at least about 75%, more preferably at least about 90% complementary. See, M. Kanehisa Nucleic Acids Res. 12:203 (1984), incorporated herein by reference.

"Duplex" means at least two oligonucleotides and/or polynucleotides that are fully or partially complementary undergo Watson-Crick type base pairing among all or most of their nucleotides so that a stable complex is formed. The terms "annealing" and "hybridization" are used interchangeably to mean the formation of a stable duplex. "Perfectly matched" in reference to a duplex means that the poly- or oligonucleotide strands making up the duplex form a double stranded structure with one another such that every nucleotide in each strand undergoes Watson-Crick base pairing with a nucleotide in the other strand. A stable duplex can include Watson-Crick base pairing and/or non-Watson-Crick base pairing between the strands of the duplex (where base pairing means the forming hydrogen bonds). In certain embodiments, a non-Watson-Crick base pair includes a nucleoside analog, such as deoxyinosine, 2, 6-diaminopurine, PNAs, LNA's and the like. In certain embodiments, a non-Watson-Crick base pair includes a "wobble base", such as deoxyinosine, 8-oxo-dA, 8-oxo-dG and the like, where by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand (wobble bases are described in further detail below). A "mismatch" in a duplex between two oligonucleotides or polynucleotides means that a pair of nucleotides in the duplex fails to undergo Watson-Crick bonding.

"Genetic locus," "locus," or "locus of interest" in reference to a genome or target polynucleotide, means a contiguous sub-region or segment of the genome or target polynucleotide. As used herein, genetic locus, locus, or locus of interest may refer to the position of a nucleotide, a gene or a portion of a gene in a genome, including mitochondrial DNA or other non-chromosomal DNA (e.g., bacterial plasmid), or it may

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refer to any contiguous portion of genomic sequence whether or not it is within, or associated with, a gene. A genetic locus, locus, or locus of interest can be from a single nucleotide to a segment of a few hundred or a few thousand nucleotides in length or more. In general, a locus of interest will have a reference sequence associated with it (see description of "reference sequence" below).

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

"Ligation" means to form a covalent bond or linkage between the termini of two or more nucleic acids, e.g. oligonucleotides and/or polynucleotides, in a template-driven reaction. The nature of the bond or linkage may vary widely and the ligation may be carried out enzymatically or chemically. As used herein, ligations are usually carried out enzymatically to form a phosphodiester linkage between a 5' carbon of a terminal nucleotide of one oligonucleotide with 3' carbon of another oligonucleotide. A variety of template-driven ligation reactions are described in the following references, which are incorporated by reference: Whiteley et al, U.S. patent 4,883,750; Letsinger et al, U.S. patent 5,476,930; Fung et al, U.S. patent 5,593,826; Kool, U.S. patent 5,426,180; Landegren et al, U.S. patent 5,871,921; Xu and Kool, Nucleic Acids Research, 27: 875-881 (1999); Higgins et al, Methods in Enzymology, 68: 50-71 (1979); Engler et al, The Enzymes, 15: 3-29 (1982); and Namsaraev, U.S. patent publication 2004/0110213.

"Multiplex Identifer" (MID) as used herein refers to a tag associated with a nucleic acid whose identity (e.g., sequence) can be used to differentiate nucleic acids in a sample. In certain embodiments, the MID on a nucleic acid is used to identify the source from which a nucleic acid is derived. For example, a nucleic acid sample may be a pool of nucleic acids derived from different sources, (e.g., nucleic acids derived from different individuals, different tissues or cells, or

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nucleic acids isolated at different times points), where the nucleic acids from each different source is tagged with a unique MID. Identification of the MID on a nucleic acid thus allows one to determine from which source the nucleic acid is derived. In other words, the MID provides a correlation between a nucleic acid and its source. In certain embodiments, MIDs are employed to uniquely tag each individual nucleic acid in a sample. Identification of the number of unique MIDs in a sample will provide a readout of how many individual nucleic acids are present in the sample (or from how many original nucleic acids a manipulated nucleic acid sample was derived; see, e.g., U.S. Patent No. 7,537,897, issued on May 26, 2009, incorporated herein by reference in its entirety). MIDs can range in length from 2 to 100 nucleotide bases or more. Exemplary nucleic acid tags that find use as MIDs are described in U.S. Patent 7,544,473, issued on June 6, 2009, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Patent 7,393,665, issued on July 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides.

"Nucleoside" as used herein includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g. as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). "Analogs" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g. described by Scheit, Nucleotide Analogs (John Wiley, New York, 1980); Uhlman and Peyman, Chemical Reviews, 90: 543-584 (1990), or the like, with the proviso that they are capable of specific hybridization. Such analogs include synthetic nucleosides designed to enhance binding properties, reduce complexity, increase specificity, and the like. Polynucleotides comprising analogs with enhanced hybridization or nuclease resistance properties are described in Uhlman and Peyman (cited above); Crooke et al, Exp. Opin. Ther. Patents, 6: 855-870 (1996); Mesmaeker et al, Current Opinion in Structual Biology, 5: 343-355 (1995); and the like. Exemplary types of polynucleotides that are capable of enhancing duplex stability include oligonucleotide N3'→P5' phosphoramidates (referred to herein as "amidates"), peptide nucleic acids (referred to herein as "PNAs"), oligo-2'-O-alkylribonucleotides, polynucleotides containing C-5 propynylpyrimidines, locked nucleic acids ("LNAs"), and like compounds. Such

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oligonucleotides are either available commercially or may be synthesized using methods described in the literature.

"Polymerase chain reaction," or "PCR," means a reaction for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. In other words, PCR is a reaction for making multiple copies or replicates of a target nucleic acid flanked by primer binding sites, such reaction comprising one or more repetitions of the following steps: (i) denaturing the target nucleic acid, (ii) annealing primers to the primer binding sites, and (iii) extending the primers by a nucleic acid polymerase in the presence of nucleoside triphosphates. Usually, the reaction is cycled through different temperatures optimized for each step in a thermal cycler instrument. Particular temperatures, durations at each step, and rates of change between steps depend on many factors well-known to those of ordinary skill in the art, e.g. exemplified by the references: McPherson et al, editors, PCR: A Practical Approach and PCR2: A Practical Approach (IRL Press, Oxford, 1991 and 1995, respectively). For example, in a conventional PCR using Taq DNA polymerase, a double stranded target nucleic acid may be denatured at a temperature >90°C, primers annealed at a temperature in the range 50-75°C, and primers extended at a temperature in the range 72-78°C. The term "PCR" encompasses derivative forms of the reaction, including but not limited to, RT-PCR, real-time PCR, nested PCR, quantitative PCR, multiplexed PCR, and the like. Reaction volumes range from a few hundred nanoliters, e.g. 200 nL, to a few hundred μL, e.g. 200 μL. "Reverse transcription PCR," or "RT-PCR," means a PCR that is preceded by a reverse transcription reaction that converts a target RNA to a complementary single stranded DNA, which is then amplified, e.g. Tecott et al, U.S. patent 5,168,038, which patent is incorporated herein by reference. "Real-time PCR" means a PCR for which the amount of reaction product, i.e. amplicon, is monitored as the reaction proceeds. There are many forms of real-time PCR that differ mainly in the detection chemistries used for monitoring the reaction product, e.g. Gelfand et al, U.S. patent 5,210,015 ("TAQMAN™"); Wittwer et al, U.S. patents 6,174,670 and 6,569,627 (intercalating dyes); Tyagi et al, U.S. patent 5,925,517 (molecular beacons); which patents are incorporated herein by reference. Detection chemistries for real-time PCR are reviewed in Mackay et al, Nucleic Acids Research, 30: 1292-1305 (2002), which is also incorporated herein by reference. "Nested PCR" means a two-stage PCR wherein the amplicon of a first PCR becomes the sample for a second PCR using a new set of primers,

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at least one of which binds to an interior location of the first amplicon. As used herein, "initial primers" in reference to a nested amplification reaction mean the primers used to generate a first amplicon, and "secondary primers" mean the one or more primers used to generate a second, or nested, amplicon. "Multiplexed PCR" means a PCR wherein multiple target sequences (or a single target sequence and one or more reference sequences) are simultaneously carried out in the same reaction mixture, e.g. Bernard et al, Anal. Biochem., 273: 221-228 (1999)(two-color real-time PCR). Usually, distinct sets of primers are employed for each sequence being amplified.

"Quantitative PCR" means a PCR designed to measure the abundance of one or more specific target sequences in a sample or specimen. Quantitative PCR includes both absolute quantitation and relative quantitation of such target sequences. Quantitative measurements are made using one or more reference sequences that may be assayed separately or together with a target sequence. The reference sequence may be endogenous or exogenous to a sample or specimen, and in the latter case, may comprise one or more competitor templates. Typical endogenous reference sequences include segments of transcripts of the following genes: β-actin, GAPDH, β_2-microglobulin, ribosomal RNA, and the like. Techniques for quantitative PCR are well-known to those of ordinary skill in the art, as exemplified in the following references that are incorporated by reference: Freeman et al, Biotechniques, 26: 112-126 (1999); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9447 (1989); Zimmerman et al, Biotechniques, 21: 268-279 (1996); Diviacco et al, Gene, 122: 3013-3020 (1992); Becker-Andre et al, Nucleic Acids Research, 17: 9437-9446 (1989); and the like.

"Polynucleotide" or "oligonucleotide" is used interchangeably and each means a linear polymer of nucleotide monomers. Monomers making up polynucleotides and oligonucleotides are capable of specifically binding to a natural polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, base stacking, Hoogsteen or reverse Hoogsteen types of base pairing, wobble base pairing, or the like. As described in detail below, by "wobble base" is meant a nucleic acid base that can base pair with a first nucleotide base in a complementary nucleic acid strand but that, when employed as a template strand for nucleic acid synthesis, leads to the incorporation of a second, different nucleotide base into the synthesizing strand. Such monomers and their internucleosidic linkages may be naturally occurring or may be analogs thereof, e.g. naturally occurring or non-naturally

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occurring analogs. Non-naturally occurring analogs may include peptide nucleic acids (PNAs, e.g., as described in U.S. Patent 5,539,082, incorporated herein by reference), locked nucleic acids (LNAs, e.g., as described in U.S. Patent 6,670,461, incorporated herein by reference), phosphorothioate internucleosidic linkages, bases containing linking groups permitting the attachment of labels, such as fluorophores, or haptens, and the like. Whenever the use of an oligonucleotide or polynucleotide requires enzymatic processing, such as extension by a polymerase, ligation by a ligase, or the like, one of ordinary skill would understand that oligonucleotides or polynucleotides in those instances would not contain certain analogs of internucleosidic linkages, sugar moieties, or bases at any or some positions. Polynucleotides typically range in size from a few monomeric units, e.g. 5-40, when they are usually referred to as "oligonucleotides," to several thousand monomeric units. Whenever a polynucleotide or oligonucleotide is represented by a sequence of letters (upper or lower case), such as "ATGCCTG," it will be understood that the nucleotides are in 5'→3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, "I" denotes deoxyinosine, "U" denotes uridine, unless otherwise indicated or obvious from context. Unless otherwise noted the terminology and atom numbering conventions will follow those disclosed in Strachan and Read, Human Molecular Genetics 2 (Wiley-Liss, New York, 1999). Usually polynucleotides comprise the four natural nucleosides (e.g. deoxyadenosine, deoxycytidine, deoxyguanosine, deoxythymidine for DNA or their ribose counterparts for RNA) linked by phosphodiester linkages; however, they may also comprise non-natural nucleotide analogs, e.g. including modified bases, sugars, or internucleosidic linkages. It is clear to those skilled in the art that where an enzyme has specific oligonucleotide or polynucleotide substrate requirements for activity, e.g. single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for the oligonucleotide or polynucleotide substrates is well within the knowledge of one of ordinary skill, especially with guidance from treatises, such as Sambrook et al, Molecular Cloning, Second Edition (Cold Spring Harbor Laboratory, New York, 1989), and like references.

"Primer" means an oligonucleotide, either natural or synthetic, that is capable, upon forming a duplex with a polynucleotide template, of acting as a point of initiation of nucleic acid synthesis and being extended from its 3' end along the template so that an extended duplex is formed. The sequence of nucleotides added during the extension

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process is determined by the sequence of the template polynucleotide. Usually primers are extended by a DNA polymerase. Primers are generally of a length compatible with its use in synthesis of primer extension products, and are usually are in the range of between 8 to 100 nucleotides in length, such as 10 to 75, 15 to 60, 15 to 40, 18 to 30, 20 to 40, 21 to 50, 22 to 45, 25 to 40, and so on, more typically in the range of between 18-40, 20-35, 21-30 nucleotides long, and any length between the stated ranges. Typical primers can be in the range of between 10-50 nucleotides long, such as 15-45, 18-40, 20-30, 21-25 and so on, and any length between the stated ranges. In some embodiments, the primers are usually not more than about 10, 12, 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, or 70 nucleotides in length.

Primers are usually single-stranded for maximum efficiency in amplification, but may alternatively be double-stranded. If double-stranded, the primer is usually first treated to separate its strands before being used to prepare extension products. This denaturation step is typically affected by heat, but may alternatively be carried out using alkali, followed by neutralization. Thus, a "primer" is complementary to a template, and complexes by hydrogen bonding or hybridization with the template to give a primer/template complex for initiation of synthesis by a polymerase, which is extended by the addition of covalently bonded bases linked at its 3' end complementary to the template in the process of DNA synthesis.

A "primer pair" as used herein refers to first and second primers having nucleic acid sequence suitable for nucleic acid-based amplification of a target nucleic acid. Such primer pairs generally include a first primer having a sequence that is the same or similar to that of a first portion of a target nucleic acid, and a second primer having a sequence that is complementary to a second portion of a target nucleic acid to provide for amplification of the target nucleic acid or a fragment thereof. Reference to "first" and "second" primers herein is arbitrary, unless specifically indicated otherwise. For example, the first primer can be designed as a "forward primer" (which initiates nucleic acid synthesis from a 5' end of the target nucleic acid) or as a "reverse primer" (which initiates nucleic acid synthesis from a 5' end of the extension product produced from synthesis initiated from the forward primer). Likewise, the second primer can be designed as a forward primer or a reverse primer.

"Readout" means a parameter, or parameters, which are measured and/or detected that can be converted to a number or value. In some contexts, readout may refer to an

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actual numerical representation of such collected or recorded data. For example, a readout of fluorescent intensity signals from a microarray is the address and fluorescence intensity of a signal being generated at each hybridization site of the microarray; thus, such a readout may be registered or stored in various ways, for example, as an image of the microarray, as a table of numbers, or the like.

"Reflex site", "reflex sequence" and equivalents are used to indicate sequences in a polynucleotide that are employed to move a domain intramolecularly from its initial location to a different location in the polynucleotide. The sequence of a reflex site can be added to a polynucleotide of interest (e.g., present in an adapter ligated to the polynucleotide), be based on a sequence naturally present within the polynucleotide of interest (e.g., a genomic sequence in the polynucleotide), or a combination of both. The reflex sequence is chosen so as to be distinct from other sequences in the polynucleotide (i.e., with little sequence homology to other sequences likely to be present in the polynucleotide, e.g., genomic or sub-genomic sequences to be processed). As such, a reflex sequence should be selected so as to not hybridize to any sequence except its complement under the conditions employed in the reflex processes herein described. As described later in this application, the complement to the reflex sequence is inserted on the same strand of the polynucleotide (e.g., the same strand of a double-stranded polynucleotide or on the same single stranded polynucleotide) in a particular location so as to facilitate an intramolecular binding event on such particular strand. Reflex sequences employed in the reflex process described herein can thus have a wide range of lengths and sequences. Reflex sequences may range from 5 to 200 nucleotide bases in length.

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

"Specific" or "specificity" in reference to the binding of one molecule to another molecule, such as a labeled target sequence for a probe, means the recognition, contact, and formation of a stable complex between the two molecules, together with substantially

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less recognition, contact, or complex formation of that molecule with other molecules. In one aspect, "specific" in reference to the binding of a first molecule to a second molecule means that to the extent the first molecule recognizes and forms a complex with another molecule in a reaction or sample, it forms the largest number of the complexes with the second molecule. Preferably, this largest number is at least fifty percent. Generally, molecules involved in a specific binding event have areas on their surfaces or in cavities giving rise to specific recognition between the molecules binding to each other. Examples of specific binding include antibody-antigen interactions, enzyme-substrate interactions, formation of duplexes or triplexes among polynucleotides and/or oligonucleotides, receptor-ligand interactions, and the like. As used herein, "contact" in reference to specificity or specific binding means two molecules are close enough that weak noncovalent chemical interactions, such as Van der Waal forces, hydrogen bonding, base-stacking interactions, ionic and hydrophobic interactions, and the like, dominate the interaction of the molecules.

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature (e.g., as measured in °C) at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are known in the art (see e.g., Anderson and Young, Quantitative Filter Hybridization, in Nucleic Acid Hybridization (1985). Other references (e.g., Allawi, H.T. & SantaLucia, J., Jr., Biochemistry 36, 10581-94 (1997)) include alternative methods of computation which take structural and environmental, as well as sequence characteristics into account for the calculation of T_m.

"Sample" means a quantity of material from a biological, environmental, medical, or patient source in which detection, measurement, or labeling of target nucleic acids is sought. On the one hand it is meant to include a specimen or culture (e.g., microbiological cultures). On the other hand, it is meant to include both biological and environmental samples. A sample may include a specimen of synthetic origin. Biological samples may be animal, including human, fluid, solid (e.g., stool) or tissue, as well as liquid and solid food and feed products and ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may include materials taken from a patient including, but not limited to cultures, blood, saliva, cerebral spinal fluid, pleural fluid, milk, lymph, sputum, semen, needle aspirates, and the like. Biological samples may be obtained from all of the various families of domestic animals, as well as feral or wild

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animals, including, but not limited to, such animals as ungulates, bear, fish, rodents, etc. Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The terms "upstream" and "downstream" in describing nucleic acid molecule orientation and/or polymerization are used herein as understood by one of skill in the art. As such, "downstream" generally means proceeding in the 5' to 3' direction, i.e., the direction in which a nucleotide polymerase normally extends a sequence, and "upstream" generally means the converse. For example, a first primer that hybridizes "upstream" of a second primer on the same target nucleic acid molecule is located on the 5' side of the second primer (and thus nucleic acid polymerization from the first primer proceeds towards the second primer).

It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or the use of a "negative" limitation.

DETAILED DESCRIPTION OF THE INVENTION

The invention is drawn to compositions and methods for intramolecular nucleic acid rearrangement that find use in various applications of genetic analysis, including sequencing, as well as general molecular biological manipulations of polynucleotide structures.

Before the present invention is described, it is to be understood that this invention is not limited to particular embodiments described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any

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other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

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. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a nucleic acid" includes a plurality of such nucleic acids and reference to "the compound" includes reference to one or more compounds and equivalents thereof known to those skilled in the art, and so forth.

The practice of the present invention may employ, unless otherwise indicated, conventional techniques and descriptions of organic chemistry, polymer technology, molecular biology (including recombinant techniques), cell biology, biochemistry, and immunology, which are within the skill of the art. Such conventional techniques include polymer array synthesis, hybridization, ligation, and detection of hybridization using a label. Specific illustrations of suitable techniques can be had by reference to the example herein below. However, other equivalent conventional procedures can, of course, also be used. Such conventional techniques and descriptions can be found in standard laboratory manuals such as *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV), *Using Antibodies: A Laboratory Manual, Cells: A Laboratory Manual, PCR Primer: A Laboratory Manual, and Molecular Cloning: A Laboratory Manual* (all from Cold Spring Harbor Laboratory Press), Stryer, L. (1995) *Biochemistry* (4th Ed.) Freeman, New York,

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Gait, "*Oligonucleotide Synthesis: A Practical Approach*" 1984, IRL Press, London, Nelson and Cox (2000), Lehninger, A., *Principles of Biochemistry* 3rd Ed., W. H. Freeman Pub., New York, N.Y. and Berg et al. (2002) *Biochemistry,* 5th Ed., W. H. Freeman Pub., New York, N.Y., all of which are herein incorporated in their entirety by reference for all purposes.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

As summarized above, aspects of the present invention are drawn to the use of a 'reflex' sequence present in a polynucleotide (e.g., in an adapter structure of the polynucleotide, in a genomic region of the polynucleotide, or a combination of both) to move a domain of the polynucleotide intra-molecularly from a first location to a second location. The reflex process described herein finds use in any number of applications, e.g., placing functional elements of a polynucleotide (e.g., sequencing primer binding sites and/or MID tags) into proximity to a desired sub-region of interest.

Nucleic Acids

The reflex process (as described in detail below) can be employed for the manipulation and analysis of nucleic acid sequences of interest from virtually any nucleic acid source, including but not limited to genomic DNA, complementary DNA (cDNA), RNA (e.g., messenger RNA, ribosomal RNA, short interfering RNA, microRNA, etc.), plasmid DNA, mitochondrial DNA, etc. Furthermore, as any organism can be used as a source of nucleic acids to be processed in accordance with the present invention, no limitation in that regard is intended. Exemplary organisms include, but are not limited to, plants, animals (e.g., reptiles, mammals, insects, worms, fish, etc.), bacteria, fungi (e.g., yeast), viruses, etc. In certain embodiments, the nucleic acids in the nucleic acid sample are derived from a mammal, where in certain embodiments the mammal is a human.

In certain embodiments, the nucleic acid sequences are enriched prior to the reflex sequence process. By enriched is meant that the nucleic acid is subjected to a process that reduces the complexity of the nucleic acids, generally by increasing the relative

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concentration of particular nucleic acid species in the sample (e.g., having a specific locus of interest, including a specific nucleic acid sequence, lacking a locus or sequence, being within a specific size range, etc.). There are a wide variety of ways to enrich nucleic acids having a specific characteristic(s) or sequence, and as such any convenient method to accomplish this may be employed. The enrichment (or complexity reduction) can take place at any of a number of steps in the process, and will be determined by the desires of the user. For example, enrichment can take place in individual parental samples (e.g., untagged nucleic acids prior to adaptor ligation) or in multiplexed samples (e.g., nucleic acids tagged with primer binding sites, MID and/or reflex sequences and pooled; MID are described in further detail below).

In certain embodiments, nucleic acids in the nucleic acid sample are amplified prior to analysis. In certain of these embodiments, the amplification reaction also serves to enrich a starting nucleic acid sample for a sequence or locus of interest. For example, a starting nucleic acid sample can be subjected to a polymerase chain reaction (PCR) that amplifies one or more region of interest. In certain embodiments, the amplification reaction is an exponential amplification reaction, whereas in certain other embodiments, the amplification reaction is a linear amplification reaction. Any convenient method for performing amplification reactions on a starting nucleic acid sample can be used in practicing the subject invention. In certain embodiments, the nucleic acid polymerase employed in the amplification reaction is a polymerase that has proofreading capability (e.g., phi29 DNA Polymerase, Thermococcus litoralis DNA polymerase, Pyrococcus furiosus DNA polymerase, etc.).

In certain embodiments, the nucleic acid sample being analyzed is derived from a single source (e.g., a single organism, virus, tissue, cell, subject, etc.), whereas in other embodiments, the nucleic acid sample is a pool of nucleic acids extracted from a plurality of sources (e.g., a pool of nucleic acids from a plurality of organisms, tissues, cells, subjects, etc.), where by "plurality" is meant two or more. As such, in certain embodiments, a nucleic acid sample can contain nucleic acids from 2 or more sources, 3 or more sources, 5 or more sources, 10 or more sources, 50 or more sources, 100 or more sources, 500 or more sources, 1000 or more sources, 5000 or more sources, up to and including about 10,000 or more sources.

In certain embodiments, nucleic acid fragments that are to be pooled with nucleic acid fragments derived from a plurality of sources (e.g., a plurality of organisms, tissues,

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cells, subjects, etc.), where by "plurality" is meant two or more. In such embodiments, the nucleic acids derived from each source includes a multiplex identifier (MID) such that the source from which the each tagged nucleic acid fragment was derived can be determined. In such embodiments, each nucleic acid sample source is correlated with a unique MID, where by unique MID is meant that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID. Any type of MID can be used, including but not limited to those described in co-pending U.S. Patent Application Serial Number 11/656,746, filed on January 22, 2007, and titled "Nucleic Acid Analysis Using Sequence Tokens", as well as U.S. Patent 7,393,665, issued on July 1, 2008, and titled "Methods and Compositions for Tagging and Identifying Polynucleotides", both of which are incorporated herein by reference in their entirety for their description of nucleic acid tags and their use in identifying polynucleotides. In certain embodiments, a set of MIDs employed to tag a plurality of samples need not have any particular common property (e.g., T_m, length, base composition, etc.), as the asymmetric tagging methods (and many tag readout methods, including but not limited to sequencing of the tag or measuring the length of the tag) can accommodate a wide variety of unique MID sets.

In certain embodiments, each individual polynucleotide (e.g., double-stranded or single-stranded, as appropriate to the methodological details employed) in a sample to be analyzed is tagged with a unique MID so that the fate of each polynucleotide can be tracked in subsequent processes (where, as noted above, unique MID is meant to indicate that each different MID employed can be differentiated from every other MID employed by virtue of at least one characteristic, e.g., the nucleic acid sequence of the MID). For example (and as described below), having each nucleic acid tagged with a unique MID allows analysis of the sequence of each individual nucleic acid using the reflex sequence methods described herein. This allows the linkage of sequence information for large nucleic acid fragments that cannot be sequenced in a single sequencing run.

Reflex Sequence Process

As summarized above, aspects of the present invention include methods and compositions for moving a domain in a polynucleotide from a first location to a second location in the polynucleotide. An exemplary embodiment is shown in Figure 1A.

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Figure 1A shows a single stranded polynucleotide 100 comprising, in a 5' to 3' orientation, a first domain (102; the domain to be moved); a reflex sequence 104; a nucleic acid sequence 106 having a site distal to the first domain (Site A), and a complement of the reflex sequence 108 (positioned at the 3' terminus of the polynucleotide). The steps of the reflex method described below will move the first domain into closer proximity to Site A. It is noted here that the prime designation in Figure 1A denotes a complementary sequence of a domain. For example, First Domain' is the complement of the First Domain.

In Step 1, the reflex sequence and its complement in the polynucleotide are annealed intramolecularly to form structure 112, with the polynucleotide folding back on itself. In this configuration, the 3' end of the complement of the reflex sequence can serve as a nucleic acid synthesis priming site. Nucleic acid synthesis from this site is then performed in extension Step 2 producing a complement of the first domain at the 3' end of the nucleic acid extension (shown in structure114; extension is indicated with dotted arrow labeled "extend").

Denaturation of polynucleotide 114 (e.g., by heat) generates linear single stranded polynucleotide 116. As shown in Figure 1, resultant structure 116 contains a complement of the first domain at a position proximal to Site A (i.e., separated by only the complement of the reflex sequence). This resultant polynucleotide may be used for any subsequent analysis or processing steps as desired by the user (e.g., sequencing, as a template for amplification (linear, PCR, etc.), sequence specific extraction, etc.).

In certain embodiments, the first domain and reflex sequence are removed from the 5' end of the double-stranded region of polynucleotide 114 (shown in structure 118; removal is shown in the dotted arrow labeled "remove"). Removal of this region may be accomplished by any convenient method, including, but not limited to, treatment (under appropriate incubation conditions) of polynucleotide structure 114 with T7 exonuclease or by treatment with Lambda exonuclease; the Lambda exonuclease can be employed so long as the 5' end of the polynucleotide is phosphorylated.

In certain embodiments, polynucleotide 116 or 118 is used as a template to produce a double stranded polynucleotide, for example by performing a nucleic acid synthesis reaction with a primer that primes in the complement of the first domain. This step is sometimes referred to as copying to reverse polarity of a single stranded polynucleotide, and in some instances, the double-stranded intermediate product of this

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copying is not shown (see, e.g., Figure 2). For example, copying to reverse the polarity of polynucleotide 116 results in single-stranded polynucleotide 120 having, in a 5' to 3' orientation, the first domain (122); the reflex sequence (124); the complement of polynucleotide 106 (oriented with the complement of Site A (Site A'; 126) proximal to the reflex sequence); the complement of the reflex sequence (128); and the complement of the first domain (130).

In certain embodiments, the first domain in the polynucleotide comprises one or more elements that find use in one or more subsequent processing or analysis steps. Such sequences include, but are not limited to, restriction enzyme sites, PCR primer binding sites, linear amplification primer sites, reverse transcription primer sites, RNA polymerase promoter sites (such as for T7, T3 or SP6 RNA polymerase), MID tags, sequencing primer sites, etc. Any convenient element can be included in the first domain and, in certain embodiments, is determined by the desires of the user of the methods described herein.

As an exemplary embodiment, suppose we want to sequence a specific polynucleotide region from multiple genomes in a pooled sample where the polynucleotide region is too long to sequence in a single reaction. For example, sequencing a polynucleotide region that is 2 kilobases or more in length using Roche 454 (Branford, CT) technology, in which the length of a single sequencing run is about 400 bases. In this scenario, we can design a set of left hand primers (A_n) and right hand primers (B_n) specific for the polynucleotide region that overlap in such a way that we can obtain direct sequences of all parts of the insert, as shown in Figure 1B. Note that the polynucleotide shown in Figure 1B (140) has a domain (142) containing a primer binding site and an MID denoting from which original sample the polynucleotide is derived. The polynucleotide also includes a reflex site (144), which can be part of the polynucleotide region itself (e.g., a genomic sequence), added in a ligated adapter domain along with the primer binding site and the MID (an artificial sequence), or a combination of both (a sequence spanning the adapter/polynucleotide junction). Note further that polynucleotide 140 does not include a 3' reflex sequence complementary to the reflex site; this will be added as detailed below (using a B_n primer).

Each A_n-B_n primer pair defines a nucleic acid region that is less than approximately 400 bases in length, which is below the sequencing run length of the current Roche 454 sequencing platform. Thus, each product from each reflex process can

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be sequenced in a single run. It is noted here that primer pairs as shown in Figure 1B can be used to define regions 1 to 5 shown in Figure 2 (described in further detail below).

In certain embodiments, to obtain the first part of the sequence of the polynucleotide region (i.e., in the original structure, that part of the polynucleotide closest to the first domain), we only need a right hand primer (e.g., B_0) and we do not need to transfer the MID as it is within reach of this sequencing primer (i.e., the MID is within 400 bases of sequencing primer B_0). All other B_n primers have the reflex sequence added to their 5' ends ("R" element shown on B primers) so that they read 5' reflex-B_n. However, in certain embodiments, the B_0 primer does include the reflex sequence and is used in the reflex process (along with a corresponding A_0 primer) as detailed below.

As described above, we obtain a single stranded polynucleotide having, in the 5' to 3' orientation, a primer binding site (e.g., for Roche 454 sequencing), a MID, a reflex sequence and the polynucleotide to be sequenced. Numerous methods for obtaining single-stranded polynucleotides of interest have been described and are known in the art, including in U.S. Patent 7,217,522, issued on May 15, 2007; U.S. Patent Application 11/377,462, filed on March 16, 2006; and U.S. Patent Application 12/432,080, filed on April 29, 2009; each of which is incorporated by reference herein in their entirety. For example, a single stranded product can be produced using linear amplification with a primer specific for the primer binding site of the template. In certain embodiments, the primer includes a binding moiety to facilitate isolation of the single stranded nucleic acid of interest, e.g., to immobilize the top strand on a binding partner of the binding moiety immobilized on a solid support. Removal of a hybridized, non-biotinylated strand by denaturation using heat or high pH (or any other convenient method) serves to isolate the biotinylated strand. Binding moieties and their corresponding binding partners are sometimes referred to herein as binding partner pairs. Any convenient binding partner pairs may be used, including but not limited to biotin/avidin (or streptavidin), antigen/antibody pairs, etc.

It is noted here that while the figures and description of the reflex process provided herein depict manipulations with regard to a single stranded polynucleotide, it is not necessarily required that the single stranded polynucleotide described or depicted in the figures be present in the sample in an isolated form (i.e., isolated from its complementary strand). In other words, double stranded polynucleotides may be used where only one strand is described/depicted, which will generally be determined by the user.

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The implementation of a single strand isolation step using the methods described above or variations thereof (or any other convenient single strand isolation step) will generally be based on the desires of the user. One example of isolating single stranded polynucleotides is shown in Figure 6. In this Figure, a starting double stranded template (with 5' to 3' orientation shown as an arrow) is denatured and primed with a biotinylated synthesis primer specific for the primer site. After extension of the primer (i.e., nucleic acid synthesis), the sample is contacted with a solid support having avidin bound to it. The biotin moiety (i.e., the binding partner of avidin) on the extended strands will bind to the solid-phase avidin. Denaturation and washing is then performed to remove all non-biotinylated polynucleotide strands. If desired, the bound polynucleotide, which can be used in subsequent reflex process steps (e.g., as a template for Bn primer extension reactions), may be eluted from the avidin support. Alternatively, the bound polynucleotide may be employed in subsequent steps of the desired process while still bound to the solid support (e.g., in solid phase extension reactions using Bn primers). This process, with minor variations depending on the template being used and the identity of the desired single stranded polynucleotide, may be employed at any of a number of steps in which a single stranded product is to be isolated. It is noted that in certain embodiments, substrate bound biotinylated polynucleotide can be used to produce and isolate non-biotinylated single stranded products (i.e., by eluting the non-biotinylated products while leaving the biotinylated templates bound to the avidin on the solid support). Thus, the specifics of how binding partners are used to isolate single stranded polynucleotides of interest will vary depending on experimental design parameters.

Additional single-stranded isolation/production methods include asymmetric PCR and the use of in-vitro transcription followed by reverse transcriptase (IVT-RT) with subsequent destruction of the RNA strand. As noted above, any convenient single stranded production/isolation method may be employed.

To the single stranded polynucleotide shown in Figure 1B we anneal one of the B_n primers having the appended reflex sequence, denoted with a capital "R" (e.g., B_1) and extend the primer under nucleic acid synthesis conditions to produce a copy of the polynucleotide that has a reflex sequence at its 5' end. A single stranded copy of this polynucleotide is then produced to reverse polarity using a primer specific for the primer binding site in the domain 118. The resulting nucleic acid has structure 100 shown in

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Figure 1A, where the first domain 102 includes the primer binding site and the MID. Site A (110) in Figure 1 is determined by the specificity of the 5' reflex-Bn primer used.

The reflex process (e.g., as shown in Figure 1) is then performed to produce a product in which the primer binding site and the MID are now in close proximity to the desired site within the original polynucleotide (i.e., the site defined by the primer used, e.g., B1). The resulting polynucleotide can be used in subsequent analyses as desired by the user (e.g., Roche 454 sequencing technology).

It is noted here that, while not shown in Figure 1A and 1B, any convenient method for adding adapters to a polynucleotide to be processed as described herein may be used in the practice of the reflex process (adapters containing, e.g., primer binding sites, polymerase sites, MIDs, restriction enzyme sites, and reflex sequences). For example, adapters can be added at a particular position by ligation. For double stranded polynucleotides, an adapter can be configured to be ligated to a particular restriction enzyme cut site. Where a single stranded polynucleotide is employed, a double stranded adapter construct that possesses an overhang configured to bind to the end of the single-stranded polynucleotide can be used. For example, in the latter case, the end of a single stranded polynucleotide can be modified to include specific nucleotide bases that are complementary to the overhang in the double stranded adaptor using terminal transferase and specific nucleotides. In other embodiments, PCR or linear amplification methods using adapter-conjugated primers is employed to add an adapter at a site of interest. Again, any convenient method for producing a starting polynucleotide may be employed in practicing the methods of the subject invention.

In certain embodiments, the nucleic acid may be sequenced directly using a sequencing primer specific for the primer binding site. This sequencing reaction will read through the MID and desired site in the insert.

In certain embodiments, the polynucleotide may be isolated (or fractionated) using an appropriate A_n primer (e.g., when using B_1 as the first primer, primer A_1 can be used). In certain embodiments, the A_n primed polynucleotide is subjected to nucleic acid synthesis conditions to produce a copy of the fragment produced in the reflex process. In certain of these embodiments, the A_n primer has appended on its 5' end a primer binding site that can be used in subsequent steps, including sequencing reactions. Providing a primer binding site in the A_n primer allows amplifying and/or sequencing from both ends of the resultant fragment: from the primer site in the first domain 118 and the primer site

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in the A_n primer (not shown in Figure 1B). Because of the position of the primer binding sites and their distance apart (i.e., less than one sequencing run apart), sequencing from both ends will usually capture the sequence of the desired site and the sequence of the MID, which can be used for subsequent bioinformatic analyses, e.g., to positively identify the sample of origin. It is noted here that while sequencing in both directions is possible, it is not necessary, as sequencing from either primer site alone will capture the sequence of interest of a polynucleotide as well as its corresponding MID sequence.

Note that in certain embodiments, the first fragment which is obtained by B_0 directly, the polarity of the sequence is reversed as there has been no transfer. In this case B_0 may have appended to it a primer binding site (e.g., at its 5' end) that can be used for subsequent amplification and/or sequencing reactions (e.g., in Roche 454 sequencing system). However, in certain embodiments, as noted above, the reflex process may be used with a corresponding B_0-A_0 primer pair as described above, i.e., using a B_0 primer having a 5' reflex sequence and a corresponding A_0 primer with its corresponding 5' adapter domain (e.g., a primer binding site).

It is noted here that because the particular sections of sequence to be analyzed are defined by the A_n-B_n primer pairs (as shown and described above), a much higher sequence specificity is achieved as compared to using previous extraction methods that employ only a single oligo binding event (e.g., using probes on a microarray).

Figure 2 provides a detailed flow chart for an exemplary embodiment that employs reflex sequences for use in sequencing multiple specific regions in a polynucleotide (i.e., regions 1, 2, 3, 4 and 5 in an 11kb region of lambda DNA).

A single parent DNA fragment 202 is generated that includes adapter domains (i.e., a Roche 454 sequencing primer site, a single MID, and a reflex sequence) and the sequence of interest. In the example shown, the sequence of interest is from lambda DNA and the reflex sequence is present on the top strand (with its complement shown in the bottom strand). Any convenient method for producing this parent DNA fragment may be used, including amplification with a primer that includes the adapter domains (e.g., using PCR) or by cloning the fragment into a vector that includes the adapter domains (e.g., a vector with the adapter domains adjacent to a cloning site). While only a single fragment with a single MID is shown, the steps in Figure 2 are applicable to samples having multiple different fragments each with a different MID, e.g., a sample having a population of homologous fragments from any number of different sources (e.g., different

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individuals). Figure 2 describes the subsequent enzymatic steps involved in creating the five daughter fragments in which regions 1, 2, 3, 4 and 5 (shown in structure 204) are rearranged to be placed within a functional distance of the adapter domains (i.e., close enough to the adapter domains to be sequenced in a single Roche 454 sequencing reaction). Note that certain steps are shown for region 4 only (206).

In step 1, the regions of interest are defined within the parent fragment (see 204) and corresponding primer pairs are designed for each (A_n-B_n primers as shown and described in Figure 1B). In step 2, sequence specific primer extensions are performed (only region 4 is shown) with corresponding B_n primers to produce single stranded polynucleotides having structure 208 (i.e., having the reflex sequence on the 5' terminus). Note that the B_n primer for region 4 will include a sequence specific primer binding site that primes at the 3'-most end of region 4 (where "3'-most" refers to the template strand). As such, the indication of regions 1 to 5 in Figure 2 is showing the entire sub-region of interest, not merely the corresponding B_n primer binding site. This structure is copied back to produce structure 210 having reversed polarity (e.g., with a primer that lands in the 454A' domain). Polynucleotide 210 has structure similar to structure 100 shown at the top of Figure 1. Step 4 depicts the result of the intramolecular priming between the reflex sequence and its complement followed by extension to produce the MID' and 454A' structures at the 3' end (structure 212). In the embodiments shown in Figure 2, structure 212 is treated with T7 exonuclease to remove double stranded DNA from the 5' end (as indicated above, this step is optional). The structure formed for region 4 is shown as 216 with structures for the other regions also shown (214).

It is noted here that the formation of each of the structures 214 may be accomplished either in separate reactions (i.e., structure with region 1 in proximity to the adapter domains is in a first sample, the structure with region 2 in proximity to the adapter region is in a second sample, etc.) or in one or more combined sample.

In step 6 the polynucleotides 214 are copied to reverse polarity to form polynucleotides 218. In step 7, each of these products are then primed with the second primer of the specific primer pair (see A_n primers as shown in Figure 1B) each having a second Roche 454 primer site (454B) and extended to form products 220.

Figure 2 shows that the reflex process produces five daughter fragments 220 of defined length (e.g., ~500 bp) defined by DNA sequences that differ in their distance from the reflex sequence in the starting structure 202 while maintaining the original MID.

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The reflex process described herein can be used to perform powerful linkage analysis by combining it with nucleic acid counting methods. Any convenient method for tagging and /or counting individual nucleic acid molecules with unique tags may be employed (see, e.g., U.S. Patent 7,537,897, issued on May 26, 2009; U.S. Patent 7,217,522, issued on May 15, 2007; U.S. Patent Application 11/377,462, filed on March 16, 2006; and U.S. Patent Application 12/432,080, filed on April 29, 2009; each of which is incorporated by reference herein in their). All of this can be conducted in parallel thus saving on the cost of labor, time and materials.

In one exemplary embodiment, a large collection of sequences is tagged with MID such that each polynucleotide molecule in the sample has a unique MID. In other words, each polynucleotide in the sample (e.g., each individual double stranded or single stranded polynucleotide) is tagged with a MID that is different from every other MID on every other polynucleotide in the sample. In general, to accomplish such molecular tagging the number of distinct MID tags to be used should be many times greater than the actual number of molecules to be analyzed. This will result in each individual nucleic acid molecule being labeled with a unique ID tag. Any sequences that then result from the reflex process on that particular molecule (e.g., as described above) will thus be labeled with the same unique MID tag and thus inherently linked. Note that once all molecules in a sample are individually tagged, they can be manipulated and amplified as much as needed for processing so long as the MID tag is maintained in the products generated.

For example, we might want to sequence one thousand viral genomes or one thousand copies of a gene present in somatic cells. After tagging each polynucleotide in the sample with a sequencing primer site, MID and reflex sequence (as shown in the figures and described above), we use the reflex process to break each polynucleotide into lengths appropriate to the sequencing procedure being used, transferring the sequencing primer site and MID to each fragment (as described above). Obtaining sequence information from all of the reflex-processed samples can be used to determine the sequence of each individual polynucleotide in the starting sample, using the MID sequence to defining linkage relationships between sequences from different regions in the polynucleotide being sequenced. Using a sequencing platform with longer read lengths can minimize the number of primers to be used (and reflex fragments generated).

The advantages noted above are shown in Figure 5. This figure shows a comparison of methods for identifying nucleic acid polymorphisms in homologous nucleic

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acids in a sample (e.g., the same region derived from a chromosomal pair of a diploid cell or viral genomes/transcripts). The top schematic shows two nucleic acid molecules in a sample (1 and 2) having a different assortment of polymorphisms in polymorphic sites A, B and C (A1, B1, C1 and C2). Standard sequencing methods using fragmentation (left side) can identify the polymorphisms in these nucleic acids but do not retain linkage information. Employing the reflex process described herein to identify polymorphisms (right side) maintains linkage information. It is noted that not all domain structures and steps are shown in the reflex process for simplicity.

Kits and Systems

Also provided by the subject invention are kits and systems for practicing the subject methods, as described above, such vectors configured to add reflex sequences to nucleic acid inserts of interest and regents for performing any steps in the cloning or reflex process described herein (e.g., restriction enzymes, nucleotides, polymerases, primers, exonucleases, etc.). The various components of the kits may be present in separate containers or certain compatible components may be precombined into a single container, as desired.

The subject systems and kits may also include one or more other reagents for preparing or processing a nucleic acid sample according to the subject methods. The reagents may include one or more matrices, solvents, sample preparation reagents, buffers, desalting reagents, enzymatic reagents, denaturing reagents, where calibration standards such as positive and negative controls may be provided as well. As such, the kits may include one or more containers such as vials or bottles, with each container containing a separate component for carrying out a sample processing or preparing step and/or for carrying out one or more steps of a nucleic acid variant isolation assay according to the present invention.

In addition to above-mentioned components, the subject kits typically further include instructions for using the components of the kit to practice the subject methods, e.g., to prepare nucleic acid samples for perform the reflex process according to aspects of the subject methods. The instructions for practicing the subject methods are generally recorded on a suitable recording medium. For example, the instructions may be printed on a substrate, such as paper or plastic, etc. As such, the instructions may be present in the kits as a package insert, in the labeling of the container of the kit or components thereof

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(i.e., associated with the packaging or sub-packaging) etc. In other embodiments, the instructions are present as an electronic storage data file present on a suitable computer readable storage medium, e.g. CD-ROM, diskette, etc. In yet other embodiments, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source, e.g. via the internet, are provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions is recorded on a suitable substrate.

In addition to the subject database, programming and instructions, the kits may also include one or more control samples and reagents, e.g., two or more control samples for use in testing the kit.

Utility

The reflex process described herein provides significant advantages in numerous applications, a few of which are noted below (as well as described above

For example, as described above, certain aspects of the reflex process define the particular sections of sequence to be analyzed by a primer pair, as in PCR (e.g., the two oligos shown as An-Bn in Figure 1B). This results in a higher sequence specificity as compared to other extraction methods (e.g., using probes on a microarray) that only use a single oligo sequence. The separation of the probes defines a length that can be relatively uniform (hence making subsequent handling including amplification more uniform) and can also be tailored to the particular sequencing platform being employed.

Further, as described above, aspects of the present invention can be used to analyze homologous genomic locations in a multiplexed sample (i.e., a sample having polynucleotides from different genomic samples) in which the polynucleotides are tagged with the MID. This is possible because the reflex process, which operates intramolecularly, maintains the MID thus linking any particular fragment to the sample from which it originates.

Finally, as the reflex processes described herein functions intramolecularly, one can determine the genetic linkage between different regions on the same large fragment that are too far apart to be sequenced in one sequence read. Such a determination of linkage may be of great value in plant or animal genetics (e.g., to decide if a particular set of variations are linked together on the same stretch of chromosome) or in viral studies

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(e.g., to determine if particular variations are linked together on the same stretch of a viral genome/transcripts, e.g., HIV, hepatitis virus, etc.).

EXAMPLES

Example I

Figures 3 and 4 provide experimental data and validation of the reflex process described herein using synthetic polynucleotide substrates.

METHODS

Substrate:

The 100 base oligonucleotide substrate (as shown diagramatically in Figure 3A) was synthesized with internal fluorescein-dT positioned between the reflex sequences. This label provides convenient and sensitive method of detection of oligonucleotide species using polyacrylamide gel electrophoresis.

Extension reactions:

Reactions were prepared which contained 1 μM of the 100 base oligonucleotide substrate, 200 μM dNTPs, + or – 1 μM competitor oligonucleotide, 0.5 μl of each polymerase: Vent (NEB), Taq (Qiagen) and Herculase (Stratagene), and made up to 50 μl with the appropriate commercial buffers for each polymerase and dH_20. For Taq titrations 0.5 μl, 1 μl, 2 μl, and 3 μl enzyme was used in the same 50 μl volume. Reactions were heated in a Biometra thermocycler to 95 °C for 15 minutes (Taq) or 5 minutes (Herculase, Vent), followed by 55 °C or 50 °C for 30 seconds, and a final incubation at 72 °C for 10 minutes.

T7 exonuclease digestions:

Reactions were prepared with 10 μl extension reactions above, 0.5 μl T7 exonuclease (NEB), and made up to 50 μl using NEB buffer 4 and dH_20. Reactions were incubated at 25 °C for 30 minutes.

Gel Electrophoresis analysis:

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An 8% denaturing polyacrylamide gel was used to analyze reaction species. 0.4 µl of extension reactions, and 2 µl of digestion reactions were loaded and ran at 800V for ~1.5 hours. Gels were analyzed for fluorescein using a Typhoon imager.

RESULTS

Figure 3A shows the structure of each stage of reflex sequence processing with the expected nucleic acid size shown on the left. The initial single stranded nucleic acid having a sequencing primer site (Roche 454 sequencing primer binding site A; listed as 454A); a MID; insert and a complement of the reflex sequence is 100 nucleotides in length. After self annealing and extension, the product is expected to be 130 nucleotides in length. After removal of the double stranded region from the 5' end, the nucleic acid is expected to be 82 bases in length.

Figure 3B shows the results of three experiments using three different nucleic acid polymerases (Vent, Herculase and Taq, indicated at the top of the lanes). The temperature at which the reactions were carried out is shown at the top of each lane (either 50°C or 55°C). The sizes of the three nucleic acids as noted above are indicated on the left and right side of the gel.

As shown in figure 3B, extension appears to be most efficient under the conditions used with Herculase (Herculase is a mixture of two enzymes: modified Pfu DNAP and Archaemax (dUTPase)). Most (or all) of the initial 100 base pair nucleic acid are converted to the 130 base pair product (see lanes 6 and 7). However, 3'-5' exonuclease activity of Herculase results in partial digestion of the desired 82 base product (note bands at and below the 82 base pairs in lanes 8 and 9).

Taq, which lacks 3'-5' exonuclease activity, shows a stronger band at the expected size of the final product (see lane 13).

Figure 4 shows the effect on the reflex process of increasing amounts of Taq polymerase as well as the use of a reflex sequence competitor (schematically shown in Figure 4A).

As shown in lanes 2 to 5, increased Taq concentration improves extension to ~90% conversion of the starting nucleic acid (see lane 5). Lanes 7 to 8 show that T7 exonuclease digestion does not leave a perfect 82 base product. This may be due to collapse of dsDNA when T7 exonuclease has nearly completed its digestion from the 5' end in the double stranded region of the fold-back structure. It is noted that in many

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embodiments, the removal of a few additional bases from the 5' end of the polynucleotide will not interfere with subsequent analyses, as nucleotide bases at the 5' end are often removed during subsequent steps.

As shown in Lanes 11-14, addition of a competitor (which can interfere with annealing of the reflex sequences to form the fold-back structure) results in a small decrease (~5-10%) of fully extended product. Although not shown, we have observed that the competitor oligonucleotide also gets extended by the same amount (5-10%). The concentration of the competitor, and the overall genetic complexity, will likely affect this. The experiments shown in Figures 3 and 4 demonstrate that the reflex processes as described herein is functional and can be implemented.

Example II

Figure 7 shows the reflex workflow (diagram at left) and exemplary results of the workflow (gel at right) for a specific region of interest (ROI). The starting material is a double stranded nucleic acid molecule (700) that contains a 454A primer site, an MID, a reflex site, and a polynucleotide of interest having 3 ROIs (1, 2 and 3) at different locations therein. This starting material was subjected to reflex processes specific for ROI 2 as shown in the diagram at the left of the figure, both with and without the use of a T7 exonuclease step (the T7 exonuclease step is shown in the diagram is indicated as "Optional").

Completion of all steps shown in the reflex process should result in a double stranded polynucleotide of 488 base pairs (702) with or without the T7 exonuclease step.

As shown in the gel on the right of Figure 7, the 488 base pair produce was produced in reflex processes with and without the T7 exonuclease step.

Figure 8 shows an exemplary protocol for a reflex process based on the results discussed above. The diagram shows specific reflex process steps with indications on the right as to where purification of reaction products is employed (e.g., using Agencourt beads to remove primer oligos). As indicated, three purification steps are indicated. Of note, the steps of reversing polarity, reflex priming and extension, and "stretch out" (or denaturation) / second reversing polarity step can be performed without intervening purification steps.

The protocol shown in Figure 8 includes the following steps:

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annealing a first primer containing the 5' reflex sequence (or reflex tail, as noted in the figure) specific for the R' region to the starting polynucleotide and extending (first denature, anneal and extend process indicated on the right);

after purification, adding a 454A primer and performing three cycles of denaturing, annealing and extending: the first cycle results in the copy-back from the 454A primer to reverse the polarity of the strand just synthesized; the second cycle breaks apart the double stranded structure produced, allows the reflex structure to form and then extend; the third cycle results in another copy-back using the same 454A primer originally added;

after purification, adding a second primer specific for the R' region having a 5' 454B tail and denaturing, annealing and extending resulting in a polynucleotide product having 454A and 454B sites surrounding the MID, the reflex sequence, and R' (the first primer specific for the R' region and the second primer specific for the R' region define its boundaries, as described above and depicted in Figure 1B);

after another purification, adding 454A and 454B primers and performing a PCR amplification reaction.

Example III

As described above, a reflex sequence can be an "artificial" sequence added to a polynucleotide as part of an adapter or can be based on a sequence present in the polynucleotide of interest being analyzed, e.g., a genomic sequence (or "non-artificial").

The data shown in prior Examples used "artificial" reflex sites. In this Example, the reflex site is a genomic sequence present in the polynucleotide being analyzed.

The starting material is a double stranded DNA containing a 454A site, an MID and a polynucleotide to be analyzed. The 454A and MID were added by adapter ligation to parent polynucleotide fragments followed by enrichment of the polynucleotide to be analyzed by a hybridization-based pull-out reaction and subsequent secondary PCR amplification (see Route 1 in Figure 10). Thus, the reflex site employed in this example is a sequence normally present at the 5' end of the subject polynucleotide (a genomic sequence). The polynucleotide being analyzed includes a region of interest distal to the 454A and MID sequences that is 354 base pairs in length.

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This starting double stranded nucleic acid is 755 base pairs in length. Based on the length of each of the relevant domains in this starting nucleic acid, the reflex process should result in a product of 461 base pairs.

Figure 9 shows the starting material for the reflex process (left panel) and the resultant product generated using the reflex process (right panel; reflex process was performed as described in Example II, without using a T7 exonuclease step). This figure shows that the 755 base pair starting nucleic acid was processed to the expected 461 base pair product, thus confirming that a "non-artificial" reflex site is effective in moving an adapter domain from one location to another in a polynucleotide of interest in a sequence specific manner.

Figure 10 shows a diagram of exemplary protocols for performing the reflex process on pools of nucleic acids, for example, pools of nucleic acids from different individuals, each of which are labeled with a unique MID. In Route 3, a pooled and tagged extended library is subjected directly to a reflex process. In Route 2, the pooled library is enriched by target-specific hybridization followed by amplification prior to performing the reflex process. In Route 1, enriched/amplified sample in Route 2 is subjected to a secondary PCR reaction followed by the reflex protocol. The choice of protocol will be dependent on the desires of the user.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof,

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are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims.

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CLAIMS

That which is claimed is:

1. A method of moving a domain in a nucleic acid, the method comprising:

a) annealing a reflex sequence in a polynucleotide strand in a sample with a complement of the reflex sequence present in the the polynucleotide strand, wherein the polynucleotide strand comprises the following in a 5' to 3' orientation:

a first domain;

the reflex sequence;

a polynucleotide of interest; and

the complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand; and

b) extending from the 3' end of the complement of the reflex sequence through the first domain to produce a double stranded region;

whereby a complement of the first domain is present at the 3' end of the polynucleotide strand, thereby moving the first domain in the polynucleotide strand.

2. The method of claim 1, wherein the first domain comprises a multiplex identifier (MID).

3. The method of claim 1, wherein the first domain comprises a primer binding site.

4. The method of claim 1, wherein the reflex sequence is from 10 to 50 nucleotides in length.

5. The method of claim 1, wherein the reflex sequence comprises an RNA polymerase binding site.

6. The method of claim 1, the method further comprising removing the first domain and the reflex sequence from the 5' end of the double stranded region.

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7. The method of claim 6, wherein the removing step comprises treating the nucleic acid of step b with T7 exonuclease.

8. The method of claim 1, wherein the first domain is present in an adapter region attached to the polynucleotide of interest.

9. The method of claim 9, wherein the reflex sequence is present in the adapter region.

10. The method of claim 9, wherein the reflex sequence is a sequence present in the 5' region of the polynucleotide of interest.

11. The method of claim 1, wherein the sample is a multiplexed sample, wherein the first domain in each of the polynucleotide strands in the multiplexed sample comprises a MID corresponding to its parental sample.

12. The method of claim 1, wherein the sample comprises multiple polynucleotide strands, and wherein each polynucleotide strand in the sample comprises a unique MID in the first domain.

13. The method of claim 1, wherein the polynucleotide strand is produced by:

annealing a nucleic acid synthesis primer to a parent polynucleotide strand, wherein the parent polynucleotide strand comprises the following in a 5' to 3' orientation:

the first domain;

the reflex sequence; and

a second domain comprising the polynucleotide of interest;

wherein the nucleic acid synthesis primer comprises:

a 3' priming domain that is complementary to a site adjacent to and 3' of the polynucleotide of interest in the second domain; and

a 5' reflex sequence;

extending the nucleic acid synthesis primer to produce an extension product; and

replicating the resultant extension product with a second nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain, thereby producing the polynucleotide strand.

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14. The method of claim 13, wherein the length of the second domain is from 0.1 to 30 kilobases.

15. The method of claim 13, wherein the parent polynucleotide strand is enriched prior to the annealing step based on its sequence.

16. The method of claim 1, further comprising the steps of:

c) replicating the polynucleotide strand of step b with a first nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain to produce a first extension product;

d) annealing a second nucleic acid synthesis primer to the first extension product, wherein the second nucleic acid synthesis primer comprises a 3' priming domain that is complementary to a site within the polynucleotide of interest in the first extension product; and

e) extending the second synthesis primer to produce a second extension product.

17. The method of claim 16, wherein the second synthesis primer comprises a 5' domain that is not complementary to the polynucleotide of interest in the first extension product.

18. The method of claim 17, wherein the first domain comprises a first primer binding site and the 5' domain of the second synthesis primer comprises a second primer binding site.

19. The method of claim 18, wherein the length of the polynucleotide of interest in the second extension product is from 50 to 10,000 nucleotides.

20. A polynucleotide strand comprising the following in a 5' to 3' orientation:

a first domain;

a reflex sequence;

a polynucleotide of interest; and

a complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand.

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COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

ABSTRACT OF THE DISCLOSURE

Aspects of the present invention are drawn to processes for moving a region of interest in a polynucleotide from a first position to a second position with regard to a domain within the polynucleotide, also referred to as a "reflex method". In certain embodiments, the reflex method results in moving a region of interest into functional proximity to specific domain elements present in the polynucleotide (e.g., primer binding sites and/or MID). Compositions, kits and systems that find use in carrying out the reflex processes described herein are also provided.

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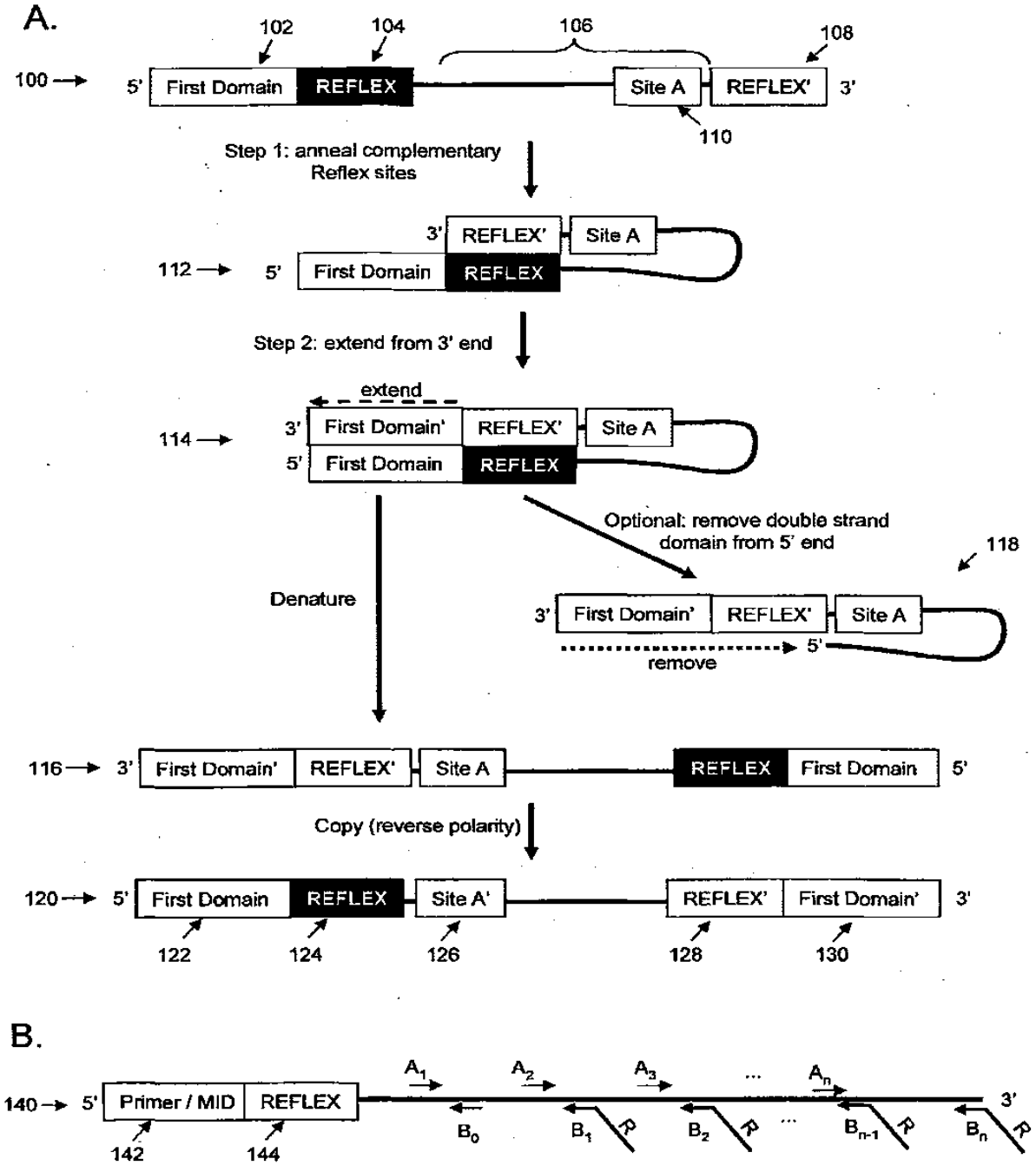
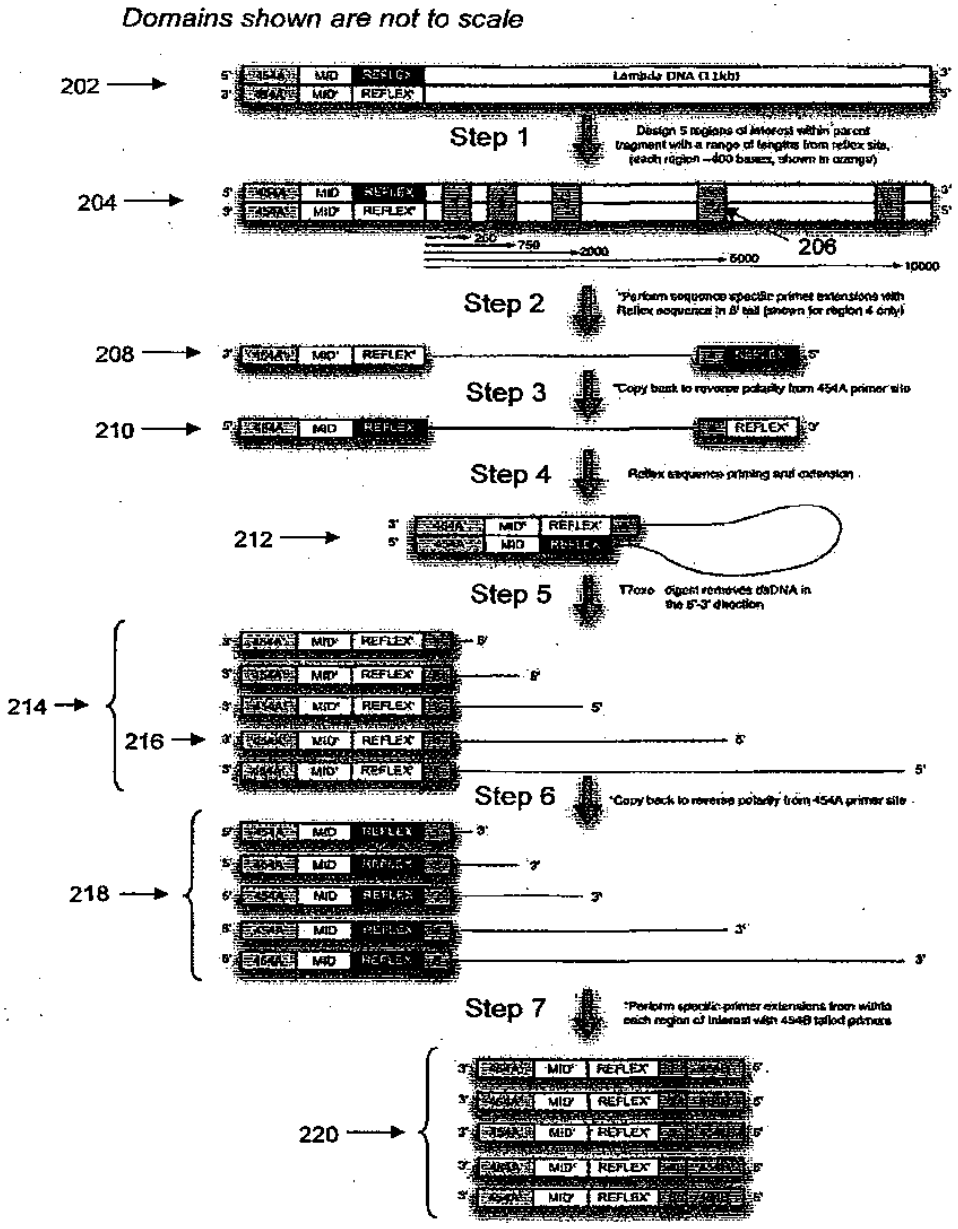


Fig. 1

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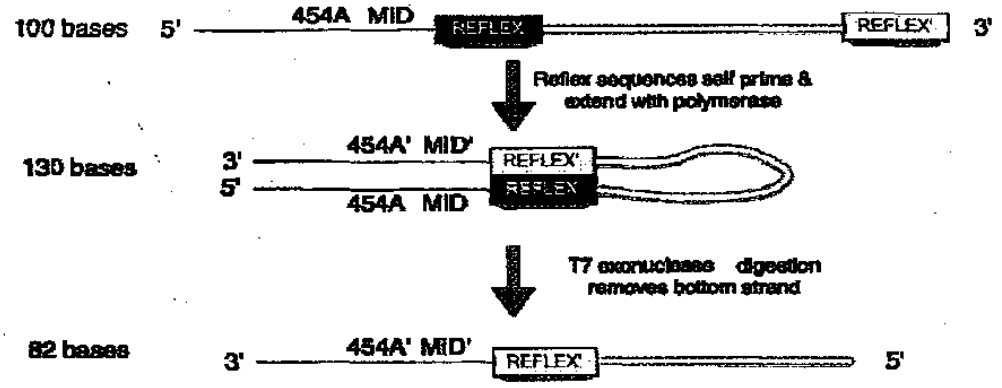


primer extensions with * may be performed such that isolation of single stranded species is facilitated (e.g., using primers with binding moieties and/or linear amplification)

Fig. 2

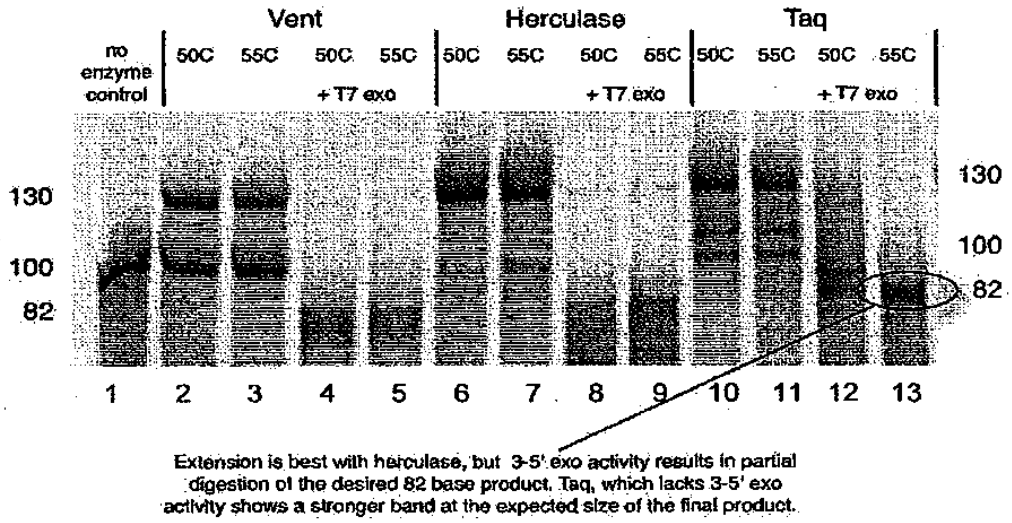
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A.



B.

Polymerase Testing at two annealing temps



Extension is best with herculase, but 3-5' exo activity results in partial digestion of the desired 82 base product. Taq, which lacks 3-5' exo activity shows a stronger band at the expected size of the final product.

Fig. 3

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A.



B.

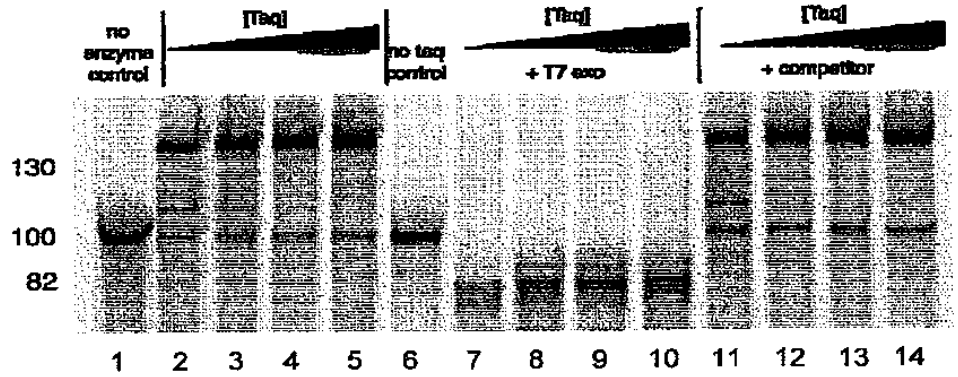


Fig. 4

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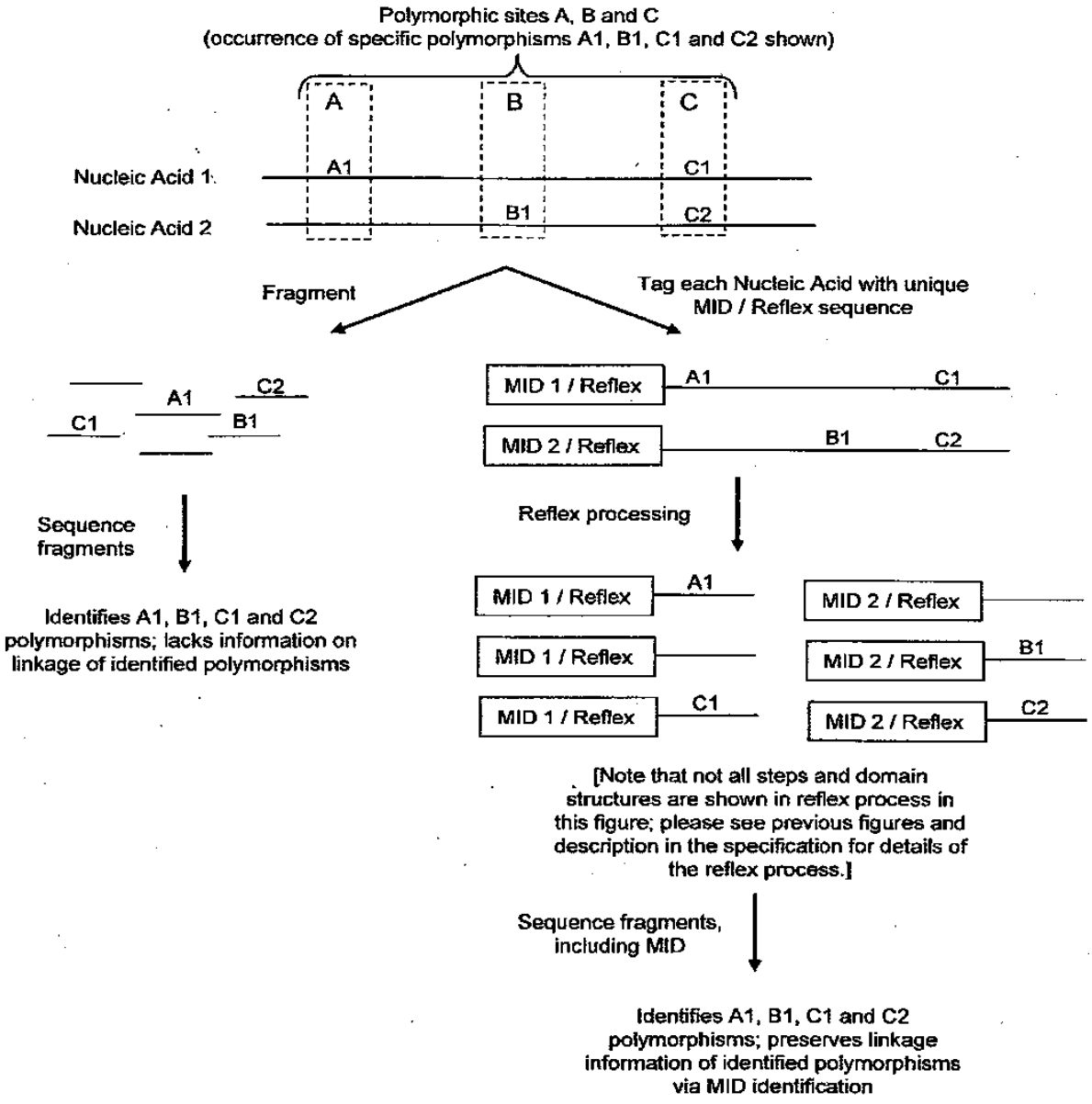


Fig. 5

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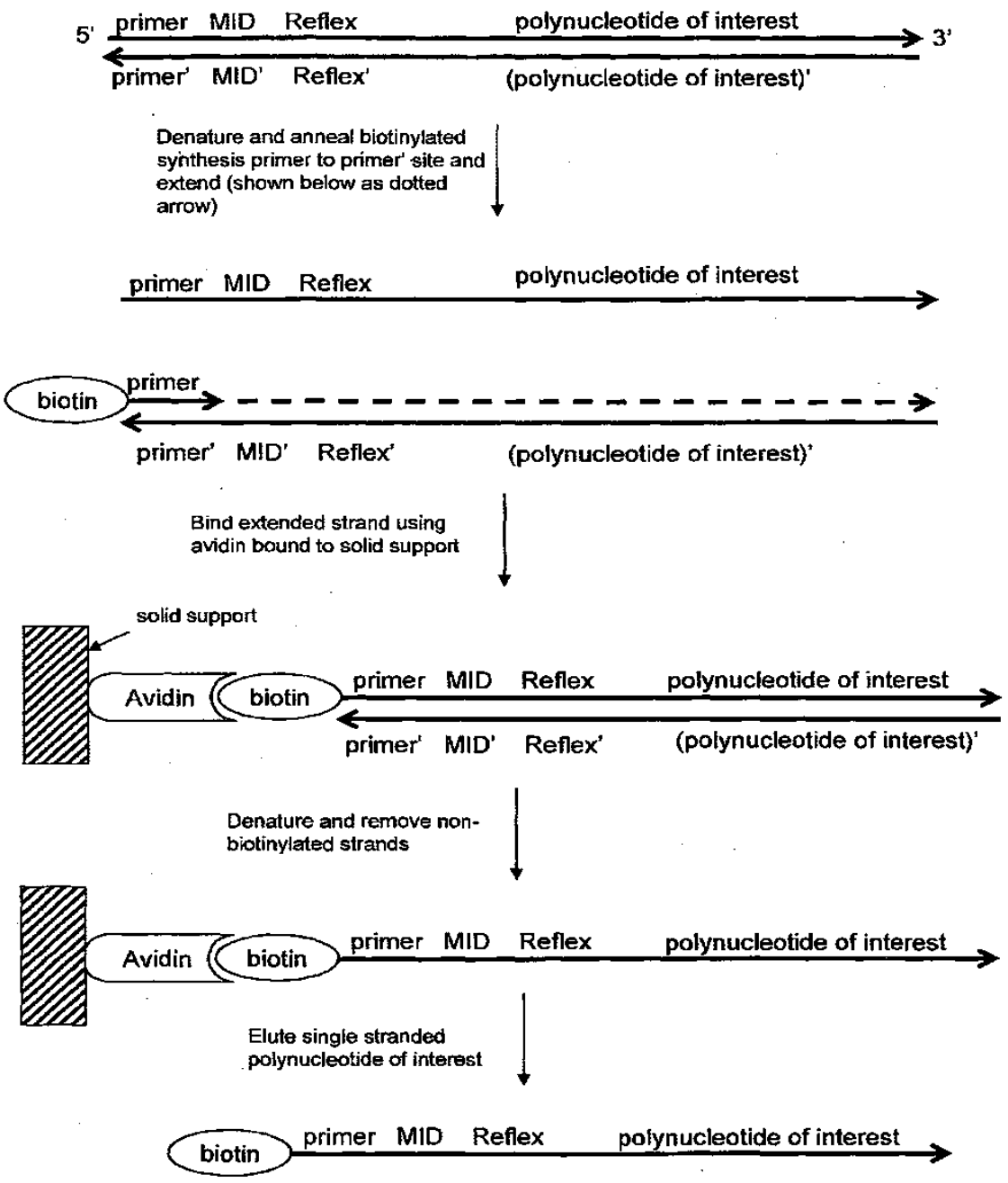


Fig. 6

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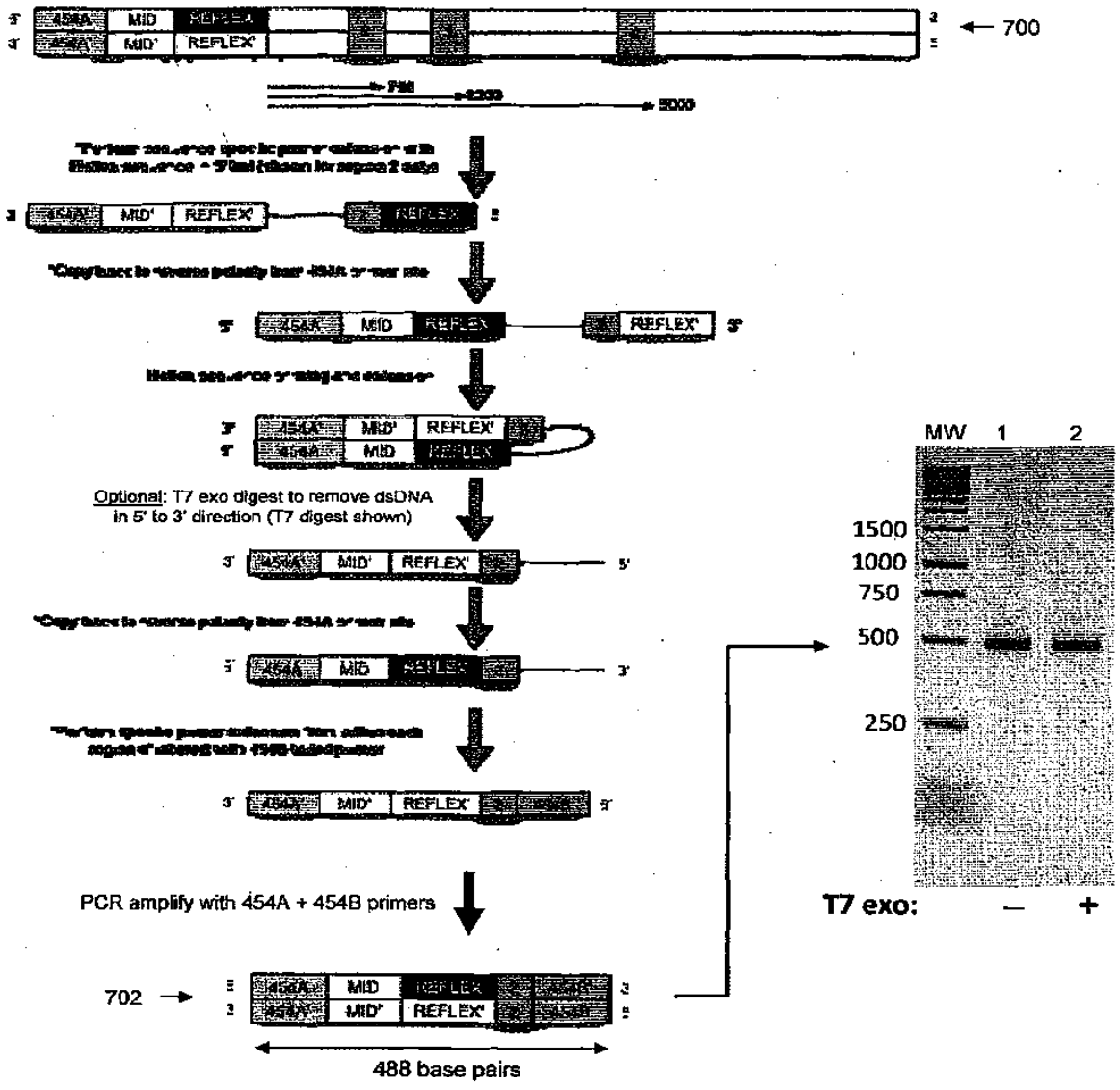


Fig. 7

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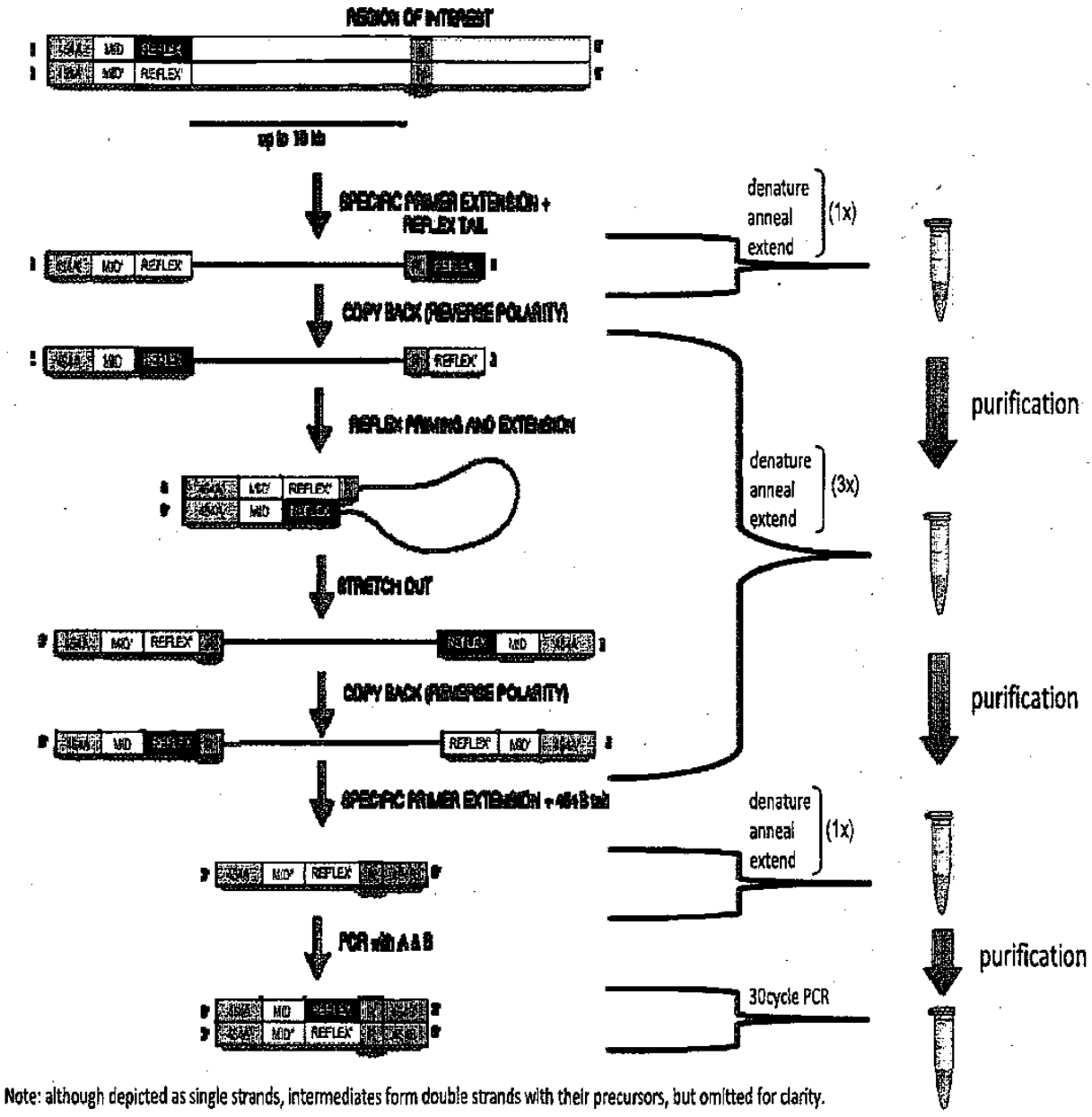


Fig. 8

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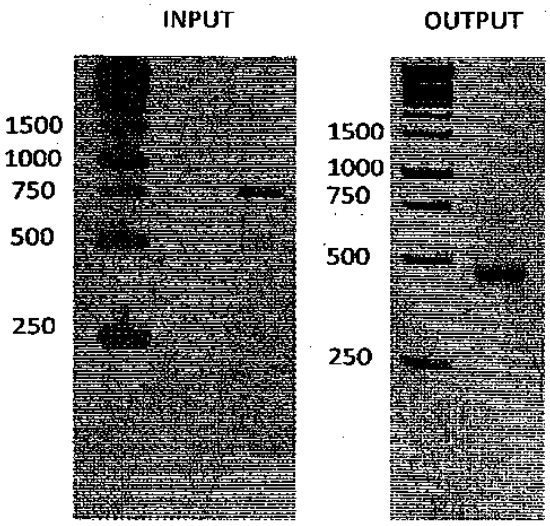


Fig. 9

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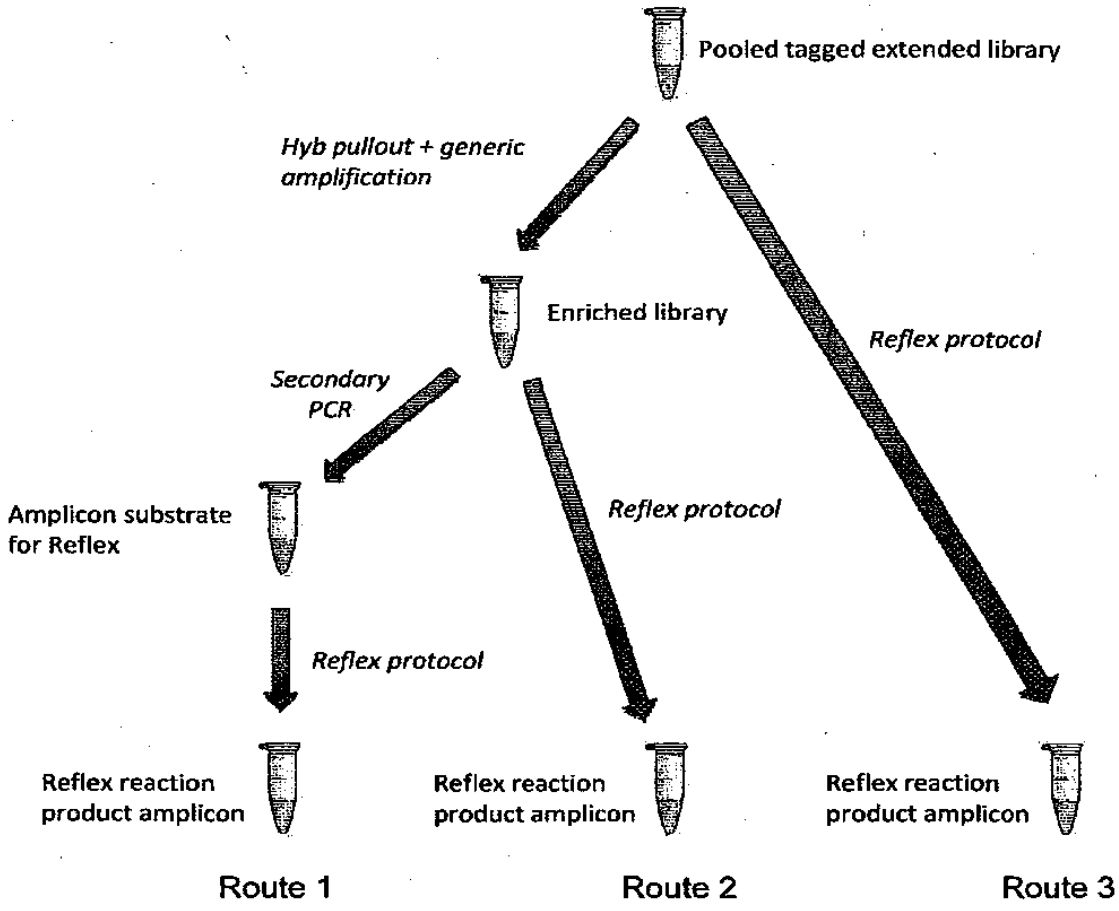


Fig. 10

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APPLICATION DATA SHEET

APPLICATION INFORMATION

Application Type::	Provisional
Title::	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
Attorney Docket Number::	CGLC-020PRV2
Request for Non-Publication?::	
Assignee for Publication::	
Total Drawing Sheets::	10
Small Entity?::	Yes
Sequence Submission?::	No
Computer Readable Form (CRF)?::	

INVENTOR INFORMATION

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Country::	UK
Citizenship Country::	GB

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State or Province of Residence::	United Kingdom
Country::	UK
Citizenship Country::	JP

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00677

APPLICATION DATA SHEET

State or Province::	United Kingdom
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City of Residence::	Great Chesterfield
State or Province of Residence::	United Kingdom
Country::	UK
Citizenship Country::	UK

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State or Province of Residence::	United Kingdom
Country::	UK
Citizenship Country::	UK

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Telephone Two::	(650) 833-7707
Fax::	(650) 327-3231
Electronic Mail::	scherer@bozpat.com

REPRESENTATIVE INFORMATION

Representative Customer Number::	24353

CONTINUITY INFORMATION

This application is a::	Provisional...
> Application One::	
Filing Date::	

PRIOR FOREIGN APPLICATIONS

Foreign Application One::	
Filing Date::	
Country::	
Priority Claimed::	

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UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

U.S. APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
13/387,343	Sydney Brenner	CGI.C-020

24353
BOZICEVIC, FIELD & FRANCIS LLP
1900 UNIVERSITY AVENUE
SUITE 200
EAST PALO ALTO, CA 94303

INTERNATIONAL APPLICATION NO.	
PCT/IB2010/002243	
I.A. FILING DATE	**PRIORITY DATE**
08/13/2010	08/20/2009

CONFIRMATION NO. 3537
371 FORMALITIES LETTER



Date Mailed: 02/07/2012

NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as a Designated Office (37 CFR 1.494):

- Indication of Small Entity Status
- Priority Document
- Copy of the International Application filed on 01/26/2012
- Copy of the International Search Report filed on 01/26/2012
- Preliminary Amendments filed on 01/26/2012
- Information Disclosure Statements filed on 01/26/2012
- Request for Immediate Examination filed on 01/26/2012
- U.S. Basic National Fees filed on 01/26/2012
- Priority Documents filed on 01/26/2012

The following items **MUST** be furnished within the period set forth below in order to complete the requirements for acceptance under 35 U.S.C. 371:

- Oath or declaration of the inventors, in compliance with 37 CFR 1.497(a) and (b), identifying the application by the International application number and international filing date.

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE OR BY 32 MONTHS FROM THE PRIORITY DATE FOR THE APPLICATION, WHICHEVER IS LATER. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

FORM PCT/DO/EO/905 (371 Formalities Notice)

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For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

MARILYN J YOUNGER

Telephone: (571) 272-8183

FORM PCT/DO/EO/905 (371 Formalities Notice)

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00680

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 13/387,343

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	190		N/A	
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	245		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	125		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	18 minus 20 =	*	x 30 =	0.00	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*	x 125 =	0.00			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			0.00			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				0.00			
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	560		TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE(S)	SMALL ENTITY ADDITIONAL FEE(S)	OR	OTHER THAN SMALL ENTITY RATE(S)	OTHER THAN SMALL ENTITY ADDITIONAL FEE(S)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x		OR	x	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x		OR	x	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE(S)	ADDITIONAL FEE(S)		RATE(S)	ADDITIONAL FEE(S)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x		OR	x	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x		OR	x	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
13387343	
Applicant(s) Sydney Brenner	

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
	Indep	Depend	Indep	Depend	Indep	Depend
1	1		1			
2		1	---	---		
3		1	---	---		
4		1		1		
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Total Indep	2		1		0	
Total Depend	18	↵	17	↵	0	↵
Total Claims	20		18		0	

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SUBMITTED ELECTRONICALLY FEBRUARY 15, 2012

PETITION UNDER 37 C.F.R.§1.102(c) Address to: **Mail Stop: Petitions** Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Attorney Docket	CGLC-020
	Confirmation No.	3537
	First Named Inventor	Sydney Brenner
	Application Number	13/387,343
	Filing Date	
	Group Art Unit	
	Examiner Name	
	Title	COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

Sir:

This is a Petition to Make Special, in accordance with 37 C.F.R. §1.102(c), on the grounds that the applicant, Sydney Brenner, is age 65 or more.

Evidence in support of this Petition submitted herewith includes:

1) A copy of Sydney Brenner's passport, showing his date of birth as January 13, 1927.

According to 37 C.F.R. §1.102(c), no fee is due with this Petition.

However, should the Commissioner deem otherwise, he is hereby authorized to charge any underpayment of fees associated with this communication, to Deposit Account No. 50-0815, order number CGLC-020.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: February 15, 2012

By: /James S. Keddie, Reg. No. 48,920/
James S. Keddie
Registration No. 48,920

BOZICEVIC, FIELD & FRANCIS LLP
1900 University Avenue, Suite 200
East Palo Alto, CA 94303
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00684

SUBMITTED ELECTRONICALLY FEBRUARY 15, 2012

PRELIMINARY AMENDMENT Under CFR 1.115	Attorney Docket No.	CGLC-020
	Confirmation No.	3537
	First Named Inventor	Sydney Brenner
	Application Number	13/387,343
	Filing Date	
	Group Art Unit	
	Examiner Name	
Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Title: "Compositions and Methods for Intramolecular Nucleic Acid Rearrangement"	

Sir:

Prior to the examination of the above-referenced application on the merits, please enter the amendments below.

AMENDMENTS TO THE CLAIMS

Please amend the claims as shown below. A complete listing of the claims in this case, including their current status identifier, is shown below.

1. (Previously Presented) A method of sample analysis, comprising:

a) annealing a reflex sequence in a polynucleotide strand in a sample with a complement of the reflex sequence present in the polynucleotide strand, wherein the polynucleotide strand comprises the following in a 5' to 3' orientation:

a first domain comprising i. a multiplex identifier (MID) whose identity can be used to differentiate polynucleotides in the sample, and ii. a primer site;

the reflex sequence;

a polynucleotide of interest having a desired site that is distal to the first domain; and

the complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand;

b) extending from the 3' end of the complement of the reflex sequence through the first domain to produce a double stranded region comprising a complement of the first domain at the 3' end of the extended polynucleotide strand, thereby moving the first domain into proximity with the desired site; and

c) sequencing the MID and the desired site using a primer that complementary to the primer site in the complement of the first domain.

2-3 (Canceled)

4. (Original) The method of claim 1, wherein the reflex sequence is from 10 to 50 nucleotides in length.

5. (Previously Presented) The method of claim 1, wherein the first domain comprises an RNA polymerase site.

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Page 00686

6. (Original) The method of claim 1, the method further comprising removing the first domain and the reflex sequence from the 5' end of the double stranded region.

7. (Original) The method of claim 6, wherein the removing step comprises treating the nucleic acid of step b with T7 exonuclease.

8. (Original) The method of claim 1, wherein the first domain is present in an adapter region attached to the polynucleotide of interest.

9. (Original) The method of claim 8, wherein the reflex sequence is present in the adapter region.

10. (Original) The method of claim 8, wherein the reflex sequence is a sequence present within the 5' region of the polynucleotide of interest.

11. (Original) The method of claim 1, wherein the sample is a multiplexed sample, wherein the first domain in each of the polynucleotide strands in the multiplexed sample comprises a MID corresponding to its parental sample.

12. (Original) The method of claim 1, wherein the sample comprises multiple polynucleotide strands, and wherein each polynucleotide strand in the sample comprises a unique MID in the first domain.

13. (Original) The method of claim 1, wherein the polynucleotide strand is produced by:

annealing a nucleic acid synthesis primer to a parent polynucleotide strand, wherein the parent polynucleotide strand comprises the following in a 5' to 3' orientation:

the first domain;

the reflex sequence; and

a second domain comprising the polynucleotide of interest;

wherein the nucleic acid synthesis primer comprises:

a 3' priming domain that is complementary to a site adjacent to and 3' of the polynucleotide of interest in the second domain; and

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a 5' reflex sequence;

extending the nucleic acid synthesis primer to produce an extension product; and

replicating the resultant extension product with a second nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain, thereby producing the polynucleotide strand.

14. (Original) The method of claim 13, wherein the length of the second domain is from 30 bases to 30 kilobases.

15. (Original) The method of claim 13, wherein the parent polynucleotide strand is enriched prior to the annealing step based on its sequence.

16. (Original) The method of claim 1, further comprising the steps of:

c) replicating the polynucleotide strand of step b with a first nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain to produce a first extension product;

d) annealing a second nucleic acid synthesis primer to the first extension product, wherein the second nucleic acid synthesis primer comprises a 3' priming domain that is complementary to a site within the polynucleotide of interest in the first extension product; and

e) extending the second synthesis primer to produce a second extension product.

17. (Original) The method of claim 16, wherein the second synthesis primer comprises a 5' domain that is not complementary to the polynucleotide of interest in the first extension product.

18. (Original) The method of claim 17, wherein the first domain comprises a first primer site and the 5' domain of the second synthesis primer comprises a second primer site.

19. (Original) The method of claim 18, wherein the length of the polynucleotide of interest in the second extension product is from 50 to 10,000 nucleotides.

20. (Previously Presented) A kit comprising:

a polynucleotide strand comprising the following in a 5' to 3' orientation:

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a first domain;

a reflex sequence;

a polynucleotide of interest; and

a complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand; and

instructions for performing the method of claim 1.

21. (New) The method of claim 1, wherein polynucleotide of interest is cDNA.

22. (New) The method of claim 1, further comprising repeating said method to obtain the nucleotide sequence of a second desired site in said polynucleotide of interest, and analyzing the sequence obtained in c) and the sequence of the second desired site to obtain linkage information about polymorphisms in said polynucleotide of interest.

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REMARKS

Formal Matters

Claims 1 and 4-20 are pending.

Claims 2-3 were previously canceled.

New claims 21 and 22 have been added. Support for new claim 21 is found at page 18, line 6. Support for new claim 22 is found at, e.g., page 30, lines 7-16. No new matter has been added.

Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number CGLC-020.

Respectfully submitted,

Date: February 15, 2012

By: /James S. Keddie, Reg. No. 48,920/
James S. Keddie, Ph.D.
Registration No. 48,920

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(650) 327-3400 main
(650) 327-3231 fax
docket@bozpat.com

10x Genomics, Inc. Exhibit 2005
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Page 00690

PTO/SB/51A (01-09)
Approved for use through 03/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☐ The attached application, or
☒ Application No. PCT/IB2010/002243 filed on August 13, 2010,

☐ As amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT International filing date of the continuation-in-part application.

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All statements made herein of my/our own knowledge are true, all statements made herein on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001, and may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF INVENTOR(S)

Inventor one: **Sydney Brenner** Date: Feb 1 2012

Signature: Sydney Brenner Citizen of: **GB**

Inventor two: **Gi Mikawa** Date: _____

Signature: _____ Citizen of: **JP**

☒ Additional inventors or a legal representative are being named on 1 additional form(s) attached hereto

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Atty Docket No: **CGLC-020**

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76) (PAGE 2)

FULL NAME OF INVENTOR(S)

Inventor one: **Robert Osborne** Date: _____

Signature: _____ Citizen of: **GB**

Inventor two: **Andrew Slatter** Date: _____

Signature: _____ Citizen of: **GB**

Privacy Act Statement

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10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00692

PTO/SB/01A (07-09)
Approved for use through 06/30/2010. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

As the below named inventor(s), I/we declare that:

This declaration is directed to:

☐ The attached application, or
☒ Application No. PCT/IB2010/002243 filed on August 13, 2010.

☐ As amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

I/we have reviewed and understand the contents of the above-identified application, including the claims, as amended by any amendment specifically referred to above;

I/we acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

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FULL NAME OF INVENTOR(S)

Inventor one: **Sydney Brenner** Date: _____

Signature: _____ Citizen of: **GB**

Inventor two: **Gi Mikawa** Date: 1-Feb-2012

Signature: _____ Citizen of: **JP**

☒ Additional inventors or a legal representative are being named on 1 additional form(s) attached hereto.

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Atty Docket No: **CGLC-020**

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76) (PAGE 2)

FULL NAME OF INVENTOR(S)

Inventor one: **Robert Osborne** Date: _____

Signature: _____ Citizen of: **GB**

Inventor two: **Andrew Slatter** Date: _____

Signature: _____ Citizen of: **GB**

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Page 00694

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☐ The attached application, or
☒ Application No. PCT/IB2010/002243 filed on August 13, 2010,

☐ As amended on _____ (if applicable);

I/we believe that I/we am/are the original and first inventor(s) of the subject matter which is claimed and for which a patent is sought;

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FULL NAME OF INVENTOR(S)

Inventor one: **Sydney Brenner** Date: _____

Signature: _____ Citizen of: GB

Inventor two: **Gi Mikawa** Date: _____

Signature: _____ Citizen of: JP

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Atty Docket No: **CGLC-020**

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76) (PAGE 2)

FULL NAME OF INVENTOR(S)	
Inventor one: **Robert Osborne**	Date: 1/2/12
Signature:	Citizen of: **GB**
Inventor two: **Andrew Statter**	Date: 1/2/12
Signature:	Citizen of: **GB**

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Atty Docket No: **CGLC-020**

Electronic Patent Application Fee Transmittal

Application Number:	13387343
Filing Date:	
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Filer:	James Samuel Keddie/David Castro
Attorney Docket Number:	CGLC-020

Filed as Small Entity

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Oath/decl > 30 mo. from priority date	2617	1	65	65
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
			Total in USD ($)	65

Electronic Acknowledgement Receipt	
EFS ID:	12080687
Application Number:	13387343
International Application Number:	
Confirmation Number:	3537
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	24353
Filer:	James Samuel Keddie/David Castro
Filer Authorized By:	James Samuel Keddie
Attorney Docket Number:	CGLC-020
Receipt Date:	15-FEB-2012
Filing Date:	
Time Stamp:	13:36:59
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$65
RAM confirmation Number	11329
Deposit Account	
Authorized User	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Petition to make special based on Age/ Health	CGLC-020_Ptn2MakeSpecial.pdf	17659	no	1
Warnings:					
Information:					
2	Petition to make special based on Age/ Health	CGLC-005CON_Copy_S_Brenner_passport.pdf	106434	no	1
Warnings:					
Information:					
3		CGLC-020_Prelim-2.pdf	31095	yes	6

Multipart Description/PDF files in .zip description

Document Description	Start	End
Preliminary Amendment	1	1
Claims	2	5
Applicant Arguments/Remarks Made in an Amendment	6	6

Warnings:					
Information:					
4	Oath or Declaration filed	CGLC-020_Dec-SIGNED.pdf	2742264	no	6
Warnings:					
Information:					
5	Fee Worksheet (SB06)	fee-info.pdf	30132	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	2927584		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
13/387,343	02/15/2012	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☒		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	02/15/2012	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 18	Minus	** 20	= 0	X $30 =	0	OR	X $ =	
	Independent (37 CFR 1.16(h))	* 1	Minus	***3	= 0	X $125 =	0	OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/ANDREW j. JAMES JR/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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www.uspto.gov

U.S. APPLICATION NUMBER NO.	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
13/387,343	Sydney Brenner	CGI.C-020

24353
BOZICEVIC, FIELD & FRANCIS LLP
1900 UNIVERSITY AVENUE
SUITE 200
EAST PALO ALTO, CA 94303

INTERNATIONAL APPLICATION NO.	
PCT/IB2010/002243	
I.A. FILING DATE	**PRIORITY DATE**
08/13/2010	08/20/2009

CONFIRMATION NO. 3537
371 ACCEPTANCE LETTER

OC000000052701434

Date Mailed: 02/23/2012

NOTICE OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C 371 AND 37 CFR 1.495

The applicant is hereby advised that the United States Patent and Trademark Office in its capacity as a Designated / Elected Office (37 CFR 1.495), has determined that the above identified international application has met the requirements of 35 U.S.C. 371, and is ACCEPTED for national patentability examination in the United States Patent and Trademark Office.

The United States Application Number assigned to the application is shown above and the relevant dates are:

02/15/2012	02/15/2012
DATE OF RECEIPT OF 35 U.S.C. 371(c)(1), (c)(2) and (c)(4) REQUIREMENTS	DATE OF COMPLETION OF ALL 35 U.S.C. 371 REQUIREMENTS

A Filing Receipt (PTO-103X) will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE " FILING DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371 (c)(1), (c)(2) and (c)(4) REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN ABOVE.** *The filing date of the above identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363).* Once the Filing Receipt has been received, send all correspondence to the Group Art Unit designated thereon.

The following items have been received:

- Indication of Small Entity Status
- Copy of the International Application filed on 01/26/2012
- Copy of the International Search Report filed on 01/26/2012
- Preliminary Amendments filed on 01/26/2012
- Information Disclosure Statements filed on 01/26/2012
- Oath or Declaration filed on 02/15/2012
- Request for Immediate Examination filed on 01/26/2012
- U.S. Basic National Fees filed on 01/26/2012
- Priority Documents filed on 01/26/2012

FORM PCT/DO/EO/903 (371 Acceptance Notice)

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00703

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

MARILYN J YOUNGER

Telephone: (571) 272-8183

FORM PCT/DO/EO/903 (371 Acceptance Notice)

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00704

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
13/387,343	02/15/2012		625	CGLC-020	18	1

CONFIRMATION NO. 3537

24353
BOZICEVIC, FIELD & FRANCIS LLP
1900 UNIVERSITY AVENUE
SUITE 200
EAST PALO ALTO, CA 94303

FILING RECEIPT

OC000000052701433

Date Mailed: 02/23/2012

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Applicant(s)

Sydney Brenner, Ely, UNITED KINGDOM;
Gi Mikawa, Cambridge, UNITED KINGDOM;
Robert Osborne, Saffron, UNITED KINGDOM;
Andrew Slatter, London, UNITED KINGDOM;

Power of Attorney: The patent practitioners associated with Customer Number 24353

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/IB2010/002243 08/13/2010
which claims benefit of 61/288,792 12/21/2009
and claims benefit of 61/235,595 08/20/2009

Foreign Applications (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.)

If Required, Foreign Filing License Granted: 02/21/2012

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 13/387,343**

Projected Publication Date: 05/31/2012

Non-Publication Request: No

Early Publication Request: No
** **SMALL ENTITY** **

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00705

Title

Compositions and Methods for Intramolecular Nucleic Acid Rearrangement

Preliminary Class

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4158).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00706

license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation and commercialization of new technologies. The USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage, facilitate, and accelerate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 13/387,343

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A	190		N/A	
SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A	245		N/A	
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A	125		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	18 minus 20 =	*	x 30 =	0.00	OR		
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*	x 125 =	0.00			
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).			0.00			
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))				0.00			
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL	560		TOTAL	

APPLICATION AS AMENDED - PART II

AMENDMENT A

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE(S)	SMALL ENTITY ADDITIONAL FEE(S)	OR	OTHER THAN SMALL ENTITY RATE(S)	OTHER THAN SMALL ENTITY ADDITIONAL FEE(S)
Total (37 CFR 1.16(i))	*	Minus	**	=	x		OR	x	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x		OR	x	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT B

	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE(S)	ADDITIONAL FEE(S)		RATE(S)	ADDITIONAL FEE(S)
Total (37 CFR 1.16(i))	*	Minus	**	=	x		OR	x	
Independent (37 CFR 1.16(h))	*	Minus	***	=	x		OR	x	
Application Size Fee (37 CFR 1.16(s))									
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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BOZICEVIC, FIELD & FRANCIS LLP
1900 UNIVERSITY AVENUE
SUITE 200
EAST PALO ALTO CA 94303

MAILED

MAR 20 2012

OFFICE OF PETITIONS

In re Application of :
Sydney BRENNER et al. :
Application No. 13/387,343 : DECISION ON PETITION
Filed: February 15, 2012 : TO MAKE SPECIAL UNDER
Attorney Docket No. CGLC-020 : 37 CFR 1.102(c)(1)
:

This is a decision on the petition under 37 CFR 1.102(c)(1), filed February 15, 2012, to make the above-identified application special based on applicant's age as set forth in M.P.E.P. § 708.02, Section IV.

The petition is **GRANTED**.

A grantable petition to make an application special under 37 CFR 1.102(c)(1) and MPEP § 708.02, Section IV: Applicant's Age must be accompanied by evidence showing that at least one of the applicants is 65 years of age, or more, such as a birth certificate or a statement by applicant. No fee is required

The instant petition includes a copy of the inventor's passport as evidence that Sydney Brenner is over 65 years of age. Accordingly, the above-identified application has been accorded "special" status.

Telephone inquiries concerning this decision should be directed to the undersigned at 571-272-4231.

All other inquiries concerning either the examination or status of the application should be directed to the Technology Center.

This application is being referred to the Office of Patent Application for processing. This application will be accorded "special" status when pre-examination processing is done.

/Michelle R. Eason/
Michelle R. Eason
Paralegal Specialist
Office of Petitions

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/387,343	02/15/2012	Sydney Brenner	CGI.C-020	3537

24353 7590 04/12/2012

BOZICEVIC, FIELD & FRANCIS LLP
1900 UNIVERSITY AVENUE
SUITE 200
EAST PALO ALTO, CA 94303

EXAMINER	
BHAT, NARAYAN KAMESHWAR	

ART UNIT	PAPER NUMBER
1634	

MAIL DATE	DELIVERY MODE
04/12/2012	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00710

Office Action Summary	Application No.	Applicant(s)	
	13/387,343	BRENNER ET AL.	
	Examiner	Art Unit	
	NARAYAN BHAT	1634	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *15 February 2012*.

2a)☐ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) *1 and 4-22* is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☐ Claim(s) _____ is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☒ Claim(s) *1 and 4-22* are subject to restriction and/or election requirement.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

12)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *1/26/12*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00711

DETAILED ACTION

This action is in response to the papers filed on February 15, 2012. Claims 1 and 4-22 are pending in this application.

Election/Restrictions

Restriction is required under 35 U.S.C. 121 and 372.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In accordance with 37 CFR 1.499, applicant is required, in reply to this action, to elect a single invention to which the claims must be restricted.

Group I claims 1, 4-19, 21 and 22 are drawn to a method of sample analysis.

Group II claim 20 is drawn to a kit.

The groups of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons. The technical feature linking groups I to II is the kit comprising a polynucleotide strand and instructions for its use of group II invention. However, the kit comprising the polynucleotide strand and instructions for its use was known in the art at the time the claimed invention was made as taught by Kanda et al (2007/0238113 published Oct. 11, 2007, cited in the IDS filed January 26, 2012).

Regarding group II invention, Kanda teaches a kit comprising a polynucleotide strand in a 5' to 3' orientation (Fig. 5) and instructions for using the kit (paragraph 0187).

With respect to the polynucleotide strand of the kit, it is noted that it merely requires a first domain, a reflex sequence and a polynucleotide of interest and a complement of the reflex sequence at the 3' end of the polynucleotide strand. Also, with regard to the reflex sequence, the instant specification recites that the reflex sequence does not hybridize any other sequences except for its complement (instant specification, pg. 14, first paragraph).

The polynucleotide strand of the kit is not novel, because it reads on the stem loop probe or the hairpin loop probe with a 5' extension, which are well established in the art.

Also, the polynucleotide strand presented in Fig. 5 of Kanda meets the limitation of the polynucleotide strand of the instant kit because the polynucleotide strand of Kanda as illustrated in Fig. 5 comprises in 5' to 3' orientation, a first domain (indicated by an arrow), and an adjacent sequence (i.e., one part of the closing stem, F1 c), a polynucleotide of interest (loop, F2) and the complement the adjacent sequence at the 3' end of the strand (F1). The adjacent sequence of Kanda does not hybridize to other sequence except for its complement sequences (Fig. 5). The adjacent sequence of Kanda is the reflex sequence of the polynucleotide strand as recited in the instant specification.

Since the kit comprising the polynucleotide strand and instructions for its use were known in the art at the time the claimed invention was made, the technical feature linking the inventions of groups I to II does not constitute a special technical feature as defined by PCT Rule 13.2, as it does not define a contribution over the prior art.

Notice of Possible Rejoinder

The examiner has required restriction between product and process claims. Where applicant elects claims directed to the product, and the product claims are subsequently found allowable, withdrawn process claims that depend from or otherwise require all the limitations of the allowable product claim will be considered for rejoinder. All claims directed to a nonelected process invention must require all the limitations of an allowable product claim for that process invention to be rejoined.

In the event of rejoinder, the requirement for restriction between the product claims and the rejoined process claims will be withdrawn, and the rejoined process claims will be fully examined for patentability in accordance with 37 CFR 1.104. Thus, to be allowable, the rejoined claims must meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103 and 112. Until all claims to the elected product are found allowable, an otherwise proper restriction requirement between product claims and process claims may be maintained. Withdrawn process claims that are not commensurate in scope with an allowable product claim will not be rejoined. See MPEP § 821.04(b). Additionally, in order to retain the right to rejoinder in accordance with the above policy, applicant is advised that the process claims should be amended during prosecution to require the limitations of the product claims. **Failure to do so may result in a loss of the right to rejoinder**. Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP § 804.01.

Applicant is advised that the reply to this requirement to be complete must include an election of invention to be examined even though the requirement is traversed (37 CFR 1.143) and (ii) identification of the claims encompassing the elected invention.

Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C.103(a) of the other invention.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to NARAYAN K. BHAT whose telephone number is (571)272-5540. The examiner can normally be reached on 8.30 am to 5 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on (571)-272-0731. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Narayan K. Bhat/
Examiner, Art Unit 1634

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (01-10)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT
(Not for submission under 37 CFR 1.99)

Application Number	
Filing Date	
First Named Inventor	Sydney Brenner
Art Unit	1634
Examiner Name	Bhat N.
Attorney Docket Number	CGLC-020

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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear
	1	20070238113	A1	2007-10-11	KANDA, e al.	
	2	20070259357	A1	2007-11-08	BRENNER	

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Examiner Initial*	Cite No	Foreign Document Number[3]	Country Code[2] i	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear	T[5]
	1	2004/061083	WO	A2	2004-07-22	AMGEN INC		☐
	2	2007/018601	WO	A1	2007-02-15	RUBICON GENOMICS INC		☐

EFS Web 2.1.17

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	
	Filing Date	
	First Named Inventor	Sydney Brenner
	Art Unit	1634
	Examiner Name	Bhat N.
	Attorney Docket Number	CGLC-020

13387343 - GAU: 1634

	3	1841879	EP	A2	2007-10-10	POPULATION GENETICS TECHNOLOGI		☐
	4	1672064	EP	A1	2006-06-21	RIKEN		☐

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NON-PATENT LITERATURE DOCUMENTS Remove

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	1	YAN, et al., "Rapid one-step construction of hairpin RNA", Biochemical and Biophysical Research Communications, 2009, 383:464-8.	☐
	2	PCT/IB2010/002243, International Search Report and Written Opinion, mailed February 9, 2011, 13pgs.	☐

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EXAMINER SIGNATURE

Examiner Signature	/Narayan Bhat/	Date Considered	03/29/2012

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /N.B./

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00718

SUBMITTED ELECTRONICALLY APRIL 30, 2012

RESPONSE TO RESTRICTION REQUIREMENT	Docket No.	CGLC-020
	Application No.	13/387,343
	Confirmation No.	3537
	Filing Date	February 15, 2012
Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Examiner	N.K. Bhat
	Group Art Unit	1634

Sir:

This communication is submitted in response to the Restriction Requirement dated May 31, 2011. The Examiner therein required election of one of the following groups of claims:

Group I: Claims 1, 4-19, 21 and 22

Group II: Claim 20

The Applicants hereby elect to prosecute the claims of **Group I, claims 1, 4-19, 21 and 22**, with traverse.

As stated in the MPEP §803, if search and examination of an entire application can be made without serious burden, the examiner must examine the entire application on the merits, even though the entire application includes claims to independent or distinct inventions. It is the Applicants' position that it would not be unduly burdensome to perform a search on all of the claims together in the present application. Accordingly, the Applicants traverse the restriction requirement.

The Applicants expressly reserve the right under 35 USC §121 to file a divisional application directed to the non-elected subject matter or any subject matter disclosed in this application during the pendency of this application.

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00719

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16 and 1.17 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815, order number CGLC-020.

Respectfully submitted,

BOZICEVIC, FIELD & FRANCIS LLP

Date: April 30, 2012

By: /James S. Keddie, Reg. No. 48,920/

James S. Keddie, Ph.D.
Reg. No. 48,920

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00720

Electronic Acknowledgement Receipt	
EFS ID:	12656023
Application Number:	13387343
International Application Number:	
Confirmation Number:	3537
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	24353
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020
Receipt Date:	30-APR-2012
Filing Date:	15-FEB-2012
Time Stamp:	18:44:57
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Response to Election / Restriction Filed	CGLC-020_Rsp2RR.pdf	25738 a19c91ba9981b92e4bd18609c6f2e88b35e1	no	2
Warnings:					
Information:					

Total Files Size (in bytes):	25738

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/387,343	02/15/2012	Sydney Brenner	CGI.C-020	3537

24353 7590 05/16/2012
BOZICEVIC, FIELD & FRANCIS LLP
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EXAMINER	
BHAT, NARAYAN KAMESHWAR	

ART UNIT	PAPER NUMBER
1634	

MAIL DATE	DELIVERY MODE
05/16/2012	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00723

Office Action Summary	Application No.	Applicant(s)	
	13/387,343	BRENNER ET AL.	
	Examiner	Art Unit	
	NARAYAN BHAT	1634	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *30 April 2012*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) *1 and 4-22* is/are pending in the application.

5a) Of the above claim(s) *20* is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1,4-19,21 and 22* is/are rejected.

8)☐ Claim(s) _____ is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *26 January 2012* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

12)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00724

DETAILED ACTION

1. This action is in response to the papers filed on April 30, 2012. Claims 1 and 4-22 are pending in this application.

Election/Restrictions

2. Applicant's election with traverse of claims 1, 4-19, 21 and 22 of Group I invention in the reply filed on April 30, 2012 is acknowledged. The traversal is on the grounds that that it would not be unduly burdensome to perform a search on all of the claims together in the present application (Remarks, pg. 1).

This is not found persuasive because as discussed in the restriction action, the groups of inventions I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons. The technical feature linking groups I to II is the kit comprising a polynucleotide strand and instructions for its use of group II invention. However, the kit comprising the polynucleotide strand and instructions for its use was known in the art at the time the claimed invention was made as taught by Kanda. Since the kit comprising the polynucleotide strand and instructions for its use were known in the art at the time the claimed invention was made, the technical feature linking the inventions of groups I to II does not constitute a special technical feature as defined by PCT Rule 13.2, as it does not define a contribution over the prior art.

Therefore arguments regarding that it would not be unduly burdensome to perform a search on all of the claims together in the present application are moot.

3. Claim 20 is withdrawn from further consideration pursuant to 37 CFR 1.142(b), as being drawn to a nonelected invention of group II there being no allowable generic or linking claim. Applicant timely traversed the restriction (election) requirement in the reply filed on April 30, 2012.

4. Claims 1, 4-19, 21 and 22 are pending in this application.

Claim Interpretation

5. The method of claim 1 is dawn to the steps of moving a domain in a single stranded nucleic acid to a different position by using a sequence, termed "reflex sequence" and its complement; the complement of the reflex sequence is then extended to have the complement of the domain, thereby moving the first domain into the proximity with the desired site. The method of claim 1 also requires a polynucleotide of interest having a desired site that is distal to the first domain. However, instant claim 1 as recited does not require that the polynucleotide of interest be a different nucleic acid from the polynucleotide strand. Therefore the polynucleotide of interest is interpreted as to encompass a site that is distal to the first domain. Also the limitation of "thereby" moving the first domain into proximity with the desired site of instant claim 1 is interpreted as encompassed by extending from 3' end of the complement of the reflex sequence.

Claim Rejections - 35 USC § 103

6. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

7. This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

8. Claims 1, 4-11 and 13-22 are rejected under 35 U.S.C. 103(a) as being unpatentable over Kanda (US 2007/0238113 published Oct. 11, 2007, cited in the previous office action).

Regarding claim 1, Kanda teaches a method of sample analysis, comprising following steps.

Regarding step 'a' of annealing a reflex sequence in a polynucleotide strand n a sample with a complement of the reflex sequence present in the polynucleotide strand, it is noted that the instant specification recites "reflex sequence" and equivalents are used to indicate sequences in a polynucleotide that are employed to move a domain

intramolecularly from its initial location to a different location in the polynucleotide (pg. 13). Thus the teaching of the prior art of a polynucleotide strand comprising complementary region at two different ends forming a double stranded region meets the limitation of reflex sequence because by forming a double stranded region moves sequence from its initial location in a polynucleotide strand to a different location in the polynucleotide.

Kanda teaches a polynucleotide 11 comprising an R1c sequence at its 5' end and its complement sequence R1 at the 3' end forming a duplex region (Fig. 4 and paragraph 0049). The R1c sequence of Kanda meets the limitation of the reflex sequence as recited in the instant specification because the polynucleotide 11 in its linear form comprises R1c and its complement R1 at different locations on the polynucleotide but the complement R1 is moved to a different location in the polynucleotide after forming the duplex. One having ordinary skill in the art would recognize that the formation of the duplex region between the R1c and R1 meets the limitation of annealing step because forming the said duplex requires an annealing step.

Regarding the first domain, Kanda also teaches that the polynucleotide 11 comprises the following in a 5' to 3' orientation a F1c/F2/F1 domain and the loop region F2 comprises sequences that is unique in the polynucleotide strand (e.g., see Fig. 5). The instant specification recites that the multiplex Identifier as used herein refers to a tag or combination of tags associated with a polynucleotide whose identity (e.g., the tag DNA sequence) can be used to differentiate polynucleotides in a sample (pg. 7, last

paragraph). One having ordinary skill in the art would recognize that the F2 sequence of Kanda meets the limitation of broadly recited multiplex identifier because F2 can be used as the tag to differentiate a polynucleotide in the sample. Kanda also teaches that the loop comprises the F1c/F2 primer binding site (Fig. 5). The Fi1c/F2/F1 domain of Kanda is the first domain because as discussed above it comprises the structural features of the first domain.

As discussed above, Kanda teaches R1c sequence (i.e., the reflex sequence) and the complement of the reflex sequence, wherein the complement of the reflex sequence R1 is present at the 3' end of the polynucleotide strand 11(Fig. 4).

Regarding a polynucleotide of interest, Kanda teaches an R2c region distal to the F1c/F2/F1 domain (i.e., the first domain) in the polynucleotide strand 11, which meets the limitation of the polynucleotide strand of interest as discussed in the claim interpretation section.

Regarding step 'b', Kanda teaches extending from the 3' end of the R1 (i.e., the 3' end of the complement reflex sequence) through the first domain by amplification (Fig. 4 and paragraph 0186). One having ordinary skill in the art would recognize that the extension of the 3' end of the reflex complement (i.e., R1 sequence) of Kanda through the first domain produces complementary sequences of the first domain at the 3' end of the extended polynucleotide strand. The step of extending the 3' end of the complement reflex sequence meets the limitation of thereby moving the first domain (F1c/F2/F1 into the proximity with the desired site R2c.

Regarding step 'c', Kanda teaches the sequencing the amplified product for confirming the nucleotide sequence (paragraph 0306). Although Kanda does not specifically teach the primer that is complementary to the primer site in the complement of the first domain for sequencing, one having ordinary skill in the art would recognize that the sequencing the amplified product using the said primer is obvious because Kanda teaches the primer binding site in the first domain.

Regarding claim 4, Kanda teaches F1c/F2 region comprises about 50 nucleotides in length (Fig. 5), which is in the range from about 10 to 50 nucleotides in length.

Regarding claims 5-7, it noted that the subject-matter differs in that the first domain and the reflex sequence from the 5' end of the double stranded region are removed. A skilled person who wanted to obtain a molecule only with the reflex sequence and the inverted region of interest would remove said first domain and the reflex sequence from the 5' end without the need of any inventive step. The use of an exonuclease would be one of the preferred options, and T7 is one of the best known for the skilled person, thus, the subject-matter of claims 6-7 would be obvious because removing nucleic acid sequence from the 5' end is established in the art.

Regarding claims 8-10, requiring first domain to be present in an adaptor region attached to the polynucleotide of interest, Kanda teaches that the polynucleotide 11 region is directly attached to the first domain (Fig. 4). One having ordinary skill in the art would recognize that based on the teachings of Kanda of polynucleotide strand 11 comprising the first domain and the reflex sequence and the polynucleotide of interest

meets the limitation of the adapter region because the adapter region does not have features that are distinct from the first domain and the reflex sequence. With respect to the limitation of the reflex sequence is a sequence present within the 5' region of the polynucleotide of interest, one having ordinary skill in the art would have recognized that it would be obvious to have the said arrangement in order to move the polynucleotide interest region closer to the first domain.

Regarding claim 11, Kanda teaches the sample comprises a target nucleic acid and further teaches that the target sequence is amplified by using primer having a specific structure (paragraph 0157). One having ordinary skill in the art would recognize that the amplified sample is the multiplexed sample and the multiplexed sample comprises an identifier (i.e., specific primer) corresponding to the parent sample because the amplified samples are identified by having said primer site.

Regarding claim 13, it is noted that instant claim 13 differs from that of claim 1 in that two primers are used, in particular, the main difference is the step of incorporating in a polynucleotide strand the region corresponding to the complement of the "reflex sequence" by extension using a primer which is complementary to the polynucleotide of interest or the second domain. This is a method step which would not represent any inventive activity for the skilled person in the field of in molecular biology because Kanda teaches the R2c region comprising the polynucleotide of interest, which is the second domain as recited in instant claim 13. Also, Kanda teaches that the polynucleotide strand 11 comprises a sequence 5' of the reflex sequence R1c and a 3' priming domain that is complementary to a site adjacent to and 3' of the polynucleotide

interest (Fig. 4, R2 domain). One having ordinary skill in the art would recognize that the steps of claim 13 are optimization of a result effective variable, which is ordinarily within the skill of the art for producing large quantities of polynucleotide strands 11 because it merely requires two primer sets for amplifying the polynucleotide strand 11.

Also, the court decided In re Boesch(205 USPQ 215) that optimization of a result effective variable is ordinarily within the skill of the art. A result effective variable is one that has well known and predictable results. In the instant case producing a polynucleotide strand from the parent strand merely requiring an amplification step requiring two primers are well established in the art and therefore would have the well-known and expected result for producing large quantities of the parent polynucleotide strands for detecting analytes from plurality of samples. It would have been within the skill of the art to modify Kanda's reference as optimization of a result effective variable and to gain the advantage of having the large quantities of the polynucleotide strands for downstream applications.

Regarding claim 14, Kanda teaches that R2 (i.e., the second domain) is about 43 nucleotides (Fig. 7).

Regarding claim 15, it is noted that the instant claim as recited requiring the step of enriching the parent nucleotide strand, would be a routine optimization because one having ordinary skill in the art would recognize that having enriched parent molecules before annealing ensures proper annealing.

Regarding claims 16-19 and 22, it is noted that the steps seem to represent a mere alternative for further amplifications and priming, which are well established in the

art as exemplified by the arts of the record (cited in the IDS of Jan. 26, 2012). Therefore the steps do not appear to involve any inventive steps over the art of the record and the general knowledge of the skilled person.

Regarding claim 21, Kanda teaches the cDNA (paragraph 0021).

9. Claims 1 and 12 are rejected under 35 U.S.C. 103(a) as being unpatentable over Kanda (US 2007/0238113 published Oct. 11, 2007, cited in the previous office action) in view of Brenner (US 2007/0259357 published Nov. 8, 2007, cited in the IDS filed Jan. 26, 2012).

Kanda and Brenner teach a method for sample analysis and therefore are analogous arts. The teachings of Brenner are applied for each polynucleotide strand in the sample comprising a unique multiplex identifier in the first domain.

Claim 12 is dependent from claim 1. The teachings of Kanda regarding claim 1 are discussed above section 6.

Regarding claim 12, Kanda teaches a polynucleotide 11 comprises the loop region F2 further comprising sequences that are unique in the polynucleotide strand (e.g., see Fig. 5). The instant specification recites that the multiplex Identifier as used herein refers to a tag or combination of tags associated with a polynucleotide whose identity (e.g., the tag DNA sequence) can be used to differentiate polynucleotides in a sample (pg. 7, last paragraph). One having ordinary skill in the art would recognize that the F2 sequence of Kanda meets the limitation of broadly recited multiplex identifier because F2 can be used a tag to differentiate a polynucleotide in the sample. Kanda

does not specifically teach that when the sample comprises multiple polynucleotide strands, each polynucleotide strand in the sample comprise a unique identifier in the first domain.

However, each polynucleotide strand in the sample comprising the unique identifier was known in the art at the time the claimed invention was made as taught by Brenner.

Brenner teaches a method for tagging nucleic acids in the sample comprising the step of tagging unique sequence tag140 to one population of polynucleotide 138 (Fig. 1 and paragraph 0080) and the unique identifier further comprises a plurality of sequence tokens, which can be identified by a variety of read out platforms (Abstract), thus teaching each nucleic acid comprises a multiplex identifier. Brenner also teaches that the sequence tokens can be added at any locations (paragraph 0058). Brenner further teaches that the availability of a convenient and efficient molecular tagging method could be used for discovery of genetic variation and/or high-throughput DNA sequencing would extend the use of these useful reagents and lead to improvements in analytical assays in many fields, including scientific and biomedical research and medicine (paragraph 0004), thus providing a motivation to one of ordinary skill in the art to include the unique multiplex identifier comprising a plurality of sequence tokens of Brenner in place of the identifier in the first domain of the polynucleotide strand of Kanda.

As discussed above, Kanda teaches a polynucleotide strand comprises an identifier. Brenner teaches a unique multiplex identifier in each polynucleotide strand

and further teaches that it can be added at any location. One having ordinary skill in the art would recognize that the step of the first domain comprising the multiplex identifier in each polynucleotide strand is obvious over Kanda in view of Brenner because Brenner teaches that the multiplex identifier can be added at any location including the first domain of Kanda.

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the unique multiplex identifier comprising a plurality of sequence tokens of Brenner in place of the identifier in the first domain of the polynucleotide strand of Kanda with a reasonable expectation of success with the expected benefit of identifying the polynucleotide of interest in the sample as taught by Brenner (paragraph 0004). An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including the multiplex identifier in the first domain of Kanda which is routinely practiced in the art as exemplified by Brenner.

Conclusion

10. No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to NARAYAN BHAT whose telephone number is (571)272-5540. The examiner can normally be reached on 8.30 am to 5 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on (571)-272-0731. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Narayan K. Bhat/
Examiner, Art Unit 1634

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	15	(osborne.in. or brenner.in. or mikawa.in. or slatter.in. or population.as.) and intramolecular	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/09 17:53
L2	3	l1 and intramolecular near3 rearrang$5	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/09 17:54
L7	1141	(identifier or barcode or "bar code" or zipcode) and (moving or transport$) near3 domain	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/09 17:57
L9	113	l7 and (identifier or barcode or "bar code" or zipcode) same (distinguish$3 or differentiat$4 or identif$6) same (polynucleotide or dna or nucleic)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/09 17:59
L10	6	(osborne.in. or brenner.in. or mikawa.in. or slatter.in. or population.as.) and reflex adj sequence	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/09 18:04
L11	2	l10 and reflex adj sequence.clm.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/09 18:05
L12	263	($5probe or oligonucleotide) near5 complementary near5 (section or segment or region) adj "3'"	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/09 18:14
L13	131	l12 and (extend$3 or elongat$3) near3 "3'"	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/09 18:15

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EAST Search History

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Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L16	81	hairpin adj adapt$4	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/10 12:35
L17	55	l16 and (anneal$3 or bind$3) near5 single near3 strand$3	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/10 12:37

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CONFIRMATION NO. 3537

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
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APPLICANTS

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Andrew Slatter, London, UNITED KINGDOM;

/NKB/

**** CONTINUING DATA ***************************

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which claims benefit of 61/288,792 12/21/2009
and claims benefit of 61/235,595 08/20/2009

/NKB/

**** FOREIGN APPLICATIONS ***************************

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Foreign Priority claimed ☐ Yes ☑ No	☐ Met after Allowance	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
35 USC 119(a-d) conditions met ☐ Yes ☐ No		UNITED KINGDOM	13	18	1
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PubMed loop mediated isothermal amplification

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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	13387343	BRENNER ET AL.
	Examiner	Art Unit
	NARAYAN BHAT	1634

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Searched for the inventors and assignee in PALM for the ODP.	5/6/12	Bhat N.
Searched for the prior arts in the filed IDS	5/6/12	Bhat N.
Searched for the topics in East and Pubmed. Search histories are enclosed.	5/6/12	Bhat N.

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	12637	osborne.in. or brenner.in. or mikawa.in. or slatter.in. or population.as.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/06 19:10
L2	17	l1 and reflex$5	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/06 19:10
L3	6	l2 and reflex adj sequence	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/06 19:11
L4	11	reflex adj sequence	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/06 19:40
L5	239	multiplex near3 (identifier or barcode or "bar code" or zipcode)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/06 19:42
L6	46	l1 and moving near3 (domain or region or segment or section)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/06 19:43
L7	1	l6 and primer and ($5nucleotide or dna or nucleic)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/06 19:44
L8	1867	multiplex same(identifier or barcode or "bar code" or zipcode)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/06 19:45
L9	200	l8 and primer and ($5nucleotide or dna or nucleic)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/06 19:45
L10	46	l9 and multiplex same(identifier or barcode or "bar code" or zipcode) same sequencing	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/05/06 19:46

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
13/387,343	02/15/2012	Sydney Brenner	CGI.C-020

CONFIRMATION NO. 3537

24353
BOZICEVIC, FIELD & FRANCIS LLP
1900 UNIVERSITY AVENUE
SUITE 200
EAST PALO ALTO, CA 94303

PUBLICATION NOTICE

OC00000054564715

Title: Compositions and Methods for Intramolecular Nucleic Acid Rearrangement

Publication No.US-2012-0135407-A1
Publication Date:05/31/2012

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Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/387,343	02/15/2012	Sydney Brenner	CGI.C-020	3537

24353 7590 07/17/2012
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EXAMINER	
BHAT, NARAYAN KAMESHWAR	
ART UNIT	PAPER NUMBER
1634	

MAIL DATE	DELIVERY MODE
07/17/2012	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

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Applicant-Initiated Interview Summary	Application No.	Applicant(s)	
	13/387,343	BRENNER ET AL.	
	Examiner	Art Unit	
	NARAYAN BHAT	1634	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Examiners: Bhat & Kapushoc*.

(2) *SPE: Nguyen*.

(3) *Representatatives: Dr. Keddie and Men Kronick*.

(4)_____.

Date of Interview: *11 July 2012*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☒ Yes ☐ No.
If Yes, brief description: *Drawings to illustrate the invention of claim 1 and an agenda for the discussion purpose only* .

Issues Discussed ☐101 ☐112 ☐102 ☒103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *1*.

Identification of prior art discussed: *Kanda*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

The representative summarized the invention of claim 1 and the structural features of the polynucleotide and presented arguments Kanda does not specifically teach step 'c' of sequencing the MID and the desired site using a primer that complementary to the primer site in the complement of the first domain. The Examiner acknowledged that Kanda does not specifically teach the primer that complementary to the primer site in the complement of the first domain. The Examiners also suggested to file the response regarding the teachings of Kanda and any amendments to the claims further clarifying the MID, which would be taken into the consideration in making the decision to withdraw Kanda's reference or maintain the rejection. No specific claim language was agreed upon during the interview .

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/Stephen Kapushoc/ Primary Examiner, Art Unit 1634	/Narayan K. Bhat/ Examiner, Art Unit 1634

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Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted.
2) an identification of the claims discussed.
3) an identification of the specific prior art discussed.
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner.
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

SUBMITTED ELECTRONICALLY JULY 25, 2012

RESPONSE TO OFFICE ACTION Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Docket No.	CGLC-020
	Application No.	13/387,343
	Confirmation No.	3537
	Filing Date	February 15, 2012
	Examiner	N.K. Bhat
	Group Art Unit	1634
	Inventor	Brenner

Sir:

This communication is responsive to the Office Action dated May 16, 2012, for which a three month period for response was given, making this amendment due by August 16, 2012. As such, this response is timely filed.

AMENDMENTS TO THE CLAIMS

Please amend the claims as shown below. A complete listing of the claims in this case, including their current status identifier, is shown below.

1. (Currently Amended) A method of sample analysis, comprising:

a) annealing a reflex sequence in a polynucleotide strand in a sample with a complement of the reflex sequence present in the polynucleotide strand, wherein the polynucleotide strand comprises the following in a 5' to 3' orientation:

a first domain comprising i. ~~a multiplex identifier (MID) whose identity can be used to differentiate polynucleotides in the sample, and ii.~~ a primer site **and ii. a multiplex identifier (MID) comprising a unique nucleotide sequence that distinguishes said polynucleotide strand from other polynucleotide strands in said sample**;

the reflex sequence;

a polynucleotide of interest having a desired site that is distal to the first domain; and

the complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand;

b) extending from the 3' end of the complement of the reflex sequence through the first domain to produce a double stranded region comprising a complement of the first domain at the 3' end of the extended polynucleotide strand, thereby moving the first domain into proximity with the desired site; and

c) sequencing the MID and the desired site using a primer that complementary to the primer site in the complement of the first domain.

2-3 (Canceled)

4. (Original) The method of claim 1, wherein the reflex sequence is from 10 to 50 nucleotides in length.

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5. (Previously presented) The method of claim 1, wherein the first domain comprises an RNA polymerase site.

6. (Original) The method of claim 1, the method further comprising removing the first domain and the reflex sequence from the 5' end of the double stranded region.

7. (Original) The method of claim 6, wherein the removing step comprises treating the nucleic acid of step b with T7 exonuclease.

8. (Original) The method of claim 1, wherein the first domain is present in an adapter region attached to the polynucleotide of interest.

9. (Original) The method of claim 8, wherein the reflex sequence is present in the adapter region.

10. (Original) The method of claim 8, wherein the reflex sequence is a sequence present within the 5' region of the polynucleotide of interest.

11. (Original) The method of claim 1, wherein the sample is a multiplexed sample, wherein the first domain in each of the polynucleotide strands in the multiplexed sample comprises a MID corresponding to its parental sample.

12. (Original) The method of claim 1, wherein the sample comprises multiple polynucleotide strands, and wherein each polynucleotide strand in the sample comprises a unique MID in the first domain.

13. (Original) The method of claim 1, wherein the polynucleotide strand is produced by:

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annealing a nucleic acid synthesis primer to a parent polynucleotide strand, wherein the parent polynucleotide strand comprises the following in a 5' to 3' orientation:

the first domain;

the reflex sequence; and

a second domain comprising the polynucleotide of interest;

wherein the nucleic acid synthesis primer comprises:

a 3' priming domain that is complementary to a site adjacent to and 3' of the polynucleotide of interest in the second domain; and

a 5' reflex sequence;

extending the nucleic acid synthesis primer to produce an extension product; and

replicating the resultant extension product with a second nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain, thereby producing the polynucleotide strand.

14. (Original) The method of claim 13, wherein the length of the second domain is from 30 bases to 30 kilobases.

15. (Original) The method of claim 13, wherein the parent polynucleotide strand is enriched prior to the annealing step based on its sequence.

16. (Original) The method of claim 1, further comprising the steps of:

c) replicating the polynucleotide strand of step b with a first nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain to produce a first extension product;

d) annealing a second nucleic acid synthesis primer to the first extension product, wherein the second nucleic acid synthesis primer comprises a 3' priming domain that is complementary to a site within the polynucleotide of interest in the first extension product; and

e) extending the second synthesis primer to produce a second extension product.

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17. (Original) The method of claim 16, wherein the second synthesis primer comprises a 5' domain that is not complementary to the polynucleotide of interest in the first extension product.

18. (Original) The method of claim 17, wherein the first domain comprises a first primer site and the 5' domain of the second synthesis primer comprises a second primer site.

19. (Original) The method of claim 18, wherein the length of the polynucleotide of interest in the second extension product is from 50 to 10,000 nucleotides.

20. (Withdrawn) A kit comprising:

a polynucleotide strand comprising the following in a 5' to 3' orientation:

a first domain;

a reflex sequence;

a polynucleotide of interest; and

a complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand; and

instructions for performing the method of claim 1.

21. (Previously presented) The method of claim 1, wherein polynucleotide of interest is cDNA.

22. (Previously presented) The method of claim 1, further comprising repeating said method to obtain the nucleotide sequence of a second desired site in said polynucleotide of interest, and analyzing the sequence obtained in c) and the sequence of the second desired site to obtain linkage information about polymorphisms in said polynucleotide of interest.

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REMARKS

Formal Matters

Claims 1 and 4-22 are pending.

Claims 1, 4-19, 21 and 22 were examined and rejected. Claim 20 was withdrawn by the Examiner.

Claim 1 is amended to clarify the order of the primer binding site and the MID. Support for this amendment is found in Fig. 6A, for example. Claim 1 has also been amended to clarify that the MID comprises a unique nucleotide sequence that distinguishes said polynucleotide strand from other polynucleotide strands in the sample, support for which can be found on page 8. No new matter is added.

Reconsideration of this application is requested in view of the comments that follow below.

Interview Summary

Examiners Bhat, Kapushoc and Ngyuen are thanked for their time in a productive interview with the undersigned and Mel Kronick (from the assignee) on July 11, 2012.

During the interview, the undersigned explained what is being claimed. The undersigned also explained the differences between the claimed method and the method described in Kanda's publication (US2007/0238113).

Applicants argued that Kanda does not teach or suggest step (c) of the rejected claims, i.e., "sequencing the MID and the desired site using a primer that complementary to the primer site in the complement of the first domain". The Examiners collectively agreed that Kanda does not teach such a step. Examiner Bhat then argued that selecting a primer that is complementary to the primer site would be an arbitrary choice for one of skill in the art. The Examiner also indicated that he could likely find a secondary reference to provide the elements that are missing from Kanda. The Applicants countered by arguing that, at a technical level, one of skill would not design a sequencing primer that hybridizes to the stem of a hairpin (such as that described by Kanda) because the hairpin would compete for primer binding and the end of hairpin would provide a second priming site for sequencing. Thus, even if Kanda

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could be combined with a secondary reference that teaches primers and/or primer binding sites, one of skill in the art would still not be lead to the claimed method. Examiner Kapushoc indicated that he agreed with this point.

The Examiner's interview summary of July 17, 2012, is essentially correct.

The arguments presented below are consistent with those presented during the interview. Withdrawal of the §103 rejections is requested.

Rejections under § 103

Claims 1, 4-11 and 13-22 are rejected under 35 U.S.C. § 103 as being obvious over Kanda (US2007/0238113). The Applicants respectfully traverse this rejection.

In order to meet its burden in establishing a rejection under 35 U.S.C. §103, the Office must first demonstrate that a prior art reference, or references when combined, teach or suggest all claim elements. *See, e.g., KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1740 (2007); *Pharmastem Therapeutics v. Viacell et al.*, 491 F.3d 1342, 1360 (Fed. Cir. 2007); MPEP § 2143(A)(1). In addition to demonstrating that all elements were known in the prior art, the Office must also articulate a reason for combining the elements. *See, e.g., KSR* at 1741; *Omegaflex, Inc. v. Parker-Hannifin Corp.*, 243 Fed. Appx. 592, 595-596 (Fed. Cir. 2007). Further, the Supreme Court in *KSR* also stated that that "a court *must* ask whether the improvement is more than the predictable use of prior art elements according to their established functions." (emphasis added) *See KSR* at 1740. Therefore, in addition to showing that all elements of a claim were known in the prior art and that one of ordinary skill in the art had reason to combine them, the Office must also provide evidence that the combination would have a reasonable expectation of success.

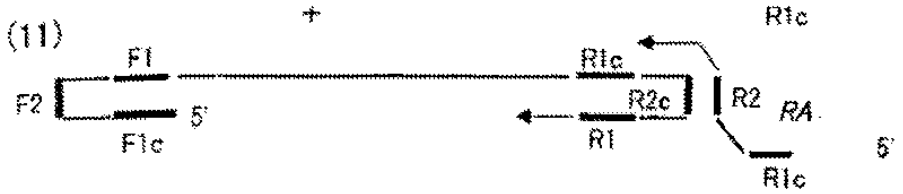
The Examiner has not shown that all elements of Claim 1 were known in the art

Kanda describes a method for amplifying a segment of DNA. This method is generally described in Kanda's Figs. 2-4. In ¶ 306, Kanda states that "the amplified nucleic acid was confirmed by sequence to have the nucleotide sequence of the CYP2C19 gene".

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As best understood from the Office Action, the Examiner believes that amplification product 11 shown at the bottom of Kanda's Fig. 4, combined with Kanda's suggestion to sequence the amplified product, renders the claims obvious.

Amplification product 11, as shown at the bottom of Kanda's Fig. 4, is shown below for reference:



The Applicants submit that Kanda does not teach step (c) of the claimed method.

Step (c) of claim 1 is not a generic sequencing step in which any region of the product of step (b) is sequenced using any primer. Rather step (c) requires that a specific region of the product of step (b) is sequenced using a specific primer. Specifically, step (c) of claim 1 recites "sequencing the MID and the desired site using a primer that complementary to the primer site in the complement of the first domain".

Kanda does not teach a primer that is complementary to the primer site (which primer would be complementary to the complement of region F1c in the above figure). Nor does Kanda teach sequencing the MID (which is alleged by the Examiner as being the same as region F2 in the above figure) and the desired site (which is alleged by the Examiner as being the same as region R2c in the above figure). Indeed, there would be no point in Kanda sequencing regions F2 and R2c because they are primer sequences.

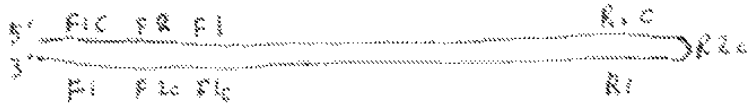
In view of the foregoing discussion, the Applicants submit that Kanda fails to teach or suggest step (c) of claim 1. Thus, this rejection should be withdrawn.

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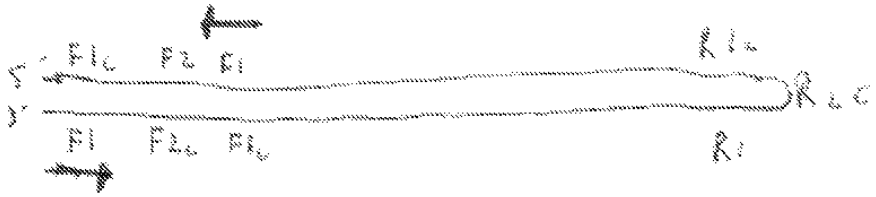
One of skill in the art would not design a sequencing primer that hybridizes to the stem of Kanda's hairpin

To the extent that the Examiner believes that he can fill the void in the teachings of Kanda with a secondary reference, the Applicants submit that one of skill in the art would not be able to sequence Kanda's molecule 11 using a primer that is complementary to the complement of region F1c.

One of skill in the art would not design a sequencing primer that hybridizes to F1 (i.e., a primer that is complementary to the complement of F1c) of molecule 11 because extension from the 3' end of molecule 11 shown above would produce the following nucleic acid:



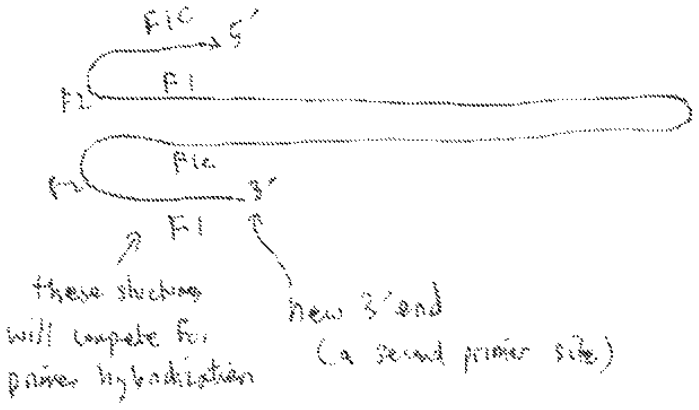
Hybridization of a primer that is complementary to the complement of region F1c to such a molecule would result in a primer hybridizing to *two* sites, where the sites in the illustration shown below are indicated by the arrows.



Such an oligonucleotide would not be used for sequencing Kanda's extension product because it hybridizes to two sites in the target molecule.

Further, as illustrated below, the intramolecular hairpins created by hybridization of F1c and F1 would compete with primer hybridization and/or create a new 3' end, either one of which would interfere with sequencing.

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In view of the foregoing discussion, the Applicants submit that Kanda fails to provide each and every element of the rejected claims. Moreover, because Kanda's molecule 11 has a hairpin at its 5' end, i.e., at its left-hand end, one of skill in the art would not design a sequence primer in that region. Thus, a *prima facie* case of obviousness has not been established, and this rejection may be withdrawn.

Withdrawal of this rejection is respectfully requested.

Claims 1 and 12 are rejected under 35 U.S.C. § 103 as being obvious over Kanda (US2007/0238113) in view of Brenner (US2007/0259357). The Applicants respectfully traverse this rejection.

Kanda is deficient for the reasons outlined above.

The Applicants submit that Brenner fails to meet Kanda's deficiency and, as such, these references, taken in any combination, cannot render the claims of this case obvious.

Withdrawal of this rejection is therefore requested.

Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees up to a strict limit of $3,000.00 beyond that authorized on the credit card, but not more than $3,000.00 in additional fees due

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with any communication for the above referenced patent application, including but not limited to any necessary fees for extensions of time, or credit any overpayment of any amount to Deposit Account No. 50-0815, order number CGLC-020.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: July 25, 2012

By: /James S. Keddie, Reg. No. 48,920/
James S. Keddie, Ph.D.
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Electronic Acknowledgement Receipt	
EFS ID:	13336415
Application Number:	13387343
International Application Number:	
Confirmation Number:	3537
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	24353
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020
Receipt Date:	25-JUL-2012
Filing Date:	15-FEB-2012
Time Stamp:	17:30:49
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		CGLC-020_Rsp2OA.pdf	334052	yes	11

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Claims	2	5
	Applicant Arguments/Remarks Made in an Amendment	6	11
Warnings:			
Information:			
	Total Files Size (in bytes):	334052	

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

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PTO/SB/06 (07-06)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 13/387,343	Filing Date 02/15/2012	☐ To be Mailed

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☒		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT 07/25/2012	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	* 20	Minus	** 20	= 0	X $30 =	0	OR	X $ =	
Independent (37 CFR 1.16(h))	* 2	Minus	***3	= 0	X $125 =	0	OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
☐ Application Size Fee (37 CFR 1.16(s))									
☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
					TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/BRENDA HINES/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/387,343	02/15/2012	Sydney Brenner	CGI.C-020	3537

24353 7590 08/09/2012
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EXAMINER	
BHAT, NARAYAN KAMESHWAR	
ART UNIT	**PAPER NUMBER**
1634	

MAIL DATE	DELIVERY MODE
08/09/2012	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00802

Office Action Summary	Application No.	Applicant(s)	
	13/387,343	BRENNER ET AL.	
	Examiner	Art Unit	
	NARAYAN BHAT	1634	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *25 July 2012*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

5)☒ Claim(s) *1 and 4-22* is/are pending in the application.

5a) Of the above claim(s) *20* is/are withdrawn from consideration.

6)☐ Claim(s) _____ is/are allowed.

7)☒ Claim(s) *1,4,5,8-12,16-18 and 21* is/are rejected.

8)☒ Claim(s) *6,7,13-15, 19 and 22* is/are objected to.

9)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☒ The drawing(s) filed on *26 January 2012* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

12)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00803

FINAL ACTION

1. This office action is written in response to the papers filed on July 25, 2012. The amendments to independent claim 1 requiring a polynucleotide comprising in 5' to 3' orientation a fist domain further comprising a primer site followed by a MID comprising a unique nucleotide sequence that distinguishes the polynucleotide strand form other polynucleotide strands in the sample changing the scope of the independent claim 1 necessitated the new grounds of rejection presented in this office action. Accordingly, ***this action is made final.***

Claim Status

2. Claims 1 and 4-22 are pending in this application. Claim 1 is amended. The amendments have been reviewed and entered. No new matter has been introduced by the claim amendments. The arguments filed on July 25, 2012 have been fully considered and are addressed below.

3. Claim 20 is withdrawn from further consideration pursuant to 37 CFR 1.142(b), as being drawn to a nonelected invention of group II there being no allowable generic or linking claim. Applicant timely traversed the restriction (election) requirement in the reply filed on April 30, 2012.

4. Claims 1, 4-19, 21 and 22 are under prosecution.

Interview Summary

5. The Examiner acknowledges the interview summary filed by the Applicant is essentially correct and appreciates the acknowledgement of the interview summary filed by the Examiner (Remarks, pg. 7).

Withdrawn Rejections and Response to the Remarks

6. The previous rejection of claims 1, 4-11, 13-19, 21 and 22 under 35 USC 103(a) as being unpatentable over Kanda has been withdrawn in view of the persuasive arguments made by the Applicant. The arguments filed on July 25, 2012 with respect to the said have been fully considered and are persuasive (Remarks, pgs. 7-9). The said rejection based on teachings of Kanda has been withdrawn.

7. The previous rejection of claims 1 and 12 under 35 USC 103(a) as being unpatentable over Kanda in view of Brenner has been withdrawn in view of the persuasive arguments made by the Applicant regarding the teachings of Kanda. The arguments with respect to the teachings of Kanda have been fully considered and are persuasive (Remarks, pg. 10). The said rejection based on teachings of Kanda has been withdrawn.

Claim Interpretation

8. The method of claim 1 is dawn to the steps of moving a domain in a single stranded nucleic acid to a different position by using a sequence, termed "reflex sequence" and its complement; the complement of the reflex sequence is then

extended to have the complement of the domain, thereby moving the first domain into the proximity with the desired site. The method of claim 1 also requires a polynucleotide of interest having a desired site that is distal to the first domain.

However, instant claim 1 as recited does not require that the polynucleotide of interest be a different nucleic acid from the polynucleotide strand. Therefore the polynucleotide of interest is interpreted as to encompass a site that is distal to the first domain.

Also, the instant specification does not provide a limiting definition for the reflex sequence. Thus, the claim has been given the broadest reasonable interpretation consistent with the teachings of the specification regarding the reflex sequence (In re Hyatt, 211 F.3d1367, 1372, 54 USPQ2d 1664, 1667 (Fed. Cir. 2000) (see MPEP 2111 [R-1]). Given the broadest reasonable interpretation the reflex sequence is interpreted as to encompass two complementary sequence within the polynucleotide strand meeting its structural requirement.

The limitation of the multiplex identifier comprising a unique nucleotide sequence that distinguishes the polynucleotide strand for other strand has been interpreted broadly to encompass any sequence that distinguishes one polynucleotide from the other polynucleotide strand.

Also the limitation of "thereby" moving the first domain into proximity with the desired site of instant claim 1 is interpreted as the result of extending from 3' end of the complement of the reflex sequence because "thereby" clause is not given weight

because it simply express the intended result of a positively recited extension step from the 3' end.

The following rejection necessitated by the amendments to claim 1.

Claim Rejections - 35 USC § 102

9. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

10. Claims 1, 4, 5, 8-12 and 16-18 are rejected under 35 U.S.C. 102(b) as being anticipated by Laird et al (PNAS, 2004, 1010, 204-209). The RNA promoter binding to an AT rich sequence present in the polynucleotide strand of Laird as applied to claim 5 is further evidenced by Imburgio et al (Biochemistry, 2000, 39, 10419-10430).

Laird teaches a method for analyzing the patterns of cytosine methylation in the individual DNA molecules , which meets the limitations of the steps of amended claim 1 as discussed in detail below.

Regarding claim 1 of step 'a' of annealing a reflex sequence in a polynucleotide strand n a sample with a complement of the reflex sequence present in the polynucleotide strand, it is noted that the instant specification recites "reflex sequence" and equivalents are used to indicate sequences in a polynucleotide that are employed to move a domain intramolecularly from its initial location to a different location in the polynucleotide (pg. 13). Thus the teaching of the prior art of a polynucleotide strand

comprising complementary region at two different ends forming a double stranded region meets the limitation of reflex sequence because by forming a double stranded region moves sequence from its initial location in a polynucleotide strand to a different location in the polynucleotide.

Regarding claim 1, Laird teaches a hairpin oligonucleotide (i.e., a polynucleotide) comprising CAGGAATTC sequence at its 5' end and its complement sequence GAATTCCTG sequence at the 3' end forming a duplex region (Fig. 1 and Panel A). The CAGGAATTC sequence of Laird meets the limitation of the reflex sequence as recited in the instant specification because the hairpin oligonucleotide in its linear form comprises CAGGAATTC and its complement GAATTCCTG at different locations on the polynucleotide but the complement GAATTCCTG is moved to a different location in the polynucleotide after forming the duplex. One having ordinary skill in the art would recognize that the formation of the duplex region between the CAGGAATTC and GAATTCCTG meets the limitation of annealing step because forming the said duplex requires an annealing step.

Regarding the first domain, Laird teaches that the hairpin polynucleotide comprises the following in a 5' to 3' orientation: a segment 5'GAAGGGCG..... CAGGAATTC comprises a primer site (e.g., as shown in Fig. 1, panel D on the complementary strand) and a CGCCCATTT sequence (Fig.1 panel A, see the legend) that is unique to the hairpin polynucleotide capable of distinguishing other nucleic acid (e.g., nucleic acid sequence in Fig. 2 panel C). The instant specification recites that the multiplex Identifier as used herein refers to a tag or combination of tags associated with

a polynucleotide whose identity (e.g., the tag DNA sequence) can be used to differentiate polynucleotides in a sample (pg. 7, last paragraph). One having ordinary skill in the art would recognize that the CGCCCATTT sequence of Laird meets the limitation of broadly recited multiplex identifier because CGCCCATTT sequence can be used as the tag to differentiate the hairpin polynucleotide in the sample. The segment 5'GAAGGGCG..... CAGGAATTC of Laird is the first domain because as discussed above in the claim interpretation section it comprises the structural features of the broadly recited first domain.

Regarding the reflex and the complement of the reflex sequence, Laird teaches CAGGAATTC sequence (i.e., the reflex sequence) and the complement GAATTCCTG sequence (i.e., the complement of the reflex sequence), wherein the complement of the reflex sequence GAATTCCTG is present at the 3' end of the hairpin polynucleotide strand (Fig. 1 and Panel A).

Regarding a polynucleotide of interest having a desired site, Laird teaches an loop region distal to the 5'GAAGGGCG..... CAGGAATTC domain (i.e., the first domain) in the hairpin polynucleotide strand and comprises the second T site, which meets the limitation of the polynucleotide strand of interest with the desired site as discussed in the claim interpretation section.

Regarding step 'b' of extending from 3' end, Laird teaches extending from the 3' end of the GAATTCCTG sequence (i.e., the 3' end of the complement reflex sequence) through the first domain to produce a double stranded region comprising a complement of the first domain at the 3'end of the extended hairpin polynucleotide strand (Fig. 1 and

Panel B, see the legend). As discussed above, the step of extending the 3' end of the complement reflex sequence meets the limitation of thereby moving the first domain (5'GAAGGGCG..... CAGGAATTC domain) into the proximity with the desired site second T in the loop because "thereby" clause is not given weight because it simply express the intended result of a positively recited extension step from the 3' end.

Regarding step 'c' of sequencing the MID and the desired site, Laird teaches the sequencing of the extended product using primers complementary to the primer site in the complement of the first domain and sequencing the entire polynucleotide (Fig. 1, Panels C and D, see the primer above the complementary strand, pg. 205, column 1, paragraph 1 and column 2, paragraph 2), which includes the MID and the desired site.

It is noted that Laird teaches sequencing of the complete hairpin polynucleotide by amplifying the polynucleotide and sequencing the PCR product (pg. 205 column 1, paragraph 1 and column 2, paragraph 2). However, instant claim 1 recited with open claim language "comprising" can include additional steps as long as it includes the steps that are claimed.

It is noted that the MPEP 2111.03 makes it clear that the transitional term "comprising" is open-ended and does not exclude additional, unrecited elements (See, e.g., > Mars Inc. v. H.J. Heinz Co., 377 F.3d 1369,1376, 71 USPQ2d 1837, 1843 (Fed. Cir. 2004). Also the term "comprising" is a term of art used in claim language which means that the named elements are essential, but other elements may be added and still form a construct within the scope of the claim (Genentech, Inc. v. Chiron Corp., 112 F.3d 495, 501, 42 USPQ2d 1608, 1613 (Fed. Cir. 1997). In the instant case, as

discussed above, Laird teaches sequencing the MID and the desired site using a primer that is complementary to the primer site in the complement of the first domain, which meet the limitation of step 'c'.

Regarding claim 4, laird teaches the "TCAAGCGATCG" sequence is 10 nucleotide in length (Fig. 2C), which meets the limitation of the reflex sequence as discussed while rejecting claim 1. The reflex sequence (i.e., the "TCAAGCGATCG" sequence) of Laird is in the range from about 10 to 50 nucleotides in length because it is 10 nucleotides long.

Regarding claim 5, Laird teaches that the first domain (5'GAAGGGCG..... CAGGAATTC domain) comprises GC rich region followed by AT rich region (e.g., AACGCCCATTT region; Fig. 1A). The AT rich region followed GC rich region comprises T7 RNA polymerase binding site as evidenced by Imburgio (Fig. 1).

Regarding claims 8 and 9, as discussed above while rejecting claim 1, Laird teaches the hairpin oligonucleotide comprises the reflex region within the 5' region (Fig. 1A), which meets the broadly recited "within the 5' region" without further specifying the distance in between. The hairpin polynucleotide of Laird also meets the limitation of first domain is in the adaptor region attached to the polynucleotide of interest because laird teaches adding hairpin linker (i.e., the adaptor) to the FMR polynucleotide of interest (Fig. 2).

Regarding claim 10, as discussed above, while rejecting claim 1, Laird teaches that the CAGGAATTC sequence (i.e., the reflex sequence) is at the 5' end of the TTTT

sequence (i.e., polynucleotide sequence of interest; Fig. 1A), which meets the limitation of reflex sequence is within (i.e., includes) the 5' region of the polynucleotide of interest.

Regarding claims 11 and 12, as discussed above, while rejecting claim 1, Laird teaches amplifying the extended product by PCR (pg. 205, column 1, paragraph 1 and column 2, paragraph 2). The amplification by PCR generates many copies of the same molecules thereby meeting the limitation of a multiplexed sample because it comprises multiple molecules of the sample and retains MID of the parental sample or multiple polynucleotide strands comprising the MID of the first domain.

Regarding claims 16-18, it is noted limitations are directed amplification of the extended product using appropriate primers to generate the amplified product includes the second extension product. As discussed above while rejecting claim 1, Laird teaches amplifying the extension products using primer that anneals at the 3' end of the complement of the first domain to produce extension product (Fig. 1B) and annealing second primer to produce second extension product (Fig. 1D). The teachings of the second primer meets the limitation of the second synthesis primer comprises a 5' domain that is not complementary to the polynucleotide of interest in the first extension product because primer is not complementary to the TTTT nucleotides (Fig. 1). The teachings of the first and the second primer location of Laird also meets the limitation of the first domain comprises a first primer site and the 5' domain of the second synthesis primer comprises a second primer site because the locations are different (Fig. 1).

Claim Rejections - 35 USC § 103

11. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

12. This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

13. Claims 1 and 21 are rejected under 35 U.S.C. 103(a) as being unpatentable over Laird et al (PNAS, 2004, 1010, 204-209) in view of Ushijima (Nature Reviews, 2005, 5, 223-231).

Laird and Ushijima teach a method for assaying methylation pattern and therefore are analogous arts. The teachings of Ushijima are applied for the specific teachings of cDNA polynucleotide for evaluating the effect of methylation on the transcription.

Claim 21 is dependent from claim 1.

Regarding claim 1 of step 'a' of annealing a reflex sequence in a polynucleotide strand n a sample with a complement of the reflex sequence present in the polynucleotide strand, it is noted that the instant specification recites "reflex sequence" and equivalents are used to indicate sequences in a polynucleotide that are employed to move a domain intramolecularly from its initial location to a different location in the polynucleotide (pg. 13). Thus the teaching of the prior art of a polynucleotide strand comprising complementary region at two different ends forming a double stranded region meets the limitation of reflex sequence because by forming a double stranded region moves sequence from its initial location in a polynucleotide strand to a different location in the polynucleotide.

Laird teaches a hairpin oligonucleotide (i.e., a polynucleotide) comprising CAGGAATTC sequence at its 5' end and its complement sequence GAATTCCTG sequence at the 3' end forming a duplex region (Fig. 1 and Panel A). The CAGGAATTC sequence of Laird meets the limitation of the reflex sequence as recited in the instant specification because the hairpin oligonucleotide in its linear form comprises CAGGAATTC and its complement GAATTCCTG at different locations on the polynucleotide but the complement GAATTCCTG is moved to a different location in the polynucleotide after forming the duplex. One having ordinary skill in the art would recognize that the formation of the duplex region between the CAGGAATTC and GAATTCCTG meets the limitation of annealing step because forming the said duplex requires an annealing step.

Regarding the first domain, Laird teaches that the hairpin polynucleotide comprises the following in a 5' to 3' orientation: a segment 5'GAAGGGCG..... CAGGAATTC comprises a primer site (e.g., as shown in Fig. 1, panel D on the complementary strand) and a CGCCCATTT sequence (Fig.1 panel A, see the legend) that is unique to the hairpin polynucleotide capable of distinguishing other nucleic acid (e.g., nucleic acid sequence in Fig. 2 panel C). The instant specification recites that the multiplex Identifier as used herein refers to a tag or combination of tags associated with a polynucleotide whose identity (e.g., the tag DNA sequence) can be used to differentiate polynucleotides in a sample (pg. 7, last paragraph). One having ordinary skill in the art would recognize that the CGCCCATTT sequence of Laird meets the limitation of broadly recited multiplex identifier because CGCCCATTT sequence can be used as the tag to differentiate the hairpin polynucleotide in the sample. The segment 5'GAAGGGCG..... CAGGAATTC of Laird is the first domain because as discussed above in the claim interpretation section it comprises the structural features of the broadly recited first domain.

Regarding the reflex and the complement of the reflex sequence, Laird teaches CAGGAATTC sequence (i.e., the reflex sequence) and the complement GAATTCCTG sequence (i.e., the complement of the reflex sequence), wherein the complement of the reflex sequence GAATTCCTG is present at the 3' end of the hairpin polynucleotide strand (Fig. 1 and Panel A).

Regarding a polynucleotide of interest having a desired site, Laird teaches an loop region distal to the 5'GAAGGGCG..... CAGGAATTC domain (i.e., the first

domain) in the hairpin polynucleotide strand and comprises the second T site, which meets the limitation of the polynucleotide strand of interest with the desired site as discussed in the claim interpretation section.

Regarding step 'b' of extending from 3' end, Laird teaches extending from the 3' end of the GAATTCCTG sequence (i.e., the 3' end of the complement reflex sequence) through the first domain to produce a double stranded region comprising a complement of the first domain at the 3'end of the extended hairpin polynucleotide strand (Fig. 1 and Panel B, see the legend). As discussed above, the step of extending the 3' end of the complement reflex sequence meets the limitation of thereby moving the first domain (5'GAAGGGCG..... CAGGAATTC domain) into the proximity with the desired site second T in the loop because "thereby" clause is not given weight because it simply express the intended result of a positively recited extension step from the 3' end.

Regarding step 'c' of sequencing the MID and the desired site, Laird teaches the sequencing of the extended product using primers complementary to the primer site in the complement of the first domain and sequencing the entire polynucleotide (Fig. 1, Panels C and D, pg. 205, column 1, paragraph 1 and column 2, paragraph 2), which includes the MID and the desired site.

Regarding claim 21, Laird does not specifically teach that the polynucleotide of interest is cDNA.

However, the cDNA was known in the art at the time the claimed invention was made as taught by Ushijima.

Ushijima teaches a method for analysis of the methylation pattern and further teaches that the polynucleotide is cDNA (pg. 226, column 2, paragraph 3), further teaches that cDNA allows to identify the role of epigenetic modification in the transcriptional regulation of the genes, which are useful cancer diagnosis and prognosis (pg. 223, column 1, paragraph 1).

It would have been prima facie obvious to one of ordinary skill in the art at the time the claimed invention was made to include the cDNA of Ushijima in the method of Laird with a reasonable expectation of success with the expected benefit of analyzing the transcription pattern useful for cancer diagnosis and prognosis as taught by Ushijima (pg. 223, column 1, paragraph 1). An artisan having ordinary skill in the art would be motivated to include the cDNA analysis which would beneficial for Laird for correlating the methylation profile of the genome with a phenotypic profile of the genome as taught by Ushijima. An artisan having ordinary skill in the art would have reasonable expectation of success because it merely involves including cDNA and microarray steps which are routinely practiced in the art as exemplified by Ushijima .

Allowable Subject Matter

14. Claims 6, 7, 13-15, 19 and 22 are objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form including all of the limitations of the base claim and any intervening claims.

Conclusion

15. No claim is allowed.

16. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to NARAYAN BHAT whose telephone number is (571)272-5540. The examiner can normally be reached on 8.30 am to 5 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on (571)-272-0731. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Narayan K. Bhat/
Examiner, Art Unit 1634

/Stephen Kapushoc/
Primary Examiner, Art Unit 1634

Notice of References Cited	Application/Control No. 13/387,343	Applicant(s)/Patent Under Reexamination BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
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	J	US-			
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	O					
	P					
	Q					
	R					
	S					
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NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Laird et al, Hairpin-bisulfite PCR: Assessing epigenetic methylation patterns on complementary strands of individual DNA molecules, 2004, PNAS, 101, 204-209.
	V	Ushijima et al, Detection and interpretation of altered methylation patterns in cancer cells, 2005, Nature reviews, 5, 223-231.
	W	Imburgio et al, Studies of Promoter Recognition and Start Site Selection by T7 RNA Polymerase Using a Comprehensive Collection of Promoter Variants, 2000, Biochemistry, 39, 10419-10430.
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	200	hairpin near2 adapt$3	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/08/01 09:54
S2	0	S1 and loop near5 (identifier or zipcode or barcode)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/08/01 09:55
S3	5	S1 and loop same (identifier or zipcode or barcode)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/08/01 09:55
S4	4	S1 and (identifier or zipcode or barcode) near5 hairpin	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/08/01 09:56
S5	1273	(hairpin or stem adj loop) near5 (adapt$3 or linker)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/08/01 09:59
S6	7	S5 and (identifier or zipcode or barcode) near5 (hairpin or stem adj loop)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/08/01 10:00
S7	3	"7855536"	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/08/01 10:52
S8	3	"7923237"	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/08/01 10:58

8/3/2012 9:29:01 AM
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Isolating human transcription factor targets by coupling chromatin immunoprecipitation and CpG island microarray analysis [HTML] from cshlp.org
... It has been postulated, on the basis of E2F overexpression and **cDNA microarray** analysis, that ... T...; Nevins JR. (1995) Cellular targets for activation by the E2F1 **transcription** factor include ... Abstract. ... ; Huang TH-M...; Perry MR...; Laux DE. (1998) **Methylation** profiling of CpG islands in ...
Cited by 436 - Related articles - BL Direct - All 10 versions

Detection and interpretation of altered methylation patterns in cancer cells [PDF] from stanford.edu
... Markers of pancreatic cancer were identified by combination of 5-aza-dC treatment and **cDNA-microarray** analysis, and used ... Another investigation of cell lines with high and low transcriptional capacities also indicated that weak **transcription** leads to promoter **methylation** ...
Cited by 284 - Related articles - All 11 versions

Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells [PDF] from nature.com
... (c) **Methylation** analysis in ... For each gene, the **transcription** start site (arrow) and first exon (open box) are shown, and regions analyzed by bisulfite sequencing are ... RT-PCR was done on **cDNA** prepared from female fibroblasts and SW48 cells and treated with 5 M 5-aza-dC for 4 ...
Cited by 1264 - Related articles - BL Direct - All 26 versions

Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays
... previously (10) that treatment of these cell lines with 5Aza-dC (1 μm) for 4 days results in marked induction of several genes silenced by aberrant **methylation** without evidence for ... Labeled cRNA was synthesized from the purified **cDNA** by in vitro **transcription** reaction using ...
Cited by 215 - Related articles - BL Direct - All 7 versions

Methylation of histone H3 Lys 4 in coding regions of active genes [HTML] from nih.gov
Full View
... the more subtle influence of HDA1 deletion on acetylation and **transcription** and is ... After quantitation, the ratio between H3 Lys 4 **methylation** and H3 acetylation in ... mRNA expression profiles were determined by conventional **cDNA microarray** analysis, in duplicate, as described ...
Cited by 443 - Related articles - BL Direct - All 22 versions

Assessing gene significance from cDNA microarray expression data via mixed models [PDF] from gatech.edu
... Not a subscriber? Get started ... Assessing Gene Significance from **cDNA Microarray** Expression Data via Mixed Models ... ABSTRACT. The determination of a list of differentially expressed genes is a basic objective in many **cDNA microarray** experiments. ...
Cited by 291 - Related articles - BL Direct - All 20 versions

Alterations of gene expression during colorectal carcinogenesis revealed by cDNA microarrays after laser-capture microdissection of tumor tissues and normal ...
... five up-regulated and five down-regulated) by semiquantitative reverse **transcription**-PCR using the ... including loss of heterozygosity, inactivation of important genes by **methylation** (7) or ... Hence, we have recruited a newly developed technique, the **cDNA microarray**, that provides ...
Cited by 284 - Related articles - BL Direct - All 7 versions

The histone modification pattern of active genes revealed through genome-wide chromatin analysis of a higher eukaryote [HTML] from nature.com
... To ensure comparability, we hybridized the same ChIP samples to the chromosomal array that were used for the **cDNA** hybridizations. ... 6). Given our observed link between Lys 4 **methylation** and **transcription**, it will be interesting to determine if enriched intergenic ...
Cited by 504 - Related articles - BL Direct - All 22 versions

Identification of genes up-regulated by histone deacetylase inhibition with cDNA microarray and exploration of epigenetic alterations on hepatoma cells
... We conducted **cDNA microarray** analysis to screen for genes induced by histone deacetylase (HDAC ... mRNA expression of several genes was examined by reverse **transcription**-polymerase chain ... Acetylated histones and **methylation** status in 5′CpG islands was assessed by ...
Cited by 63 - Related articles - All 8 versions

Inhibition of DNA methyltransferase stimulates the expression of signal transducer and activator of transcription 1, 2, and 3 genes in colon tumor cells [HTML] from pnas.org
Full View
... observation that the expression of IFN-γ can be regulated by DNA **methylation** (40–42 ... measured by reverse **transcription**–PCR along with gapdh to confirm equivalent **cDNA** input. ... Because STAT **transcription** factors are effectors of IFN signaling (43), we next examined whether 5 ...

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Cited by 123 - Related articles - BL Direct - All 11 versions

Identification of genes associated with head and neck carcinogenesis by cDNA microarray comparison between matched primary normal epithelial and squamous ...
AM Al-Mulla, MA Abdulla, ... - Oncology ..., 2007 - Wiley Online Library
... of nine genes involved in cell-cell interaction, using Western blot and/or reverse **transcription** (R1)-PCR of ... **cDNA microarray**: gene expression head and neck cancer, ... by genetic and epigenetic changes including loss of heterozygosity, gene inactivation by **methylation**, and/or ...
Cited by 180 - Related articles - BL Direct - All 3 versions

[PDF] A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer [PDF] from 80.239.175.167
JG Suzuki, B Gonzalez, W Chen ... - Nature ..., 2002 - nature.com
... Here we have used **cDNA microarray** analysis to screen for genes that are epigenetically silenced ... aberrantly hypermethylated CpG islands in gene promoters, with loss of **transcription** of the ... Recognition of this promoter hyper- **methylation** has fostered a growing effort to screen ...
Cited by 640 - Related articles - View as HTML - All 8 versions

A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors [HTML] from aacrjournals.o
MF Fraga, M Herranz ... - Cancer research, 2004 - AACR
... the bisulfite genomic sequencing and **methylation**-specific PCR primers according to genomic sequences around presumed **transcription** start sites ... Primer sequences and PCR conditions for **methylation** analysis are included in supplementary web data ... Mouse **cDNA Microarray**. ...
Cited by 101 - Related articles - BL Direct - All 6 versions

[HTML] Analysis of gene expression profiles in pancreatic carcinoma by using cDNA microarray [HTML] from hindawi.com
XJ Yu, J Hong, SL Pei, GH Xiong, ... - Hepatobiliary Pancreatic Dis ..., 2004 - scholar.google.com
... The **microarray** of **cDNA** clones covers a wide range of genes and is suitable for gene screening in a large scale. ... [3-5] We conclude that promoter excessive **methylation** adjusts the **transcription** of anti-oncogene negatively, and MBD1 plays a vital role in this process. ...
Cited by 17 - Related articles - Direct - All 5 versions

Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription [PDF] from berkeley.edu
D Zilberman, M Gehring, RK Tran, T Ballinger, ... - Nature genetics, 2007 - nature.com
... frequently methylated, and we show a direct relationship between gene **methylation** and **transcription**. ... further illustrate the ancient and conserved functions of DNA **methylation** in eukaryotic ... into double-stranded DNA using the SuperScript Double-Stranded **cDNA** Synthesis ...
Cited by 451 - Related articles - BL Direct - All 16 versions

Coordinated genetic regulation of growth and lignin revealed by quantitative trait locus analysis of cDNA microarray data in an interspecific backcross of eucalyptus [HTML] from plantphysiol.o
M Kirst, AA Myburg, JPG De Leon, ME Kirst, ... - Plant ..., 2004 - Am Soc Plant Biol
... revealed that the expression of genes involved in lignin biosynthesis and associated **methylation** pathways is ... 6. Zhu Y, Wang X, Keeps JA, Kay SA (2000) Orchestrated **transcription** of key ... JE, Shenton E, Lee N, Quackenbush J (2003) A concise guide to **cDNA microarray** analysis ...
Cited by 132 - Related articles - BL Direct - All 13 versions

Identification of unknown target genes of human transcription factors using chromatin immunoprecipitation [PDF] from ucdavis.edu
NS Weinmann, ... - Methods, 2002 - Elsevier
... regions of DNA [1]. For example, the specific acetylation or **methylation** of the ... regulatory regions contained within the human genome, strategies to examine **transcription** factor–DNA ... is to couple the overexpression or underexpression of that factor to **cDNA microarray** analysis [3 ...
Cited by 305 - Related articles - BL Direct - All 19 versions

Genomic binding by the Drosophila Myc, Max, Mad/Mnt transcription factor network [HTML] from cshlp.org
A Orian, B van Steensel, J Delrow, ... - Genes & ..., 2003 - genesdev.cshlp.org
... 2A). DamID analysis of binding by GAF, a **transcription** factor unrelated to dMax network proteins ... profile data sets of the dMax network proteins derived from the entire **cDNA** array, ... diagram in Figure 2F. Previous work showed that significant and specific **methylation** by tethered ...
Cited by 283 - Related articles - BL Direct - All 15 versions

cDNA microarray analysis of gene expression during Fe-deficiency stress in barley suggests that polar transport of vesicles is implicated in phytosiderophore ...
T Negishi, H Nishizawa, J Ogawa, ... - The Plant ..., 2002 - Wiley Online Library
... is an enzyme in the pathway for tetrahydrofolate production, which mediates **methylation** of homocysteine ... In this scenario, diurnally regulated **transcription** of calreticulin may regulate vesicle ... Using a rice **cDNA microarray**, we demonstrated that methionine synthesis through the ...
Cited by 122 - Related articles - BL Direct - All 8 versions

Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen [HTML] from aacrjournals.o
M Widschwendter, KD Siegmund, PW Müller, ... - Cancer research, 2004 - AACR
... Before **cDNA** synthesis, RNA samples were treated with DNase to ensure removal of contaminating genomic DNA. TATA box–binding protein served as the reference gene. ... **Methylation** levels were measured immediately downstream of the **transcription** start site of ...
Cited by 166 - Related articles - BL Direct - All 6 versions

[HTML] Profiling and verification of gene expression patterns in normal and malignant human prostate tissues by cDNA microarray analysis [HTML] from nih.gov
H Luo, GF Greene, RM Hughes, ... - Neoplasia (New York ..., 2001 - Nature Publishing Group
... cancer, and **transcription** of the gene is downregulated via promoter **methylation** in some ... Expression of the basic **transcription** element binding protein 2 (BTEB2) was upregulated 3.1 x ... normal and three malignant human prostate tissues using the same **cDNA microarray** used ...

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Cited by 66 - Related articles - BL Direct - All 9 versions

Use of cDNA microarrays to monitor transcriptional responses of the chestnut blight fungus Cryphonectria parasitica to infection by virulence-attenuating hypoviruses [HTML] from nih.gov
[authors] - [publication info] - 2003 - nih.gov
... to 13.4% of the unique genes contained on the C. parasitica **cDNA microarray** chip. ... rates of DNA mutations and genome stability due to changes in DNA **methylation** patterns have ... such as receptors, that are involved in initiating signaling pathways and **transcription** factors that ...
Cited by 71 - Related articles - BL Direct - All 8 versions

E-cadherin transcriptional downregulation by promoter **methylation** but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines [HTML] from nih.gov
[authors] - [publication info] - 2005 - nih.gov
... Altered expression of these **transcription** factors seems to be also associated with an ... **Methylation**-specific fragments were sequenced to determine **methylation** of CpGs between the primer ... **cDNA** clones were amplified to generate PCR products for the **cDNA microarray** from a ...
Cited by 170 - Related articles - BL Direct - All 6 versions

Mapping global histone **methylation** patterns in the coding regions of human genes [HTML] from nih.gov
[authors] - [publication info] - 2004 - Am Soc Microbiol
... **transcription** networks (24, 27, 33). This principle has also been utilized to map histone acetylation and **methylation** in yeast (4, 42). Mapping histone modifications in humans is particularly hampered by the fact that the majority of the available human DNA **microarrays** are **cDNA** ...
Cited by 74 - Related articles - All 10 versions

Development of cDNA microarray for expression profiling of estrogen-responsive genes [PDF] from endocrinology-j
[authors] - [publication info] - 2003 - Soc Endocrinology
... 1998). In normal and malignant mammary cells, estrogen receptor alpha (ERα), one of the nuclear receptor **transcription** ... For elucidation of the scheme of estrogen signaling and improvement of clinical decisions, expression profiling analysis using **cDNA microarray** tech- nology ...
Cited by 176 - Related articles - BL Direct - All 6 versions

Discovery of novel **methylation** biomarkers in cervical carcinoma by global demethylation and microarray analysis
[authors] - [publication info] - 2006 - AACR
... A probability associated with **methylation** pattern of candidate genes in tOC-positive and tOC-negative exfoliated cervical cell ... Real-time Reverse **Transcription**-PCR. ... Each **cDNA** sample was additionally prepared in the absence of enzyme and used as a negative control to ...
Cited by 61 - Related articles - All 8 versions

The human let-7a-3 locus contains an epigenetically regulated microRNA gene with oncogenic function
[authors] - [publication info] - 2007 - AACR
... DNA **methylation** analysis. ... Total RNA was isolated from cells using Trizol (Invitrogen) and **cDNA** was synthesized using the ThermoScript reverse **transcription**-PCR (RT-PCR) system (Invitrogen). ... Hybridizations were done on genome-wide **cDNA microarrays**. ...
Cited by 242 - Related articles - BL Direct - All 7 versions

Transcript profiling in Arabidopsis reveals complex responses to global inhibition of DNA **methylation** and histone deacetylation [HTML] from pb.org
[authors] - [publication info] - 2004 - Publish
... 3. Genes up-regulated by aza-dC are also up-regulated in mutants affecting cytosine **methylation** or **methylation**-related gene silencing. ... DNA-free RNA was converted to **cDNA** by reverse **transcription** (Applied Biosystems). ...
Cited by 87 - Related articles - All 5 versions

Dissecting complex epigenetic alterations in breast cancer using CpG island microarrays
[authors] - [publication info] - 2004 - AACR
... The **cDNA microarray** approach requires targets derived from mRNA, which is more labile and difficult to isolate directly from clinical ... 4 The abbreviations used are: DMH, differential **methylation** hybridization; GPC3, glypican 3; RT-PCR, reverse **transcription**-PCR; ER/PR ...
Cited by 236 - Related articles - BL Direct - All 7 versions

The expression and function of estrogen receptor alpha and beta in human breast cancer and its clinical application [PDF] from endocrinology-j
[authors] - [publication info] - 2002 - Soc Endocrinology
... Furthermore, **methylation** of the ERα gene promoters also contribute to the regulation of gene **transcription**. ... growth of breast cancer cells and to develop a diagnostic tool, we have analyzed the gene expression profiles of estrogen-responsive genes using **cDNA microarray**. ...
Cited by 101 - Related articles - BL Direct - All 2 versions

[HTML] Microarray and cDNA sequence analysis of transcription during nerve-dependent limb regeneration [HTML] from biomedcentral
[authors] - [publication info] - 2003 - BMC
... These results further support the idea that **transcription** during limb regeneration is ... Many of these changes were also detected by 454 **cDNA** sequence analysis ... are associated with epigenetic phenomena, including chromatin remodeling, DNA **methylation**, and transcriptional ...
Cited by 35 - Related articles - Direct - All 24 versions

Genome-wide analysis of histone lysine **methylation** variations caused by diabetic conditions in human monocytes [HTML] from jbc.org
[authors] - [publication info] - 2007 - ASBMB
... recently use a ChIP-**cDNA** array approach to profile and analyze histone lysine **methylation** patterns in ... We used human 12K **cDNA** and 12K CpG island arrays to cover these regions ... of the CpG islands found in the human genome with ~60% located near a **transcription** start site ...
Cited by 42 - Related articles - All 8 versions

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Mutant p53 and aberrant cytosine methylation cooperate to silence gene expression
MM Ozturk, SS Wade, RJ Wamsley, DJ Lee... - Oncogene, 2005 - nature.com
... We performed two-color fluorescence **cDNA microarray** experiments, described herein, to profile gene expression patterns ... promoter region and found wt p53 to be a positive **transcription** factor in ... have shown MASPIN to be silenced in association with aberrant **methylation** of its ...
Cited by 78 - Related articles - BL Direct - All 6 versions

Gene expression analysis in human gastric cancer cell line treated with trichostatin A and S-Adenosyl-L-homocysteine using cDNA microarray [PDF] from sharif.ir
HS Lee, MH Park, JK Hong, HY Jung... - Biological and ..., 2004 - [illegible]
... in **transcription** 18) Moreover, physical and functional interactions between deacetylation (HDAC1) and **methylation** (SUVAR3-9) clearly indicate the close connection between histone deacetylation and methyla- tion 19) Fast advances in **cDNA microarray** technologies have en ...
Cited by 18 - Related articles - BL Direct - All 5 versions

Microarray analysis of gene regulation in the Hepa1c1c7 cell line following exposure to the DNA methylation inhibitor 5-aza-2'-deoxycytidine and 2, 3, 7, 8, ...
B Lee, S Park, DH Kim... - Toxicology in Vitro, 2004 - Elsevier
... Approximately 3–6% of cytosine residues in mammalian genomic DNA are present as 5-methylcytosine. DNA **methylation** is known to silence gene **transcription** and there are two widely ... **cDNA microarray** technology has become an important tool in toxicogenomics. ...
Cited by 17 - Related articles - All 5 versions

Construction of a zebrafish cDNA microarray: gene expression profiling of the zebrafish during development [PDF] from 144.206.159.17
C You, D Shanmugam, YJ Davis... - Biochemical and ..., 2003 - Elsevier
... dHAND is a basic helix–loop–helix **transcription** factor that is expressed in cardiac mesoderm. ... Our study represents the first application of **cDNA microarray** to the zebrafish and provides a global view of gene expression patterns in the zebrafish during embryonic development. ...
Cited by 50 - Related articles - BL Direct - All 3 versions

[HTML] Identification of novel high-frequency DNA methylation changes in breast cancer [HTML] from plos.org
SB Fackler, MA Stukenberg, N Matsumura... - PLoS One, 2007 - dx.plos.org
... 7). Four breast IDC samples (>90% neoplastic cellularity) were analyzed for both DNA **methylation** and **transcription**. ... of GHSR1a (see Discussion) was undetectable in all four tumor samples while expression was detected at 1:10 dilution of the normal breast **cDNA**. ...
Cited by 64 - Related articles - Cached - All 9 versions

Genome-wide transcription analyses in rice using tiling microarrays [PDF] from nature.com
L Li, X Wang, V Stolc, X Li, D Zhang, N Su... - Nature genetics, 2006 - nature.com
... homologous regions do not necessarily represent annotated genes. we assayed **transcription** in the ... Average probe fluorescence intensity in each **cDNA** region and its counterpart were calculated ... Tiling **microarray** design and experimental data are available in the NCBI Gene ...
Cited by 105 - Related articles - BL Direct - All 21 versions

Identification of potential diagnostic markers of prostate cancer and prostatic intraepithelial neoplasia using cDNA microarray [PDF] from nih.gov
JH Luo, C Ding, H Chen, G Mas... - Human pathology, 2003 - nih.gov
... prostate cancer and prostatic intraepithelial neoplasia using **cDNA microarray** ... We describe the assembly and use of a 1577-member **microarray** representing **cDNA** clones from a range of prostate cancer stages and grades, precursor lesions and normal tissue. ...
Cited by 56 - Related articles - BL Direct - All 6 versions

Deacetylase activity is required for recruitment of the basal transcription machinery and transactivation by STAT5 [HTML] from nih.gov
A Nakao, JA Imasaki, H ... - Molecular and cellular ..., 2004 - Am Soc Microbiol
... In concert with covalent histone modification (acetylation, phosphorylation, and **methylation**), it controls ... We have shown that a deacetylase activity is required for **transcription** activation by ... Arrays containing 16,700 **cDNA** elements were hybridized with five probe pairs (see ...
Cited by 32 - Related articles - BL Direct - All 8 versions

Development of anonymous cDNA microarrays to study changes to the Senecio floral transcriptome during hybrid speciation
MJ Hegarty, GM Hiscock, JD Wilson... - Molecular ..., 2005 - Wiley Online Library
... 2005) and alterations to DNA **methylation** state (Madlung et al ... Efficiency of the reverse **transcription** step for each taxon was assessed by amplification using the pHUM2 ... it was decided that no further treatment (ie subtraction) was necessary prior to preparing the **cDNA** inserts for ...
Cited by 79 - Related articles - All 8 versions

A tiling microarray expression analysis of rice chromosome 4 suggests a chromosome-level regulation of transcription [HTML] from plantcell.org
Y Jiao, P Xu, X Wang, N Su, S Yu... - The Plant Cell ..., 2005 - Am Soc Plant Biol
... As a control, our detection rate of full-length **cDNA**-supported gene models was 86%. ... functions to maintain the structure of chromatin, and rice also uses DNA **methylation** and histone ... than the organ identity, may be the signal for the chromosome-level regulation of **transcription** ...
Cited by 46 - Related articles - All 24 versions

Oligonucleotide-based microarray for DNA methylation analysis: Principles and applications
H Sun, J Shortt, J Saunthararajah, P Hansbro... - Journal of ..., 2010 - Wiley Online Library
... The **transcription** start site of RASSF1A is indicated by an arrow. ... **METHYLATION**-BASED TUMOR CLASSIFICATION AND EPIGENETIC THERAPIES. ... Gene expression profiles from **cDNA microarrays** have been used to differentiate different cancer types (Golub et al., 1999 ...
Cited by 170 - Related articles - BL Direct - All 5 versions

Identifying differentially expressed genes in cDNA microarray experiments
PA Baggerly, KA Coombes... - Journal of ..., 2002 - [illegible]

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... During the process of reverse **transcription**, ...orescent dyes or radioactively labeled nucleotides can be ... to label different samples, thus allowing simultaneous monitoring of two samples on the same **microarray**. After the labeled **cDNA** in a solution is obtained, it is placed over the ...
Cited by 106 - Related articles - BL Direct - All 6 versions

Microarray analysis of epigenetic silencing of gene expression in the KAS-6/1 multiple myeloma cell line
... - ... [PDF] from ... - Cancer research, 2003 - AACR
... KAS-6/1 cells, we decided to perform a systematic study on genes epigenetically regulated by zebularine using total cell RNA as a probe in the expression **cDNA microarray** technique. This approach has already been used to study **methylation**-regulated gene **transcription**. eg, 5 ...
Cited by 60 - Related articles - BL Direct - All 5 versions

Genome-wide profiling of gene expression in 29 normal human tissues with a cDNA microarray
... - [PDF] from ... - Nucleic ... - Oxford Univ Press
... Tissues with a **cDNA Microarray** ... (Received 14 February 2002; revised 28 March 2002; Abstract We have performed a comprehensive analysis of the expression profiles in 25 adult and 4 fetal human tissues by means of a **cDNA microarray** consisting of 23,040 human genes, ...
Cited by 70 - Related articles - BL Direct - All 5 versions

Diminished expression of S100A2, a putative tumor suppressor, at early stage of human lung carcinogenesis [HTML] from aacrjournals.o
... - ... - Clinical cancer ..., 2001 - AACR
... Abstract/FREE Full Text ... Xiong Z., Laird PW COBRA: a sensitive and quantitative DNA **methylation** assay. ... of **cDNA** subtraction and **microarray** analysis. ... Medline ... Stoll SW, Zhao X, Elder JT EGF stimulates **transcription** of CaN19 (S100A2) in HaCaT keratinocytes. J. investig. ...
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Genome-wide profiling of DNA methylation reveals transposon targets of CHROMOMETHYLASE3 [PDF] from 119.3%.223.1?)
... - ... [PDF] from ... - Current ... by ... - Elsevier
... retrotransposons are targeted by CMT3 is consistent with the activation of **transcription** of certain ... a consequence of our blast+ filtering to exclude repetitive sequences from the **microarray**. ... Table 1. Loci Displaying Significant **Methylation** Changes in cmt3 and met1 Mutant Plants. ...
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Blocking sp1 transcription factor broadly inhibits extracellular matrix gene expression in vitro and in vivo: implications for the treatment of tissue fibrosis
... - ... [PDF] from ... - Journal of ... - ...
... Also, CpG islands remained **methylation** free and active chromatin was formed at the global loci. ... It should be noted that despite little Sp1 **transcription** factor, clone 8 had growth ... of anti-sense Sp1 on fibroblast gene expression profiles as measured by **cDNA microarray** analysis. ...
Cited by 78 - Related articles - BL Direct - All 6 versions

Limited gene activation in tumor and normal epithelial cells treated with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine [HTML] from aspetjournals.
... - ... [PDF] from ... - Molecular ... - ...
... GAPDH amplification confirmed equivalent **cDNA** input. ... Critically, a recent study showed that **methylation** of a CpG in the context of an Ets site prevents binding and activation by Ets family **transcription** factors (Maier et al., 2003). ...
Cited by 73 - Related articles - BL Direct - All 6 versions

Suppressed expression of genes involved in transcription and translation in in vitro compared with in vivo cultured bovine embryos [HTML] from reproduction-o
... - ... [PDF] from ... - Biol Reprod, 2003 - Soc Reprod Fertility
... Differences in efficiency of **cDNA** production were corrected for by adjusting expression values (0.1 ... is unknown. It is suggested that DOT1L regulates telomeric silencing through **methylation** of this ... FKHRL1 is a member of the FOXO family of Forkhead **transcription** factors and is ...
Cited by 58 - Related articles - BL Direct - All 7 versions

Intraperitoneal dissemination of Ad12-induced undifferentiated neuroectodermal hamster tumors: de novo methylation and transcription patterns of integrated viral and ...
... - ... [PDF] from ... - Virus research, 2002 - Elsevier
... Tumor size and de novo **methylation** of the integrated Ad12 genomes are correlated events in some of the tumors. Cellular gene **transcription** patterns among individual tumors have shown similarities and differences. ... (b)Hybridization to mouse **cDNA microarrays** carrying 1980 ...
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The use of cDNA microarray to identify differentially expressed labor-associated genes within the human myometrium during labor
... - ... [PDF] from ... - American Journal of ... - 2004 - Elsevier
... Real-time RT-PCR was used to verify up-regulation of several genes of interest that were identified using **cDNA microarray**. Reverse **transcription** was performed using superscript II RT enzyme (Invitrogen, Carlsbad, Calif) on total RNA obtained from 11 laboring and 14 ...
Cited by 43 - Related articles - All 7 versions

"Promoter Array" Studies Identify Cohorts of Genes Directly Regulated by Methylation, Copy Number Change, or Transcription Factor Binding in Human Cancer Cells
... - ... [PDF] from ... - Annals of the New ... - 2003 - Wiley Online Library
... of unknown target genes of human **transcription** factors using chromatin immunoprecipitation. Methods 26: 37–47. 13 Shi, H. et al. 2003. Triple analysis of the cancer epigenome: an integrated **microarray** system for assessing gene expression, DNA **methylation**, and histone ...
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High-throughput methylation profiling by MCA coupled to CpG island microarray [HTML] from cshlp.org
... - ... - ... - genome.cshlp.org

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... Four CpG sites close to the **transcription** start site were pyrosequenced, and a consistent pattern of high levels of ... (2009) Aberrant CpG-island **methylation** has non ... PO.; Bittner, ML.; Meltzer, PS.; Ray, M.; Chen, Y.; Su, YA.; Trent, JM. (1998) Use of a **cDNA microarray** to analyse gene ...
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Using DNA microarrays to study natural variation [PDF] from nchsage.edu
Y Gilad... - Current opinion in genetics & development, 2008 - Elsevier
... identification of novel genomic regions that are transcribed, identification of promoters that bind specific **transcription** factors, and the study of epigenetic events such as **methylation**. ... This solution is not feasible for **cDNA** arrays, in which the probes are 300–1000 bp in length ...
Cited by 34 - Related articles - All 10 versions

Gene dosage alterations revealed by cDNA microarray analysis in cervical cancer: identification of candidate amplified and overexpressed genes
S Natrajan, V Srinivasan, S Ghazani... - Genes, ..., 2007 - Wiley Online Library
... COPS6, Regulation of **transcription** in response to DNA damage, ... 19q13.3, and 20q13.1) (Table 3). A number of recurrent over represented **cDNA** clones, however ... Figure 2. Comparison of expression levels by Affymetrix **microarray** and semiquantitative RT-PCR analyses of ...
Cited by 80 - Related articles - BL Direct - All 4 versions

Monitoring gene expression along pear fruit development, ripening and senescence using cDNA microarrays
S Pavanello, L Arnoldo, A Zanella, S Pamboni, A Balbo... - Plant Science, 2004 - Elsevier
... to evaluate changes in mRNA level during ripening: differential screening of **cDNA** libraries [4 ... genome and receptacle maturation using DNA **microarray** technology [7]. DNA **microarrays** are powerful ... processes and diverse stages of plant development at the **transcription** level [8 ...
Cited by 43 - Related articles - All 8 versions

A novel variant of Impact is imprinted in brain, and its expression is correlated with differential methylation of an internal CpG island [HTML] from nih.gov
SD Oliver, SA Lewis-Mueller, AJ Morris... - ... and molecular biology, 2005 - Am Soc Microbiol
... Control reverse **transcription**-PCR (RT-PCR) of an unrelated sequence confirmed the presence of ... was obtained by using 5′ RACE, followed by sequencing; the **cDNA** and genomic ... Differential **methylation** in the brain-specific imprinted transcript.CpG analysis of the mouse ...
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Comparison of gene expression profiles between diffuse-and intestinal-type gastric cancers using a genome-wide cDNA microarray
N Jinawath, V Vasoontara, S Yongchaiyud, M Hu... - Cancer letters, 2004 - Elsevier
... is often a feature of diffuse-type gastric cancers: somatic mutations of CDH1, or **methylation** in its ... in differentiation (NOTCH2), cytoskeleton (TUBB2), metabolism (FACL5 and ACAA1), signal transduction (EPS15), **transcription**, or protein ... **cDNA microarray** and analysis of data. ...
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Characterizing the physical genome [PDF] from stanford.edu
JR Pollack, VR Iyer - nature genetics, 2002 - nature.com
... and DAC-induced gene expression, assuming that sufficiently processive reverse-**transcription**-based labeling of ... This approach has been used to identify CpG **methylation** patterns that can ... **cDNA microarrays** offer a readily available alternative [36, 41], where averaging the ...
Cited by 61 - Related articles - BL Direct - All 15 versions

Cytosine methylation and human cancer
PW Laird, R Jaenisch - Current opinion in genetics ..., 1994 - journals.lww.com
... **Methylation** of promoter regions represses **transcription** when **methylation** density is high, and heavily methylated ... Transformed cell lines exhibit widespread and progressive **methylation** of genes that are not essential for viability in culture [16], and **microarray** scanning of ...
Cited by 56 - Related articles - BL Direct - All 3 versions

DOT1L/KMT4 recruitment and H3K79 methylation are ubiquitously coupled with gene transcription in mammalian cells [HTML] from nih.gov
D Steger, M Lin, J Thomas... - ... and cellular biology, 2008 - Am Soc Microbiol
... Cell maturation is accompanied by robust activation and repression of gene **transcription** and thus represents an ideal system for studying potential changes in histone **methylation**. Using Affymetrix **microarray** data generated with **cDNA** prepared from 3134.1 cells before and ...
Cited by 101 - Related articles - BL Direct - All 10 versions

SMYD3 encodes a histone methyltransferase involved in the proliferation of cancer cells
H Hamamoto, Y Hamamoto, M Kimura, Y Nakao... - Nature cell ..., 2004 - nature.com
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Identification of epigenetically silenced genes in tumor endothelial cells [HTML] from aacrjournals.o
DMH Hellebrekers, M Melotte, E Vire... - Cancer research, 2007 - AACR
... These enzymes exhibit **methylation**-independent **transcription** repressor functions by acting as transcriptional repressors themselves ... was greater than by TSA, which may be due to **methylation** of these ... PHD **microarray**, however, is that in the latter an initial **cDNA** subtraction step ...
Cited by 68 - Related articles - BL Direct - All 10 versions

Transcriptome analysis in blastocyst hatching by cDNA microarray [HTML] from oxfordjournals
HW Chan, SW Chan, JL Ko-Tsang, PL Yang... - Human ..., 2005 - ...
... GV) oocyte to blastocyst have already been published via in vitro **transcription** and **microarray** ... play the role in histone deacetylation and lead to DNA **methylation** (Ahringer 2000 ... the T7 RNA polymerase-based in vitro linear RNA amplification and **cDNA microarray** as compared ...
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Translation of microarray data into clinically relevant cancer diagnostic tests using gene expression ratios in lung cancer and mesothelioma [HTML] from aacrjournals.o
GJ Gordon, RV Jensen, LL Hsiao, SR Gullans... - Cancer research, 2002 - AACR
... reverse **transcription**-PCR; BWH, Brigham and Women's Hospital; TITF-1, thyroid **transcription** factor-1 ... L. Mulligan J. Monitoring gene expression profile changes in ovarian carcinomas using **cDNA microarrays** ... Asbert S., Nutini M., Herzenberg LA DNA **methylation** prevents the ...
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The major 8p22 tumor suppressor DLC1 is frequently silenced by methylation in both endemic and sporadic nasopharyngeal, esophageal, and cervical carcinomas, ...
T Healy, CW Lee, YJ Li... - Clinical Cancer Res..., 2006 - clincancerres.aacrjournals.org
... After cloning the subtracted **cDNA**, randomly chosen clones representing candidate genes possibly downregulated ... All three constructs could drive the **transcription** of target gene, although at different ... Frequent silencing of DLC1 by **methylation** in cell lines of NPC, esophageal ...
Cited by 213 - Related articles - BL Direct - All 10 versions

Arginine methylation provides epigenetic transcription memory for retinoid-induced differentiation in myeloid cells [HTML] from nih.gov
BL Bales, A Barnett, A Mays, DM Benso... - ... and cellular biology, 2008 - Am Soc Microbiol
... Subsequently, the HMRT1L2 **cDNA** was subcloned with BamHI and EcoRI into pcDNA3.1-B ... is the consequence of the decrease in the retinoid-dependent activation of **transcription** of this ... we have obtained point to a strong correlation between H4 arginine 3 **methylation** and the ...
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Analysis of variance for gene expression microarray data [PDF] from bu.edu
MK Kerr, M Martin... - Journal of Computational ..., 2000 - Taylor & Francis
... Aug 2010; Vol. 80, No. 8; 825-840. CrossRef. Background adjustment of **cDNA microarray** images by Maximum Entropy distributions. Christos Argyropoulos, Antonis Daskalaki, George C. Nikiforidis, George C. Sakellaropoulos. Journal of Biomedical Informatics. Aug 2010; Vol ...
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Mouse cDNA microarray analysis uncovers Slug targets in mouse embryonic fibroblasts [PDF] from cshl.edu
S Barrallo-Rodríguez, M Pérez-Pom... - Oncogene, 2005 - Nature Publishing Group
... labeled RNA from control MEFs versus the Slug-deficient MEFs to a mouse **cDNA microarray** containing 15,000 clones. Overall the expression of the majority of the spotted genes was not altered in Slug-deficient cells. The expression of both the related **transcription** factor Snail ...
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[CITATION] Replacing cRNA targets with cDNA reduces microarray cross-hybridization
AC Hurley, VK Tarasova, P Perin... - Nature biotechnology, 2005 - nature.com
... triplicate, either with a standard one-round T7 in vitro **transcription** protocol to ... ling array experiments searching for previously unidentified transcripts used labeled **cDNA** as target ... based antisense therapy, RNA-mediated chromatin remodeling and DNA **methylation**, which may ...
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Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma [HTML] from aacrjournals.o
N Sato, A Fukushima, M Iacobuzio... - ... Cancer Research, 2003 - AACR
... P450 (CYP1B1), uncoupling protein homologue, ubiquitin 1B, and thyroid **transcription** factor-1 ... many of them have been previously described as harboring **methylation** in normal ... Gene expression data from oligonucleotide or **cDNA microarray** and SAGE databases showed no ...
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[HTML] Genomic profiles in stage I primary non small cell lung cancer using comparative genomic hybridization analysis of cDNA microarrays [HTML] from nih.gov
R Garg, J Yoo, NP Ostrowski, RJ... - Neoplasia (New York, NY ..., 2004 - Neoplasia.com
... In our study, **cDNA microarray** CGH analysis provided a clear genomic profile of genes in primary ADCAs that is distinctly different from that in ... not surprising for several reason: 1) genomic DNA is a different mixture from the mRNA representation of cells; 2) **transcription** of genes ...
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Comprehensive DNA methylation profiling in a human cancer genome identifies novel epigenetic targets [HTML] from oxfordjournals
JM Ordway, A Bedi, RDK Adra... - ..., 2006 - Oxford Univ Press
... **cDNA** from the cell lines and tumor samples was produced using the oligo dT primers provided in the Superscript III ... **Methylation**-dependent DNA fractionation. ... To identify epigenetic changes in gene regulation, our efforts were focused largely upon the **transcription** start sites of ...
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Expressed CpG island sequence tag microarray for dual screening of DNA hypermethylation and gene silencing in cancer cells [HTML] from aacrjournals.o
H Shi, PS Yan, CM Shah, P Ramakrishnan... - Cancer research, 2003 - AACR
... 3 The abbreviations used are: DMH, differential **methylation** hybridization; ECIST ... amino-allyl dUTP; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SAM, significant analysis of **microarray**; RACE, rapid amplification of **cDNA** ends; RT-PCR, reverse **transcription**-PCR. ...
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cDNA microarray analysis of early gene expression profiles associated with hepatitis B virus X protein-mediated hepatocarcinogenesis
RR Hu, CY Chen, CMY Lee, SKW Tsui... - Biochemical and ..., 2004 - Elsevier
... candidate genes may be affected by the quality of the starting RNA. **cDNA** labelling, hybridization ... In addition, abnormal DNA **methylation** by Dnmt3a might be associated with silencing the tumor ... Estrogen receptor Esr1, which can act as a **transcription** factor, was found to be ...
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Genomic microarrays in the spotlight [PDF] from dmm.cn
CF Bastecky-Jo, PD Dunkley, P Hall, de Sous... - TRENDS in ..., 2004 - Elsevier

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... Such **cDNA**-based **microarray** analyses have identified other genes that are important in the pathogenesis of several disorders [19], [20], [21], [22] and [23]. ... First, **cDNA microarray** analysis enables only the detection of aberrations in known genes. ...
Cited by 103 - Related articles - All 11 versions

[HTML] A systematic search for new mammalian noncoding RNAs indicates little conserved intergenic transcription [HTML] from biomedcentral
... D, Schaid EE, Dark matter in the genome: evidence of widespread **transcription** detected by ... Y, Okazaki Y: Systematic expression profiling of the mouse transcriptome using RIKEN **cDNA microarrays**. ... Lowe TM, Eddy SR: A computational screen for **methylation** guide snoRNAs in ...
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Identification of carboxypeptidase E and γ-glutamyl hydrolase as biomarkers for pulmonary neuroendocrine tumors by cDNA microarray
... of selected differentially expressed genes for each tumor subtype were internally validated by real-time reverse-**transcription** polymerase chain ... Thus, **cDNA microarray** analysis led to the identification of 2 novel biomarkers that should facilitate molecular diagnosis and further ...
Cited by 38 - Related articles - All 6 versions

[HTML] A genome-wide screen for promoter methylation in lung cancer identifies novel methylation markers for multiple malignancies [HTML] from plos.org
... random from the original 132, and primers were designed using the following criteria: **methylation**-specific PCR (MSP) primers targeted a region within 250 bp of the annotated **transcription** start site ... QPCR was performed in a reaction volume of 20 μl including 1 μl of **cDNA** ...
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Downregulation of connexin 26 in human lung cancer is related to promoter methylation
... the treated cells were harvested for total RNA isolation and reverse **transcription** polymerase chain ... of Colonies from H2170 and H226 Revealed a Heterogeneous **Methylation** status ... for the clustering/classification of primary lung carcinomas using **cDNA microarrays** revealing its ...
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Meta-analysis of microarrays [PDF] from dtu.dk
... 18] , and TIMP3, an inhibitor of metalloproteinases that is silenced by **methylation** in cancers ... more complete cell signaling and enzymatic pathways, as well as **transcription** factor networks ... ML, Chen Y., Trent JM, Meltzer PS Expression profiling in cancer using **cDNA microarrays**. ...
Cited by 819 - Related articles - BL Direct - All 14 versions

[HTML] Dynamic usage of transcription start sites within core promoters [HTML] from biomedcentral
... which were identified by differential expression dependent upon chromosomal parent of origin using **cDNA microarrays** [30]. ... maternal **methylation** of imprinted genes starts at different genomic locations and that CpG **methylation** gradients may influence **transcription**. ...
Cited by 30 - Related articles - Cached - All 21 versions

Long-term low-dose arsenic exposure induces loss of DNA methylation [HTML] from nih.gov
... Rb hypophosphorylation, arsenic inhibits E2F/DP1 activity and consequently DNMT1 **transcription**. ... associate environmental factors with changes in DNA **methylation** and epigenetic ... with arsenic-induced malignant transformation: applications of **cDNA microarray** technology. ...
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DNA microarray and cancer
... Because many genetic alterations occur in the chromosomal regions not covered by **cDNA** and because current technology is unable to array the whole genome at ... Although **transcription** is altered in a large number of genes, suggesting a link between **methylation** and gene ...
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The functions of E (Z)/EZH2-mediated methylation of lysine 27 in histone H3
... Until in vivo evidence is obtained for EZH2- and E(Z)-mediated H3-K9 **methylation**, EZ42 and E(Z) should be regarded as a H3-K27 methyltransferase. H3-K27 **methylation** and Hox gene silencing ... H3-K27 **methylation** and X chromosome inactivation. ...
Cited by 452 - Related articles - All 7 versions

[HTML] Identification of novel imprinted genes in a genome-wide screen for maternal methylation [HTML] from cw.com.tw
... 8 ST matches are contained within ABM1586, the full insert sequence of brain **cDNA** clone MNCb ... ABM1586 are indicated as black lines and form part of the same **transcription** unit as ... The probe used for **methylation** analysis by Southern blot hybridization is indicated as a grey ...
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Laser capture microdissection and cDNA microarrays used to generate gene expression profiles of the rapidly expanding fibre initial cells on the surface of cotton ...
... They were able to identify a large number of **transcription** factor genes or- thologous to genes in Arabidopsis, some known to be in- volved in Arabidopsis trichome formation, but have ... 2003) used laser-capture micro- dissection (LCM) coupled with **cDNA microarrays** to establish ...
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Possible involvement of RUNX3 silencing in the peritoneal metastases of gastric cancers
C Sat Kawa, F Hasegawa, M Sasaki ... - Clinical cancer ..., 2005 - AACR
... **Methylation**-specific PCR was done as reported previously (12, 13). ... from each gastric cancer cell line was labeled by incorporating Cy3 during random-primed reverse **transcription**. **cDNA** derived from the reference pool, which we labeled with Cy5, was used as the expression ...
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Chemical genomic screening for methylation-silenced genes in gastric cancer cell lines using 5-aza-2'-deoxycytidine treatment and oligonucleotide microarray
S Yamashita, Y Tsujino, K Moriguchi... - Cancer ..., 2006 - Wiley Online Library
... From the double-stranded **cDNA**, biotin-labeled cRNA was prepared using a BioArray High-Yield RNA transcript labeling kit ... Table 1. Primers for **methylation**-specific polymerase chain reaction, ... **Transcription** start site = 0. All primers were designed on the top strand sequences. ...
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Tiny RNAs associated with transcription start sites in animals [PDF] from pasteur.edu.uy
RJ Taft, EA Glazov, N Cloonan, CL Simons... - Nature genetics, 2009 - nature.com
... marks 35 , we found that 96% of tsRNAs in ovary overlap with H3K4 **methylation** marks in ... **cDNA** was prepared by cleavage of the linkers and purification of the insert by electrophoresis and ... of small RNA 5' ends that map to bases 0 to +10 relative to the **transcription** start (either a ...
Cited by 142 - Related articles - All 16 versions

Novel patterns of gene expression in polyploid plants [PDF] from aseanbiodiversity
AR Leitch, IJ Leitch - Trends in Genetics, 2008 - Elsevier
... plant polyploids. Soltis et al.[28] examined expression patterns in natural allopolyploid Tragopogon by AFLP-**cDNA** to determine if ... (a) Read-out **transcription** from a ... was used by Wang et al.[21] for silencing methyltransferase gene (met1) to inhibit **methylation** of hypermethylated ...
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Transcriptome profiling of Lilium longiflorum generative cells by cDNA microarray
Y Okada, MN Singh,... - Plant cell reports, 2010 - Springer
... 100% overexpression relative to ovaries or gonadectomized free using testis **cDNA microarrays** (Andrews et al. ... B (2000) Gene discovery using computational and micro- array analysis of **transcription** in the ... H3 variants in male gametic cells of lily and H3 **methylation** in mature ...
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Arabidopsis transcriptome analysis under drought, cold, high-salinity and ABA treatment conditions using a tiling array [HTML] from oxfordjournals
A Matsui, J Ishida, T Morosawa ... - Plant and cell ..., 2008 - Oxford Univ Press Plant Physiol
... other hand, we used the oligo(dT) primer for the first-strand **cDNA** synthesis and ... processed by some enzymes using the sense DNA as a template after **transcription** of the ... gene silencing. RNA stability, alternative splicing, RNA editing, RNA masking and **methylation** (Hastings et ...
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[PDF] Normalizing DNA microarray data [PDF] from tandfonline.com
M Bakker, LK Goertzen, S Pond... - Current issues in ..., 2003 - caister.com
... Global analysis of herpes simplex virus type 1 **transcription** using an oligonucleotide-based DNA **microarray**. J. Virol. 74: 9918–9927. ... Genome-wide expression profiling of mid-gestation placenta and embryo using a 15 000 mouse developmental **cDNA microarray**. Proc. Natl. ...
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Characterization of the vernalization response in Lolium perenne by a cDNA microarray approach [HTML] from oxfordjournals
S Dubcovsky, J Ramakrishna, C Gallagher... - Plant molecular ..., 2005 - Oxford Univ Press Plant Physiol
... a comprehensive analysis of the vernalization process in L. perenne by a **cDNA microarray** approach ... probably lead to silencing of the floral repressor FLC by deacetylation and **methylation** of regulatory ... the fact that it is a member of the same family of **transcription** factors suggest ...
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Conserved homeodomain proteins interact with MADS box protein Mcm1 to restrict ECB-dependent transcription to the M/G1 phase of the cell cycle [HTML] from nih.gov
S Pramila, S Mead, D Dalla Betta... - Genes & ..., 2002 - genesdev.cshlp.org
... Defects in the tagged proteins would lead to constitutive **transcription** of MCM3 through the cell cycle (see Fig. ... For **microarray** analysis through the cell cycle, cheyb1yhp1 cells were synchronized with α factor in YEP ... Thirty micrograms of total RNA was used for **cDNA** synthesis. ...
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RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays [HTML] from cshlp.org
JC Marioni, CE Mason, SM Mane... - Genome ..., 2008 - genome.cshlp.org
... 2007), and DNA **methylation** (Cokus et al. ... other applications have thus far, focused on detecting the absence or presence of an event, such as a **transcription** factor binding ... a 25-μL reaction containing 2× SYBR master mix (Sigma), 0.2 μM each primer, and 1 μL of **cDNA** template ...
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[HTML] Differential DNA methylation correlates with differential expression of angiogenic factors in human heart failure [HTML] from plos.org
M Movassagh, MK Choy, M Goddard, MR Bennett ... - PLoS One, 2010 - dx.plos.org
... heart failure, regardless of its initiating cause [2]. As with **cDNA microarray** experiments utilizing ... hold together DNA conformations to form chromatin centres of active **transcription** or transcriptional ... by altering access to DNA-binding proteins. different DNA **methylation** states of ...
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1. Comparative pair-wise domain-combinations for screening the clade specific domain-architectures in metazoan genomes.
   Kawashima S, Kawashima T, Putnam NH, Rokhsar DS, Wada H, Kanehisa M.
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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	13387343	BRENNER ET AL.
	Examiner	Art Unit
	NARAYAN BHAT	1634

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
Searched for the inventors and assignee in PALM for the ODP.	8/3/12	Bhat N.
Searched for the topics in East, Pubmed and Google scholar. Search histories are enclosed.	8/3/12	Bhat N.

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

/NARAYAN BHAT/ Examiner.Art Unit 1634	

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	60	(hairpin or stem near3 loop) near3 (probe or oligonucleotide or adapt$3) same (address$3 or zipcode or identifier or barcode)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/07/11 08:43
S2	2	S1 and (identif$4 or detect$3) near5 (address$3 or zipcode or identifier or barcode)near5 sequencing	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/07/11 08:44
S3	50	(hairpin or stem near3 loop) near3 (probe or oligonucleotide or adapt$3) and (address$3 or zipcode or identifier or barcode)near3 (section or region or segment)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/07/11 09:10
S4	938	(probe or oligonucleotide or adapt$3) near5 (address$3 or zipcode or identifier or barcode)near5 (section or region or segment)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/07/11 10:05
S5	108	S4 and complementary same (stem or duplex)same (probe or oligonucleotide or adapt$3)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/07/11 10:07

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SUBMITTED ELECTRONICALLY

RESPONSE TO OFFICE ACTION Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Docket No.	CGLC-020
	Application No.	13/387,343
	Confirmation No.	3537
	Filing Date	February 15, 2012
	Examiner	N.K. Bhat
	Group Art Unit	1634
	Inventor	Brenner

Sir:

This communication is responsive to the Office Action dated August 9, 2012, for which a three month period for response was given, making this amendment due by November 9, 2012. As such, this response is timely filed.

AMENDMENT TO THE SPECIFICATION

Please amend the paragraph starting at line 15 of page 20 as follows:

Figure 1A shows a single stranded polynucleotide 100 comprising, in a 5' to 3' orientation, a first domain (102; the domain to be moved); a reflex sequence 104; a nucleic acid sequence 106 having a site distal to the first domain (Site A), and a complement of the reflex sequence 108 (positioned at the 3' terminus of the polynucleotide). **Nucleic acid sequence 106 may be from 30 bases to 30 kilobases in length.** The steps of the reflex method described below will move the first domain into closer proximity to Site A. It is noted here that the prime designation in Figure 1A denotes a complementary sequence of a domain. For example, First Domain' is the complement of the First Domain.

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AMENDMENTS TO THE CLAIMS

Please amend the claims as shown below.

1. (Currently Amended) A method of sample analysis, comprising:

a) annealing a reflex sequence in a polynucleotide strand in a sample with a complement of the reflex sequence present in the polynucleotide strand, wherein the polynucleotide strand comprises the following in a 5' to 3' orientation:

a first domain comprising i. a primer site and ii. a multiplex identifier (MID) comprising a unique nucleotide sequence that distinguishes said polynucleotide strand from other polynucleotide strands in said sample;

the reflex sequence;

a polynucleotide of interest **that is from 30 bases to 30 kilobases in length, wherein said polynucleotide of interest has** ~~having~~ a desired site that is distal to the first domain; and

the complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand;

b) extending from the 3' end of the complement of the reflex sequence through the first domain to produce a double stranded region comprising a complement of the first domain at the 3' end of the extended polynucleotide strand, thereby moving the first domain into proximity with the desired site; and

c) sequencing the MID and the desired site using a primer that **is** complementary to the primer site in the complement of the first domain.

2-3. (Canceled)

4. (Original) The method of claim 1, wherein the reflex sequence is from 10 to 50 nucleotides in length.

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5. (Previously presented) The method of claim 1, wherein the first domain comprises an RNA polymerase site.

6. (Original) The method of claim 1, the method further comprising removing the first domain and the reflex sequence from the 5' end of the double stranded region.

7. (Original) The method of claim 6, wherein the removing step comprises treating the nucleic acid of step b with T7 exonuclease.

8. (Original) The method of claim 1, wherein the first domain is present in an adapter region attached to the polynucleotide of interest.

9. (Original) The method of claim 8, wherein the reflex sequence is present in the adapter region.

10. (Original) The method of claim 8, wherein the reflex sequence is a sequence present within the 5' region of the polynucleotide of interest.

11. (Original) The method of claim 1, wherein the sample is a multiplexed sample, wherein the first domain in each of the polynucleotide strands in the multiplexed sample comprises a MID corresponding to its parental sample.

12. (Original) The method of claim 1, wherein the sample comprises multiple polynucleotide strands, and wherein each polynucleotide strand in the sample comprises a unique MID in the first domain.

13. (Original) The method of claim 1, wherein the polynucleotide strand is produced by:

annealing a nucleic acid synthesis primer to a parent polynucleotide strand, wherein the parent polynucleotide strand comprises the following in a 5' to 3' orientation:

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the first domain;

the reflex sequence; and

a second domain comprising the polynucleotide of interest;

wherein the nucleic acid synthesis primer comprises:

a 3' priming domain that is complementary to a site adjacent to and 3' of the polynucleotide of interest in the second domain; and

a 5' reflex sequence;

extending the nucleic acid synthesis primer to produce an extension product; and

replicating the resultant extension product with a second nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain, thereby producing the polynucleotide strand.

14. (Cancelled).

15. (Original) The method of claim 13, wherein the parent polynucleotide strand is enriched prior to the annealing step based on its sequence.

16. (Original) The method of claim 1, further comprising the steps of:

c) replicating the polynucleotide strand of step b with a first nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain to produce a first extension product;

d) annealing a second nucleic acid synthesis primer to the first extension product, wherein the second nucleic acid synthesis primer comprises a 3' priming domain that is complementary to a site within the polynucleotide of interest in the first extension product; and

e) extending the second synthesis primer to produce a second extension product.

17. (Original) The method of claim 16, wherein the second synthesis primer comprises a 5' domain that is not complementary to the polynucleotide of interest in the first extension product.

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18. (Original) The method of claim 17, wherein the first domain comprises a first primer site and the 5' domain of the second synthesis primer comprises a second primer site.

19. (Original) The method of claim 18, wherein the length of the polynucleotide of interest in the second extension product is from 50 to 10,000 nucleotides.

20. (Cancelled)

21. (Previously presented) The method of claim 1, wherein polynucleotide of interest is cDNA.

22. (Previously presented) The method of claim 1, further comprising repeating said method to obtain the nucleotide sequence of a second desired site in said polynucleotide of interest, and analyzing the sequence obtained in c) and the sequence of the second desired site to obtain linkage information about polymorphisms in said polynucleotide of interest.

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REMARKS

Formal Matters

Claims 1, 4-13, 15-19, 21 and 22 are pending.

Claims 1, 4-19, 21 and 22 were examined and rejected. Claim 20 was withdrawn by the Examiner.

Solely to expedite prosecution and without any intention to acquiesce to any rejection, claim 1 is amended to recite "a polynucleotide of interest that is from 30 bases to 30 kilobases in length", support for which can be found in claim 14, now cancelled. Claim 1 is also amended to correct a typographical error. Claims 14 and 20 are cancelled without prejudice.

Reconsideration of this application is requested in view of the comments that follow below.

Amendment to claim 1

The Applicants submit that, for the reasons set forth below, none of the claims are anticipated or rendered obvious by Laird because Laird does not disclose or teach step (c) of the rejected claims. As such, even if the Examiner determines that the amendment cannot be entered because it would create new issues, all rejections of record should nevertheless still be withdrawn and the application should be allowed.

Amendment to the Specification

While reviewing the specification to identify support for the amendment to claim 1, the Applicants noticed that that the body of the instant specification does not explicitly disclose a polynucleotide of interest having a length of "30 bases to 30 kilobases". Therefore, the specification has been amended to state that "Nucleic acid sequence 106 may be from 30 bases to 30kb in length", support for which is found in claim 14, as originally filed.

The Applicants note that the claims as filed in the original specification are part of the disclosure and therefore, if an application as originally filed contains a claim disclosing material not disclosed in the remainder of the specification, the applicant may amend the specification to include the claimed

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subject matter. *In re Benno*, 768 F.2d 1340, 226 USPQ 683 (Fed. Cir. 1985). As such, this amendment to the specification does not introduce new matter. See MPEP § 2163.06.

Rejections under § 102

Claims 1, 4, 5, 8-12 and 16-18 are rejected under 35 U.S.C. § 102 as being anticipated by Laird (Proc. Natl. Acad. Sci. 2004 1010: 204-209). The Applicants respectfully traverse this rejection.

The standard for anticipation under section 102 is one of strict identity. An anticipation rejection requires a showing that each limitation of a claim be found in a single reference, *Atlas Powder Co. v. E.I. DuPont de Nemours & Co.*, 224 U.S.P.Q. 409, 411 (Fed. Cir. 1984). Further, an anticipatory reference must be enabling, see *Akzo N.V. v. United States Int'l Trade Comm'n* 808 F.2d 1471, 1479, 1 U.S.P.Q.2d 1241, 1245 (Fed. Cir. 1986), *cert denied*, 482 U.S. 909 (1987), so as to place one of ordinary skill in possession of the claimed invention. To anticipate a claim, a prior art reference must disclose every feature of the claimed invention, either explicitly or inherently. *Glaxo v. Novopharm, Ltd.* 334 U.S. P.Q.2d 1565 (Fed. Cir. 1995).

This rejection is traversed on the basis that Laird does not teach step (c) of claim 1, which requires that a specific region of the product of step (b) is sequenced using a specific primer. Specifically, step (c) of claim 1 recites "sequencing the MID and the desired site using a primer that is complementary to the primer site in the complement of the first domain".

In Laird, the PCR products are not sequenced using "a primer that complementary to the primer site in the complement of the first domain". Rather, in Laird, the PCR products are cloned into a TOPO vector and then presumably sequenced using primers that hybridize to that vector. See Laird p. 205, col. 2, 2nd full ¶, copied below.

> 72°C for 1 min; a final extension was at 72°C for 7 min. All PCR products were analyzed by agarose gel electrophoresis; further cloning and sequencing of appropriately sized products was with TOPO vectors (Invitrogen Life Technologies); sequencing reactions were carried out with fluorescent dideoxy nucleotides (BIGDYE Terminator 3.0, Applied Biosystems), at the DNA Sequencing Facility, Department of Biochemistry, University of Washington. Each sequence was proofread against the electro-

In other words, in Laird's method the PCR products are actually sequenced using vector primers, not using "a primer that complementary is to the primer site in the complement of the first domain", as required by the rejected claims.

Because Laird's method is different to that being claimed, Laird cannot anticipate the claims. Withdrawal of this rejection is therefore requested.

To the extent that the Examiner believes that he can fill the void in the teachings of Laird with a secondary reference, the Applicants submit that one of skill in the art would not sequence Laird's PCR products directly (i.e., using a primer that hybridizes to the PCR products) because Laird's PCR products contain a population of different sequences (caused by differing methylation patterns in original template). Because Laird's PCR products contain a population of different sequences, they need to be sequenced separately, and that is why they are cloned. Sequencing the products *en masse* using a primer that hybridizes to the PCR products would not reveal the sequences of the individual molecules.

Rejections under § 103

Claims 1 and 21 are rejected under 35 U.S.C. § 103 as being obvious over Laird, *supra*, and Ushijima (Nature Reviews 2005 5 223-231). The Applicants respectfully traverse this rejection.

In order to meet its burden in establishing a rejection under 35 U.S.C. §103, the Office must first demonstrate that a prior art reference, or references when combined, teach or suggest all claim elements. *See, e.g., KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 1740 (2007); *Pharmastem Therapeutics v. Viacell et al.*, 491 F.3d 1342, 1360 (Fed. Cir. 2007); MPEP § 2143(A)(1). In addition to demonstrating that all elements were known in the prior art, the Office must also articulate a reason for combining the elements. *See, e.g., KSR* at 1741; *Omegaflex, Inc. v. Parker-Hannifin Corp.*, 243 Fed. Appx. 592, 595-596 (Fed. Cir. 2007). Further, the Supreme Court in *KSR* also stated that that "a court *must* ask whether the improvement is more than the predictable use of prior art elements according to their established functions." (emphasis added) *See KSR* at 1740. Therefore, in addition to showing that all elements of a

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claim were known in the prior art and that one of ordinary skill in the art had reason to combine them, the Office must also provide evidence that the combination would have a reasonable expectation of success.

Laird is deficient for the reason outlined above. Ushijima's cDNA fails to meet Laird's deficiency and, as such, the cited references, taken in any combination, fail to teach each and every element of the rejected claims.

Withdrawal of this rejection is therefore requested.

Applicant submits that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees up to a strict limit of $3,000.00 beyond that authorized on the credit card, but not more than $3,000.00 in additional fees due with any communication for the above referenced patent application, including but not limited to any necessary fees for extensions of time, or credit any overpayment of any amount to Deposit Account No. 50-0815, order number CGLC-020.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: August 28, 2012

By: /James S. Keddie, Reg. No. 48,920/
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Electronic Acknowledgement Receipt	
EFS ID:	13609534
Application Number:	13387343
International Application Number:	
Confirmation Number:	3537
Title of Invention:	Compositions and Methods for Intramolecular Nucleic Acid Rearrangement
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	24353
Filer:	James Samuel Keddie/Sarah Zizzo
Filer Authorized By:	James Samuel Keddie
Attorney Docket Number:	CGLC-020
Receipt Date:	28-AUG-2012
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Time Stamp:	18:15:13
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		CGLC-020_Rsp2OA.pdf	355692 2d0011ae4e881a7f2e6ed8436d4de6e755e7731f0f88aa85	yes	10

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment/Req. Reconsideration-After Non-Final Reject	1	1
	Specification	2	2
	Claims	3	6
	Applicant Arguments/Remarks Made in an Amendment	7	10
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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (07-06)
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U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 13/387,343	Filing Date 02/15/2012	☐ To be Mailed

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☒		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	08/28/2012	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 18	Minus	** 20	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	* 1	Minus	***3	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/KATRINA . TURNER/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

24353 7590 09/12/2012
BOZICEVIC, FIELD & FRANCIS LLP
1900 UNIVERSITY AVENUE
SUITE 200
EAST PALO ALTO, CA 94303

EXAMINER	
BHAT, NARAYAN KAMESHWAR	
ART UNIT	PAPER NUMBER
1634	

DATE MAILED: 09/12/2012

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/387,343	02/15/2012	Sydney Brenner	CGLC-020	3537

TITLE OF INVENTION: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$870	$300	$0	$1170	12/12/2012

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

B. If the status above is to be removed, check box 5b on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above, or

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check box 5a on Part B - Fee(s) Transmittal and pay the PUBLICATION FEE (if required) and 1/2 the ISSUE FEE shown above.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PTOL-85 (Rev. 02/11)

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00855

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

24353 7590 09/12/2012
BOZICEVIC, FIELD & FRANCIS LLP
1900 UNIVERSITY AVENUE
SUITE 200
EAST PALO ALTO, CA 94303

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/387,343	02/15/2012	Sydney Brenner	CGLC-020	3537

TITLE OF INVENTION: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$870	$300	$0	$1170	12/12/2012

EXAMINER	ART UNIT	CLASS-SUBCLASS
BHAT, NARAYAN KAMESHWAR	1634	435-006100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) the names of up to 3 registered patent attorneys or agents OR, alternatively, 1
(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed. 2 3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:
- ☐ Issue Fee
- ☐ Publication Fee (No small entity discount permitted)
- ☐ Advance Order - # of Copies

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**
- ☐ A check is enclosed.
- ☐ Payment by credit card. Form PTO-2038 is attached.
- ☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
- ☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.
- ☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature Date

Typed or printed name Registration No.

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00856

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/387,343	02/15/2012	Sydney Brenner	CGILC-020	3537

24353 7590 09/12/2012
BOZICEVIC, FIELD & FRANCIS LLP
1900 UNIVERSITY AVENUE
SUITE 200
EAST PALO ALTO, CA 94303

EXAMINER	
BHAT, NARAYAN KAMESHWAR	
ART UNIT	PAPER NUMBER
1634	

DATE MAILED: 09/12/2012

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 0 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 0 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

PTOL-85 (Rev. 02/11)

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
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Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Applicant-Initiated Interview Summary	**Application No.** 13/387,343	**Applicant(s)** BRENNER ET AL.	
	Examiner NARAYAN BHAT	**Art Unit** 1634	

All participants (applicant, applicant's representative, PTO personnel):

(1) *Examiner: Bhat*. (3)_____.

(2) *Representative:Keddie*. (4)_____.

Date of Interview: *22 August 2012*.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: _____.

Issues Discussed ☐101 ☐112 ☒102 ☐103 ☐Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: *1*.

Identification of prior art discussed: *Laird*.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

The representative Mr. Keddie contacted the Examiner on August 22, 2012 to discuss the rejection of independent claim 1 as being anticipated by Laird. For compact prosecution, the representative suggested that claim 1 will be amended to include all the limitations of claim 14, (which was indicated as free of the art of record in the final office action) into the independent claim 1 to place the pending claims in better condition for allowance. The Examiner informed the representative that though the amendments appear to be in the right direction, needs to consult with the primary Examiner Kapushoc. The Examiner upon consultation with Examiner Kapushoc contacted the representative on August 28, 2012 to inform that the amended independent claim 1 as suggested by the representative would place the pending claims in better condition for allowance and requested fax the draft of amended claim 1 for an informal review. The Examiner also informed the representative that cancelation of claims 14 and 20 is needed to place the pending claims in better condition for allowance pending further search and other considerations. The representative informed the Examiner that the response to the final action will be filed along with the claim amendments.

The Examiner contacted the representative on September 6, 2012 to inform that the support for "30 bases" in amended claim 1 is not found in 61/288,792 and 61/235,595 provisional applications. However, support for 100 bases (i.e., 0.1 kb) is found in the said provisional applications. The representative, Mr. Keddie authorized to amend claim 1 in the claim listing of August 28, 2012 to include "100 bases" instead of "30 bases" on September 7, 2012 .

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/Narayan K. Bhat/ Examiner, Art Unit 1634	

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00860

Summary of Record of Interview Requirements

Manual of Patent Examining Procedure (MPEP), Section 713.04, Substance of Interview Must be Made of Record
A complete written statement as to the substance of any face-to-face, video conference, or telephone interview with regard to an application must be made of record in the application whether or not an agreement with the examiner was reached at the interview.

Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews
Paragraph (b)

In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for reply to Office action as specified in §§ 1.111, 1.135. (35 U.S.C. 132)

37 CFR §1.2 Business to be transacted in writing.
All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete an Interview Summary Form for each interview held where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below. Where the substance of an interview is completely recorded in an Examiners Amendment, no separate Interview Summary Record is required.

The Interview Summary Form shall be given an appropriate Paper No., placed in the right hand portion of the file, and listed on the "Contents" section of the file wrapper. In a personal interview, a duplicate of the Form is given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephone or video-conference interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the interview rather than with the next official communication.

The Form provides for recordation of the following information:
- Application Number (Series Code and Serial Number)
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (telephonic, video-conference, or personal)
- Name of participant(s) (applicant, attorney or agent, examiner, other PTO personnel, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). Note: Agreement as to allowability is tentative and does not restrict further action by the examiner to the contrary.
- The signature of the examiner who conducted the interview (if Form is not an attachment to a signed Office action)

It is desirable that the examiner orally remind the applicant of his or her obligation to record the substance of the interview of each case. It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview.

A complete and proper recordation of the substance of any interview should include at least the following applicable items:
1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of the specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the Examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner,
   (The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he or she feels were or might be persuasive to the examiner.)
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Summary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete and accurate, the examiner will give the applicant an extendable one month time period to correct the record.

Examiner to Check for Accuracy

If the claims are allowable for other reasons of record, the examiner should send a letter setting forth the examiner's version of the statement attributed to him or her. If the record is complete and accurate, the examiner should place the indication, "Interview Record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

Notice of Allowability	Application No. 13/387,343	Applicant(s) BRENNER ET AL.	
	Examiner NARAYAN BHAT	Art Unit 1634	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *August 28, 2012*.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are *1,4-13,15-19,21 and 22*.

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
   a) ☐ All b) ☐ Some* c) ☐ None of the:
      1. ☐ Certified copies of the priority documents have been received.
      2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
      3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ A SUBSTITUTE OATH OR DECLARATION must be submitted. Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL PATENT APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient.

6. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   (a) ☐ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached
      1) ☐ hereto or 2) ☐ to Paper No./Mail Date _____.
   (b) ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

7. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Notice of Draftperson's Patent Drawing Review (PTO-948)
3. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____
4. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
5. ☐ Notice of Informal Patent Application
6. ☒ Interview Summary (PTO-413), Paper No./Mail Date *8/22/12* .
7. ☒ Examiner's Amendment/Comment
8. ☒ Examiner's Statement of Reasons for Allowance
9. ☒ Other *SPEC-8/28/12 enter document*.

/Stephen Kapushoc/ Primary Examiner, Art Unit 1634	/Narayan K. Bhat/ Examiner, Art Unit 1634

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NOTICE OF ALLOWANCE

Interview Summary

The representative Mr. Keddie contacted the Examiner on August 22, 2012 to discuss the rejection of independent claim 1 as being anticipated by Laird. For compact prosecution, the representative suggested that claim 1 will be amended to include all the limitations of claim 14, (which was indicated as free of the art of record in the final office action) into the independent claim 1 to place the pending claims in better condition for allowance. The Examiner informed the representative that though the amendments appear to be in the right direction, needs to consult with the primary Examiner Kapushoc. The Examiner upon consultation with Examiner Kapushoc contacted the representative on August 28, 2012 to inform that the amended independent claim 1 as suggested by the representative would place the pending claims in better condition for allowance and requested fax the draft of amended claim 1 for an informal review. The Examiner also informed the representative that cancelation of claims 14 and 20 is needed to place the pending claims in better condition for allowance pending further search and other considerations. The representative informed the Examiner that the response to the final action will be filed along with the claim amendments.

The Examiner contacted the representative on September 6, 2012 to inform that the support for "30 bases" in amended claim 1 is not found in 61/288,792 and 61/235,595 provisional applications. However, support for 100 bases (i.e., 0.1 kb) is found in the said provisional applications. The representative, Mr. Keddie authorized to

amend claim 1 in the claim listing of August 28, 2012 to include "100 bases" instead of "30 bases" on September 7, 2012.

Claim Status

This office action is in response to the papers filed on August 28, 2012. Claims 1, 4-13, 15-19, 21 and 22 are pending in this application. Claim 1 is amended. Claims 14 and 20 are cancelled. The representative Mr. Keddie authorized on September 7, 2012 to delete "30 bases" in the claim listing of August 28, 2012 and insert in its place "100 bases." The claim amendments have been reviewed and entered. No new matter has been introduced by the claim amendments. With this office action it is reiterated that claims 1, 4-13, 15-19, 21 and 22 of August 28, 2012 are entered and all of the previous rejections have been withdrawn. All the references in the IDS of January 26, 2012 have been considered by the Examiner. Claims 1, 4-13, 15-19, 21 and 22 are allowed.

The papers in the instant application, in the PCT/IB2010/002243 and in the 61/288,792 and 61/235,595 provisional applications have been reviewed for the support for the newly added limitations of a polynucleotide of interest that is from 100 bases to 30 kilobases in length, wherein said polynucleotide of interest has having a desired site that is distal to the first domain. The support has been found in said applications. No new matter has been added by the amendments of claim 1.

Amendments to Specification and Claims are entered

The amendments to the specification and claims made on August 28, 2012 have been reviewed and entered. No new matter has been introduced by the said amendments because claim 14 at the time of filing had the support for "30 bases."

Priority

Receipt is acknowledged of papers submitted under 35 U.S.C. 119(a)-(d), which papers have been placed of record in the file. The certified copy of the PCT/IB2010/002243 filed in Great Britain has been received by the office. The support for the instant pending claims is found in the instant application, in the PCT/IB2010/002243 and in the 61/288,792 and 61/235,595 provisional applications.

Withdrawn Rejections and Response to the Remarks

The previous rejection of claims 1,4, 5, 8-12 and 16-18 under 35 U.S.C. 102(b) as being anticipated by Laird has been withdrawn in view of the persuasive arguments made by the Applicant that Laird does not specifically anticipate step 'c' (Remarks, pgs. 8 and 9). The arguments regarding the said rejection have been fully considered. The arguments of Laird does not discloses step 'c' of claim 1, which requires that a specific region of the product of step (b) is sequenced using a specific primer that is complementary to the primer site in the complement of the first domain, but rather sequencing the cloned fragments (Remarks, pg. 8). The arguments are persuasive to the extent Laird does not anticipate step 'c' because sequencing is not done directly on

the extended product. The arguments are moot in view of the withdrawn rejection necessitated by the claim amendments.

The Examiner acknowledges that Laird does not teach the newly added limitations of "a polynucleotide interest that is from 100 bases to 30 kilobases in length, wherein said polynucleotide of interest has a desired site that is distal to the first domain.

The previous rejection of claims 1 and 21 under 35 U.S.C. 103(a) as being unpatentable over Laird in view of Ushijima has been withdrawn in view of the persuasive arguments made by the Applicant (Remarks, pgs. 9 and 10) and also necessitated by the claim amendments. The arguments of filling the void in the teachings of Laird with a secondary reference, the Applicants submit that one of skill in the art would not sequence Laird's PCR products directly (i.e., using a primer that hybridizes to the PCR products) because Laird's PCR products contain a population of different sequences (caused by differing methylation patterns in original template). Because Laird's PCR products contain a population of different sequences, they need to be sequenced separately, and that is why they are cloned. Sequencing the products en masse using a primer that hybridizes to the PCR products would not reveal the sequences of the individual molecules have been fully considered (Remarks, pg. 9, paragraph 3). The Examiner acknowledges that the Laird teaches sequencing of the extended product by amplification and cloning as argued by the Applicant.

However, Laird also teaches the concept of using a primer that is complementary to the complement of the first domain to amplify the sequence by PCR (Fig. 1 steps B

and C). The sequencing of PCR product without cloning is well understood, conventional activity routinely practiced in the art to verify the amplified PCR product contains the amplified product. Therefore it would have been obvious to one having ordinary skill in the art to sequence the PCR products directly without the bisulfite treatment and cloning using the primer complementary to the extended PCR product.

However, the Examiner acknowledges that it would not have been obvious to one having ordinary skill in the art to modify the hairpin linker of Laird to include polynucleotide of interest in the loop having a length of 100 bases to 30 kilobases as required in the amended independent claim 1.

EXAMINER'S AMENDMENT

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Mr. Keddie on September 7, 2012.

In the claim listing of August 28, 2012, delete "30 bases" in line 9 of claim 1 and insert "100 bases" in the place of deleted "30 bases."

Claims allowed

Claims 1, 4-13, 15-19, 21 and 22 are allowed.

In the claim listing of August 28, 2012, claims 1, 4-13, 15-19, 21 and 22 have been renumbered as claims 1-18 according to 37 C.F.R. 1.126 (see MPEP 608.01 (j) and 608.01 (n) IV).

REASONS FOR ALLOWANCE

The following is an examiner's statement of reasons for allowance:

Laird alone or in combination with the art of the record does not disclose or make it obvious the claimed method steps 'a' to 'c', wherein the polynucleotide strand comprises in a 5' to 3' orientation: a first domain comprising (i) a primer site and (ii) a multiplex identifier (MID) comprising a unique nucleotide sequence that distinguishes said polynucleotide strand from other polynucleotide strands in said sample; the reflex sequence; a polynucleotide of interest that is from 100 bases to 30 kilobases in length, wherein said polynucleotide of interest has a desired site that is distal to the first domain and sequencing the MID and the desired site using a primer that is complementary to the primer site in the complement of the first domain.

The instant method has several advantages including defining the particular sections of nucleic acid sequence to be analyzed by a primer pair, as in PCR resulting in higher sequence specificity as compared to other extraction methods (e.g., using probes on a microarray) that only use a single oligonucleotide sequence, allows to analyze homologous genomic locations in a multiplexed sample (i.e., a sample having polynucleotides from different genomic samples) in which the polynucleotides are tagged with the multiplex identifier domain. This is possible because the reflex process,

which operates intramolecularly, maintains the multiplex identifier domain thus linking any particular fragment to the sample from which it originates and also allows determining the genetic linkage between different regions on the same large fragment that are too far apart to be sequenced in one sequence read.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Conclusion

Claims 1, 4-13, 15-19, 21 and 22 are allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Narayan K. Bhat whose telephone number is (571)-272-5540. The examiner can normally be reached on 8.30 am to 5 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Dave Nguyen can be reached on (571)-272-0731. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for

published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Narayan K. Bhat/

Examiner, Art Unit 1634

/Stephen Kapushoc/

Primary Examiner, Art Unit 1634

PubMed hairpin AND extension

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AMENDMENTS TO THE CLAIMS

Please amend the claims as shown below.

1. (Currently Amended) A method of sample analysis, comprising:

a) annealing a reflex sequence in a polynucleotide strand in a sample with a complement of the reflex sequence present in the polynucleotide strand, wherein the polynucleotide strand comprises the following in a 5' to 3' orientation:

a first domain comprising i. a primer site and ii. a multiplex identifier (MID) comprising a unique nucleotide sequence that distinguishes said polynucleotide strand from other polynucleotide strands in said sample;

the reflex sequence;

a polynucleotide of interest **that is from 30 bases to 30 kilobases in length, wherein said polynucleotide of interest has** ~~having~~ a desired site that is distal to the first domain; and

the complement of the reflex sequence, wherein the complement of the reflex sequence is present at the 3' end of the polynucleotide strand;

b) extending from the 3' end of the complement of the reflex sequence through the first domain to produce a double stranded region comprising a complement of the first domain at the 3' end of the extended polynucleotide strand, thereby moving the first domain into proximity with the desired site; and

c) sequencing the MID and the desired site using a primer that **is** complementary to the primer site in the complement of the first domain.

2-3. (Canceled)

4. (Original) The method of claim 1, wherein the reflex sequence is from 10 to 50 nucleotides in length.

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5. (Previously presented) The method of claim 1, wherein the first domain comprises an RNA polymerase site.

6. (Original) The method of claim 1, the method further comprising removing the first domain and the reflex sequence from the 5' end of the double stranded region.

7. (Original) The method of claim 6, wherein the removing step comprises treating the nucleic acid of step b with T7 exonuclease.

8. (Original) The method of claim 1, wherein the first domain is present in an adapter region attached to the polynucleotide of interest.

9. (Original) The method of claim 8, wherein the reflex sequence is present in the adapter region.

10. (Original) The method of claim 8, wherein the reflex sequence is a sequence present within the 5' region of the polynucleotide of interest.

11. (Original) The method of claim 1, wherein the sample is a multiplexed sample, wherein the first domain in each of the polynucleotide strands in the multiplexed sample comprises a MID corresponding to its parental sample.

12. (Original) The method of claim 1, wherein the sample comprises multiple polynucleotide strands, and wherein each polynucleotide strand in the sample comprises a unique MID in the first domain.

13. (Original) The method of claim 1, wherein the polynucleotide strand is produced by:

annealing a nucleic acid synthesis primer to a parent polynucleotide strand, wherein the parent polynucleotide strand comprises the following in a 5' to 3' orientation:

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the first domain;

the reflex sequence; and

a second domain comprising the polynucleotide of interest;

wherein the nucleic acid synthesis primer comprises:

a 3' priming domain that is complementary to a site adjacent to and 3' of the polynucleotide of interest in the second domain; and

a 5' reflex sequence;

extending the nucleic acid synthesis primer to produce an extension product; and

replicating the resultant extension product with a second nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain, thereby producing the polynucleotide strand.

14. (Cancelled).

15. (Original) The method of claim 13, wherein the parent polynucleotide strand is enriched prior to the annealing step based on its sequence.

16. (Original) The method of claim 1, further comprising the steps of:

c) replicating the polynucleotide strand of step b with a first nucleic acid synthesis primer that anneals at the 3' end of the complement of the first domain to produce a first extension product;

d) annealing a second nucleic acid synthesis primer to the first extension product, wherein the second nucleic acid synthesis primer comprises a 3' priming domain that is complementary to a site within the polynucleotide of interest in the first extension product; and

e) extending the second synthesis primer to produce a second extension product.

17. (Original) The method of claim 16, wherein the second synthesis primer comprises a 5' domain that is not complementary to the polynucleotide of interest in the first extension product.

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18. (Original) The method of claim 17, wherein the first domain comprises a first primer site and the 5' domain of the second synthesis primer comprises a second primer site.

19. (Original) The method of claim 18, wherein the length of the polynucleotide of interest in the second extension product is from 50 to 10,000 nucleotides.

20. (Cancelled)

21. (Previously presented) The method of claim 1, wherein polynucleotide of interest is cDNA.

22. (Previously presented) The method of claim 1, further comprising repeating said method to obtain the nucleotide sequence of a second desired site in said polynucleotide of interest, and analyzing the sequence obtained in c) and the sequence of the second desired site to obtain linkage information about polymorphisms in said polynucleotide of interest.

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... Other blocks are more stable but also more expensive. Zhou et al. [2008] elegantly solved this issue by using a so-called **snapback primer**, that is, a 50 tailed primer including a loop region and a sequence complementary to its extension product, covering the variant to scan. ...

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... Closed-tube analysis eliminates the risk of PCR con- tamination. Furthermore, **Snapback primer** annealing is rapid and compatible with the fastest PCR protocols. ... [0019] FIG. 2 shows the intramolecular hybridization of a **Snapback primer**. [0020] FIG. ...

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L32	7691	l31 and (polynucleotide or dna or nucleic) near5 (domain or segment or region or section) near5 complementary	USPAT; UPAD	OR	ON	2012/09/02 14:07
L34	1040	l32 and exten$5 near5 ("3'" or "3 '")same (domain or segment or region or section)	USPAT; UPAD	OR	ON	2012/09/02 14:07
L35	6	l34 and (mid or barcode or zipcode or token or identifier adj multiplex)near5 (domain or segment or region or section)	USPAT; UPAD	OR	ON	2012/09/02 14:08
L36	69	l34 and (mid or barcode or zipcode or token or identifier adj multiplex)same(domain or segment or region or section)	USPAT; UPAD	OR	ON	2012/09/02 14:08
L37	2	l36 and sequencing same (mid or barcode or zipcode or token or identifier adj multiplex)	USPAT; UPAD	OR	ON	2012/09/02 14:09

9/2/2012 2:10:52 PM
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Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	13387343	BRENNER ET AL.
	Examiner	Art Unit
	NARAYAN BHAT	1634

SEARCHED			
Class	Subclass	Date	Examiner
435	6.1, 6.11, 6.12, 91.2 and 287.2	9/2/12	Bhat N.
536	23.1, 24.3 and 24.33	9/2/12	Bhat N.

SEARCH NOTES		
Search Notes	Date	Examiner
Searched for the inventors and assignee in PALM for the ODP.	9/2/12	Bhat N.
Searched for the topics in East, Pubmed and Google scholar. Search histories are enclosed.	9/2/12	Bhat N.
Reviewed papers in the PCT/IB2010/002243, 61288,792 and 61/235,595 provisional applications	9/2/12	9/2/12

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner
	Searched for key words in East. Search history is enclosed.	9/2/12	Bhat N.

/NARAYAN BHAT/ Examiner.Art Unit 1634	

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Issue Classification	Application/Control No.	Applicant(s)/Patent Under Reexamination
	13387343	BRENNER ET AL.
	Examiner	Art Unit
	NARAYAN BHAT	1634

ORIGINAL						INTERNATIONAL CLASSIFICATION											
CLASS			SUBCLASS			CLAIMED						NON-CLAIMED					
435			6.1			C	1	2	O	1 / 68 (2006.01.01)							
CROSS REFERENCE(S)						C	1	2	P	19 / 34 (2006.01.01)							
						C	0	7	H	21 / 02 (2006.01.01)							
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					C	0	7	H	21 / 04 (2006.01.01)							
435	6.11	6.12	91.2	91.53	287.2												
536	23.1	24.2	24.3	24.33													

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original
	1		21														
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	18																
	19																

/NARAYAN BHAT/ Examiner.Art Unit 1634	09/02/2012	Total Claims Allowed:
(Assistant Examiner)	(Date)	18
/STEPHEN KAPUSHOC/ Primary Examiner.Art Unit 1634	09/06/2012	O.G. Print Claim(s): 1 / O.G. Print Figure: None
(Primary Examiner)	(Date)	

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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	13387343	BRENNER ET AL.
	Examiner	Art Unit
	NARAYAN BHAT	1634

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original									
	1	=								
	2	-								
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	4	=								
	5	=								
	6	=								
	7	=								
	8	=								
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	21	=								
	22	=								

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AMENDMENT TO THE SPECIFICATION

Please amend the paragraph starting at line 15 of page 20 as follows:

Figure 1A shows a single stranded polynucleotide 100 comprising, in a 5' to 3' orientation, a first domain (102; the domain to be moved); a reflex sequence 104; a nucleic acid sequence 106 having a site distal to the first domain (Site A), and a complement of the reflex sequence 108 (positioned at the 3' terminus of the polynucleotide). **Nucleic acid sequence 106 may be from 30 bases to 30 kilobases in length.** The steps of the reflex method described below will move the first domain into closer proximity to Site A. It is noted here that the prime designation in Figure 1A denotes a complementary sequence of a domain. For example, First Domain' is the complement of the First Domain.

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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
S1	16305	brenner.in. or mikawa.in. or osborne.in. or slater.in. or population.as.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/09/02 13:23
S2	254	S1 and (primer or oligonucleotide or probe) near5 (anneal$3 or bind$3 or hybridi$6) near5 (polynucleotide or dna or nucleic)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/09/02 13:26
S3	17	S2 and (polynucleotide or dna or nucleic) near5 (domain or segment or region or section) same complementary same loop	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:28
S4	13	S3 and exten$5 near5 ("3'" or "3'")	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:30
S5	0	S4 and loop near5 (mid or barcode or zipcode or token or identifier adj multiplex)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:31
S6	2	S4 and (mid or barcode or zipcode or token or identifier adj multiplex)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:31
S7	458	S1 and (mid or barcode or zipcode or token or identifier adj multiplex)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:35
S8	7	S7 and loop same (mid or barcode or zipcode or token or identifier adj multiplex)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:36
S9	2	S7 and (polynucleotide or dna or nucleic) near5 (domain or segment or region or section) same complementary same loop	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:36
S10	29	S7 and (polynucleotide or dna or nucleic) near5 (domain or segment or region or section) same complementary	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:37

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S11	18	S10 and exten$5 near5 ("3'" or "3'")	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:37
S12	704	(polynucleotide or dna or nucleic) near5 (mid or barcode or zipcode or token or identifier adj multiplex) and (adapt$3 or linker)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:41
S13	0	S12 and identifier adj multiplex near5 (domain or segment or section or region)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:42
S14	0	S12 and identifier adj multiplex same (domain or segment or section or region)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:42
S15	43	S12 and (polynucleotide or dna or nucleic) near5 (domain or segment or region or section) same complementary same loop	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:43
S16	0	(mid or barcode or zipcode or token or identifier adj multiplex) same (adapt$3 or linker) adj (hairpin or stem adj loop)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:47
S17	14	(mid or barcode or zipcode or token or identifier adj multiplex) and (adapt$3 or linker) adj (hairpin or stem adj loop)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:47
S18	1093	(adapt$3 or linker or probe or oligonucleotide) adj (hairpin or stem adj loop)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:50
S19	99	S18 and (mid or barcode or zipcode or token or identifier adj multiplex) and primer near5 (hairpin or stem adj loop)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:51
S20	84	S19 and exten$5 near5 ("3'" or "3'")	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:51
S21	842	S18 and (domain or segment or region or section) same complementary	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:54
S22	422	S21 and exten$5 near5 ("3'" or "3'")	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:54

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S23	27	S22 and sequencing same primer same complementary same exten$3	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 13:56
S24	320583	"435"/$.ccls. or "536"/$.ccls.	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 14:00
S25	67122	S24 and (primer or oligonucleotide or probe) near5 (anneal$3 or bind$3 or hybridi$6) near5 (polynucleotide or dna or nucleic)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	OFF	2012/09/02 14:01
S26	25697	S25 and (polynucleotide or dna or nucleic) near5 (domain or segment or region or section) same complementary	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 14:01
S27	3654	S26 and exten$5 near5 ("3'" or "3 '")same (domain or segment or region or section)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 14:02
S28	189	S27 and (mid or barcode or zipcode or token or identifier adj multiplex)same (domain or segment or region or section)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 14:03
S29	165	S28 and sequencing	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 14:03
S30	7	S29 and sequencing near5 (mid or barcode or zipcode or token or identifier adj multiplex)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 14:04
S31	2	"snap back" near5 (dna or nucleic or polynucleotide) near5 exten$4 near3 ("3'" or "3 '")	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 14:31
S32	6	"snap back" same (dna or nucleic or polynucleotide) same exten$4 same ("3'" or "3 '")	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 14:32
S33	13	"snap back" same (dna or nucleic or polynucleotide) same exten$4	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 14:39
S40	9	"snap back" near5 primer	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 16:13

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S41	42	("snap back" or snapback)near5 primer	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 16:37
S42	87	("snap back" or snapback)near5 (primer or probe or oligonucleotide)	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/04 09:50
S43	36	S42 and exten$5 near5 ("3'" or "3'")	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/04 09:51

9/6/2012 11:22:27 AM
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EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L40	13	"snap back" same (dna or nucleic or polynucleotide) same exten$4	US-PGPUB; USPAT; FPRS; EPO; JPO; DERWENT; IBM_TDB	OR	ON	2012/09/02 14:39

9/ 2/ 2012 2:50:01 PM
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"hairpin primer" domain moving

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Herpes simplex virus processivity factor UL42 imparts increased DNA-binding specificity to the viral DNA polymerase and decreased dissociation from primer-...
F Weisshart, DS Chow, DM Coen - Journal of Virology, 1995 - Am Soc Microbiol
... Pol, consistent with previous results obtained by Gottlieb and Challberg by using **hairpin primer**-template DNA ... Certain mutations that affect this **domain** impair DNA polymerase activity and in vivo ... instead of Pol/UL42 **moving** along the DNA, the newly synthesized DNA loops and ...
Cited by 54 Related articles All 6 versions

[HTML] from nih.gov

The TE promoter element of the histone H1t gene is essential for transcription in transgenic mouse primary spermatocytes
HP Ranger, SA Wesley, SR Grimes Jr - Biology of reproduction, 1999 - Soc Study Reprod
... First, the plasmid pPS3–18 was constructed by **moving** the entire 6.85-kb rat genomic EcoRI ... mice were used with the H1TAC box primer and the rat post-**hairpin primer** (Materials and ... Crystal structure of globular **domain** of histone H5 and its implications for nucleosome binding. ...
Cited by 10 Related articles All 5 versions

[HTML] from nichegprod.org

Linear diffusion on DNA despite high-affinity binding by a DNA polymerase processivity factor
KP Koller, EM Geertsema, GN Duda - Nanomaterials, 2021 - mdpi.com
... Figure 2. Footprinting of Pol/UL42 after Chain Elongation. (A) Experimental design. The sequence of the **hairpin primer**-template, HP96-MT, is shown. HP96-MT was radiolabeled at the 5′ end (indicated by asterisk). The primer ...
Cited by 21 Related articles All 6 versions

Mechanisms for replicating DNA
CK Suzuki, TJ Kelly - DNA replication in eukaryotic cells, 2021 - books.google.com
... by a duplex hairpin at the terminus of the genome (see below), there remains the problem of replicating the **hairpin primer** sequence itself. ... as in vitro experiments have shown that the phage T4 replication apparatus can bypass an RNA polymerase complex **moving** in either ...
Cited by 27 Related articles All 3 versions

[PDF] from usf.edu

In-house HIV-1 RNA real-time RT-PCR assays: principle, available tests and usefulness in developing countries
F Rouet, H Montcho, J Viljoen, ... - Expert review of ..., 2010 - Taylor & Francis
... In the absence of the target, scorpions constitute a nonfluorescent complex (the quencher absorbs the fluorescence emitted by the fluorophore). During annealing the **hairpin primer** binds to the template and is then extended ...
Cited by 14 Related articles All 7 versions

Mostly Natural DNA Sequencing by Synthesis
N Hagag, E Kools - US Patent App. 12/263,041, 2010 - Google Patents
... platforms, so that reagents can be added and removed from a given sample without **moving** the platform ... In the third read, a **hairpin primer** is attached to the 3' end of the extended strand ... Family B polymerases typically have a single catalytic **domain** with polymerase and 3' to 5 ...
Cited by 4

Escherichia coli DnaA protein loads a single DnaB helicase at a DnaA box hairpin
KM Carr, JL Kaguni - Journal of Biological Chemistry, 2001 - ASBMB
... with the nucleotide serving to alter the conformation of an N-terminal **domain** of DnaC ... Following the release of DnaC, DnaB is then free to **move** on the ssDNA. ... **Primer** extension assays were performed as described under "Experimental Procedures" with the indicated proteins ...
Cited by 32 Related articles All 6 versions

[HTML] from jbc.org

Uracil recognition in archaeal DNA polymerases captured by X-ray crystallography
SJ Fogg, J Kilkenny, D Hopkins, PA Lewis, ... - Journal of molecular ..., 2014 - Elsevier
... The best diffracting crystals were obtained with a 26-mer **hairpin primer**–template mimic containing uracil at the critical − 4 ... When analysed on a **domain**-by-**domain** basis, the comparison, summarized in Table 2, illustrates which domains **move** most significantly (Fig ...
Cited by 23 Related articles All 6 versions

DNA virus replication
SK Weller - Trends and Advances in Microbiology and Molecular ..., 2016 - Wiley Online Library
... Replication bubbles could be observed which consisted of two replication forks **moving** in opposite directions. ... Considerable effort has been given to understanding the structure and function of the 708 residues of SV40 large T. The **domain** structure of this multifunctional protein ...
Cited by 2 Related articles All 2 versions

HIV-1 Reverse Transcriptase Inhibitors and Mechanisms of Resistance
B Marchand, SG Sarafianos - Viral Genome Replication, 2009 - Springer
Page 1. Chapter 24 HIV-1 Reverse Transcriptase Inhibitors and Mechanisms of Resistance Bruno Marchand and Stefan G. Sarafianos Due to its vital role in the viral life cycle and to the lack of a mammalian equivalent, the reverse ...
Related articles

[PDF] Oligonucleotide Design and Codon Optimization for PCR-based Gene Synthesis
PJ Rayner, 10/30 - gsurat.utexas.edu

[PDF] from mit.edu

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Page 1. Oligonucleotide Design and Codon Optimization for PCR-based Gene Synthesis by Paul Jamesen Steiner SB Computer Science and Engineering MIT. Submitted to the Department of Electrical Engineering and Computer ...
Related articles - View as HTML - Library System

Multi-stage amplification reactions by control of sequence replication times
I Kozma, S Jeremias - US Patent App. 11/746,240, 2007 - Google Patents
Page 1. (19) United States (12) Patent Application Publication Kozma et al. US 20080102496A1 (10) Pub. No.: US 2008/0102496 A1 (43) Pub. Date: May 1, 2008 (54) MULTI-STAGE AMPLIFICATION REACTIONS BY CONTROL ...
Related articles - All 5 versions

The replication of cowpea mosaic virus
A Van Kammen, HR Diggelen - Biochimica et Biophysica Acta (BBA) ..., 1976 - Wiley Online Library
... of M-RNA following in vitro inoculation of cowpea protoplasts, B-RNA cannot **move** to adjacent cells ... The viral replicase starting elongation at the **hairpin primer** then further transcribes the viral RNA template ... cleavage carried out by the proteolytic activity in the 24K **domain** of the ...
Cited by 6 - Related articles

Pseudo-cyclic oligonucleobases
S Agrawal, EP Kandimalla - US Patent 6,583,266, 2003 - Google Patents
... Anti-sen se- **domain** JCGCACGG \ GCGTGCC 3c C ggtcactc < .GGN GG8 L Protective **domain** CGG ! CACYCCTCCGTGCG 'ACCGCCGCCAGUGAGGAGGCACGCAGCCUU FIGURE 1 ... These include TaqMan (40 41), molecular beacon (42), **hairpin-primer** (43), and scorpion (44 ...
Cited by 18 - Related articles - All 3 versions

A Synthetic DNA Used in Amplification Reactions
US Patent - AutoScout DNA - Instruments and Accessories, 2007 - Instrumentation.com
... or any other desired sequence is introduced on the target sequence by one primer after opposite primer extension.(B) **Hairpin primer** for detection ... can be reduced either by performing the reaction in narrow capillaries instead of tubes (eg. Reference 45) or by **moving** the sample ...
Related articles

Methods For Preparing Sequencing Libraries
M Adil, BE Burnham, YE Makarenkov - US Patent App. 12/503,910, 2010 - Google Patents
... 2 Hm 2 O Meis! g Nil QE—!0xb4 6: , § M:.-=2 v5 g !1<zf2 Ei.! §, m02 O ¡ Ruxxi § Tali Z .! !"!" " " !": ! tab !Ikb **Domain** Size (log scale) FIG 3C Page 10. Patent Application Publication Aug. ... In one embodiment, the primer is a **hairpin primer**. ...
All 3 versions

Systems and methods for multiplex analysis of PCR in real time
DF Wiemann, DJ Dailey - US Patent 7,141,377, 2006 - Google Patents
... In the case of superparamagnetism, this return to random orientation of the domains is nearly instantaneous, while paramagnetic materials will retain **domain** alignment for some period of time after removal of the magnetic field. ...
Related articles - All 5 versions

Closed-system multi-stage nucleic acid amplification reactions
J Ching, R Cheng, DB Dailey, SF Cheng, ... - US Patent App. 11/..., 2006 - Google Patents
Page 1. US 20060177844A1 (19) United States (12) Patent Application Publication (10) Pub. No.: US 2006/0177844 A1 Ching et al. (43) Pub. Date: Aug. 10, 2006 (54) CLOSED-SYSTEM MULTI-STAGE NUCLEIC ACID AMPLIFICATION ...
Cited by 1 - Related articles - All 2 versions

NUCLEIC ACID AMPLIFICATION EMPLOYING TEMPERATURE OSCILLATION
M Lee, A Inventor - US Patent 20,100,099,100, 2010 - freepatentsonline.com
... In a particular embodiment, the temperature is allowed to **move** in a downward direction. ... This means that another portion (a target closing **domain**) of the torch that does not hybridise to ... least not to the same extent in the amplicon as compared to the **hairpin primer**, the existence ...
Cached

Napiwotzky, Daniel [PDF] from uni-tuebingen.d
... Figure 2: The semaphorin family 6 subclasses of semaphorins are described with a total of 20 proteins. Semaphorins are proteins with a typical amino-terminal, cystein-rich 'Sema' **domain**. ... SEMA7A is a polypeptid which contains a 391 AA 'SEMA' **domain** 49-50 and a ...
Related articles - All 2 versions

Peptide Nucleic Acid (PNA) Hybridization to Nucleic Acid Targets [PDF] from idtl.org
... - 2001 - repository.tamu.edu
Page 1. PEPTIDE NUCLEIC ACID (PNA) HYBRIDIZATION TO NUCLEIC ACID TARGETS APPROVED BY SUPERVISORY COMMITTEE ..... David R. Corey, Ph.D., Advisor ...
Related articles - All 5 versions

Real-Time Quantitative PCR: Theory and Practice
CT Bustin - Encyclopedia of Molecular Cell Biology and ..., 2005 - Wiley Online Library
... This trend means that the technology is **moving** from one found primarily in core laboratories to the bench tops of individual investigators. ... The plate is scanned once for each fluo- rescent dye with the filter wheels **moving** in tandem to bring the new filter pair into the optical path ...
Cited by 1 - Related articles - All 2 versions

Cellular and molecular studies of postembryonic muscle fibre recruitment in zebrafish (Danio rerio L.) [PDF] from st-andrews.ac.u
... - 2012 - research-repository.st-andrews.ac.uk
... Its protein encompassed a single conserved **domain** (DUF410) of unknown function although knock-down of cee in C. elegans and yeast have suggested a role in regulating ... Page 10. 5 In the wild, zebrafish generally inhabit in shallow and slow-**moving** water varying ...

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Library Search - All 3 versions

[PDF] Structural determinants of murine leukemia virus(MLV) reverse transcriptase(RT) important for fidelity and drug-resistance in vivo.[electronic resource] [PDF] from wvu.edu
[author block] - [year] - [publication info]
... Reverse transcription continues, **moving** from the 3' end of the RNA genome towards the PBS and ... This involves both the αH and αI helices of the thumb **domain** that bind to the primer and ... involve the β12-β3 **hairpin** (**primer** grip) and regions including portions of the fingers, ...
Related articles - View as HTML - All 4 versions

[CITATION] Viral RNA synthesis
[author block] - [publication info]
Cited by 60 - Related articles

Biochemical characterization of adeno-associated virus rep68 DNA helicase and ATPase activities [HTML] from nih.gov
[author block] - [publication info]
... as a terminal-repeat-unwinding protein which catalyzes the conversion of a duplex end to a **hairpin** **primer**. ... In 3 min, Rep68 would **move** 1,035 bases, assuming a rate of translocation of 345 ... and Kodin (20a) reported that a fusion protein of the maltose binding **domain** and Rep52 ...
Cited by 68 - Related articles - BL Direct - All 4 versions

Insights into DNA polymerization mechanisms from structure and function analysis of HIV-1 reverse transcriptase
[author block] - [publication info]
... The polymerase **domain** was found to contain four distinct subdomains (fingers, palm, thumb, and connection). ... Other interactions occur between the β12-β3 **hairpin** (**primer** grip) and p4, αB, β6-αE, and p5a (template grip) and the base pairs closest to the polymerase active ...
Cited by 171 - Related articles - BL Direct - All 4 versions

Invasive cleavage of nucleic acids
[author block] - [publication info]
Cited by 56 - Related articles - All 4 versions

Detection of nucleic acids by multiple sequential invasive cleavages
[author block] - [publication info]
The present invention relates to means for the detection and characterization of nucleic acid sequences, as well as variations in nucleic acid sequences. The present invention also relates to methods.
Cited by 67 - Related articles - Cached - All 3 versions

Cleavage of nucleic acid acid using thermostable methanococcus jannaschii FEN-1 endonucleases
[author block] - [publication info]
The present invention relates to means for cleaving a nucleic acid cleavage structure in a site-specific manner. Structure-specific nucleases, including 5' nucleases, thermostable FEN-1 endonucleases,
Cited by 111 - Related articles - Cached

[PDF] Direct antigen presentation by medullary thymic epithelial cells is essential for CD4 T cell tolerance [PDF] from ulaval.ca
[author block] - [year] - [publication info]
... Structural analysis of AIRE shed some light on how AIRE might exert its function. First, AIRE contains a SAND **domain** (Sp100 AIRE-1 Nuc/P41/75 DEAF-1) that was ... Second, the PHD finger (plant homeo **domain**), a typical protein-protein interaction site, was speculated to ...
Cited by 1 - Related articles - View as HTML

Cleavage of nucleic acids
[author block] - [publication info]
The present invention relates to means for the detection and characterization of nucleic acid sequences, as well as variations in nucleic acid sequences. The present invention also relates to methods.
Cited by 58 - Related articles - Cached - All 4 versions

Cleavage agents
[author block] - [publication info]
The present invention relates to means for the detection and characterization of nucleic acid sequences, as well as variations in nucleic acid sequences. The present invention also relates to improved.
Cited by 50 - Related articles - Cached

Nucleic acid detection kits
[author block] - [publication info]
The present invention relates to means for the detection and characterization of nucleic acid sequences, as well as variations in nucleic acid sequences. The present invention also relates to methods.
Cited by 7 - Related articles - Cached

FEN-1 endonuclease, mixtures and cleavage methods
[author block] - [publication info]
The present invention relates to means for the detection and characterization of nucleic acid sequences, as well as variations in nucleic acid sequences. The present invention also relates to improved.
Cited by 2 - Related articles - Cached

Methods and compositions for detecting target sequences
[author block] - [publication info]

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00912

The present invention relates to compositions and methods for the detection and characterization of nucleic acid sequences and variations in nucleic acid sequences. The present invention relates to me...
Cited by 12 - Related articles - Cached

FEN-1 endonucleases, mixtures and cleavage methods
MW Kaiser, VI Lyamichev, ... - US Patent ..., 2003 - freepatentsonline.com
The present invention relates to means for the detection and characterization of nucleic acid sequences, as well as variations in nucleic acid sequences. The present invention also relates to improved...
Cited by 4 - Related articles - Cached

Comparison of the replication of positive-stranded RNA viruses of plants and animals
KW Buck - Advances in virus research, 1996 - Elsevier
... Since there is evidence that plant proteins and/or mRNAs **move** through plasmodesmata during the course of normal plant development (Lucas ... Mutations in the central polymerase-like **domain** of the brome mosaic virus 2a protein encompassing motif IV and flanking sequences ...
Cited by 383 - Related articles - BL Direct - All 4 versions

[BOOK] Studies of structure, hydration and dynamics of biological macromolecules by use of NMR spectroscopy
S Xia - 2002 - scholarbridge.com
... of biomolecules from NMR constraints EDTA Ethylenediamine tetraacetic acid FID Free induction decay γ Gyromagnetic ratio GFT G-matrix Fourier transform GLOMSA Global method for stereospecific assignments η Viscosity HB Hydrogen Bond HP **Hairpin primer** HPLC High ...
All 3 versions

Invasive cleavage of nucleic acids
C Hall, D Jeffs, P Lyamichev, MW Kaiser, ... - EP Patent ..., 2011 - freepatentsonline.com
<p>The present invention relates to a composition for detecting a target nucleic acid, comprising:
 a) a purified thermostable FEN-1 endonuclease, and
 b) a probe oligonucleotide, wherein a t...
Cached

Invasive cleavage of nucleic acids
JG Hall, N Lyamichev, MW Kaiser, ... - EP Patent ..., 2003 - freepatentsonline.com
<p>The present invention relates to a set of reagents for the detection and characterization of nucleic acid sequences, as well as variations in nucleic acid sequences. The present invention also rela...
Related articles - Cached

Overview of real-time RT-PCR strategies for quantification of gene rearrangements in the myeloid malignancies
L Pallisgaard, M Dahl, J Hansen, ... - Methods in Molecular Medicine, 2004 - Springer
... The 3' sequence specifically anneals to a **hairpin primer**. ... This DNAzyme has a catalytic **domain** of 15 deoxynucleotides flanked by two substrate-recognition domains (arms). ... The polymerase continues to **move** from the now free end to the new bifurcation, and the 5'-3' ...
Cited by 10 - Related articles - BL Direct - All 3 versions

Invasive cleavage of nucleic acids
JG Hall, N Lyamichev, MW Kaiser, ... - EP Patent ..., 2008 - freepatentsonline.com
Complete Patent Searching Database and Patent Data Analytics Services.
Related articles - Cached

FEN-1 endonucleases, mixtures and cleavage methods
MW Kaiser, VI Lyamichev, ... - EP Patent ..., 2008 - freepatentsonline.com
<p>The present invention relates to means for the detection and characterization of nucleic acid sequences, as well as variations in nucleic acid sequences. The present invention also relates to impro...
Related articles - Cached

FEN-1 ENDONUCLEASES, MIXTURES AND CLEAVAGE METHODS
MW Kaiser, VI Lyamichev, ... - EP Patent ..., 2008 - freepatentsonline.com
Complete Patent Searching Database and Patent Data Analytics Services.
Related articles - Cached

Fen-1 endonucleases, mixtures and cleavage methods
MW Kaiser, VI Lyamichev, ... - EP Patent ..., 2011 - freepatentsonline.com
... ends. A first region along a nucleic acid strand is said to be upstream of another region if the 3' end of the first region is before the 3' end of the second region when **moving** along a strand of nucleic acid in a 5' to 3' direction. ...
Cached

DNA replication in viruses
S Weitz - Annual Review of genetics, 1982 - Web - Annualreviews.org
... Its N-terminal region contains varying amounts of phosphate, which affect the quality of ssDNA-binding to the C-terminal **domain** (Klein et al 1979). ... While rep protein moves along the ssDNA tail in a 3'→5' direction helicase I, II and III **move** in a 5'→3' direction. ...
Cited by 175 - Related articles - All 5 versions

Telomerase catalytic subunit
KB Beattie, T Kilian, ... - Gene & cancer (Lett. Pub. Co., 2001), 2001 - Google Patents
Cited by 45 - Related articles - All 2 versions

Create alert

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00913

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00914

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

24353 7590 09/12/2012
BOZICEVIC, FIELD & FRANCIS LLP
1900 UNIVERSITY AVENUE
SUITE 200
EAST PALO ALTO, CA 94303

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/387,343	02/15/2012	Sydney Brenner	CGLC-020	3537

TITLE OF INVENTION: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

APPLN. TYPE	SMALL ENTITY	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	YES	$870	$300	$0	$1170	12/12/2012

EXAMINER	ART UNIT	CLASS-SUBCLASS
BHAT, NARAYAN KAMESHWAR	1634	435-006100

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.
1 Bozicevic, Field & Francis LLP
2 James S. Keddie, Ph.D.
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: Population Genetics Technologies LTD
(B) RESIDENCE: (CITY and STATE OR COUNTRY): Cambridge, United Kingdom

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:
☒ Issue Fee
☒ Publication Fee (No small entity discount permitted)
☐ Advance Order - # of Copies

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**
☐ A check is enclosed.
☒ Payment by credit card. Form PTO-2038 is attached.
☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number 50-0815 (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
☐ a. Applicant claims SMALL ENTITY status. See 37 CFR 1.27.
☐ b. Applicant is no longer claiming SMALL ENTITY status. See 37 CFR 1.27(g)(2).

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature /James S. Keddie, Ph.D., Reg. No. 48,920/ Date September 19, 2012
Typed or printed name James S. Keddie, Ph.D. Registration No. 48,920

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

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Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00915

Filed Electronically September 19, 2012

AMENDMENT UNDER 37 C.F.R. §1.312 Address to: Mail Stop Issue Fee Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Attorney Docket	CGLC-020
	Confirmation No.	3537
	First Named Inventor	Brenner, Sydney
	Application Number	13/387,343
	Filing Date	February 15, 2012
	Group Art Unit	1634
	Examiner Name	Bhat, Narayan Kameshwar
	Title "Compositions and Methods for Intramolecular Nucleic Acid Rearrangement"	

Sir:

This amendment is responsive to the Notice of Allowance and Fees Due dated December 12, 2012. Applicants submit that the amendments set forth below raise no new issues. Entry of this amendment is thus respectfully requested.

AMENDMENTS

IN THE SPECIFICATION

Please amend the paragraph starting at line 15 of page 20 as follows:

Figure 1A shows a single stranded polynucleotide 100 comprising, in a 5' to 3' orientation, a first domain (102; the domain to be moved); a reflex sequence 104; a nucleic acid sequence 106 having a site distal to the first domain (Site A), and a complement of the reflex sequence 108 (positioned at the 3' terminus of the polynucleotide). **Nucleic acid sequence 106 may be from 100 bases to 30 kilobases in length.** The steps of the reflex method described below will move the first domain into closer proximity to Site A. It is noted here that the prime designation in Figure 1A denotes a complementary sequence of a domain. For example, First Domain' is the complement of the First Domain.

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00917

Formal Matters

Claims 1, 4-13, 15-19, 21 and 22 are allowed.

The specification is amended to say that the nucleic acid sequence 106 (which corresponds to the polynucleotide of interest in the claims) may be from 100 bases to 30 kilobases in length. Support for this amendment is found at, e.g., page 3, lines 12-13 of 61/235,595 and page 37, lines 1-2 of 61/288,792, which are incorporated by reference into the instant application. Support for the amendment is also implicitly found in the instant specification in the combination of claim 14 as originally filed (which describes a second domain that is of 30 bases to 30 kilobases in length) and page 27, lines 27-28 (which describes a DNA fragments that are of 100 to 5000 base pairs in length).

In the Notice of Allowance, the Examiner stated the introduction of "100 bases to 30 kilobases" into claim 1 does not represent new matter. See, e.g., the last paragraph of page 3. As such, this amendment should not generate new issues for the Examiner.

Interview Summary

The Examiner's characterization of the interview are essential correct.

The amendment to claim 1 should not be interpreted as a acquiescence to the correctness of any rejection. Rather, the amendment to claim 1 was to expedite prosecution and receive an early allowance.

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00918

CONCLUSION

The Commissioner is hereby authorized to charge any underpayment of fees up to a strict limit of $3,000.00 beyond that authorized on the credit card, but not more than $3,000.00 in additional fees due with any communication for the above referenced patent application, including but not limited to any necessary fees for extensions of time, or credit any overpayment of any amount to Deposit Account No. 50-0815, order number CGLC-020.

Respectfully submitted,

BOZICEVIC, FIELD & FRANCIS LLP

Date: September 19, 2012

By: /James S. Keddie, Ph.D., Reg. No. 48,920/
James S. Keddie, Ph.D.
Registration No. 48,920

BOZICEVIC, FIELD & FRANCIS, LLP
1900 University Avenue, Suite 200
East Palo Alto, California 94303
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00919

Electronic Patent Application Fee Transmittal

Application Number:	13387343
Filing Date:	15-Feb-2012
Title of Invention:	COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT
First Named Inventor/Applicant Name:	Sydney Brenner
Filer:	James Samuel Keddie/Lori Lynn Millsaps
Attorney Docket Number:	CGLC-020
Filed as Small Entity	

U.S. National Stage under 35 USC 371 Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Utility Appl issue fee	2501	1	870	870
Publ. Fee- early, voluntary, or normal	1504	1	300	300

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Miscellaneous:				
			Total in USD ($)	1170

Electronic Acknowledgement Receipt	
EFS ID:	13784800
Application Number:	13387343
International Application Number:	
Confirmation Number:	3537
Title of Invention:	COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	24353
Filer:	James Samuel Keddie/Lori Lynn Millsaps
Filer Authorized By:	James Samuel Keddie
Attorney Docket Number:	CGLC-020
Receipt Date:	19-SEP-2012
Filing Date:	15-FEB-2012
Time Stamp:	17:44:53
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	Credit Card
Payment was successfully received in RAM	$1170
RAM confirmation Number	4601
Deposit Account	
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)

1	Issue Fee Payment (PTO-85B)	CGLC-020_Issue.pdf	97620 6f9d9e841c891b4d015bbbfd96a1ebe97f8	no	1
Warnings:					
Information:					
2		CGLC-020_312_Amend.pdf	120297 4ffe8831bf76fbfeeeb30b2ad5e8f8ba2e57a	yes	4

Multipart Description/PDF files in .zip description		
Document Description	**Start**	**End**
Amendment after Notice of Allowance (Rule 312)	1	1
Specification	2	2
Applicant Arguments/Remarks Made in an Amendment	3	4

Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	31614 1d0d9166e6ed1906f6aaf7a8c05e1cafb9d1ac	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	249531		

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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

OK TO ENTER: /N.B./

Filed Electronically September 19, 2012

AMENDMENT UNDER 37 C.F.R. §1.312 Address to: Mail Stop Issue Fee Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Attorney Docket	CGLC-020
	Confirmation No.	3537
	First Named Inventor	Brenner, Sydney
	Application Number	13/387,343
	Filing Date	February 15, 2012
	Group Art Unit	1634
	Examiner Name	Bhat, Narayan Kameshwar
	Title "Compositions and Methods for Intramolecular Nucleic Acid Rearrangement"	

Sir:

This amendment is responsive to the Notice of Allowance and Fees Due dated December 12, 2012. Applicants submit that the amendments set forth below raise no new issues. Entry of this amendment is thus respectfully requested.

Response to Rule 312 Communication	Application No.	Applicant(s)
	13/387,343	BRENNER ET AL.
	Examiner	Art Unit
	NARAYAN BHAT	1634

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

1. ☒ The amendment filed on *19 September 2012* under 37 CFR 1.312 has been considered, and has been:

a) ☒ entered.

b) ☐ entered as directed to matters of form not affecting the scope of the invention.

c) ☐ disapproved because the amendment was filed after the payment of the issue fee.
Any amendment filed after the date the issue fee is paid must be accompanied by a petition under 37 CFR 1.313(c)(1) and the required fee to withdraw the application from issue.

d) ☐ disapproved. See explanation below.

e) ☐ entered in part. See explanation below.

/Narayan K. Bhat/
Examiner, Art Unit 1634

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00925

AMENDMENTS

IN THE SPECIFICATION

Please amend the paragraph starting at line 15 of page 20 as follows:

Figure 1A shows a single stranded polynucleotide 100 comprising, in a 5' to 3' orientation, a first domain (102; the domain to be moved); a reflex sequence 104; a nucleic acid sequence 106 having a site distal to the first domain (Site A), and a complement of the reflex sequence 108 (positioned at the 3' terminus of the polynucleotide). **Nucleic acid sequence 106 may be from 100 bases to 30 kilobases in length.** The steps of the reflex method described below will move the first domain into closer proximity to Site A. It is noted here that the prime designation in Figure 1A denotes a complementary sequence of a domain. For example, First Domain' is the complement of the First Domain.

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
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Page 00926

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UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/387,343	10/30/2012	8298767	CGLC-020	3537

24353 7590 10/10/2012

BOZICEVIC, FIELD & FRANCIS LLP
Bozicevic, Field & Francis
1900 UNIVERSITY AVENUE
SUITE 200
EAST PALO ALTO, CA 94303

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site http://pair.uspto.gov for additional applicants):

Sydney Brenner, Ely, UNITED KINGDOM;
Gi Mikawa, Cambridge, UNITED KINGDOM;
Robert Osborne, Saffron, UNITED KINGDOM;
Andrew Slatter, London, UNITED KINGDOM;

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	PATENT NUMBER	GROUP ART UNIT	FILE WRAPPER LOCATION
13/387,343	8298767	1634	9200

OC000000057239135

Correspondence Address/Fee Address Change

The following fields have been set to Customer Number 93726 on 10/22/2012

- **Correspondence Address**

The address of record for Customer Number 93726 is:

93726
EPA - Bozicevic Field & Francis LLP
Bozicevic, Field & Francis
1900 University Ave
Suite 200
East Palo Alto, CA 94303

PTO/SB/47 (03-09)
Approved for use through 03/31/2012. OMB 0651-0016
U.S. Patent and Trademark Office: U. S. DEPARTMENT OF COMMERCE
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"FEE ADDRESS" INDICATION FORM

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Alexandria, VA 22313-1450

Fax to:
571-273-6500

- OR -

INSTRUCTIONS: The issue fee must have been paid for application(s) listed on this form. In addition, only an address represented by a Customer Number can be established as the fee address for maintenance fee purposes (hereafter, fee address). A fee address should be established when correspondence related to maintenance fees should be mailed to a different address than the correspondence address for the application. **When to check the first box below:** If you have a Customer Number to represent the fee address. **When to check the second box below:** If you have no Customer Number representing the desired fee address, in which case a completed Request for Customer Number (PTO/SB/125) must be attached to this form. For more information on Customer Numbers, see the Manual of Patent Examining Procedure (MPEP) § 403.

For the following listed application(s), please recognize as the "Fee Address" under the provisions of 37 CFR

1.363 the address associated with:

☒ Customer Number: **00197**

OR

☐ The attached Request for Customer Number (PTO/SB/125) form.

PATENT NUMBER (if known)	APPLICATION NUMBER
8,298,767	**13/387,343**

Completed by (check one):

☐ Applicant/Inventor

☒ Attorney or Agent of record **48,920** (Reg. No.)

☐ Assignee of record of the entire interest. See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96)

☐ Assignee recorded at Reel _____ Frame _____

/James S. Keddie, Reg. No. 48,920/
Signature

James S. Keddie, Ph.D.
Typed or printed name

(650) 833-7723
Requester's telephone number

January 11, 2013
Date

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more that one signature is required, see below*.

☒ * **Total of 1 forms are submitted.**

This collection of information is required by 37 CFR 1.363. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 5 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop M Correspondence, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt	
EFS ID:	14672053
Application Number:	13387343
International Application Number:	
Confirmation Number:	3537
Title of Invention:	COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	93726
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020
Receipt Date:	11-JAN-2013
Filing Date:	15-FEB-2012
Time Stamp:	13:24:52
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Change of Address	CGLC-020-Fee_Address_Change.pdf	25451	no	1
Warnings:					
Information:					

Total Files Size (in bytes):	25451

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Filed Electronically

CHANGE OF CORRESPONDENCE ADDRESS		
	Attorney Docket	CGLC-020
	First Named Inventor	Brenner, Sydney
	Application Number	13/387,343
	Confirmation No.	3537
	Filing Date	February 15, 2012
	Group Art Unit	1634
Address to: Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Examiner Name	Bhat, Narayan Kameshwar
	Title: "Compositions and Methods for Intramolecular Nucleic Acid Rearrangement"	

Please change the Correspondence Address for the above-identified application to:

☒ **The address associated with Customer Number 43850**

Firm Name	Morgan, Lewis & Bockius LLP		
Individual Name			
Address	One Market, Spear Street Tower		
City, State, Zip	San Francisco, California 94105		
Country	U.S.A.		
Telephone	(415) 442-1000	Facsimile	(415) 442-1001

SIGNATURE OF ATTORNEY/AGENT OF RECORD

Name/Reg. No.	James S. Keddie, 48,920		
Signature	/James S. Keddie, Reg. No. 48,920/	Date	07-27-2016

Electronic Acknowledgement Receipt

EFS ID:	26471052
Application Number:	13387343
International Application Number:	
Confirmation Number:	3537
Title of Invention:	COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	197
Filer:	James Samuel Keddie
Filer Authorized By:	
Attorney Docket Number:	CGLC-020
Receipt Date:	27-JUL-2016
Filing Date:	15-FEB-2012
Time Stamp:	19:21:54
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Change of Address	CGLC-020-Change_Corresp.pdf	20040 205c99ff120bd6ee9aee630ecd3ab6009b92e1d	no	1

Warnings:

Information:	
Total Files Size (in bytes):	20040

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/AIA/80 (07-12)
Approved for use through 11/30/2014. OMB 0651-0035
U.S. Patent and Trademark Office, U.S DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

I hereby appoint:

■ Practitioners associated with Customer Number: 43850

OR

☐ Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

Name	Registration Number	Name	Registration Number

As attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignments documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:

■ The address associated with Customer Number: 43850

OR

☐	Firm or Individual Name		
	Address		
	City	State	Zip
	Country		
	Telephone	Email	

Assignee Name and Address: 10X Genomics, Inc.
7068 Koll Center Parkway, Suite 401
Pleasanton, CA 94566

A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is required to be Filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of The practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.

SIGNATURE of Assignee of Record

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee

Signature	[signature]	Date	3/13/2015
Name	Matthew Murphy	Telephone	(925) 401-7495
Title	Vice President, General Counsel		

This collection of information is required by 37 CFR 1.31, 1.32 and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt	
EFS ID:	28637620
Application Number:	13387343
International Application Number:	
Confirmation Number:	3537
Title of Invention:	COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	43850
Filer:	Gargi Talukder/Sheila Gloss
Filer Authorized By:	Gargi Talukder
Attorney Docket Number:	104371-5016-US
Receipt Date:	15-MAR-2017
Filing Date:	15-FEB-2012
Time Stamp:	19:56:22
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		US_POA.pdf	211036 818836a81174124a6ddd4fcc90f87f3a58d3ac3	yes	2

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Assignee showing of ownership per 37 CFR 3.73	1	1
	Power of Attorney	2	2
Warnings:			
Information:			
	Total Files Size (in bytes):	211036	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/96 (07-09)
Approved for use through 07/31/2012. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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STATEMENT UNDER 37 CFR 3.73(b)

Applicant/Patent Owner: 10X GENOMICS, INC.

Application No./Patent No.: 8,298,767 Filed/Issue Date: October 30, 2012

Titled: COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT

10X GENOMICS, INC., a corporation
(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.

states that it is:

1. ☒ the assignee of the entire right, title, and interest in;

2. ☐ an assignee of less than the entire right, title, and interest in (The extent (by percentage) of its ownership interest is _____ %); or

3. ☐ the assignee of an undivided interest in the entirety of (a complete assignment from one of the joint inventors was made)

the patent application/patent identified above, by virtue of either:

A. ☒ An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel 039162, Frame 0389, or for which a copy therefore is attached.

OR

B. ☐ A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____
   The document was recorded in the United States Patent and Trademark Office at
   Reel _____, Frame _____, or for which a copy thereof is attached.

2. From: _____ To: _____
   The document was recorded in the United States Patent and Trademark Office at
   Reel _____, Frame _____, or for which a copy thereof is attached.

3. From: _____ To: _____
   The document was recorded in the United States Patent and Trademark Office at
   Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☒ As required by 37 CFR 3.73(b)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (*i.e.*, a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

Gargi Talukder — Signature — 3/15/17 Date

Gargi Talukder — Printed or Typed Name — Attorney of record — Title

This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
13/387,343	02/15/2012	Sydney Brenner	104371-5016-US

CONFIRMATION NO. 3537

43850
MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105

POA ACCEPTANCE LETTER

OC000000090078017

Date Mailed: 03/23/2017

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 03/15/2017.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/sphouminh/

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00939

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
13/387,343	02/15/2012	Sydney Brenner	104371-5016-US

CONFIRMATION NO. 3537

43850
MORGAN, LEWIS & BOCKIUS LLP (SF)
One Market, Spear Street Tower, Suite 2800
San Francisco, CA 94105

POWER OF ATTORNEY NOTICE



Date Mailed: 03/23/2017

NOTICE REGARDING CHANGE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 03/15/2017.

- The Power of Attorney to you in this application has been revoked by the assignee who has intervened as provided by 37 CFR 3.71. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/sphouminh/

10x Genomics, Inc. Exhibit 2005
Parse Biosciences, Inc. v. 10x Genomics, Inc.
IPR2023-00955
Page 00940

PTO/SB/123 (04-15)
Approved for use through 01/31/2018. OMB 0651-0035
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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CHANGE OF CORRESPONDENCE ADDRESS
Patent

Address to:
Mail Stop Post Issue
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Patent Number	8,298,767
Issue Date	October 30, 2012
Application Number	13/387,343
Filing Date	February 15, 2012
First Named Inventor	Sydney BRENNER
Attorney Docket Number	104371-5016-US

Please change the Correspondence Address for the above-identified patent to:

☒ The address associated with Customer Number: 156830

OR

☐ **Firm *or* Individual Name**

Address

City	**State**	**ZIP**

Country

Telephone	**Email**

This form cannot be used to change the data associated with a Customer Number. To change the data associated with an existing Customer Number use "Request for Customer Number Data Change" (PTO/SB/124).

This form will not affect any "fee address" provided for the above-identified patent. To change a "fee address" use the "Fee Address Indication Form" (PTO/SB/47).

I am the:

☐ Patentee.

☐ Assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

☒ Attorney or agent of record. Registration Number 61,368.

Signature	/Gargi Talukder/
Typed or Printed Name	Gargi Talukder
Date	August 10, 2018
Telephone	415.442.1000

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below*.

☐ *Total of _____ forms are submitted.

This collection of information is required by 37 CFR 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop Post Issue, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt	
EFS ID:	33437595
Application Number:	13387343
International Application Number:	
Confirmation Number:	3537
Title of Invention:	COMPOSITIONS AND METHODS FOR INTRAMOLECULAR NUCLEIC ACID REARRANGEMENT
First Named Inventor/Applicant Name:	Sydney Brenner
Customer Number:	43850
Filer:	Gargi Talukder/Sheila Gloss
Filer Authorized By:	Gargi Talukder
Attorney Docket Number:	104371-5016-US
Receipt Date:	10-AUG-2018
Filing Date:	15-FEB-2012
Time Stamp:	17:05:04
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Change of Address	104371-5016-US_Corr.pdf	63866 7a58a374e81f2059ed6c6c8417250113659c 44b	no	1

Warnings:

Information:	
Total Files Size (in bytes):	63866

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY.DOCKET NO./TITLE	REQUEST ID
13/387,343	02/15/2012	Sydney Brenner	104371-5016-US	107357

Acknowledgement of Loss of Entitlement to Entity Status Discount

The entity status change request below filed through Private PAIR on 03/10/2020 has been accepted.

CERTIFICATIONS:

Change of Entity Status:

X Applicant changing to regular undiscounted fee status.

NOTE: Checking this box will be taken to be notification of loss of entitlement to small or micro entity status, as applicable.

This portion must be completed by the signatory or signatories making the entity status change in accordance with 37 CFR 1.4(d)(4).

Signature:	/Brett Lovejoy/
Name:	Brett A. Lovejoy
Registration Number:	42813