(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 May 2015 (28.05.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/076919 A1

(51) International Patent Classification:
C12Q 1/68 (2006.01) *C12M 1/00* (2006.01)
C12N 15/11 (2006.01)

(21) International Application Number:
PCT/US2014/056151

(22) International Filing Date:
17 September 2014 (17.09.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

61/908,027	22 November 2013 (22.11.2013)	US
14/214,850	15 March 2014 (15.03.2014)	US
PCT/US2014/030034	15 March 2014 (15.03.2014)	US
62/001,050	20 May 2014 (20.05.2014)	US

(71) Applicant: **THERANOS, INC.** [US/US]; 1601 S. California ave, Palo Alto, California 94304 (US).

(72) Inventors: **BELHOCINE, Kamila**; 1601 S. California Ave, Palo Alto, California 94304 (US). **LEE, Josephine**; 1601 S. California Ave, Palo Alto, California 94304 (US). **PATEL, Pranav**; 1601 S. California Ave, Palo Alto, California 94304 (US). **RICHARDSON, Aaron**; 1601 S. California Ave, Palo Alto, California 94304 (US). **TABAKMAN, Scott**; 1601 S. California Ave, Palo Alto, California 94304 (US).

(74) Agents: **MOYER, Stephen** et al.; Theranos, Inc., 1601 S. California Ave, Palo Alto, California 94304 (US).

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

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

Declarations under Rule 4.17:

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

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

WO 2015/076919 A1

(54) Title: Nucleic Acid Amplification

(57) Abstract: Methods and compositions for the amplification of nucleic acids and generation of concatemers are disclosed. Amplification methods provided herein may be performed under isothermal conditions. Methods and compositions may include reagents such as nucleic acid polymerases and primers.

NUCLEIC ACID AMPLIFICATION

BACKGROUND

[0001] There is an increasing need for methods and reagents for the amplification of nucleic acids. Generation of multiple copies of a particular nucleic acid is often necessary or helpful in order for the nucleic acid to be used for a given application. For example, in order to analyze the nucleotide sequence of a nucleic acid of interest, frequently, the nucleic acid is replicated to increase its copy number before the sequence is analyzed. In another example, in order to determine the presence or absence of a particular nucleic acid in a sample, a sample may be treated under conditions such that if the particular nucleic acid is present in the sample, it may be amplified. In another example, a nucleic acid for use as a probe may be copied repeatedly to generate a large number of nucleic acids containing the same sequence as the original nucleic acid template, thereby generating many copies of the nucleic acid which may be used as a probe.

[0002] A variety of methods for the amplification of nucleic acids are known. For example, polymerase chain reaction ("PCR") (see, e.g. U.S. Patent No. 4,683,202) is a popular method for the amplification of nucleic acids. To successfully perform a PCR reaction, the reaction must be performed at multiple different temperatures, which are repeated for multiple cycles. This requires hardware or other mechanisms for repeatedly changing the temperature of the PCR reaction. Another method for amplification of nucleic acids is referred to as loop-mediated isothermal amplification ("LAMP") (see, e.g. U.S. Patent No. 6,410,278). LAMP reactions may be performed isothermally, but typically involve the use of four different primers which recognize a total of six distinct sequences on the target nucleic acid.

[0003] To facilitate the generation of amplified nucleic acids for the many and growing number of applications which use amplified nucleic acids, new methods and reagents for the amplification of nucleic acids are desired.

INCORPORATION BY REFERENCE

[0004] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. However, in the event of a conflict between the content of the present express

disclosure and the content of a document incorporated by reference herein, the content of the present express disclosure controls.

SUMMARY

[0005] Provided herein are methods and compositions relating to the amplification of nucleic acids and the generation of concatemers.

[0006] In some embodiments, provided herein is a method for generating a concatemer comprising two or more copies of a double-stranded nucleic acid template, the method comprising: (A) treating a primary double-stranded nucleic acid comprising the double-stranded nucleic acid template with a first copy of a first primer and a polymerase under conditions such that an extension product of the first copy of the first primer is synthesized which is annealed to a first strand of the double-stranded nucleic acid template, wherein the first primer comprises a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions: (i) a tail region comprising: (a) the 5' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide (c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and (ii) a template-binding region comprising (a) the 3' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide (c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and the template-binding region of the first copy of the first primer anneals to the first strand of the double-stranded nucleic acid template, (B) treating the extension product of the first copy of the first primer of step (A) with a second primer and a polymerase under conditions such that an extension product of the second primer is synthesized which is annealed to the extension product of the first copy of the first primer of step (A), wherein the second primer comprises a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions: (i) a tail region comprising (a) the 5' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide (c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and (ii) a template-binding region comprising (a) the 3' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide (c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, the tail region of the second

primer contains a nucleotide sequence which is complementary to the nucleotide sequence of the tail region of the first primer, if the sequences are aligned such that the 5' terminal nucleotide of the second primer is aligned with the innermost nucleotide of the tail region of the first primer and the 5' terminal nucleotide of the first primer is aligned with the innermost nucleotide of the tail region of the second primer, the template-binding region of the second primer anneals to the extension product of the first copy of the first primer of step (A), and the extension product of the second primer contains a 5' terminal nucleotide, a 3' terminal nucleotide, and a 3' terminal region comprising the 3' terminal nucleotide, wherein the 3' terminal region contains the same nucleotide sequence as the nucleotide sequence of the tail region of the second primer read in the 5' to 3' direction, and the final nucleotide of the 3' terminal region is the 3' terminal nucleotide of the extension product of the second primer, (C) treating the extension product of the second primer of step (B) with a second copy of the first primer and a polymerase under conditions such that an extension product of the second copy of the first primer is synthesized which is annealed to the extension product of the second primer of step (B), to produce a first copy of a secondary nucleic acid comprising the extension product of the second primer of step (B) and the extension product of the second copy of the first primer, wherein the extension product of the second copy of the first primer contains a 5' terminal nucleotide, a 3' terminal nucleotide, and a 3' terminal region comprising the 3' terminal nucleotide, wherein the 3' terminal region contains the same nucleotide sequence as the nucleotide sequence of the tail region of the first primer read in the 5' to 3' direction, and the final nucleotide of the 3' terminal region is the 3' terminal nucleotide of the extension product of the second primer, (D) repeating at least step (C) one or more addition times to generate at least a second copy of the secondary nucleic acid of step (C), (E) treating the first copy of the secondary nucleic acid of step (C) and the second copy of the secondary nucleic acid of step (D) under conditions such that the 3' terminal region of the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid anneals to the 3' terminal region of the extension product of the second primer of the second copy of the secondary nucleic acid, to produce a cross-over structure comprising the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid and the extension product of the second primer of the second copy of the secondary nucleic acid, (F) treating the cross-over structure of step (E) with a polymerase under conditions such that an extension product of the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid is synthesized and an

extension product of the extension product of the second primer of the second copy of the secondary nucleic acid is synthesized, to produce a concatemer comprising two copies of the double-stranded nucleic acid template of step (A), wherein the concatemer comprises the extension product of the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid and the extension product of the extension product of the second primer of the second copy of the secondary nucleic acid.

[0007] In some embodiments, provided herein is a method for generating a concatemer comprising two or more copies of a polynucleotide template or an analogous sequence thereof, the method comprising, (A) treating a primary nucleic acid comprising the polynucleotide template with a first copy of a first primer and a polymerase under conditions such that an extension product of the first copy of the first primer is synthesized which is annealed to the polynucleotide template, wherein the first primer comprises a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions: (i) a tail region comprising (a) the 5' terminal nucleotide of the primer, (b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide (c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and (ii) a template-binding region comprising (a) the 3' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide (c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and the template-binding region of the first copy of the first primer anneals to the polynucleotide template, (B) treating the extension product of the first copy of the first primer of step (A) with a second primer and a polymerase under conditions such that an extension product of the second primer is synthesized which is annealed to the extension product of the first copy of the first primer of step (A), wherein the second primer comprises a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions: (i) a tail region comprising (a) the 5' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide (c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides (ii) a template-binding region comprising (a) the 3' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide (c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, the tail region of the second primer contains a nucleotide sequence which is complementary to

the nucleotide sequence of the tail region of the first primer, if the sequences are aligned such that the 5' terminal nucleotide of the second primer is aligned with the innermost nucleotide of the tail region of the first primer and the 5' terminal nucleotide of the first primer is aligned with the innermost nucleotide of the tail region of the second primer, the template-binding region of the second primer anneals to the extension product of the first copy of the first primer of step (A), and the extension product of the second primer contains a 5' terminal nucleotide, a 3' terminal nucleotide, and a 3' terminal region comprising the 3' terminal nucleotide, wherein the 3' terminal region contains the same nucleotide sequence as the nucleotide sequence of the tail region of the second primer read in the 5' to 3' direction, and the final nucleotide of the 3' terminal region is the 3' terminal nucleotide of the extension product of the second primer, (C) treating the extension product of the second primer of step (B) with a second copy of the first primer and a polymerase under conditions such that an extension product of the second copy of the first primer is synthesized which is annealed to the extension product of the second primer of step (B), to produce a first copy of a secondary nucleic acid comprising the extension product of the second primer of step (B) and the extension product of the second copy of the first primer, wherein the extension product of the second copy of the first primer contains a 5' terminal nucleotide, a 3' terminal nucleotide, and a 3' terminal region comprising the 3' terminal nucleotide, wherein the 3' terminal region contains the same nucleotide sequence as the nucleotide sequence of the tail region of the first primer read in the 5' to 3' direction, and the final nucleotide of the 3' terminal region is the 3' terminal nucleotide of the extension product of the second primer, (D) repeating at least step (C) one or more additional times to generate at least a second copy of the secondary nucleic acid comprising the extension product of the second primer of step (B) and the extension product of the second copy of the first primer of step (C), (E) treating the first copy of the secondary nucleic acid of step (C) and the second copy of the secondary nucleic acid of step (D) under conditions such that the 3' terminal region of the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid anneals to the 3' terminal region of the extension product of the second primer of the second copy of the secondary nucleic acid, to produce a cross-over structure comprising the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid and the extension product of the second primer of the second copy of the secondary nucleic acid, (F) treating the cross-over structure of step (E) with a polymerase under conditions such that an extension product of the extension product of the second copy of the first primer of the first

copy of the secondary nucleic acid is synthesized and an extension product of the extension product of the second primer of the second copy of the secondary nucleic acid is synthesized, to produce a concatemer comprising two copies of the polynucleotide template of step (A), wherein the concatemer comprises the extension product of the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid and the extension product of the extension product of the second primer of the second copy of the secondary nucleic acid. In some embodiments, the nucleic acid polymerase of step (A) is a DNA polymerase. In some embodiments, the nucleic acid polymerase of step (A) is a reverse transcriptase.

[0008] In some embodiments, provided herein is a method for generating a concatemer comprising two or more copies of a double-stranded nucleic acid template, the method comprising, (A) preparing a reaction mixture comprising: (i) a primary nucleic acid comprising the double-stranded nucleic acid template (ii) an isolated nucleic acid polymerase, (iii) a first primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions: (a) a tail region comprising (1) the 5' terminal nucleotide of the primer (2) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide (3) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and (b) a template-binding region comprising (1) the 3' terminal nucleotide of the primer (2) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide (3) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, wherein the template-binding region is complementary to a first strand of the nucleic acid template, (iv) a second primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions: (a) a tail region comprising (1) the 5' terminal nucleotide of the primer (2) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide (3) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and (b) a template-binding region comprising (1) the 3' terminal nucleotide of the primer (2) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide (3) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and wherein the template-binding region is complementary to a second strand of the nucleic acid template, and wherein the tail region of the second primer contains a nucleotide sequence which is complementary to the nucleotide sequence of the tail region of

the first primer, if the sequences are aligned such that the 5' terminal nucleotide of the second primer is aligned with the innermost nucleotide of the tail region of the first primer and the 5' terminal nucleotide of the first primer is aligned with the innermost nucleotide of the tail region of the second primer, and (B) incubating the reaction mixture for at least 3 minutes without thermocycling.

[0009] In some embodiments, provided herein is a method for generating a concatemer comprising two or more copies of a polynucleotide template, the method comprising, (A) preparing a reaction mixture comprising: (i) a nucleic acid comprising the polynucleotide template (ii) an isolated nucleic acid polymerase, (iii) a first primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions: (a) a tail region comprising (1) the 5' terminal nucleotide of the primer (2) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide (3) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and (b) a template-binding region comprising (1) the 3' terminal nucleotide of the primer (2) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide (3) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and wherein the template-binding region is complementary to the polynucleotide template, (iv) a second primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions: (a) a tail region comprising (1) the 5' terminal nucleotide of the primer (2) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide (3) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and (b) a template-binding region comprising (1) the 3' terminal nucleotide of the primer (2) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide (3) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and wherein the template-binding region is complementary to a nucleotide sequence complementary to the polynucleotide template, and wherein the tail region of the second primer contains a nucleotide sequence which is complementary to the nucleotide sequence of the tail region of the first primer, if the sequences of the primers are aligned such that the 5' terminal nucleotide of the second primer is aligned with the innermost nucleotide of the tail region of the first primer and the 5' terminal nucleotide of the first primer is aligned with the innermost nucleotide of the tail

region of the second primer, and (B) incubating the reaction mixture at a temperature of no greater than 80 C for at least 3 minutes..

[0010] In some embodiments, provided herein is a method for generating a concatemer comprising two or more copies of a double-stranded nucleic acid template, the method comprising incubating together a first copy and a second copy of a double-stranded nucleic acid molecule comprising the double-stranded nucleic acid template and a polymerase, wherein the double-stranded nucleic acid molecule comprises a first strand and a second strand, each containing a plurality of nucleotides, the first strand comprises a 5' terminal nucleotide and a 3' terminal nucleotide and contains the general format of regions in the 5' to 3' direction: A1-B-A2, the second strand comprises a 5' terminal nucleotide and a 3' terminal nucleotide and contains the general format of regions in the 5' to 3' direction: C1-D-C2, region B comprises the nucleotide sequence a first strand of the double-stranded nucleic acid template, region D comprises the nucleotide sequence of a second strand of the double-stranded nucleic acid template, in the double-stranded nucleic acid molecule, region A1 is annealed to C2, B is annealed to D, and A2 is annealed to C1, a cross-over structure comprising the first strand of the first copy of the double-stranded nucleic acid molecule and the second strand of the second copy of the double-stranded nucleic acid molecule is generated, wherein the A2 region of the first strand is annealed to the C2 region of the second strand, an extension product of the first strand of the cross-over structure is synthesized and an extension product of the second strand of the cross-over structure is synthesized, to produce a concatemer comprising the extension product of the first strand of the cross-over structure annealed to the extension product of the second strand of the cross-over structure, wherein the concatemer contains two copies of the double-stranded nucleic acid template.

[0011] In embodiments, provided herein is a method of copying a polynucleotide template, the method comprising: incubating the polynucleotide template in a reaction mixture comprising multiple copies of a first primer and multiple copies of a second primer, wherein: the first primer comprises a first region and a second region, wherein the second region of the first primer comprises a nucleotide sequence which is complementary to a first portion of the polynucleotide template; the second primer comprises a first region and a second region, wherein the second region of the second primer comprises a nucleotide sequence which is complementary to a partner nucleotide sequence, wherein the partner nucleotide sequence is complementary to a second portion of the polynucleotide template; and upon incubation of

the polynucleotide template with the multiple copies of the first primer and the multiple copies of the second primer, at least one concatemer strand is formed, wherein the concatemer strand comprises a 5' end and a 3' end, and comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', wherein: C' represents the nucleotide sequence of the first region of the second primer, T represents the nucleotide sequence of the polynucleotide template or an analogous sequence thereof, and X represents any number and sequence of nucleotides.

[0012] In some embodiments, in a method provided herein involving the formation of a concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', the concatemer strand is a first concatemer strand, and a second concatemer strand is also formed, wherein the second concatemer strand comprises a 5' end and a 3' end, and comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C-X'-T'-C-T'-C, wherein: C represents the nucleotide sequence of the first region of the first primer, T' represents a nucleotide sequence which is complementary to the polynucleotide template, and X' represents a nucleotide sequence which is complementary to the nucleotide sequence of X.

[0013] In embodiments, provided herein is method of assaying for a target polynucleotide template in a biological sample, the method comprising: A) incubating the biological sample or portion thereof in a reaction mixture comprising multiple copies of a first primer and multiple copies of a second primer, wherein: the first primer comprises a first region and a second region, wherein the second region of the first primer comprises a nucleotide sequence which is complementary to a first portion of the polynucleotide template; the second primer comprises a first region and a second region, wherein the second region of the second primer comprises a nucleotide sequence which is complementary to a partner nucleotide sequence, wherein the partner nucleotide sequence is complementary to a second portion of the polynucleotide template; and upon incubation of the polynucleotide template with the multiple copies of the first primer and the multiple copies of the second primer, at least one concatemer strand is formed, wherein the concatemer strand comprises a 5' end and a 3' end, and comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', wherein: C' represents the nucleotide sequence of the first region of the second primer, T represents the nucleotide sequence of the polynucleotide template or an analogous sequence thereof, and X represents any number and sequence of nucleotides; and

B) measuring an amount of amplified nucleic acid in the reaction mixture of A) at one or more points after the initiation of the incubating step of A). In embodiments, the measuring an amount of amplified nucleic acid in the reaction mixture may comprise determining a level of fluorescence in the reaction mixture. In embodiments, the method may further comprise determining an inflection time of nucleic acid amplification in the reaction mixture.

[0014] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', X may contain a sequence having the general structure in the 5' to 3' direction of [(C'-T)$_N$] wherein C' represents the nucleotide sequence of the first region of the second primer, T represents the nucleotide sequence of the polynucleotide template or an analogous sequence thereof, and N is any integer between 0 and 2000. In embodiments, N may be any integer between 0 and 10, 0 and 100, 0 and 1000, 0 and 5000, 0 and 10,000 1 and 10, 1 and 100, 1 and 1000, 1 and 2000, 1 and 5000, or 1 and 10,000.

[0015] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', X may contain no more than 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 500, 1000, 10,000, 50,000, 100,000, or 500,000 nucleotides. In embodiments, in a method, vessel, or kit provided herein involving a concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', X may contain at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 500, 1000, 10,000, 50,000, 100,000, or 500,000 nucleotides. In embodiments, in a method, vessel, or kit provided herein involving a concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', X may contain at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 500, 1000, 10,000, 50,000, 100,000, nucleotides, and no more than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 500, 1000, 10,000, 50,000, 100,000, or 500,000 nucleotides.

[0016] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', between at least one C' and T, one or more extra nucleotides are present which are not part of the C' or T sequence. The one or

more extra nucleotides may be, for example, between 1 and 10, 1 and 20, 1 and 100, or 1 and 1000 nucleotides.

[0017] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', at least one C' or T sequence may be missing one or more nucleotides. In the event that 2 or more nucleotides are missing, the missing nucleotides may be contiguous, or may be at separate locations. The one or more missing nucleotides may be, for example, between 1 and 10, 1 and 20, 1 and 100, or 1 and 1000 nucleotides.

[0018] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', at least one C' or T sequence may have one or more point mutations. In the event that two or more point mutations are present, the point mutations may be contiguous, or may be at separate locations. The one or more point mutations may be, for example, between 1 and 10, 1 and 20, 1 and 100, or 1 and 1000 point mutations.

[0019] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', the nucleotide sequence has two or all three of the following characteristics: i) between at least one C' and T, one or more extra nucleotides are present which are not part of the C' or T sequence; ii) at least one C' or T sequence is missing one or more nucleotides; and iii) at least one C' or T sequence contains one or more point mutations.

[0020] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', in embodiments which the polynucleotide template is an RNA molecule, the T may represent the nucleotide sequence of a DNA sequence which is analogous to the RNA sequence of the polynucleotide template.

[0021] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', the concatemer strand further

comprises one or more nucleotides to the 5' of the 5'-most situated C' sequence. The one or more nucleotides may be, for example, between 1 and 10, 1 and 20, 1 and 100, or 1 and 1000 nucleotides.

[0022] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', the concatemer strand further comprises one or more nucleotides to the 3' of the 3'-most situated C' sequence. The one or more nucleotides may be, for example, between 1 and 10, 1 and 20, 1 and 100, or 1 and 1000 nucleotides.

[0023] In embodiments, provided herein is a method of generating a concatemer comprising at least two copies of a double stranded nucleic acid template, the method comprising: incubating in a reaction mixture at least a first template molecule and a second template molecule, wherein: the first template molecule comprises a first nucleic acid strand and a second nucleic acid strand, wherein: the first nucleic acid strand of the first template molecule comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: H'-S-Y_1-H', wherein: H' represents the nucleotide sequence of a first homology sequence, S represents the nucleotide sequence of a first strand of the double stranded nucleic acid template, and Y_1 represents any number and sequence of nucleotides; and the second nucleic acid strand of the first template molecule comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: H-Y_1'-S'-H, wherein: H represents the nucleotide sequence of a second homology sequence, wherein the first homology sequence and second homology sequence are complementary to each other, Y_1' represents a nucleotide sequence which is complementary to the nucleotide sequence of Y_1, and S' represents the nucleotide sequence of a second strand of the double stranded nucleic acid template, wherein the first strand and second strand of the double stranded nucleic acid template are complementary to each other; and the second template molecule comprises a first nucleic acid strand and a second nucleic acid strand, wherein: the first nucleic acid strand of the second template molecule comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: H'-S-Y_2-H', wherein: H' represents the nucleotide sequence of the first homology sequence, S represents the nucleotide sequence of the first strand of the double stranded nucleic acid template, and Y_2 represents any number and sequence of nucleotides; and the second nucleic acid strand of the first template molecule comprises a nucleotide

sequence having the general structure in the 5' to 3' direction of: H-Y_2'-S'-H, wherein: H represents the nucleotide sequence of the second homology sequence, Y_2' represents a nucleotide sequence which is complementary to the nucleotide sequence of Y_2, and S' represents the nucleotide sequence of the second strand of the double stranded nucleic acid template; and upon incubation of the first template molecule with the second template molecule in the reaction mixture, at least one concatemer comprising at least two copies of the double stranded nucleic acid template is formed, wherein the concatemer comprises a first concatemer strand and a second concatemer strand, wherein the first concatemer strand comprises a 5' end and a 3' end, and comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: H'-S-Y_2-H'-S-Y_1-H', wherein each of H', Y_1, S, and Y_2 represent nucleotide sequences as described above; and wherein the second concatemer strand comprises a 5' end and a 3' end, and comprises a sequence having the general structure in the 5' to 3' direction of: H-Y_1'-S'-H-Y_2'-S'-H, wherein each of H', Y_1, S, and Y_2 represent nucleotide sequences as described above.

[0024] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: H'-S-Y_2-H'-S-Y_1-H', at least one of or both Y_1 and Y_2 may represent 0 nucleotides.

[0025] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: H'-S-Y_2-H'-S-Y_1-H', Y_1 may contain a sequence having the general structure in the 5' to 3' direction of [(H'-S)$_{N1}$] wherein H' and S represent nucleotide sequences as described above, and N1 is any integer between 0 and 2000.

[0026] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first concatemer strand which comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: H'-S-Y_2-H'-S-Y_1-H', Y_2 may contain a sequence having the general structure in the 5' to 3' direction of [(H'-S)$_{N2}$] wherein H' and S represent nucleotide sequences as described above, and N2 is any integer between 0 and 2000.

[0027] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first template molecule and a second template molecule, the first template molecule and second template molecule are both double-stranded DNA molecules.

[0028] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first nucleic acid strand of a first template molecule comprises, wherein the first nucleic acid strand comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: H'-S-Y_1-H', wherein H' represents the nucleotide sequence of a first homology sequence, the first homology sequence may contain no more than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, the first homology sequence may contain at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, the first homology sequence may contain at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, or 100 and no more than 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, or 200 nucleotides.

[0029] In embodiments, a reaction mixture, vessel, or kit provided herein comprises a nucleic acid polymerase. In embodiments, a nucleic acid polymerase is a DNA polymerase having strand-displacement activity. In embodiments, a nucleic acid polymerase is an RNA polymerase. In embodiments, a nucleic acid polymerase is a reverse transcriptase. In embodiments, a reaction mixture, vessel, or kit comprises more than one kind of nucleic acid polymerase, such as both a DNA polymerase having strand displacement activity and a reverse transcriptase. In embodiments, a reaction mixture, vessel, or kit provided herein comprises nucleotides and buffer.

[0030] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a polynucleotide template, the polynucleotide template is a single-stranded molecule. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a polynucleotide template, the polynucleotide template comprises one strand of a double-stranded nucleic acid template. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a polynucleotide template, the polynucleotide template is a DNA or RNA molecule.

[0031] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a nucleic acid template, the nucleic acid template is an RNA or DNA molecule. In embodiments, a nucleic acid template may be a single-stranded or double-stranded molecule.

[0032] In embodiments, in a method provided herein involving incubation of a reaction mixture, during the incubation of the reaction mixture, the temperature of the reaction mixture does not exceed 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 37, 35, 30, 25, or 20 C. In

embodiments, in a method provided herein, all steps of the method are performed at a temperature of no greater than 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 37, 35, 30, 25, or 20 C. In embodiments, a reaction mixture, vessel, or kit provided herein is maintained at a temperature of no greater than 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 37, 35, 30, 25, or 20 C. In embodiments, a method provided herein is performed without thermocycling.

[0033] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a polynucleotide template comprising a first portion, the first portion contains no more than 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, or 200 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a polynucleotide template comprising a first portion, the first portion contains at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, or 200 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a polynucleotide template comprising a first portion, the first portion contains at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, or 100 and no more than 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, or 200 nucleotides.

[0034] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a primer comprising a first region, the first region contains at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a primer comprising a first region, the first region contains no more than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a primer comprising a first region, the first region contains at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, or 90 and no more than 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first primer comprising a first region and a second primer comprising a first region, both the first primer and second primer may have any of the features described above. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first primer comprising a first region and a second primer comprising a first region, the first region of the first primer and the first region of the second primer may contain the same number of nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first primer comprising a first region and a

second primer comprising a first region, the first region of the first primer and the first region of the second primer may contain a different number of nucleotides.

[0035] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a primer comprising a second region, the second region contains at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a primer comprising a second region, the second region contains no more than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a primer comprising a second region, the second region contains at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, or 90 and no more than 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first primer comprising a second region and a second primer comprising a second region, both the first primer and second primer may have any of the features described above. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first primer comprising a second region and a second primer comprising a second region, the second region of the first primer and the second region of the second primer may contain the same number of nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first primer comprising a second region and a second primer comprising a second region, the second region of the first primer and the second region of the second primer may contain a different number of nucleotides.

[0036] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a second primer containing a second region and a polynucleotide template comprising a second portion, the nucleotide sequence of the second region of the second primer is the same as the nucleotide sequence of the second portion of the polynucleotide template.

[0037] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a primer comprising a tail region, the tail region contains at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a primer comprising a tail region, the tail region contains no more than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25,

30, 35, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a primer comprising a tail region, the tail region contains at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, or 90 and no more than 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first primer comprising a tail region and a second primer comprising a tail region, both the first primer and second primer may have any of the features described above. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first primer comprising a tail region and a second primer comprising a tail region, the tail region of the first primer and the tail region of the second primer may contain the same number of nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first primer comprising a tail region and a second primer comprising a tail region, the tail region of the first primer and the tail region of the second primer may contain a different number of nucleotides.

[0038] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a primer comprising a template-binding region, the template-binding region contains at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a primer comprising a template-binding region, the template-binding region contains no more than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a primer comprising a template-binding region, the template-binding region contains at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, or 90 and no more than 5, 6, 7, 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, or 100 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first primer comprising a template-binding region and a second primer comprising a template-binding region, both the first primer and second primer may have any of the features described above. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first primer comprising a template-binding region and a second primer comprising a template-binding region, the template-binding region of the first primer and the template-binding region of the second primer may contain the same number of nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a first primer comprising a template-binding region and a second

primer comprising a template-binding region, the template-binding region of the first primer and the template-binding region of the second primer may contain a different number of nucleotides.

[0039] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a polynucleotide template, the polynucleotide template may contain at least 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 200, 500, 1000, 2000, or 5000 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a polynucleotide template, the polynucleotide template may contain no more than 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 200, 500, 1000, 2000, 5000, or 10,000 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a polynucleotide template, the polynucleotide template may contain at least 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 200, 500, 1000, 2000, or 5000, and no more than 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 200, 500, 1000, 2000, 5000, or 10,000 nucleotides.

[0040] In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a double-stranded nucleic acid template, each strand of the double-stranded nucleic acid template may contain at least 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 200, 500, 1000, 2000, or 5000 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a double-stranded nucleic acid template, each strand of the double-stranded nucleic acid template may contain no more than 8, 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 200, 500, 1000, 2000, 5000, or 10,000 nucleotides. In embodiments, in a method, reaction mixture, vessel, or kit provided herein involving a double-stranded nucleic acid template, each strand of the double-stranded nucleic acid template may contain at least 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 200, 500, 1000, 2000, or 5000, and no more than 9, 10, 11, 12, 13,14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 200, 500, 1000, 2000, 5000, or 10,000 nucleotides.

[0041] In embodiments, a reaction mixture, vessel, or kit provided herein does not contain a recombinase enzyme.

[0042] In embodiments, in a method, reaction mixture, vessel, or kit provided herein may contain or involve multiple copies of a primer. The multiple copies may be, for example, at least 5, 10, 15, 20, 50, 100, 500, 1000, 10,000, 100,000, or 1,000,000 copies of the primer.

[0043] In embodiments, a reaction mixture or vessel provided herein may comprise at least a portion of a biological sample from a subject. The biological sample may be, for example, saliva, blood, urine, a cheek swab, or a nasal swab. The subject may be a human.

[0044] In some embodiments, all of the steps of a method provided herein are performed at a temperature of no greater than 70, 65, 60, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, or 10 C. In some embodiments, some of the steps of a method provided herein are performed at a temperature of no greater than 70, 65, 60, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, or 10 C.

[0045] In some embodiments, two or more steps of a method provided herein are performed simultaneously in the same reaction mixture. In some embodiments, all of the steps of a method provided herein are performed simultaneously in the same reaction mixture.

[0046] In some embodiments, in a method provided herein, a nucleic acid template is amplified at least 10, 100, 1000, 10,000, 100,000, or 1,000,000-fold within 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 120, or 180 minutes of initiation of the method. In some embodiments, in a method provided herein, the number of copies of a nucleic acid template in a reaction mixture is increased least 10, 100, 1000, 10,000, 100,000, or 1,000,000-fold within 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 120, or 180 minutes of initiation of the method.

[0047] In embodiments, a nucleic acid template provided herein may be a single-stranded or a double-stranded nucleic acid template.

[0048] In embodiments, provided herein is a vessel, comprising in fluid communication therein: a first primer, wherein the first primer comprises a first region and a second region, and wherein the second region of the first primer comprises a nucleotide sequence which is complementary to a first portion of a polynucleotide template; a second primer, wherein the second primer comprises a first region and a second region, and wherein the second region of the second primer comprises a nucleotide sequence which is complementary to a partner nucleotide sequence, wherein the partner nucleotide sequence is complementary to a second portion of the polynucleotide template; and at least one concatemer strand, wherein the concatemer strand comprises a 5' end and a 3' end, and comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', wherein: C' represents the nucleotide sequence of the first region of the second primer, T represents the nucleotide sequence of the polynucleotide template or an analogous sequence thereof, and X represents any number and sequence of nucleotides.

[0049] In some embodiments, provided herein is a vessel, comprising in fluid communication therein: (A) an isolated nucleic acid polymerase, (B) a nucleic acid template comprising at least a first strand, (C) a first primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions: (i) a tail region comprising (a) the 5' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide (c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and (ii) a template-binding region comprising (a) the 3' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide (c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, wherein the template-binding region is complementary to a first strand of the nucleic acid template, and (D) a second primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions: (i) a tail region comprising (a) the 5' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide (c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and (ii) a template-binding region comprising (a) the 3' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide (c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and wherein the template-binding region is complementary to a nucleotide sequence complementary to first strand of the nucleic acid template, and wherein the tail region of the second primer contains a nucleotide sequence which is complementary to the nucleotide sequence of the tail region of the first primer, if the sequences of the primers are aligned such that the 5' terminal nucleotide of the second primer is aligned with the innermost nucleotide of the tail region of the first primer and the 5' terminal nucleotide of the first primer is aligned with the innermost nucleotide of the tail region of the second primer.

[0050] In embodiments, provided herein is a kit comprising two or more fluidically isolated containers, the containers collectively comprising: a first primer, wherein the first primer comprises a first region and a second region, and wherein the second region of the first primer comprises a nucleotide sequence which is complementary to a first portion of a polynucleotide template; a second primer, wherein the second primer comprises a first region and a second region, and wherein the second region of the second primer comprises a

nucleotide sequence which is complementary to a partner nucleotide sequence, wherein the partner nucleotide sequence is complementary to a second portion of the polynucleotide template; and an isolated DNA polymerase having strand-displacement activity; wherein: the first region of the first primer and the first region of the second primer are complementary.

[0051] In some embodiments, provided herein is a kit for detecting a target nucleic acid of interest comprising at least a first strand, the kit comprising two or more fluidically isolated containers, the containers collectively comprising: (A) an isolated nucleic acid polymerase, (B) a first primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions: (i) a tail region comprising (a) the 5' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide (c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and (ii) a template-binding region comprising (a) the 3' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide (c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, wherein the template-binding region is complementary to the first strand of the target nucleic acid, and (C) a second primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions: (i) a tail region comprising (a) the 5' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide (c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and (ii) a template-binding region comprising (a) the 3' terminal nucleotide of the primer (b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide (c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and wherein the template-binding region is complementary to a nucleotide sequence complementary to the first strand of the target nucleic acid, and wherein the tail region of the second primer contains a nucleotide sequence which is complementary to the nucleotide sequence of the tail region of the first primer, if the sequences of the primers are aligned such that the 5' terminal nucleotide of the second primer is aligned with the innermost nucleotide of the tail region of the first primer and the 5' terminal nucleotide of the first primer is aligned with the innermost nucleotide of the tail region of the second primer.

[0052] In some embodiments, a kit provided herein comprises a nucleic acid having the nucleotide sequence of the target nucleic acid of interest.

[0053] In some embodiments, a reaction mixture, vessel or kit provided herein comprises a nucleic acid dye.

[0054] In some embodiments, in a vessel or kit provided herein comprising an isolated nucleic acid polymerase, the isolated nucleic acid polymerase is a DNA polymerase. In some embodiments, in a vessel or kit provided herein comprising an isolated nucleic acid polymerase, the isolated nucleic acid polymerase is a reverse transcriptase. In some embodiments, in a vessel or kit provided herein comprising an isolated nucleic acid polymerase, the vessel or kit comprises both a DNA polymerase and a reverse transcriptase.

[0055] In some embodiments, in a method, vessel, or kit provided herein comprising a nucleic acid polymerase, the nucleic acid polymerase has strand displacement activity.

[0056] In some embodiments, a method provided herein comprises treating one or more of the reaction components or steps of the method with a nucleic acid dye.

[0057] In some embodiments, in a method, vessel, or kit provided herein comprising a nucleic acid template, the template is a DNA molecule. In some embodiments, in a method, vessel, or kit provided herein comprising a nucleic acid template, the template is an RNA molecule.

[0058] In some embodiments, in a method, vessel, or kit provided herein comprising a first primer, the tail region of the first primer comprises at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides. In some embodiments, in a method, vessel, or kit provided herein comprising a first primer, the tail region of the first primer comprises no more than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, or 60 nucleotides.

[0059] In some embodiments, in a method, vessel, or kit provided herein comprising a second primer, the tail region of the second primer comprises at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides. In some embodiments, in a method, vessel, or kit provided herein comprising a second primer, the tail region of the second primer comprises no more than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, or 60 nucleotides.

[0060] In some embodiments, in a method, vessel, or kit provided herein comprising a first primer, the template-binding region of the first primer comprises at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides. In some embodiments, in a method, vessel, or kit provided herein comprising a first primer, the template-binding region of the first primer comprises no more than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, or 60 nucleotides.

[0061] In some embodiments, in a method, vessel, or kit provided herein comprising a second primer, the template-binding region of the second primer comprises at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides. In some embodiments, in a method, vessel, or kit provided herein comprising a second primer, the template-binding region of the second primer comprises no more than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, or 60 nucleotides.

[0062] In some embodiments, in methods and compositions provided herein wherein an RNA molecule is the template molecule or primary nucleic acid, amplification of the template may refer to generation of copies of DNA strands corresponding to the RNA molecule.

[0063] In some embodiments, a method provided herein comprises measuring a fluorescent signal from an assay comprising the method.

[0064] In some embodiments, a nucleic acid ligase may be included with a method or composition provided herein. In some embodiments, a nucleic acid template may be amplified more rapidly with a method provided herein when a ligase is included in a reaction mixture for a method provided herein, as compared to if a nucleic acid ligase is not included in the reaction. In embodiments, a reaction mixture, vessel, or kit provided herein may contain an enzyme having ligase activity.

[0065] In embodiments, provided herein is a method of assaying for a pathogen in a sample, the method comprising performing a method as provided herein to amplify nucleic acid from the pathogen. In embodiments, the target nucleic acid used in a composition or method provided herein may be nucleic acid from a pathogen. In embodiments, the first and second primer used in a method provided herein may each contain regions which are complementary to a sequence in the nucleic acid of the pathogen, or which are complementary to a sequence which is complementary to a sequence in the nucleic acid of the pathogen. In embodiments, the nucleic acid of the pathogen may be DNA or RNA. Pathogens may include, without

limitation, viruses, bacteria, fungi, and protists. A sample may be from a subject, and may have any of the sample characteristics described elsewhere herein.

[0066] In embodiments, a method provided herein for amplification of a nucleic acid may be used for a diagnostic method externally of a human or animal body. For example, a sample may be obtained from a human or animal, and the sample may be assayed for a target nucleic acid of interest with a method provided herein for amplification of nucleic acid.

[0067] In embodiments, a method provided herein may include: a) providing one or more reagents for performing a method as provided herein (e.g. one or more of first primer, second primer, nucleic acid template, nucleic acid polymerase, nucleotides, buffer, water, etc.) in a reaction mixture, and b) incubating the reaction mixture at a substantially isothermal temperature, wherein the temperature of the reaction mixture does not diverge from a central temperature by more or less than 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 degree Celsius during the incubation. In embodiments, a central temperature may be, for example, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, or 80 degrees Celsius.

[0068] In embodiments, a method provided herein may be performed at a substantially isothermal temperature. In embodiments, a substantially isothermal temperature may be any of 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, or 80 degrees Celsius, plus or minus 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 degree Celsius.

[0069] In embodiments, a method provided herein may be performed at one or more temperatures, none or which exceed 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, or 25 degrees Celsius.

[0070] In embodiments, methods provided herein may be performed without thermocycling / reaction mixtures may be incubated under conditions without thermocycling (i.e. without cycles of raising and lowering incubation temperatures to separate strands or allow hybridization of primers as is used in, for example, PCR-based methods).

[0071] In compositions and methods provided herein involving a first primer comprising a first region and a second primer comprising a first region, wherein the first region of the first primer is complementary to the first region of the second primer, in embodiments, the first region of the first primer and the first region of the second primer contain nucleotide sequences such that a double stranded structure which would be formed by the annealing of

the first region of the first primer to the first region of the second primer according to Watson-Crick base pairing rules would not form a restriction enzyme recognition sequence.

[0072] In compositions and methods provided herein involving a nucleic acid polymerase, in embodiments, the nucleic acid polymerase has 3' to 5' exonuclease activity.

[0073] In embodiments, provided herein is a method for amplifying a target nucleic acid strand, the method comprising: incubating a reaction mixture comprising the target nucleic acid strand, a first primer, and a second primer under substantially isothermal conditions, wherein: the target nucleic acid strand comprises a first portion and a second portion; the first primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to the first portion of the nucleic acid strand; the second primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to the second portion of the nucleic acid strand; the first region of the first primer is complementary to the first region of the second primer; and the target nucleic acid strand is amplified. Optionally, the target nucleic acid strand may further comprise a third portion, wherein the third portion is situated in the target nucleic acid strand between the first portion and the second portion, and wherein the first region of the second primer is complementary to the third portion of the target nucleic acid strand.

[0074] In embodiments, in any of the methods provided herein, a, polynucleotide template, target nucleic acid strand or the like may contain an internal motif, wherein the tail region of a primer provided herein to amplify the target nucleic acid strand is complementary to the internal motif.

[0075] In embodiments, a method provided herein may further comprise adding to a reaction mixture provided herein a peptide-nucleic acid (PNA) probe and a dye which binds to DNA-PNA hybrid, wherein the PNA probe is complementary to a target nucleic acid for amplification in the reaction mixture, or a complement thereof.

[0076] In embodiments, a reaction mixture provided herein may comprise a DNA polymerase having strand-displacement activity.

[0077] When a nucleic acid is described herein as being "amplified" or the like, the nucleic acid may also be described as being "copied" or the like.

[0078] In embodiments, in a primer described herein as having a first region and a second region, the first region may contain the 5' end of the primer and the second region may contain the 3' end of the primer.

[0079] In embodiments, in a primer described herein as having a tail region and a template-binding region, the first region may contain the 5' end of the primer and the second region may contain the 3' end of the primer.

[0080] In embodiments, provided herein is method for copying a polynucleotide template, the method comprising: incubating a reaction mixture comprising the polynucleotide template, a first primer, and a second primer under conditions without thermocycling, wherein: the polynucleotide template comprises a first portion and a second portion; the first primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to the first portion of the polynucleotide template; the second primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to the second portion of the polynucleotide template; the first region of the first primer is complementary to the first region of the second primer; and multiple copies of the polynucleotide template are generated. Optionally, the polynucleotide template may further comprise a third portion, wherein the third portion is situated in the polynucleotide template between the first portion and the second portion, and wherein the first region of the second primer is complementary to the third portion of the polynucleotide template.

[0081] In embodiments, provided herein is a method for amplifying a polynucleotide template, the method comprising incubating the polynucleotide template in a reaction mixture comprising a first primer and a second primer, wherein: the polynucleotide template comprises a first portion, a second portion and a third portion, wherein the third portion is situated in the polynucleotide template between the first portion and the second portion; the first primer comprises a first region and a second region, wherein the second region of the first primer is complementary to the first portion of the polynucleotide template; and the second primer comprises a first region and a second region, wherein the second region of the second primer is complementary to a sequence which is complementary to the second portion of the polynucleotide template, the first region of the second primer is complementary to the

first region of the first primer, and the first region of the second primer is complementary to the third portion of the polynucleotide template.

[0082] In embodiments, provided herein is a method for assessing the identity of a nucleotide at a position of interest in a nucleotide sequence in a polynucleotide template, the method comprising: A) providing copies of the polynucleotide template in each of at least a first reaction mixture and a second reaction mixture, wherein: the polynucleotide template comprises a first portion, a second portion and a third portion, wherein the third portion is situated in the polynucleotide template between the first portion and the second portion and wherein the position of interest is in the third portion; the first reaction mixture comprises copies of the polynucleotide template, a first primer, and a second primer, wherein: the first primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a first portion of the polynucleotide template; the second primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to a second portion of the polynucleotide template; the first region of the first primer is complementary to the first region of the second primer; and the first region of the second primer is complementary to the third portion of the polynucleotide template; the second reaction mixture comprises copies of the polynucleotide template, a third primer, and a fourth primer, wherein: the third primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a first portion of the polynucleotide template; the fourth primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to a second portion of the polynucleotide template; the first region of the third primer is complementary to the first region of the fourth primer; and the first region of the fourth primer is complementary to the third portion of the polynucleotide template; and the nucleotide sequence of the first region of the second primer differs from the nucleotide sequence of first region of the fourth primer by a single nucleotide, wherein the position of the different nucleotide in the second and fourth primers corresponds to the position of the nucleotide of interest in the polynucleotide template if the nucleotide sequence of the first region of the second primer or fourth primer is oriented with

the nucleotide sequence of the third portion of the polynucleotide template for maximum complementation of the sequences; B) incubating the first reaction mixture and second reaction mixture under conditions without thermocycling; and C) comparing the rate or amount of amplification of the polynucleotide template in the first reaction mixture to the rate or amount of amplification of the polynucleotide template in the second reaction mixture, wherein the rate or amount of amplification of the polynucleotide template is indicative of the degree of complementation between first region of the second or fourth primer and the nucleotide sequence of the third portion of the polynucleotide template.

[0083] Optionally, in embodiments provided herein involving a polynucleotide template, the polynucleotide template is a DNA strand.

[0084] Optionally, in embodiments provided herein involving a polynucleotide template, the polynucleotide template is an RNA strand.

[0085] Optionally, in embodiments provided herein involving a polynucleotide template, the polynucleotide template is one strand of a duplex nucleic acid molecule. In embodiments, the duplex nucleic acid molecule is a duplex DNA molecule or a duplex RNA molecule.

[0086] Optionally, in embodiments provided herein involving a reaction mixture, vessel, or kit, the reaction mixture, vessel, or kit comprises a DNA polymerase having strand displacement activity.

[0087] Optionally, in embodiments provided herein involving a reaction mixture, vessel, or kit, the reaction mixture, vessel, or kit comprises a reverse transcriptase.

[0088] Optionally, in embodiments provided herein involving a reaction mixture, vessel, or kit, the reaction mixture, vessel, or kit comprises a nucleic acid dye, nucleotides, or buffers.

[0089] Optionally, in embodiments provided herein involving a polynucleotide template containing a first portion, a second portion, and a third portion, the polynucleotide template may have the general structure of elements, in the 3' to 5' direction, of: 1P-1S-3P-2S-2P, wherein "1P" is the first portion, "1S" is a first space, "3P" is the third portion, "2S" is a second space, and "2P" is the second portion. The "first space" and "second space" are portions of the polynucleotide template which contain nucleotides which are not part of the first portion, second portion, or third portion. In embodiments, the first portion may have, for example, between 6 and 30 nucleotides or any other number of nucleotides as described elsewhere herein. In embodiments, the first space may, for example, between 2 and 30

nucleotides or any other number of nucleotides as described elsewhere herein. In embodiments, the third portion may have, for example, between 4 and 14 nucleotides or any other number of nucleotides as described elsewhere herein. In embodiments, the first space may, for example, between 2 and 30 nucleotides or any other number of nucleotides as described elsewhere herein. In embodiments, the first portion may have, for example, between 6 and 30 nucleotides or any other number of nucleotides as described elsewhere herein.

[0090] In embodiments, in methods provided herein involving the incubation of a polynucleotide template in a reaction mixture, a concatemer strand comprising at least 2, 3, 4, 5, or 10 copies of the polynucleotide template may be generated during the incubation of the reaction mixture.

[0091] In embodiments, provided herein is a method for amplifying a double stranded nucleic acid molecule, the method comprising incubating the double stranded nucleic acid molecule in a reaction mixture comprising a first primer and a second primer, wherein: the double stranded nucleic acid molecule comprises a first strand and a second strand, wherein the first strand comprises a first portion and a third portion and the second strand comprises a second portion; the first primer comprises a first region and a second region, wherein the second region of the first primer is complementary to the first portion of the first strand; and the second primer comprises a first region and a second region, wherein the second region of the second primer is complementary to the second portion of the second strand, the first region of the second primer is complementary to the third portion of the first strand, and the first region of the second primer is complementary to the first region of the first primer.

[0092] In embodiments, provided herein is a reaction mixture comprising: a polynucleotide template, a first primer, and a second primer, wherein: the polynucleotide template comprises a first portion, a second portion and a third portion, wherein the third portion is situated in the polynucleotide template between the first portion and the second portion; the first primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to the first portion of the polynucleotide template; the second primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to the second portion of the

polynucleotide template; the first region of the first primer is complementary to the first region of the second primer; and the first region of the second primer is complementary to the third portion of the polynucleotide template.

[0093] In embodiments, provided herein is a kit for the amplification of a polynucleotide template, the kit comprising: a first primer and a second primer, wherein: the first primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a first portion of the polynucleotide template; the second primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to a second portion of the polynucleotide template; the first region of the first primer is complementary to the first region of the second primer; and the first region of the second primer is complementary to a third portion of the polynucleotide template, wherein the third portion is situated in the polynucleotide template between the first portion and the second portion.

[0094] Optionally, in embodiments provided herein involving a kit, the components of the kit may be distributed between at least two separate fluidically isolated containers.

[0095] In embodiments, provided herein is a kit comprising two or more fluidically isolated containers, the containers collectively comprising: a first primer, a second primer, a third primer, and a fourth primer, wherein: the first primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a first portion of a polynucleotide template; the second primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to a second portion of the polynucleotide template; the third primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to the first portion of the polynucleotide template; the fourth primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to a second portion of the

polynucleotide template; the first region of the first primer is complementary to the first region of the second primer; the first region of the third primer is complementary to the first region of the fourth primer; and the nucleotide sequence of the first region of the first primer differs from the nucleotide sequence of first region of the third primer by a single nucleotide. Optionally, the first region of the second primer and the first region of the fourth primer are both complementary to a third portion of the polynucleotide template, wherein the third portion is situated in the polynucleotide template between the first portion and the second portion. Optionally, the second region of the each of the first primer, second primer, third primer, and fourth primers is between 6 and 30 nucleotides in length. Optionally, the second region of the each of the first primer, second primer, third primer, and fourth primers is between 6 and 30 nucleotides in length.

[0096] In embodiments provided herein involving a kit, the kit may comprises a control nucleic acid strand comprising the nucleotide sequence of a polynucleotide template to be detected with reagents of the kit.

[0097] In methods and compositions provided herein, optionally, a PNA probe and the dye $DiSc_2(5)$ may be included. The PNA probe may specifically anneal to a target sequence for amplification in the relevant reaction, and copies thereof, such that DNA-PNA duplexes are formed. Methods provided herein may include detecting a color change of the dye $DiSc_2(5)$ upon the binding of the dye to DNA-PNA duplexes. In embodiments, a PNA probe and dye may be added to a reaction mixture provided herein after the initiation or after the completion of the reaction. In embodiments, a PNA probe and dye may be added to a reaction mixture provided herein at least 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90 or 100 minutes after the initiation of the reaction. In embodiments, a PNA probe and dye may be added to a reaction mixture provided herein no more than 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 minutes after the initiation of the reaction.

[0098] References herein to generating a copy of or amplifying a polynucleotide template or nucleic acid template include generating a copy which contains the sequence of the polynucleotide template / nucleic acid template, as well as generating a copy which contains an analogous sequence of the polynucleotide template / nucleic acid template, unless the context clearly dictates otherwise. For instance, if a polynucleotide template is RNA, generating a copy of the template can include generating a copy which is a DNA molecule

which contains the DNA version of the RNA sequence of the polynucleotide template (i.e. in the DNA sequence, contains "T"s instead of "U"s).

BRIEF DESCRIPTION OF THE DRAWINGS

[0099] In the drawings,

[0100] FIG. 1 is a general schematic of a method provided herein; panels 1A-1K depict steps or features of the method. FIG. 1 includes the following nucleotide sequences: 103: AACGGTTGCTC (SEQ ID NO: 1); 104: GAGCAACCGTT (SEQ ID NO: 2); 105: ATGGGAGC (SEQ ID NO: 3); 106: CCATAACG (SEQ ID NO: 4); 111: ATGGGAGCAACCGTT (SEQ ID NO: 5); 112: CCATAACGGTTGCTCCCAT (SEQ ID NO: 6); 113: ATGGGAGCAACCGTTATGG (SEQ ID NO: 7); 121: CCATAACGGTTGCTCCCATAACGGTTGCTCCCAT (SEQ ID NO: 8); 122: ATGGGAGCAACCGTTATGGGAGCAACCGTTATGG (SEQ ID NO: 9).

[0101] FIG. 2A shows nucleotide sequences of certain primers used with methods provided herein. FIG. 2A includes the following nucleotide sequences: P1 primer, 37 nucleotides: CGCCGGATGGCTCTTGGGAAACCAAACCGTACCAACC (SEQ ID NO: 10); P1 primer, 35 nucleotides: CCGGATGGCTCTTGGGAAACCAAACCGTACCAACC (SEQ ID NO: 11); P1 primer, 33 nucleotides: GGATGGCTCTTGGGAAACCAAACCGTACCAACC (SEQ ID NO: 12); P1 primer, 31 nucleotides: ATGGCTCTTGGGAAACCAAACCGTACCAACC (SEQ ID NO: 13); P1 primer, 30 nucleotides: TGGCTCTTGGGAAACCAAACCGTACCAACC (SEQ ID NO: 14); P1 primer, 29 nucleotides: GGCTCTTGGGAAACCAAACCGTACCAACC (SEQ ID NO: 15); P1 primer, 28 nucleotides: GCTCTTGGGAAACCAAACCGTACCAACC (SEQ ID NO: 16); P1 primer, 27 nucleotides: CTCTTGGGAAACCAAACCGTACCAACC (SEQ ID NO: 17); P1 primer, 26 nucleotides: TCTTGGGAAACCAAACCGTACCAACC (SEQ ID NO: 18); P1 primer, 25 nucleotides: CTTGGGAAACCAAACCGTACCAACC (SEQ ID NO: 19); P1 primer, 24 nucleotides: TTGGGAAACCAAACCGTACCAACC (SEQ ID NO: 20); P1 primer, 23 nucleotides: TGGGAAACCAAACCGTACCAACC (SEQ ID NO: 21); P2 primer, 36 nucleotides: GTTTCCCAAGAGCCATCCGGCGATGCGGAATGTACC (SEQ ID NO: 22); P2 primer, 34 nucleotides: GTTTCCCAAGAGCCATCCGGATGCGGAATGTACC (SEQ ID NO: 23); P2 primer, 32 nucleotides: GTTTCCCAAGAGCCATCCATGCGGAATGTACC (SEQ ID NO: 24); P2 primer, 30 nucleotides: GTTTCCCAAGAGCCATATGCGGAATGTACC (SEQ ID NO: 25);

P2 primer, 29 nucleotides: GTTTCCCAAGAGCCAATGCGGAATGTACC (SEQ ID NO: 26); P2 primer, 28 nucleotides: GTTTCCCAAGAGCCATGCGGAATGTACC (SEQ ID NO: 27); P2 primer, 27 nucleotides: GTTTCCCAAGAGCATGCGGAATGTACC (SEQ ID NO: 28); P2 primer, 26 nucleotides: GTTTCCCAAGAGATGCGGAATGTACC (SEQ ID NO: 29); P2 primer, 25 nucleotides: GTTTCCCAAGAATGCGGAATGTACC (SEQ ID NO: 30); P2 primer, 24 nucleotides: GTTTCCCAAGATGCGGAATGTACC (SEQ ID NO: 31); P2 primer, 23 nucleotides: GTTTCCCAAATGCGGAATGTACC (SEQ ID NO: 32); and P2 primer, 22 nucleotides: GTTTCCCAATGCGGAATGTACC (SEQ ID NO: 33).

[0102] FIG. 2B is a graph depicting results from reactions performed according to a method provided herein.

[0103] FIGs. 3A and 3B shows nucleotide sequences of certain primers used with methods provided herein. FIG. 3A includes the following nucleotide sequences: C5: ATGGCTCTTGGGAAACTGAAACCGTACCAACC (SEQ ID NO: 34); D5: GTTTCCCAAGAGCCATGGATGCGGAATGTACC (SEQ ID NO: 35); C6: GGCTCTTGGGAAACTGAAACCGTACCAACC (SEQ ID NO: 36); D6: GTTTCCCAAGAGCCGGATGCGGAATGTACC (SEQ ID NO: 37); C7: CTCTTGGGAAACTGAAACCGTACCAACC (SEQ ID NO: 38); D7: GTTTCCCAAGAGGGATGCGGAATGTACC (SEQ ID NO: 39); C8: TCTTGGGAAACTGAAACCGTACCAACC (SEQ ID NO: 40); D8: GTTTCCCAAGAGGATGCGGAATGTACC (SEQ ID NO: 41); C9: CTTGGGAAACTGAAACCGTACCAACC (SEQ ID NO: 42); D9: GTTTCCCAAGGGATGCGGAATGTACC (SEQ ID NO: 43); C10: TTGGGAAACTGAAACCGTACCAACC (SEQ ID NO: 44); D10: GTTTCCCAAGGATGCGGAATGTACC (SEQ ID NO: 45); C11: TGGGAAACTGAAACCGTACCAACC (SEQ ID NO: 46); D11: GTTTCCCAGGATGCGGAATGTACC (SEQ ID NO: 47); C12: GGGAAACTGAAACCGTACCAACC (SEQ ID NO: 48); and D12: GTTTCCCGGATGCGGAATGTACC (SEQ ID NO: 49). FIG. 3B includes the following nucleotide sequences: A1: ATGGCTCTTGGGAAACTGCCTGAAACCGTACCAACC (SEQ ID NO: 50); B1: GTTTCCCAAGAGCCATACAGGGATGCGGAATGTACC (SEQ ID NO: 51); A2: GGCTCTTGGGAAACTGCCTGAAACCGTACCAACC (SEQ ID NO: 52); B2: GTTTCCCAAGAGCCACAGGGATGCGGAATGTACC (SEQ ID NO: 53); A3:

CTCTTGGGAAACTGCCTGAAACCGTACCAACC (SEQ ID NO: 54); B3: GTTTCCCAAGAGACAGGGATGCGGAATGTACC (SEQ ID NO: 55); A4: TCTTGGGAAACTGCCTGAAACCGTACCAACC (SEQ ID NO: 56); B4: GTTTCCCAAGAACAGGGATGCGGAATGTACC (SEQ ID NO: 57); A5: CTTGGGAAACTGCCTGAAACCGTACCAACC (SEQ ID NO: 58); B5: GTTTCCCAAGACAGGGATGCGGAATGTACC (SEQ ID NO: 59); A6: TTGGGAAACTGCCTGAAACCGTACCAACC (SEQ ID NO: 60); B6: GTTTCCCAAACAGGGATGCGGAATGTACC (SEQ ID NO: 61); A7: TGGGAAACTGCCTGAAACCGTACCAACC (SEQ ID NO: 62); B7: GTTTCCCAACAGGGATGCGGAATGTACC (SEQ ID NO: 63); A8: GGGAAACTGCCTGAAACCGTACCAACC (SEQ ID NO: 64); and B8: GTTTCCCACAGGGATGCGGAATGTACC (SEQ ID NO: 65).

[0104] FIGs. 3C and 3D are graphs depicting results from reactions performed according to a method provided herein.

[0105] FIG. 4 is a graph depicting results from reactions performed according to a method provided herein.

[0106] FIG. 5 is a graph depicting results from reactions performed according to a method provided herein.

[0107] FIG. 6 is a graph depicting results from reactions performed according to a method provided herein.

[0108] FIG. 7 is a sequence alignment depicting the nucleotide sequences of products generated in reactions performed according to a method provided herein. FIG. 7 includes the following nucleotide sequences (in order from the top sequence in the alignment to the bottom sequence in the alignment): SEQ ID NO: 66: TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAA GACCAAATTCTTGAGA; SEQ ID NO: 67: TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAA GACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT; SEQ ID NO: 68: TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAA GACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT; SEQ ID

NO: 69:
TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAA
GACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT; SEQ ID
NO: 70:
TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACTGAAGGGGAA
GACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT; SEQ ID
NO: 71:
TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAA
GACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT; SEQ ID
NO: 72:
TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAA
GACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT; SEQ ID
NO: 73:
TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAA
GACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT; SEQ ID
NO: 74:
TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAA
GACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT; SEQ ID
NO: 75:
TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAA
GACCAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT; SEQ ID
NO: 76:
GAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAAGACCAAAT
GAACCCACTAACAGTAGAAGTACCATACATTT; SEQ ID NO: 77:
TCTTGAGAGAACCCACTAACTCTTGAGAGAACCCACTAAC; and SEQ ID NO: 78:
GGAAGACCAAATTCTTGAGA.

[0109] FIGS. 8A-8C are general schematics of features of embodiments of molecules provided herein.

[0110] FIG. 9 is a general schematic of features of embodiments of molecules provided herein.

[0111] FIGS. 10A-10C are general schematics of features of embodiments of molecules provided herein.

[0112] FIG. 11 is a graph depicting results from reactions performed according to a method provided herein.

[0113] FIG. 12 is a graph depicting results from reactions performed according to a method provided herein.

[0114] It is noted that the drawings and elements therein are not necessarily drawn to shape or scale. For example, the shape or scale of elements of the drawings may be simplified or modified for ease or clarity of presentation. It should further be understood that the drawings and elements therein are for exemplary illustrative purposes only, and not be construed as limiting in any way.

DETAILED DESCRIPTION

[0115] While the invention includes various modifications and alternative forms, specific embodiments thereof are shown by way of example in the drawings and will herein be described in detail. It should be understood, however, that there is no intent to limit the invention to the particular forms disclosed, but on the contrary, the invention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention as defined by the claims.

[0116] In some embodiments, provided herein are methods and compositions relating to the amplification of nucleic acids and the generation of concatemers.

[0117] In methods provided herein, generation of nucleic acid concatemers also amplifies the number of copies of the nucleic acid template / particular nucleic acid in the concatemer. Accordingly, references herein to methods and compositions for the generation of concatemers also apply to the amplification of nucleic acids.

[0118] As used herein, a "polynucleotide" refers to a polymeric chain containing two or more nucleotides. "Polynucleotides" includes primers, oligonucleotides, nucleic acid strands, etc. A polynucleotide may contain standard or non-standard nucleotides. Typically, a polynucleotide contains a 5' phosphate at one terminus ("5' terminus") and a 3' hydroxyl group at the other terminus ("3' terminus) of the chain. The most 5' nucleotide of a polynucleotide may be referred to herein as the "5' terminal nucleotide" of the polynucleotide. The most 3' nucleotide of a polynucleotide may be referred to herein as the "3' terminal nucleotide" of the polynucleotide.

[0119] The term "downstream" as used herein in the context of a polynucleotide containing a 5' terminal nucleotide and a 3' terminal nucleotide refers to a position in the polynucleotide which is closer to the 3' terminal nucleotide than a reference position in the polynucleotide. For example, in a primer having the sequence: 5' ATAAGC 3', the "G" is downstream from the "T" and all of the "A"s.

[0120] The term "upstream" as used herein in the context of a polynucleotide containing a 5' terminal nucleotide and a 3' terminal nucleotide, refers to a position in the polynucleotide which is closer to the 5' terminal nucleotide than a reference position in the polynucleotide. For example, in a primer having the sequence: 5' ATAAGC 3', the "T" is upstream from the "G", the "C", and the two "A"s closest to the "G".

[0121] As used herein, "nucleic acid" includes both DNA and RNA, including DNA and RNA containing non-standard nucleotides. A "nucleic acid" contains at least one polynucleotide (a "nucleic acid strand"). A "nucleic acid" may be single-stranded or double-stranded.

[0122] As used herein, a "concatemer" refers to a nucleic acid molecule which contains within it two or more copies of a particular nucleic acid, wherein the copies are linked in series. Within the concatemer, the copies of the particular nucleic acid may be linked directly to each other, or they may be indirectly linked (e.g. there may be nucleotides between the copies of the particular nucleic acid). In an example, the particular nucleic acid may be that of a double-stranded nucleic acid template, such that a concatemer may contain two or more copies of the double-stranded nucleic acid template. In another example, the particular nucleic acid may be that of a polynucleotide template, such that a concatemer may contain two or more copies of the polynucleotide template.

[0123] As used herein, a "target" nucleic acid or molecule refers to a nucleic acid of interest. A target nucleic acid / molecule may be of any type, including single-stranded or double stranded DNA or RNA (e.g. mRNA).

[0124] As used herein, "complementary" sequences refer to two nucleotide sequences which, when aligned anti-parallel to each other, contain multiple individual nucleotide bases which can pair with each other according to standard base-pairing rules (e.g. A-T, G-C, or A-U), such that molecules containing the sequences can specifically anneal to each other. It is not necessary for every nucleotide base in two sequences to be capable of pairing with each other

for the sequences to be considered "complementary". Sequences may be considered complementary, for example, if at least 30%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, or 100% of the nucleotide bases in two sequences can pair with each other when the sequences are optimally aligned for complementation. In addition, sequences may still be considered "complementary" when the total lengths of the two sequences are significantly different from each other. For example, a primer of 15 nucleotides may be considered "complementary" to a longer polynucleotide containing hundreds of nucleotides if multiple individual nucleotide bases of the primer can pair with nucleotide bases in the longer polynucleotide when the primer is aligned anti-parallel to a particular region of the longer polynucleotide. Additionally, "complementary" sequences may contain one or more nucleotide analogs or nucleobase analogs. As used herein, "perfectly complementary" or "perfect complementation" or the like refers two sequences which are 100% complementary to each other (i.e. where there are no mis-matches between the nucleotides of the sequences when the sequences are paired for maximum complementation).

[0125] As used herein, the term "isolated" as applied to proteins, nucleic acids, or other biomolecules refers to a molecule that has been purified or separated from a component of its naturally-occurring environment (e.g. a protein purified from a cell in which it was naturally produced). An "isolated" molecule may be in contact with other molecules (for example, as part of a reaction mixture). As used herein, "isolated" molecules also include recombinantly-produced proteins or nucleic acids which have an amino acid or nucleotide sequence which occurs naturally. "Isolated" nucleic acids include polypeptide-encoding nucleic acid molecules contained in cells that ordinarily express the polypeptide where, for example, the nucleic acid molecule is at a chromosomal location different from that of natural cells. In some embodiments, "isolated" polypeptides are purified to at least 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 100% homogeneity as evidenced by SDS-PAGE of the polypeptides followed by Coomassie blue, silver, or other protein staining method.

[0126] As used herein, a nucleic acid molecule which is described as containing the "sequence" of a template or other nucleic acid may also be considered to contain the template or other nucleic acid itself (e.g. a molecule which is described as containing the sequence of a template may also be described as containing the template), unless the context clearly dictates otherwise.

[0127] As used herein, when a first polynucleotide is described as "annealed", "annealing" or the like to a second polynucleotide, the entirety of the first polynucleotide or any portion thereof may anneal to the second polynucleotide, and vice versa.

[0128] As used herein, a reference to "treating" a given object to a condition or other object or the like refers to directly or indirectly exposing the given object to the recited condition or other object. Thus, while a "treating" step may involve a distinct related action (e.g. adding an enzyme to a vessel, shaking a vessel, etc.), not every "treating" step requires a distinct related action. For example, a reaction involving one or more reagents can be set up in a vessel, and once the reaction has been initiated, multiple events or steps may occur in the vessel without further human or mechanical intervention with the contents of the vessel. One or more of these multiple events or steps in the vessel may be described as "treating" an object in the vessel, even if no separate intervention with the contents of the vessel occurs after the initiation of the reaction.

[0129] Embodiments of methods and compositions provided herein may be described with reference to Figure 1. A primary nucleic acid 101 may be provided (Fig. 1A). The primary nucleic acid 101 may contain the sequence of a nucleic acid template 102. The nucleic acid template 102 may be a double-stranded nucleic acid containing a first strand 103 and a second strand 104. In Figure 1, the first strand 103 of the double-stranded nucleic acid template has an exemplary nucleotide sequence in the 5' to 3' direction of: AACGGTTGCTC (SEQ ID NO: 1). The first strand 103 also contains a 5' terminal nucleotide (the 5'-most A) and a 3' terminal nucleotide (the 3'-most C). The second strand 104 has an exemplary nucleotide sequence in the 5' to 3' direction of: GAGCAACCGTT (SEQ ID NO: 2). The second strand 104 also contains a 5' terminal nucleotide (the 5'-most G) and a 3' terminal nucleotide (the 3'-most T).

[0130] A first primer 105 and a second primer 106 may also be provided (Fig. 1B). In Figure 1, the first primer 105 has an exemplary nucleotide sequence in the 5' to 3' direction of: ATGGGAGC (SEQ ID NO: 3). The first primer also contains a 5' terminal nucleotide (the 5'-most A) and a 3' terminal nucleotide (the 3'-most C). The second primer 106 has an exemplary nucleotide sequence in the 5' to 3' direction of: CCATAACG (SEQ ID NO: 4). The second primer also contains a 5' terminal nucleotide (the 5'-most C) and a 3' terminal nucleotide (the 3'-most G). Although nucleic acid template 102 of Figure 1 is 11 nucleotides

in length and has an exemplary nucleotide sequence, these are exemplary, and not to be considered as limiting.

[0131] The first primer 105 may comprise two regions: i) a tail region and ii) a template-binding region ("temp" region). The tail region of the first primer 105 may contain three components: a) the 5' terminal nucleotide of the primer, b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide, and c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides. In Figure 1, the tail region of the first primer 105 has an exemplary nucleotide sequence in the 5' to 3' direction of: ATGG, of which the 5' terminal nucleotide of the primer is the A, the middle section is the middle TG, and the innermost nucleotide is the 3'-most G. The template-binding region of the first primer 105 may contain three components: a) the 3' terminal nucleotide of the primer, b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide, and c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides. In Figure 1, the template-binding region of the first primer 105 has an exemplary nucleotide sequence in the 5' to 3' direction of: GAGC, of which the 3' terminal nucleotide of the primer is the C, the middle section is the middle AG, and the innermost nucleotide is the 5'-most G.

[0132] The second primer 106 may comprise two regions: i) a tail region and ii) a template-binding region. The tail region of the first primer 106 may contain three components: a) the 5' terminal nucleotide of the primer, b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide, and c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides. In Figure 1, the tail region of the second primer 106 has an exemplary nucleotide sequence in the 5' to 3' direction of: CCAT, of which the 5' terminal nucleotide of the primer is the 5'-most C, the middle section is the middle CA, and the innermost nucleotide is the T. The template-binding region of the second primer 106 may contain three components: a) the 3' terminal nucleotide of the primer, b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide, and c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides. In Figure 1, the template-binding region of the second primer 106 has an exemplary nucleotide sequence in the 5' to 3' direction of: AACG, of which the 3' terminal nucleotide of the

primer is the G, the middle section is the middle AC, and the innermost nucleotide is the 5'-most A.

[0133] In some embodiments, the tail region of the second primer may contain a nucleotide sequence which is complementary to the nucleotide sequence of the tail region of the first primer, if the sequences of the primers are aligned such that the 5' terminal nucleotide of the second primer is aligned with the innermost nucleotide of the tail region of the first primer and the 5' terminal nucleotide of the first primer is aligned with the innermost nucleotide of the tail region of the second primer. For example, in Figure 1, the tail region of the second primer 106 has a nucleotide sequence in the 5' to 3' direction of CCAT, and the tail region of the first primer 105 has a nucleotide sequence in the 5' to 3' direction of ATGG. These sequences are complementary when the 5' terminal nucleotide of the second primer (C) is aligned with the innermost nucleotide of the tail region of the first primer (G) and the 5' terminal nucleotide of the first primer (A) is aligned with the innermost nucleotide of the tail region of the second primer (T) 107 (Fig. 1B).

[0134] It should be noted that although the tail region of the second primer may contain a nucleotide sequence which is complementary to the nucleotide sequence of the tail region of the first primer, typically, products formed by the annealing of the first primer to the second primer are not desirable or useful for methods or compositions provided herein. Accordingly, in some embodiments, steps may be taken to minimize the formation of first primer – second primer annealed products. Such steps may include, for example, not pre-incubating a first primer and a second primer under conditions where the primers may interact for an extended period of time before initiating a method provided herein.

[0135] The primary nucleic acid 101 may be treated with a first copy of the first primer 105a and a polymerase 108 under conditions such that the template-binding region of the first copy of the first primer 105a anneals to the first strand of the nucleic acid template 103 (Fig. 1C) and an extension product of the first copy of the first primer 111 is formed (Fig. 1D). The polymerase 108 may catalyze the formation of the extension product of the first copy of the first primer 111. The polymerase may have strand displacement activity. During the synthesis of the extension product of the first copy of the first primer 111, the polymerase may displace the second strand of the nucleic acid template 104 from the first strand of the nucleic acid template 103. Typically, the extension product is generated from the 3' end of the first copy of the first primer 105a. During the generation of the extension product of the

first copy of the first primer 111, the first copy of the first primer 105a may be covalently linked to the synthesized extension product, such that the first copy of the first primer becomes part of the molecule described herein as the "extension product of the first copy of the first primer". The extension product of the first copy of the first primer 111 is complementary to the first strand of the nucleic acid template 103. In some embodiments, when the first copy of the first primer 105a anneals to the first strand of the nucleic acid template 103, the template-binding region but not the tail region of the first copy of the first primer 105a anneals to the first strand of the nucleic acid template.

[0136] In some embodiments, conditions such that the template-binding region of the first copy of the first primer 105a anneals to the first strand of the nucleic acid template 103 and an extension product of the first copy of the first primer 111 is formed may include any condition sufficient to support polymerase-based nucleic acid synthesis. Example conditions for polymerase-based nucleic acid synthesis are known in the art and are provided, for example, in Molecular Cloning: A Laboratory Manual, M.R. Green and J. Sambrook, Cold Spring Harbor Laboratory Press (2012), which is herein incorporated by reference in its entirety. In embodiments, the template-binding region of the first primer or second primer may support template-dependent extension of the primer by a nucleic acid polymerase, which may extend the primer from the primer's 3' end.

[0137] The extension product of the first copy of the first primer 111 may be treated with the second primer 106 and a polymerase 108 under conditions such that the template-binding region of the second primer 106 anneals to the extension product of the first copy of the first primer 111 (Fig. 1E) and an extension product of the second primer 112 is formed (Fig. 1F). The polymerase 108 may catalyze the formation of the extension product of the second primer 106. The polymerase may have strand displacement activity. The polymerase may be the same type of polymerase as is used to generate an extension product of the first copy of the first primer 111, or it may be different. During the synthesis of the extension product of the second primer 112, the polymerase may displace the first strand of the nucleic acid template 103 from the extension product of the first copy of the first primer 111. Typically, the extension product is generated from the 3' end of the second primer 106. During the generation of the extension product of the second primer 112, the second primer 106 may be covalently linked to the synthesized extension product, such that the second primer becomes part of the molecule described herein as the "extension product of the second primer". The

extension product of the second primer 112 is complementary to the extension product of the first copy of the first primer 111. In some embodiments, when the second primer 106 anneals to the extension product of the first copy of the first primer 111, the template-binding region but not the tail region of the second primer 106 anneals to the extension product of the first copy of the first primer 111. The extension product of the second primer 112 contains a 5' terminal nucleotide, a 3' terminal nucleotide, and a 3' terminal region. The final nucleotide of the 3' terminal region (T) is the 3' terminal nucleotide of the extension product of the second primer (T), and the 3' terminal region contains the same nucleotide sequence as the nucleotide sequence of the tail region of the second primer read in the 5' to 3' direction (CCAT).

[0138] In some embodiments, conditions such that the template-binding region of the second primer 106 anneals to the extension product of the first copy of the first primer 111 and an extension product of the second primer 112 is formed may include any condition sufficient to support polymerase-based nucleic acid synthesis, including any condition discussed elsewhere herein. The conditions may be the same as used to generate an extension product of the first copy of the first primer 111, or they may be different.

[0139] The extension product of the second primer 112 may be treated with a second copy of the first primer 105b and a polymerase 108 under conditions such that the template-binding region of the second copy of the first primer 105b anneals to the extension product of the second primer 112 (Fig. 1G) and an extension product of the second copy of the first primer 113 is formed (Fig. 1H). The polymerase 108 may catalyze the formation of the extension product of the second copy of the first primer 113. The polymerase may have strand displacement activity. The polymerase may be the same type of polymerase as is used to generate an extension product of the first copy of the first primer 111 or an extension product of the second primer 112, or it may be different. During the synthesis of the extension product of the second copy of the first primer 113, the polymerase may displace the extension product of the first copy of the first primer 111 from the extension product of the second primer 112. Typically, the extension product is generated from the 3' end of the second copy of the first primer 105b. During the generation of the extension product of the second copy of the first primer 113, the second copy of the first primer 105b may be covalently linked to the synthesized extension product, such that the second copy of the first primer becomes part of the molecule described herein as the "extension product of the second copy

of the first primer". The extension product of the second copy of the first primer 113 is complementary to the extension product of the second primer 112. In some embodiments, when the second copy of the first primer 105b anneals to the extension product of the second primer 112, both the template-binding region and the tail region of the second copy of the first primer 105b anneals to the extension product of the second primer 112. The extension product of the second copy of the first primer 113 contains a 5' terminal nucleotide, a 3' terminal nucleotide, and a 3' terminal region. The final nucleotide of the 3' terminal region (G) is the 3' terminal nucleotide of the extension product of the second primer (G), and the 3' terminal region contains the same nucleotide sequence as the nucleotide sequence of the tail region of the first primer read in the 5' to 3' direction (ATGG).

[0140] In some embodiments, conditions such that the template-binding region of the second copy of the first primer 105b anneals to the extension product of the second primer 112 and an extension product of the second copy of the first primer 113 is formed may include any condition sufficient to support polymerase-based nucleic acid synthesis, including any condition discussed elsewhere herein. The conditions may be the same as used to generate an extension product of the first copy of the first primer 111 or an extension product of the second primer 112, or they may be different.

[0141] Generation of the extension product of the second copy of the first primer 113 may result in the generation of a molecule comprising the extension product of the second copy of the first primer 113 and the extension product of the second primer 112, which may be referred to herein as the "secondary nucleic acid" 115. Within the secondary nucleic acid 115, the extension product of the second copy of the first primer 113 may be annealed to the extension product of the second primer 112. In addition, the secondary nucleic acid 115 contains the sequence of the nucleic acid template 102. The secondary nucleic acid 115 has a greater nucleotide length than the nucleic acid template 102 alone, as in addition to the sequence of the nucleic acid template 102, it may include the sequence of the tail regions of the first primer and the second primer, and complementary sequences thereof. Specifically, a secondary nucleic acid 115 may have a first end region 116 and a second end region 117. The first end region 116 may comprise the 3' terminal region of the extension product of the second primer 112, and the complement thereof. The second end region 117 may comprise the 3' terminal region of the extension product of the second copy of the first primer 113, and the complement thereof.

[0142] A first copy 115a and a second copy 115b of the secondary nucleic acid 115 may be formed or provided (Fig. 1I). The first copy 115a and second copy 115b of the secondary nucleic acid 115 may be generated by any process whereby a nucleic acid having the general structure of the secondary nucleic acid 115 may be generated, including any process discussed elsewhere herein. For example, the full process as described above for generating a secondary nucleic acid 115 from a primary nucleic acid 101 may be repeated two times, in order to generate the first copy 115a and the second copy 115b of the secondary nucleic acid. In another example, the first copy 115a and the second copy 115b of the secondary nucleic acid may be generated from a single copy of an extension product of a first copy of first primer 111. In this example, two copies 112a, 112b of an extension product of the second primer may be generated from the single copy of the extension product of a first copy of a first primer 111, which may occur if a first copy of the extension product of the second primer 112a is displaced from the single copy of the extension product of the first copy of the first primer 111, thereby permitting the generation of a second copy of the extension product of the second primer 112b from the single copy of the extension product of a first copy of a first primer 111. Then, for example, an extension product of the second copy of the first primer 113a, 113b may be generated from each copy of the extension product of the second primer 112a, 112b, respectively. Additional procedures for the generation of a first copy 115a and a second copy 115b of the secondary nucleic acid may also be performed and used with methods provided herein.

[0143] The first copy 115a and second copy 115b of the secondary nucleic acid may be treated under conditions such that the 3' terminal region of the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid 113a anneals to the 3' terminal region of the extension product of the second primer of the second copy of the secondary nucleic acid 112b, to produce a cross-over structure 118 comprising these strands (Fig. 1J) (for simplicity, strands 113b and 112a are not shown). The cross-over structure 118 may be further treated with a polymerase under conditions such that extension products of both component strands 112b, 113a are formed, the extension products which may be referred to herein as a "first concatemer strand" 121 and a "second concatemer strand" 122, respectively. The first concatemer strand 121 and the second concatemer strand 122 may be annealed together, and may be collectively referred to as a concatemer 120 (Fig. 1K). The concatemer 120 may contain two or more copies of the nucleic acid template 102. Within the concatemer 120, some or all of the two or more copies of the nucleic acid template 102 may

be separated from each other by the sequences of the tail regions of the first primer 105 and second primer 106, where the sequences of the tail regions are annealed to each other. For example, in the concatemer 120 of Fig. 1K, the first copy of the first strand of the double-stranded nucleic acid template is separated from the second copy of the first strand of the double-stranded nucleic acid template by the sequence, in 5'-3' order: CCAT. CCAT is also the sequence, in 5'-3' order, of the tail region of the second primer 106. Similarly, in the concatemer 120 of Fig. 1K, the first copy of the second strand of the double-stranded nucleic acid template is separated from the second copy of the second strand of the double-stranded nucleic acid template by the sequence, in 5'-3' order: ATGG. ATGG is also the sequence, in 5'-3' order, of the tail region of the first primer 105. Within the concatemer, the CCAT of the first concatemer strand is annealed to the ATGG of the second concatemer strand.

[0144] In some embodiments, conditions such that a cross-over structure 118 or extension products of the strands of a cross-over structure are formed may include any condition sufficient to support polymerase-based nucleic acid synthesis, including any condition discussed elsewhere herein. The conditions may be the same as used to generate an extension product of the first copy of the first primer 111, an extension product of the second primer 112, or an extension product of the second copy of the first primer 111, or they may be different.

[0145] In some embodiments, concatemers provided herein may further increase in length according to the process generally outlined in Fig 1I-1K. For example, two of the concatemer molecules 120 as shown in Figure 1K may be treated such that they form a cross-over structure similar to that shown in Figure 1J (except with longer strands), followed by generation of a larger concatemer molecule containing four copies of the nucleic acid template 102. In another example, a secondary nucleic acid 115 and a concatemer 120 may form a cross-over structure, followed by generation of a larger concatemer molecule containing three copies of the nucleic acid template 102. In some embodiments, in methods provided herein, multiple different concatemers of multiple different lengths may be simultaneously generated.

[0146] In embodiments, methods and compositions provided herein may be described with reference to Figure 8. A polynucleotide template 800 (Fig. 8A) may be a target for amplification. The polynucleotide template 800 is a single nucleic acid strand. The polynucleotide template strand may exist as a free, single-stranded molecule, or it may exist

as part of a double-stranded molecule, in which the polynucleotide template strand is annealed to a complementary strand thereof. The polynucleotide template 800 may be DNA or RNA.

[0147] The polynucleotide template 800 may contain at least a first portion 801 (also indicated with an "(i)") and a second portion 802 (also indicated with an "(ii)"). The first portion 801 and second portion 802 may each contain any number of nucleotides, such as less than 1000, 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20, 15, 10, 9, 8, 7, 6, 5, 4, or 3 nucleotides each or at least 1000, 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20, 15, 10, 9, 8, 7, 6, 5, 4, or 3 nucleotides each. Typically, the first portion 801 and second portion 802 may each contain between 10 and 30 nucleotides, although the first portion 801 and second portion 802 may be other lengths as described elsewhere herein, as well. The first portion 801 and second portion 802 may contain the same or a different number of nucleotides. In some embodiments, the polynucleotide template 800 may contain other portions in addition to the first portion 801 and second portion 802. In other embodiments, the first portion 801 and second portion 802 may constitute the entirety of the polynucleotide template 800. The polynucleotide template 800 may have the length of a nucleic acid template described elsewhere herein. The polynucleotide template 800 may be a portion of a primary nucleic acid. The polynucleotide template may be present in a sample obtained from a subject.

[0148] The polynucleotide template 800 may be incubated in a reaction mixture containing a first primer 810 and a second primer 820 (Fig. 8B). Typically, the reaction mixture contains multiple copies of each primer, such as, for example, at least 4, 5, 6, 10, 15, 20, 50, 100, 1000, 10,000, 100,000, or 1 million copies or more of each of the primers. The first primer 810 may have a first region 811 (also indicated with an "(i)") and a second region 812 (also indicated with an "(ii)"). The first region of the first primer 811 may have a 5' end, and the second region of the first primer 812 may have a 3' end. The first region of the first primer may have a nucleotide sequence which is different from the nucleotide sequence of the second region of the first primer. The second primer 820 may have a first region 821 (also indicated with a "(i)") and a second region 822 (also indicated with a "(ii)"). The first region of the second primer 821 may have a 5' end, and the second region of the second primer 822 may have a 3' end. The first region of the second primer may have a nucleotide sequence which is different from the nucleotide sequence of the second region of the second primer.

[0149] The second region of the first primer 812 may have a nucleotide sequence which is complementary to the nucleotide sequence of the first portion of the polynucleotide template 801. In Fig. 8, nucleotide sequence of the first portion of the polynucleotide template 801 is represented by the letter A, and the nucleotide sequence of the second region of the first primer 812 is represented by the letter A', where A and A' are complementary to each other. The second region of the second primer 822 may have a nucleotide sequence which is complementary to a partner nucleotide sequence 830, wherein the partner nucleotide sequence 830 is complementary to the nucleotide sequence of the second portion of the polynucleotide template 802. In Fig. 8, the nucleotide sequence of the second portion of the polynucleotide template 802 is represented by the letter B, the nucleotide sequence of the partner nucleotide sequence 830 is represented by the letter B', and the nucleotide sequence of the second region of the second primer 822 is represented by the letter B, where B and B' are complementary to each other. In other words, as indicated by Fig. 8, the nucleotide sequence of the second portion of the polynucleotide template 802 (B) may be the same as the nucleotide sequence of the second region of the second primer 822 (B). With compositions and methods provided herein, a partner nucleotide sequence 830 may be generated, for example, as part of an extension product from the 3' end of the first primer. In embodiments, the first region of the first primer 811 may be complementary to the first region of the second primer 821. In Fig. 8, the nucleotide sequence of the first region of the first primer is represented by the letter C, and the nucleotide sequence of the first region of the second primer is represented by the letter C', where C and C' are complementary to each other.

[0150] In embodiments, the first primer and second primer of Fig. 8 may have any of the characteristics of the first primer and second primer described elsewhere herein (e.g. as in Fig. 1), respectively. Also, in embodiments, the first region of the first primer of Fig. 8 may have any of the characteristics of the tail region of a first primer described elsewhere herein. In embodiments, the second region of the first primer of Fig. 8 may have any of the characteristics of the template binding region of a first primer described elsewhere herein. In embodiments, the first region of the second primer of Fig. 8 may have any of the characteristics of the tail region of a second primer described elsewhere herein. In embodiments, the second region of the second primer of Fig. 8 may have any of the characteristics of the template binding region of a second primer described elsewhere herein.

[0151] In embodiments, a reaction mixture containing a polynucleotide template 800 and at least two copies of a first primer 810 and at least two copies of a second primer 820 may contain one or more other components which may support polymerase-based nucleic acid synthesis as described elsewhere herein (e.g. polymerases, nucleotides, buffers, water, etc.). Similarly, a reaction mixture containing a polynucleotide template 800 and at least two copies of a first primer 810 and at least two copies of a second primer 820 may be incubated at any of the reaction conditions described elsewhere herein for supporting the amplification of a nucleic acid template or the generation of concatemers.

[0152] In embodiments, upon incubation of a polynucleotide template 800 and at least two copies of a first primer 810 and at least two copies of a second primer 820 in a reaction mixture as described herein, one or more concatemer strands may be formed (Fig. 8C). In embodiments, a formed concatemer strand 840 may have a 5' end and a 3' end, and may have a sequence containing the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', wherein C' is the nucleotide sequence of the first region of the second primer, T is the sequence of the polynucleotide template or an analogous sequence thereof, and X is any number and sequence of nucleotides. Thus, as indicated by the general structure of the concatemer strand, a concatemer strand may contain at least two copies of the sequence of the polynucleotide template or an analogous sequence thereof, where the copies of the sequence of the polynucleotide template are separated by at least the sequence of the first region of the second primer. In embodiments, T may be an analogous sequence of the polynucleotide template. As used herein, an "analogous sequence" of a polynucleotide template refers to a sequence which contains a similar sequence as the polynucleotide template, but which contains one or more analogous nucleotides to a nucleotide in the polynucleotide template. Thus, for example, if the polynucleotide template contains an RNA sequence, an analogous sequence thereof may be a DNA version of the same sequence (i.e. the uracils in the RNA sequence are thymines in the analogous DNA sequence), or vice-versa. Continuing with the example, if the polynucleotide template contains an RNA sequence of 5' UACCUG 3', an analogous sequence is the DNA sequence of 5' TACCTG 3'.

[0153] In embodiments, in a sequence containing the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', X is zero nucleotides (i.e. the T and C sequence on either side of the X are directly linked). In other embodiments, X is less than 10,000, 5000, 4000, 3000, 2000, 1000, 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 40, 30, 20, 15, 10, 9, 8, 7, 6, 5, 4, or 3

nucleotides. The nucleotides (if any) in X may have any sequence. In embodiments, X contains a sequence having the general structure in the 5' to 3' direction of [(C'-T)$_N$] wherein C' is the nucleotide sequence of the first region of the second primer, T is the sequence of the polynucleotide template or analogous sequence thereof, and N is any integer less than 1000, 500, 200, 100, 50, or 10, such as an integer between 1 and 500, 1 and 200, 1 and 100, 1 and 50, or 1 and 10. For example, if "N" is 3 when X contains a sequence having the general structure in the 5' to 3' direction of [(C'-T)$_N$], there are 3 sequential repeats of (C'-T) in the X position of the structure C'-T-C'-T -X-C', such that the corresponding structure could be written as: C'-T-C'-T–<u>C'-T–C'-T –C'-T</u>-C', where the underlined C's and Ts are the C'-T repeats of the formula [(C'-T)$_3$]. Thus, for example, according to compositions and methods provided herein, concatemer strands may be formed which contain tens or hundreds of copies of the polynucleotide template. In embodiments, a concatemer strand as provided in Fig. 8 may have any of the characteristics of a concatemer strand described elsewhere herein. Also, in some embodiments, during the generation of a concatemer strand, at one or more positions of a C' or T in a sequence having the general structure C'-T-C'-T -X-C', a small number of nucleotides (e.g. 15 or less, 10 or less, 5 or less, or 3 or less) may be inserted between a C' and T. Also, in some embodiments, during the generation of a concatemer strand, at one or more positions of a C' or T in a sequence having the general structure C'-T-C'-T -X-C' or a small number of nucleotides (e.g. 15 or less, 10 or less, 5 or less, or 3 or less) may be deleted from a C' or T sequence. Thus, concatemer strands generated according to methods provided herein may include strands which have a general structure as provided herein, but which may have a small number of nucleotides added or removed at one or more junction points between different sequence components of the concatemer strand (i.e. junctions between template sequence and primer sequence / T and C'). Also, in embodiments, a concatemer strand as provided herein may contain additional nucleotides (e.g. up to 3, 5, 10, 20, 50, 100, 500, 1000 or more additional nucleotides) at the 5' or 3' end of a molecule containing a concatemer having a sequence with the general structure C'-T-C'-T -X-C'. In embodiments, a C' of the general structure C'-T-C'-T -X-C' may have any of the characteristics (e.g. length, sequence) of the tail region of a second primer described elsewhere herein. In embodiments, a T of the general structure C'-T-C'-T -X-C' may have any of the characteristics (e.g. length, sequence) of a polynucleotide template strand described elsewhere herein.

[0154] In embodiments, a concatemer strand 840 generated according to method provided herein may be part of a single-stranded molecule. In other embodiments, a concatemer strand

840 generated according to method provided herein may be part of a double-stranded concatemer, in which the concatemer strand 840 is annealed to a strand which is complementary to the concatemer strand 850. While Fig. 8C depicts concatemer strands having a sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C' (strand 840) or C-T'-C-T'-X'-C (strand 850), wherein X is 0 nucleotides, a concatemer strand generated according to the schematic of Fig. 8 may have any of the characteristics of a concatemer strand described above or elsewhere herein. Also, as relating to Fig. 8C, sequences A and B are part of the template strand, and thus are not separately notated in the general structure C'-T-C'-T -X-C' or C-T'-C-T'-X'-C.

[0155] To further indicate the relationship of elements in embodiments of Figure 8, blocks 860, 862, 864, 866, and 868 are provided in Figure 8. Block 860 (with a backslash-like fill) indicates the relative position of the total polynucleotide template sequence (i.e. element 800), or its complementary sequence. Block 862 (with vertical line fill) indicates the relative position of the sequence of first portion of the polynucleotide template (i.e. the relative position of the sequence of element 801), or its complementary sequence. Thus, block 862 also represents the sequence of the second region of the first primer (i.e. element 812), which is complementary to element 801. Block 864 (with vertical and horizontal line fill) indicates the relative position of the sequence of the second portion of the polynucleotide template (i.e. the relative position of the sequence of element 802), or its complementary sequence. Thus, block 864 also represents the sequence of the partner nucleotide sequence (i.e. element 830), which is complementary to element 802. Block 864 also indicates the relative position of the sequence of the second region of the second primer 822, which may be the same sequence as the sequence of the second portion of the polynucleotide template (element 802), or a similar sequence. In other words, in embodiments the nucleotide sequence of 822 may be the same as or similar to nucleotide sequence of 802. As depicted, for example, in Figure 8A, in embodiments, there are nucleotides in the polynucleotide template which are not part of the first portion or the second portion of the polynucleotide template. For instance, in one example, the first portion of the polynucleotide template may contain 15 nucleotides, the second portion of the polynucleotide template may contain 15 nucleotides, and between the first portion and second portion of the polynucleotide template are 25 nucleotides. Typically, the first portion of the polynucleotide template and the second portion of the polynucleotide template are each between 10 and 30 nucleotides in length, although other numbers are also contemplated. For example, each of the first portion and second portion of the

polynucleotide template may be at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, or 100 nucleotides, no more than 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, or 100 nucleotides, or at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, or 50 nucleotides and no more than 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, or 100 nucleotides (with the upper limit being above the lower limit). In embodiments, in the polynucleotide template between the first portion and second portion, there may be, for example, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 100, 200, or 500 nucleotides, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 100, 200, or 500 nucleotides, or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 100, or 200 nucleotides and no more than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 100, 200, or 500 nucleotides (with the upper limit being above the lower limit). Block 866 (with wavy line fill) indicates the relative position of the sequence of the first region of the first primer (i.e. the relative position of the sequence of element 811 / region "C"), or its complementary sequence. Thus, block 866 also represents the sequence of the first region of the second primer (element 821 / region "C'"), which is complementary to element 811. Block 868 (with diamond fill) indicates the relative position of the sequence of the concatemer strand (element 840), or its complementary sequence. Thus, block 868 also represents the sequence of a strand which is complementary to the concatemer strand (element 850). As depicted in Figure 8C, within the concatemer strand or its complement (868), two copies of the polynucleotide template sequence or its complement (860) are present, separated by the nucleotide sequence of the first region of the first primer or its complement (866). In addition, within the concatemer strand or its complement (868), the nucleotide sequence of the first region of the first primer or its complement (866) flank the polynucleotide template sequence or its complement (860) on both ends. Also, within the concatemer strand or its complement (868), there are two copies of the sequence of the second region of the first primer or its complement (862) and two copies of the sequence of the second region of the second primer or its complement (864), which are both present in two copies as part of the two copies of the polynucleotide template sequence or its complement. Blocks 860, 862, 864, 866, and 868 are not to be interpreted to as to dictate any particular required size relationship between the elements of Figure 8; rather blocks 860, 862, 864, 866, and 868 are

simply to help indicate the general position of the elements of Figure 8 relative to each other in certain embodiments provided herein.

[0156] Concatemers generated according to methods and compositions provided herein may be of any length of nucleotides. In some embodiments, concatemer molecules generated herein may be at least 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, 15,000, 20,000, or 25,000 nucleotides in length. In some embodiments, concatemer molecules generated herein may be no more than 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, 15,000, 20,000, or 25,000 nucleotides in length. In some embodiments, concatemer molecules generated herein may have a length selected from a range having a minimum value of 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, 15,000, or 20,000 nucleotides in length, and a maximum value of 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, 15,000, 20,000, or 25,000 nucleotides in length. In some embodiments, at least some concatemers generated according to a method or composition provided herein have characteristics described above. In some embodiments, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of concatemers generated according to a method or composition provided herein have characteristics described above.

[0157] Concatemers generated according to methods and compositions provided herein may contain any number of copies of a nucleic acid template or particular nucleic acid. In some embodiments, concatemer molecules generated herein may contain at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 copies of a nucleic acid template or particular nucleic acid. In some embodiments, concatemer molecules generated herein may contain no more than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 copies of a nucleic acid template or particular nucleic acid. In some embodiments, concatemer molecules generated herein may have a number of copies of a nucleic acid template or particular nucleic acid selected from a range having a minimum value of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, or 90 copies, and a maximum value of 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40,

45, 50, 60, 70, 80, 90, or 100 copies. In some embodiments, at least some concatemers generated according to a method or composition provided herein have characteristics described above. In some embodiments, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% of concatemers generated according to a method or composition provided herein have characteristics described above.

[0158] Progress of a method provided herein may be monitored in multiple different ways. In embodiments, a reaction may be assayed for a nucleic acid amplification product (e.g. for the amount of the product or the rate of its generation). In other embodiments, a reaction may be assayed for the activity of a polymerase along a nucleic acid template (e.g. for movement of a polymerase along a template strand). Thus, in some embodiments, events of a method provided herein may observed due to the accumulation of product from a method (which may be during or after completion of steps of the method), or due to detectable events occurring during the steps of a method.

[0159] The presence of amplified nucleic acids can be assayed, for example, by detection of reaction products (amplified nucleic acids or reaction by-products) or by detection of probes associated with the reaction progress.

[0160] In some embodiments, reaction products may be identified by staining the products with a dye. In some embodiments, a dye may have greater fluorescence when bound to a nucleic acid than when not bound to a nucleic acid. In embodiments, a dye may intercalate with a double-stranded nucleic acid or it may bind to an external region of a nucleic acid. Nucleic acid dyes that may be used with methods and compositions provided herein include, for example, cyanine dyes, PicoGreen ®, OliGreen ®, RiboGreen ®, SYBR® dyes, SYBR ® Gold, SYBR ® Green I, SYBR ® Green II, ethidium bromide, dihydroethidium, BlueView™, TOTO ® dyes, TO-PRO ® dyes, POPO ® dyes, YOYO ® dyes, BOBO ® dyes, JOJO ® dyes, LOLO ® dyes, SYTOX ® dyes, SYTO ® dyes, propidium iodide, hexidium iodide, methylene blue, DAPI, acridine orange, quinacrine, acridine dimers, 9-amino-6-chloro-2-methoxyacridine, bisbenzimide dyes, Hoechst dyes, 7-aminoactinomycin D, actinomycin D, hydroxystilbamidine, pyronin Y, Diamond ™ dye, GelRed™, GelGreen™ and LDS 751.

[0161] In some embodiments, reaction products may be identified by analysis of turbidity of amplification reactions. For example, in embodiments, increased turbidity may be correlated

with formation of reaction products and reaction by-products (e.g. pyrophosphate complexed with magnesium).

[0162] In some embodiments, reaction products may be identified by separating a reaction performed according to a method herein by gel electrophoresis, followed by staining of the gel with a dye for nucleic acids. The dye may be any nucleic acid dye disclosed herein or otherwise known in the art.

[0163] In some embodiments, any method or composition known in the art for the detection of nucleic acids or for the generation of nucleic acids may be used with methods and compositions provided herein.

[0164] In some embodiments, a nucleic acid probe which contains a nucleotide sequence complementary to a portion of a nucleic acid template strand (or a strand having a similar or identical sequence) and which contains one or both of a fluorescent reporter (fluorophore) and a quencher are included in a reaction provided herein.

[0165] In an example, a nucleic acid probe may contain a fluorescent reporter at its 5' or 3' terminus, and a quencher at the other terminus. The probe may further have a nucleotide sequence containing, in order, at least a first, second, and third region, where the first and third regions are complementary to each other, and where at least a portion of the second region is complementary to a portion of a strand of the nucleic acid template (the probe "detection sequence"). In some embodiments, the length of the second region may be greater than the length of the first or third regions. In some embodiments, the length of the second region may be between 10 and 40 nucleotides, and the length of first and third regions may be between 4 and 10 nucleotides. The probe may have at least two different conformations: (A) a conformation where the probe is not annealed to its detection sequence and where the first and third regions are annealed to each other; this conformation may be a "stem-loop" structure, where the first and third regions form the stem and the second region forms the loop, and (B) a conformation where the probe is annealed to its detection sequence; in this conformation, the second region or a portion thereof is annealed to its detection sequence and the first and third regions are not annealed to each other. In conformation (A) of the probe, the fluorescent reporter and quencher (which are located at opposite termini of the probe / at the outer ends of the first and third regions) may be in close proximity to each other (both being at the end of the stem structure formed by the annealing of the first and third regions), such that the fluorescent reporter is quenched. In conformation (B) of the probe, the

fluorescent reporter and quencher may not be in close proximity to each other, such that the fluorescent reporter is not quenched. The probe may be used to monitor accumulation of a selected reaction product, for example, under reaction conditions where the probe may either form a stem-loop structure or anneal to its detection sequence. In some embodiments, if the detection sequence is present, the probe may anneal to the detection sequence, and the probe may fluoresce in response to light of a wavelength of the fluorophore's excitation spectrum. In contrast, if the detection sequence is not present, the probe may form a stem-loop structure, and not fluoresce in response to light of a wavelength of the fluorophore's excitation spectrum.

[0166] In another example, a nucleic acid probe may contain a fluorescent reporter at its 5' or 3' terminus, and it may be annealed to a nucleic acid primer containing a quencher. The nucleic acid primer containing a quencher may contain the quencher at a position in the primer such that when the nucleic acid probe is annealed to the primer, the fluorescent reporter is quenched. The probe may be used to monitor accumulation of a selected reaction product, for example, under reaction conditions where the probe may either anneal to the primer or anneal to its detection sequence in the reaction product. In some embodiments, if the detection sequence is present, the probe may anneal to the detection sequence, and the probe may fluoresce in response to light of a wavelength of the fluorophore's excitation spectrum (thus indicating the presence of the reaction product). In contrast, if the detection sequence is not present, the probe may remain paired with the primer, and not fluoresce in response to light of a wavelength of the fluorophore's excitation spectrum.

[0167] In probes containing a fluorescent reporter and quencher pair, the fluorescent reporter and quencher may be selected so that the quencher can effectively quench the reporter. In some embodiments, a fluorescent reporter is paired with a quencher where the emission maximum of the fluorescent reporter is similar to the absorption maximum of the quencher. Fluorophores that may be used as the fluorescent reporter include, for example, CAL Fluor Gold, CAL Fluor Orange, Quasar 570, CAL Fluor Red 590, CAL Fluor Red 610, CAL Fluor Red 610, CAL Fluor Red 635, Quasar 670 (Biosearch Technologies), VIC, NED (Life Technologies), Cy3, Cy5, Cy5.5 (GE Healthcare Life Sciences), Oyster 556, Oyster 645 (Integrated DNA Technologies), LC red 610, LC red 610, LC red 640, LC red 670, LC red 705 (Roche Applies Science), Texas red, FAM, TET, HEX, JOE, TMR, and ROX. Quenchers that may be used include, for example, DDQ-I, DDQ-II (Eurogentec), Eclipse

(Epoch Biosciences), Iowa Black FQ, Iowa Black RQ (Integrated DNA Technologies), BHQ-1, BHQ-2, BHQ-3 (Biosearch Technologies), QSY-7, QSY-21 (Molecular Probes), and Dabcyl.

[0168] In some embodiments, a probe used with a composition or method provided herein may be a peptide nucleic acid (PNA) probe. Peptide nucleic acids are synthetic DNA analogs in which, for example, the phosophodiester backbone is replaced by repetitive units of N-(2-aminoethyl) glycine and to which the purine and pyrimidine bases are attached via a methyl carbonyl linker. Optionally, a PNA probe may be provided in a reaction provided herein which further contains a dye which binds to PNA-DNA duplexes, and which changes color upon binding to PNA-DNA duplexes. An example of such a dye is 3,3'-diethylthiadicarbocyanine iodide [$DiSc_2(5)$]. $DiSc_2(5)$ changes color from blue to purple upon binding to PNA-DNA duplexes, and this color change may be observed, for example in a spectrophotometer, by image analysis, or by an unaided human eye. The use of PNA probes and $DiSc_2(5)$ for detection of PNA-DNA duplexes is described, for example in Wilhelmsson et. al, Nucleic Acids Research, 2002, Vol. 30, No.2 e3, which is hereby incorporated by references for all purposes. In embodiments using a PNA probe, typically, the PNA probe will be designed to be complementary to a sequence that is to be amplified in the relevant amplification reaction. Thus, if the sequence is successfully amplified, the PNA probe will anneal to DNA strands containing the amplified sequence, and this annealing can be detected (e.g. by observing the color change in $DiSc_2(5)$ when it binds to PNA-DNA duplexes). Use of a PNA probe with embodiments provided herein thus may aid in accurately determining reactions in which a sequence is successfully amplified, and in differentiating these reactions from reactions which have non-specific amplification of nucleic acids. To use a PNA probe with embodiments provided herein, optionally, the PNA probe may be included in a reaction mixture provided herein at the start of the reaction. In other examples, the PNA probe may be added to the reaction mixture at a time after the initiation of the reaction or after the completion of the reaction. A dye may be added to a reaction mixture at the same time a PNA probe is added, or at a different time. For example, in embodiments, a dye may be added to the reaction mixture at the start of the reaction, and the PNA may be added at point after the initiation of the reaction. In embodiments, a kit provided herein may further contain a PNA probe which is complementary to a nucleotide sequence to be amplified by reagents of the kit, and dye which binds to PNA-DNA duplexes.

[0169] In some embodiments, a reaction performed according to a method provided herein may be monitored in an apparatus containing a light source and an optical sensor. In some situations, the reaction may be positioned in the path of light from the light source, and light absorbed by the sample (e.g. in the case of a turbid reaction), scattered by the sample (e.g. in the case of a turbid reaction), or emitted by the sample (e.g. in the case of a reaction containing a fluorescent molecule) may be measured. In some embodiments, a method provided herein may be performed or monitored in a device or module therein as disclosed in U.S. Pat. App. Ser. No. 13/769,779, filed February 18, 2013, which is herein incorporated by reference in its entirety.

[0170] Using methods provided herein, specific amplification products of a nucleic acid template of interest may be identified within, for example, 30 seconds, 1 minute, 3 minutes, 5 minutes, 10 minutes, 15 minutes, 20 minutes, 30 minutes, 45 minutes, 60 minutes, 90 minutes, 120 minutes, 180 minutes, or 240 minutes of initiation of an amplification reaction. In other examples, using methods provided herein, amplification reactions which are positive for a nucleic acid template of interest may be identified when as few as 10, 50, 100, 500, 1000, 5000, 10,000, 50,000, 100,000, 500,000, or 1,000,000 copies of the template are generated. In other examples, using methods provided herein, the presence of a nucleic acid template of interest in a sample containing as few as 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, 100, 200, 500, 1000, 5000, or 10,000 copies of the template of interest at the start of the method may be identified.

[0171] In embodiments, methods provided herein may be used to assay a sample for a target nucleic acid of interest. In certain embodiments, the presence or quantity of a target nucleic acid of interest in a sample may be determined by a method involving determining an inflection time for nucleic acid amplification in a reaction. An inflection time / inflection point is a time or a point where an amplification reaction is determined as being positive for a nucleic acid template. An inflection time / point may be identified by one or more indicators, such as for example, the time post-initiation of a reaction when a selected quantity of nucleic acid has been generated in the reaction, the time when the rate of amplification in a reaction changes from a baseline phase to an exponential phase, or the time when the rate of amplification in a reaction changes from an exponential phase to a plateau phase, etc. In embodiments, an inflection time / point may be identified based on a change in fluorescence or absorbance of a reaction, or upon the fluorescence or absorbance of a reaction reaching a

selected value. In certain embodiments, the presence or quantity of a target nucleic acid of interest in a sample may be determined by a method involving comparison of an inflection time for nucleic acid amplification of a reaction of which has an unknown amount of target nucleic acid of interest versus one or both of: i) a reaction which is known to lack the target nucleic acid of interest (i.e. a negative control) or ii) a reaction which is known to contain the target nucleic acid of interest (i.e. a positive control). In embodiments, both a reaction which contains the target nucleic acid of interest and a reaction which does not contain the target nucleic acid may be measured for a selected inflection time. In embodiments, the presence of a target nucleic acid of interest in a sample may be determined based on a method which involves evaluation of the difference in time between inflection of a reaction containing a sample which may or may not contain a target nucleic acid of interest, and a time of inflection of one or more reactions with known target nucleic acid of interest status (e.g. which are known to contain or not contain the target nucleic acid of interest). For example, a sample may be identified as containing a target nucleic acid of interest if the inflection time of the reaction according to a method provided herein is at least 3, 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, or 180 minutes earlier than a corresponding reaction which is known to not contain the target nucleic acid of interest. In another example, a sample may be identified as containing a target nucleic acid of interest if the inflection time of the reaction according to a method provided herein is no more than 3, 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, or 180 minutes later than a corresponding reaction which is known to contain the target nucleic acid of interest.

[0172] Methods provided herein may be performed for any length of time. Typically, the method will be performed for a length of time sufficient to monitor, for example, the rate of nucleic acid replication, the occurrence of polymerase activity, or the accumulation of amplification product. In some embodiments, a method provided herein may be performed for a total of less than 10 seconds, 30 seconds, 1 minute, 5 minutes, 10 minutes, 20 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 8 hours, 12 hours, 16 hours, or 24 hours, by which time the rate of nucleic acid replication, the occurrence of polymerase activity, or the accumulation of amplification product is measured.

[0173] Methods provided herein may be terminated in various ways. In one embodiment, steps of a method may end upon the reduction in concentration or complete consumption of one or more reagents involved in one or more steps of the method (e.g. dNTPs). In another

embodiment, steps of a method may end upon inactivation of one or more enzymes involved in one or more steps of the method (e.g. polymerases). Enzymes may be inactivated by various ways. For example, enzymes may gradually lose enzymatic activity over time due to random events that affect the structure of the enzyme, or enzymes may be exposed to a condition to accelerate the inactivation of the enzyme activity (e.g. high heat, extreme pH, etc.).

[0174] In some embodiments, a primary nucleic acid may be single stranded or double-stranded. A single stranded primary nucleic acid may also be referred to herein as a "primary polynucleotide". A primary nucleic acid may be linear or circular. A primary nucleic acid may comprise a nucleic acid template. In some embodiments, the entirety of a primary nucleic acid may be a nucleic acid template. In other embodiments, a primary nucleic acid may contain one or more nucleotides which are not part of a nucleic acid template (e.g. the primary nucleic acid may be of a greater length than a nucleic acid template contained within the primary nucleic acid). In some embodiments, a primary nucleic acid may contain two or more copies of a nucleic acid template. A primary nucleic acid may contain DNA, RNA, or a mixture thereof. A double-stranded linear primary nucleic acid may have blunt ends or sticky ends ("sticky ends" refer to ends having an overhanging strand having one or more unpaired nucleotides).

[0175] A primary nucleic acid may be of any length of nucleotides. For example, a primary nucleic acid may be at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, 1000, or 1500 nucleotides in length. In another example, a primary nucleic acid may be between 2 and 100,000, between 5 and 100,000, between 10 and 100,000, between 15 and 100,000, between 20 and 100,000, between 25 and 100,000, between 30 and 100,000, between 50 and 100,000, between 70 and 100,000, between 100 and 100,000, between 200 and 100,000, between 2 and 10,000, between 5 and 10,000, between 10 and 10,000, between 15 and 10,000, between 20 and 10,000, between 25 and 10,000, between 30 and 10,000, between 50 and 10,000, between 70 and 10,000, between 100 and 10,000, between 200 and 10,000, between 2 and 5,000, between 5 and 5,000, between 10 and 5,000, between 15 and 5,000, between 20 and 5,000, between 25 and 5,000, between 30 and 5,000, between 50 and 5,000, between 70 and 5,000, between 100 and 5,000, between 200 and 5,000, between 2 and 3,000, between 5 and 3,000, between 10 and 3,000, between 15 and 3,000, between 20 and 3,000, between 25 and 3,000, between 30

and 3,000, between 50 and 3,000, between 70 and 3,000, between 100 and 3,000, between 200 and 3,000, between 2 and 1,000, between 5 and 1,000, between 10 and 1,000, between 15 and 1,000, between 20 and 1,000, between 25 and 1,000, between 30 and 1,000, between 50 and 1,000, between 70 and 1,000, between 100 and 1,000, between 200 and 1,000, between 2 and 500, between 5 and 500, between 10 and 500, between 15 and 500, between 20 and 500, between 25 and 500, between 30 and 500, between 50 and 500, between 70 and 500, between 100 and 500, or between 200 and 500 nucleotide bases in length.

[0176] In some embodiments, a nucleic acid template may be single stranded or double-stranded. A single strand of a nucleic acid template may be referred to herein as a "polynucleotide template". A "polynucleotide template" as referred to herein is not precluded from binding to a complementary sequence thereof. In other words, a "polynucleotide template" may be, for example, the entirety of a single-stranded nucleic acid template, or it may be one strand of a double-stranded nucleic acid template. A nucleic acid template may be contained in a primary nucleic acid molecule. In some embodiments, a nucleic acid template may constitute the entirety of a primary nucleic acid molecule. In other embodiments, a nucleic acid template may be contained in a primary nucleic acid which contains one or more nucleotides which are not part of the nucleic acid template (e.g. the nucleic acid template may be of a shorter length than the primary nucleic acid which contains the nucleic acid template).

[0177] A nucleic acid template may be of any length of nucleotides. For example, a nucleic acid template may be at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, 1000, or 1500 nucleotides in length. In another example, a nucleic acid template may be between 2 and 100,000, between 5 and 100,000, between 10 and 100,000, between 15 and 100,000, between 20 and 100,000, between 25 and 100,000, between 30 and 100,000, between 50 and 100,000, between 70 and 100,000, between 100 and 100,000, between 200 and 100,000, between 2 and 10,000, between 5 and 10,000, between 10 and 10,000, between 15 and 10,000, between 20 and 10,000, between 25 and 10,000, between 30 and 10,000, between 50 and 10,000, between 70 and 10,000, between 100 and 10,000, between 200 and 10,000, between 2 and 5,000, between 5 and 5,000, between 10 and 5,000, between 15 and 5,000, between 20 and 5,000, between 25 and 5,000, between 30 and 5,000, between 50 and 5,000, between 70 and 5,000, between 100 and 5,000, between 200 and 5,000, between 2 and 3,000, between 5 and 3,000, between 10

and 3,000, between 15 and 3,000, between 20 and 3,000, between 25 and 3,000, between 30 and 3,000, between 50 and 3,000, between 70 and 3,000, between 100 and 3,000, between 200 and 3,000, between 2 and 1,000, between 5 and 1,000, between 10 and 1,000, between 15 and 1,000, between 20 and 1,000, between 25 and 1,000, between 30 and 1,000, between 50 and 1,000, between 70 and 1,000, between 100 and 1,000, between 200 and 1,000, between 2 and 500, between 5 and 500, between 10 and 500, between 15 and 500, between 20 and 500, between 25 and 500, between 30 and 500, between 50 and 500, between 70 and 500, between 100 and 500, or between 200 and 500 nucleotide bases in length.

[0178] A "primer" as used herein may refer to a polynucleotide which is i) capable of hybridizing to an original nucleic acid strand and ii) acting as a point of initiation for the synthesis of a new nucleic acid strand, wherein the new nucleic acid strand is an extension product of the primer and is complementary to the original strand. A primer may have a free –OH group at its 3' terminus, which may serve as the origin of synthesis for the extension product.

[0179] A primer may contain standard nucleotides [e.g. standard DNA deoxyribonucleotides (deoxyadenosine monophosphate, deoxyguanosine monophosphate, thymidine monophosphate, deoxycytidine monophosphate) or standard RNA ribonucleotides (adenosine monophosphate, guanosine monophosphate, uridine monophosphate, cytidine monophosphate)], alternative nucleotides (e.g. inosine), modified nucleotides, nucleotide analogs, or a combination thereof. For example, an oligonucleotide primer may include peptide nucleic acids, morpholinos (e.g. phosphorodiamidate morpholino oligos), locked nucleic acids [see, for example, Kaur, H, et. al, Biochemistry 45 (23), 7347-55 (2006)], glycol nucleic acids, or threose nucleic acids. A primer may have a backbone, including, for example, phosphodiester linkages, phosphorothioate linkages (a non-bridging O is replaced with sulfur), or peptide linkages (as part of a peptide nucleic acid). Alternative nucleotides, modified nucleotides, and nucleotide analogs may be referred to collectively herein as "non-standard nucleotides."

[0180] The presence of a non-standard nucleotide in a primer may affect various properties of the primer. In some embodiments, inclusion of a non-standard nucleotide in a primer may increase or decrease the thermodynamic stability of a primer to a complementary sequence thereof. For example, a primer having increased thermodynamic stability may contain a locked nucleic acid. A primer having decreased thermodynamic stability may contain, for

example, inosine (described by Auer et al., Nucl. Acids Res. 24; 5021-5025 (1996)) or a negatively charged chemical group, such as a carboxylic acid.

[0181] A first primer or a second primer provided herein may be of any length. The first primer and second primer may contain the same number of nucleotides, or a different number of nucleotides. In some embodiments, a first or second primer may be at least 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, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, 1000, or 1500 nucleotides in length. In some embodiments, a first or second primer may be no more than 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, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, 1000, or 1500 nucleotides in length. In some embodiments, a first or second primer may have a length selected from a range having a minimum value of 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, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, or 1000 nucleotides in length, and a maximum value of 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, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, 1000, or 1500 nucleotides in length.

[0182] The tail region of a first primer or a second primer provided herein may be of any length. Typically, the tail regions of a first primer and a second primer directed to the same template contain the same number of nucleotides. In some embodiments, the tail region of a first or second primer may be at least 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, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, 1000, or 1500 nucleotides in length. In some embodiments, the tail region of a first or second primer may be no more than 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, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, 1000, or 1500 nucleotides in length. In some embodiments, the tail region of a first or second primer may have a length selected from a range having a minimum value of 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, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, or 1000 nucleotides in length, and a maximum value of 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, 35, 40,

45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, 1000, or 1500 nucleotides in length.

[0183] The template-binding region of a first primer or a second primer provided herein may be of any length. The template-binding regions of a first primer and a second primer directed to the same template may contain the same number of nucleotides, or a different number of nucleotides. In some embodiments, the template-binding region of a first or second primer may be at least 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, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, 1000, or 1500 nucleotides in length. In some embodiments, the template-binding region of a first or second primer may be no more than 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, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, 1000, or 1500 nucleotides in length. In some embodiments, the template-binding region of a first or second primer may have a length selected from a range having a minimum value of 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, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, or 1000 nucleotides in length, and a maximum value of 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, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 500, 750, 1000, or 1500 nucleotides in length.

[0184] In some embodiments, a primer may be of any length and contain any nucleotide sequence which permits sufficiently stable and specific annealing of the primer to its complement at the temperature being used for a method or step thereof involving the primer. The exact length desired of a primer may depend on a variety of factors, including the temperature of a reaction, the chemical composition of the primer, and the reaction involving the primer. In some embodiments, the template-binding region of a primer may be of any length and contain any nucleotide sequence which permits sufficiently stable and specific annealing of the template-binding region of the primer to its complement at the temperature being used for a method or step thereof involving the primer. The exact length desired of the template-binding region of a primer may depend on a variety of factors, including the temperature of a reaction, the chemical composition of the template-binding region of the primer, and the reaction involving the primer. The inclusion of one or more non-standard nucleotides in the primer may change the desired length of the primer for use in a method

provided herein, as compared to the length of a corresponding primer lacking a non-standard nucleotide. For example, if with a method provided herein it is desired to have a primer with a certain melting temperature ("Tm"), in some embodiments, a primer with the selected Tm may be of a shorter length if the primer contains at least some non-standard nucleotides, as compared to if the primer contains only standard nucleotides. Generally, "melting temperature" of a nucleotide sequence refers to the temperature at which 50% of nucleic acids having the nucleotide sequence are based paired to a complementary sequence thereof (i.e. are in a double-stranded molecule), and 50% of nucleic acids having the nucleotide sequence are in single-stranded form.

[0185] In some embodiments, one or both of a first primer or second primer provided herein may be designed to contain a nucleotide sequence in the tail region of the primer which is complementary to an internal motif (i.e. a nucleotide sequence) within a target nucleic acid sequence. For example, referring again to Figure 1, the tail region of a first primer 105 may contain a nucleotide sequence which is complementary to an internal motif in a second strand of a double-stranded nucleic acid template 104, an extension product of a first copy of the first primer 111, or an extension product of a second copy of the first primer 113. Similarly, referring still to Figure 1, the tail region of a second primer 106 may contain a nucleotide sequence which is complementary to an internal motif in a first strand of a double-stranded nucleic acid template 103 or an extension product of the second primer 112. In at least some circumstances, having a nucleotide sequence in the tail region of a primer which is complementary to an internal motif within a target nucleic acid sequence may increase the speed or specificity of a reaction as described herein.

[0186] Without being bound by theory, it is believed that having a nucleotide sequence in the tail region of a primer which is complementary to an internal motif within a target nucleic acid sequence may increase the speed or specificity of a reaction according the mechanism outlined in Figure 9. For brevity, Figure 9 and this mechanism is described only with reference to a first copy of a first primer, but equally may apply to a second copy of a first primer, a second primer, or the like, as appropriate. Also, Figure 9 and this mechanism is described only with reference to an extension product of a first copy of the first primer 111 which is annealed to an extension product of the second primer 112, but equally may apply to other molecules containing an extension product of a primer described herein. Turning now to Figure 9, at least a portion of the tail region of a first primer may contain a nucleotide

sequence 901 (checkerboard fill) which is complementary to an internal motif 902 (backslash-like fill) in an extension product of a first copy of the first primer 111 and that first primer may be integrated into the extension product of the first copy of the first primer (as described elsewhere herein) 111. The extension product of the first copy of the first primer 111 may be annealed to an extension product of the second primer 112. During the "breathing" of a duplex nucleic acid containing the extension product of the first copy of the first primer 111 and the extension product of the second primer 112, the tail region of the primer 901 may move towards and may temporarily anneal to the internal motif 902 present in the extension product of a first copy of the first primer. After temporarily annealing to the internal motif 902 in the extension product of the first copy of the first primer, the tail region of the primer 901 may move back to its original position annealed to the extension product of the second primer 112, and optionally, it may repeatedly move back and forth between these configurations (depicted by the arrows in Figure 9). During the time that the nucleotide sequence of the tail region 901 is annealed to the internal motif 902, one or more nucleotides at the 3' terminus of the extension product of the second primer 112 may be un-annealed, and available for binding to a new copy of a first primer or to a complementary sequence in another duplex nucleic acid (e.g. a secondary nucleic acid). In the event that one or more nucleotides at the 3' terminus of the extension product of the second primer 112 binds to a new copy of a first primer or to a complementary sequence in another duplex nucleic acid (e.g. a secondary nucleic acid), either of these events may increase the rate of a reaction as described elsewhere herein, for example, by the generation of a new extension product of the first primer (which displaces the original first primer extension product during its generation). Also, even without there being a nucleotide sequence 901 in the first primer which is complementary to an internal motif 902, there may still be "breathing" between complementary sequences in a duplex nucleic acid in methods provided herein. However, the amount of time during which a duplex nucleic acid may "breathe" may be increased when there is a nucleotide sequence 901 in the first primer which is complementary to an internal motif 902, and this may in turn increase the rate of a reaction according to a method provided herein.

[0187] In embodiments, an internal motif may contain at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 75, or 100 nucleotides. In embodiments, an internal motif may contain no more than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 75, or 100 nucleotides. In embodiments, an internal motif

may contain at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, or 75 and no more than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 75, or 100 nucleotides (in which the upper value is above the lower value). Typically, the nucleotide sequence in the primer which is complementary to the internal motif contains the same number of nucleotides as in the internal motif. Also, the nucleotide sequence in the primer which is complementary to the internal motif may be perfectly complementary to the internal motif, or there may be one or more mismatched nucleotides between the nucleotide sequence in the primer and the internal motif. The start of the internal motif may be separated by any distance from the 3' terminal nucleotide of the primer which is extended to form the primer extension product which contains the internal motif, but typically may be separated from the 3' terminal nucleotide of the primer, for example, by at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 75, or 100 nucleotides. In embodiments, the start of the internal motif may be separated from the 3' terminal nucleotide of the primer which is extended to form the primer extension product which contains the internal motif by no more than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 75, or 100 nucleotides. In embodiments, the start of the internal motif may be separated from the 3' terminal nucleotide of the primer which is extended to form the primer extension product which contains the internal motif by at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, or 75 and no more than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 75, or 100 nucleotides (where the upper number is greater than the lower number).

[0188] As used herein, in the context of a single target nucleic acid strand / polynucleotide template, an "internal motif" is a portion of the target nucleic acid strand to which the tail region of one primer of a primer pair to amplify the target nucleic acid provided herein is complementary, and which has the same or a similar nucleotide sequence as the tail region of the other primer of the primer pair. Thus, for example, if the internal motif in a target nucleic acid has the nucleotide sequence, in the 5' to 3' direction of GGGATGGA, the tail / first region of one primer of a primer pair used to amplify the target nucleic acid according to a method provided herein will have the nucleotide sequence, in the 5' to 3' direction of TCCATCCC (or a similar sequence), and the tail / first region of the other primer of the primer pair will have the nucleotide sequence, in the 5' to 3' direction of GGGATGGA (or a similar sequence). In the context of a double stranded target nucleic acid, the term "internal

motif" refers to the relevant region of either of the two strands, as described above for a single strand. In the context of a primer extension product described herein, an "internal motif" refers to the portion of the extension product which is complementary to the portion of the primer extension product which corresponds to the tail region of the primer that was extended to form that particular primer extension product. Also, if reference is to be made to a nucleotide sequence in a different strand which is complementary to the "internal motif" in a particular strand, that complementary sequence may be referred to as herein as an "internal motif complement". Of note, in situations where two complementary nucleic acid strands are present (e.g. as in a double-stranded molecule containing two complementary primer extension products), each strand will have a different "internal motif" (the "internal motif's of the different strands will be complementary to each other), so the description of an "internal motif" provided herein may be qualified with reference to the relevant strand or primer, where appropriate. For example, a double stranded nucleic acid may be described, wherein the first strand of the nucleic acid has, in 5'-3' order, portions C'-B-C-A-C', and the second strand of the nucleic acid has, in 5'-3' order, portions C-A'-C'-B'-C, where portion C' of the first strand is annealed to portion C of the second strand, portion A of the first strand is annealed to portion A' of the second strand, etc. If portion C' of the first strand corresponds to the tail region of a primer provided herein, and portion C' is complementary to portion C, then portion C of the first strand may be referred to as the "internal motif" of the first strand, and portion C' of the second strand may be referred to as the "internal motif complement" in relation to the first strand. At the same time, if portion C of the second strand also corresponds to the tail region of a primer provided here, and it is complementary to portion C', then portion C' of the second strand may be referred to as the "internal motif" of the second strand, and portion C of the first strand may be referred to as the "internal motif complement" in relation to the second strand.

[0189] In embodiments, methods and compositions provided herein may be described with reference to Figure 10. Features of Figure 10 may be described in generally the same way as Figure 8, except that the template of Figure 10 also includes an internal motif.

[0190] A polynucleotide template 1000 (Fig. 10A) may be a target for amplification. The polynucleotide template 1000 is a single nucleic acid strand. The polynucleotide template strand may exist as a free, single-stranded molecule, or it may exist as part of a double-

stranded molecule, in which the polynucleotide template strand is annealed to a complementary strand thereof. The polynucleotide template 1000 may be DNA or RNA.

[0191] The polynucleotide template 1000 may contain at least a first portion 1001 (also indicated with an "(i)" and letter "A") and a second portion 1002 (also indicated with an "(ii)" and letter "B"). The first portion 1001 and second portion 1002 may each contain any number of nucleotides, such as is described for the first portion and second portion of the template in Figure 8. The first portion 1001 and second portion 1002 may contain the same or a different number of nucleotides. In addition, the polynucleotide template 1000 may contain a third portion 1003 (also indicated with an "(iii)" and letter "C"), which may also be referred to as an "internal motif." The internal motif typically contains between 6 and 10 nucleotides, although optionally may contain other numbers of nucleotides, such as at least 4, 5, 6, 7, 8, 9, 10, 11, or 12 nucleotides, no more than 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides, or at least 4, 5, 6, 7, 8, 9, 10, 11, or 12 nucleotides and no more than 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides (with the upper value being larger than the lower value). The internal motif 1003 typically is separated from the first portion 1001 and the second portion 1002 on either side by between 2 and 25 nucleotides, although may be separated by other numbers of nucleotides such as at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, or 50 nucleotides, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, or 60 nucleotides, or between 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, or 50 nucleotides and 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, or 60 nucleotides (with the upper value being larger than the lower value). In embodiments, there may be the same number of nucleotides between the first portion 1001 and the internal motif 1003, as compared to between the second portion 1002 and the internal motif 1003 (e.g. there may be 6 nucleotides between the first portion and the internal motif and 6 nucleotides between the second portion and the internal motif). In other embodiments, there may be a different number of nucleotides between the first portion 1001 and the internal motif 1003 than between the second portion 1002 and the internal motif 1003 (e.g. there may be 6 nucleotides between the first portion and the internal motif and 8 nucleotides between the second portion and the internal motif). In embodiments, there are no nucleotides between the internal motif and the first portion 1001 or the second portion 1002 (e.g. in embodiments, the internal motif may be directly next to one or both of the first portion 1001 or second portion 1002).

[0192] The polynucleotide template 1000 may be incubated in a reaction mixture containing a first primer 1010 and a second primer 1020 (Fig. 10B). Typically, the reaction mixture contains multiple copies of each primer, such as, for example, at least 4, 5, 6, 10, 15, 20, 50, 100, 1000, 10,000, 100,000, or 1 million copies or more of each of the primers. The first primer 1010 may have a first region 1011 (also indicated with an "(i)") and a second region 1012 (also indicated with an "(ii)"). The first region of the first primer 1011 may have a 5' end, and the second region of the first primer 1012 may have a 3' end. The first region of the first primer may have a nucleotide sequence which is different from the nucleotide sequence of the second region of the first primer. The second primer 1020 may have a first region 1021 (also indicated with a "(i)") and a second region 1022 (also indicated with a "(ii)"). The first region of the second primer 1021 may have a 5' end, and the second region of the second primer 1022 may have a 3' end. The first region of the second primer may have a nucleotide sequence which is different from the nucleotide sequence of the second region of the second primer.

[0193] The second region of the first primer 1012 may have a nucleotide sequence which is complementary to the nucleotide sequence of the first portion of the polynucleotide template 1001. In Fig. 10, nucleotide sequence of the first portion of the polynucleotide template 1001 is represented by the letter A, and the nucleotide sequence of the second region of the first primer 1012 is represented by the letter A', where A and A' are complementary to each other. The second region of the second primer 1022 may have a nucleotide sequence which is complementary to a partner nucleotide sequence 1030, wherein the partner nucleotide sequence 1030 is complementary to the nucleotide sequence of the second portion of the polynucleotide template 1002. In Fig. 10, the nucleotide sequence of the second portion of the polynucleotide template 1002 is represented by the letter B, the nucleotide sequence of the partner nucleotide sequence 1030 is represented by the letter B', and the nucleotide sequence of the second region of the second primer 1022 is represented by the letter B, where B and B' are complementary to each other. In other words, as indicated by Fig. 10, the nucleotide sequence of the second portion of the polynucleotide template 1002 (B) may be the same as the nucleotide sequence of the second region of the second primer 1022 (B). With compositions and methods provided herein, a partner nucleotide sequence 1030 may be generated, for example, as part of an extension product from the 3' end of the first primer. In embodiments, the first region of the first primer 1011 may be complementary to the first region of the second primer 1021. In Fig. 10, the nucleotide sequence of the first region of

the first primer is represented by the letter C, and the nucleotide sequence of the first region of the second primer is represented by the letter C', where C and C' are complementary to each other.

[0194] In addition, the first region of the second primer 1021 may be complementary to the internal motif 1003 in the polynucleotide template. In Figure 10, the nucleotide sequence of the internal motif 1003 of the polynucleotide template is represented by the letter C, where C and C' are complementary to each other. Thus, as indicated by Figure 10, in embodiments, the first region of the first primer 1011 may have the same nucleotide sequence as the internal motif 1003 of the polynucleotide template, while the first region of the second primer 1021 has a sequence which is complementary to the internal motif 1003. In addition, the first region of the second primer 1021 is complementary to both the first region of the first primer 1011 and the internal motif, while the first region of the first primer 1011 is complementary to the first region of the second primer 1011 and to the internal motif complement.

[0195] In embodiments, the first primer and second primer of Fig. 10 may have any of the characteristics of the first primer and second primer described elsewhere herein (e.g. as in Fig. 1 or 8), respectively. Also, in embodiments, the first region of the first primer of Fig. 10 may have any of the characteristics of the tail region of a first primer described elsewhere herein. In embodiments, the second region of the first primer of Fig. 10 may have any of the characteristics of the template binding region of a first primer described elsewhere herein. In embodiments, the first region of the second primer of Fig. 10 may have any of the characteristics of the tail region of a second primer described elsewhere herein. In embodiments, the second region of the second primer of Fig. 10 may have any of the characteristics of the template binding region of a second primer described elsewhere herein.

[0196] In embodiments, a reaction mixture containing a polynucleotide template 1000 and at least two copies of a first primer 1010 and at least two copies of a second primer 1020 may contain one or more other components which may support polymerase-based nucleic acid synthesis as described elsewhere herein (e.g. polymerases, nucleotides, buffers, water, etc.). Similarly, a reaction mixture containing a polynucleotide template 1000 and at least two copies of a first primer 1010 and at least two copies of a second primer 1020 may be incubated at any of the reaction conditions described elsewhere herein for supporting the amplification of a nucleic acid template or the generation of concatemers.

[0197] In embodiments, upon incubation of a polynucleotide template 1000 and at least two copies of a first primer 1010 and at least two copies of a second primer 1020 in a reaction mixture as described herein, one or more concatemer strands may be formed (Fig. 10C). In embodiments, a formed concatemer strand 1040 may have a 5' end and a 3' end, and may have a sequence containing the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', wherein C' is the nucleotide sequence of the first region of the second primer, T is the sequence of the polynucleotide template or an analogous sequence thereof, and X is any number and sequence of nucleotides. As further indicated by Fig. 10C, within the sequence of the polynucleotide template of strand 1040, in the 5' to 3' direction, the sequence of the following portions of the template are present: B (the second portion of the polynucleotide template), C (the internal motif), and A (the first portion of the polynucleotide template). Thus, as shown in Fig. 10C, the sequence of the internal motif / internal motif complement occurs at multiple positions in a concatemer strand in embodiments provided herein. Specifically, in strand 1040, the sequence of the internal motif complement (C') appears in the concatemer strand between the two copies of the sequence of the polynucleotide template and at the ends of the strand, and the sequence of the internal motif (C) occurs in the same strand within the sequence of the polynucleotide template. Thus, as indicated by the general structure of the concatemer strand 1040, a concatemer strand may contain at least two copies of the sequence of the polynucleotide template or an analogous sequence thereof, where the copies of the sequence of the polynucleotide template are separated by at least the sequence of the first region of the second primer. In embodiments, T in the general structure C'-T-C'-T -X-C' provided above may be an analogous sequence of the polynucleotide template.

[0198] In embodiments, a concatemer strand 1040 generated according to method provided herein may be part of a single-stranded molecule. In other embodiments, a concatemer strand 1040 generated according to method provided herein may be part of a double-stranded concatemer, in which the concatemer strand 1040 is annealed to a strand which is complementary to the concatemer strand 1050. While Fig. 10C depicts concatemer strands having a sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C' (strand 1040) or C-T'-C-T'-X'-C (strand 1050), wherein X is 0 nucleotides, a concatemer strand generated according to the schematic of Fig. 10 may have any of the characteristics of a concatemer strand described above or elsewhere herein. Also, as shown in Fig. 10C, sequences A and B and C are part of the template strand, and thus are not separately notated in the general structure C'-T-C'-T -X-C' or C-T'-C-T'-X'-C.

[0199] To further indicate the relationship of elements in embodiments of Figure 10, blocks 1060, 1062, 1064, 1066, and 1068 are provided in Figure 10. Block 1060 (with a backslash-like fill) indicates the relative position of the total polynucleotide template sequence (i.e. element 1000), or its complementary sequence. Block 1062 (with vertical line fill) indicates the relative position of the sequence of first portion of the polynucleotide template (i.e. the relative position of the sequence of element 1001), or its complementary sequence. Thus, block 1062 also represents the sequence of the second region of the first primer (i.e. element 1012), which is complementary to element 1001. Block 1064 (with vertical and horizontal line fill) indicates the relative position of the sequence of the second portion of the polynucleotide template (i.e. the relative position of the sequence of element 1002), or its complementary sequence. Thus, block 1064 also represents the sequence of the partner nucleotide sequence (i.e. element 1030), which is complementary to element 1002. Block 1064 also indicates the relative position of the sequence of the second region of the second primer 1022, which may be the same sequence as the sequence of the second portion of the polynucleotide template (element 1002), or a similar sequence. In other words, in embodiments the nucleotide sequence of 1022 may be the same as or similar to nucleotide sequence of 1002. As depicted, for example, in Figure 10A, in embodiments, there are nucleotides in the polynucleotide template which are not part of the first portion, second portion, or third portion (internal motif) of the polynucleotide template. Block 1066 (with wavy line fill) indicates the relative position of the sequence of the first region of the first primer (i.e. the relative position of the sequence of element 1011 / region "C"), or its complementary sequence. Thus, block 1066 also represents the sequence of the first region of the second primer (element 1021 / region "C'"), which is complementary to element 1011. In addition, block 1066 also represents the sequence of the internal motif or its complementary sequence. Of note, the sequence of the internal motif or its complementary sequence is both in the template sequence, as well as in the tails of the first and second primers. As described elsewhere herein, the internal motif may be directly adjacent to one or both of the first portion or the second portion of the polynucleotide template, or there may be additional nucleotides between the internal motif and the first portion or the second portion. Block 1068 (with diamond fill) indicates the relative position of the sequence of the concatemer strand (element 1040), or its complementary sequence. Thus, block 1068 also represents the sequence of a strand which is complementary to the concatemer strand (element 1050). As depicted in Figure 10C, within the concatemer strand or its complement (1068), two copies of

the polynucleotide template sequence or its complement (1060) are present, separated by the nucleotide sequence of the first region of the first primer or its complement (1066). This sequence separating the copies of the polynucleotide template is also the sequence of the internal motif or its complement (1066). In addition, within the concatemer strand or its complement (1068), the nucleotide sequence of the first region of the first primer or its complement (1066) flank the polynucleotide template sequence or its complement (1060) on both ends. Also, within the concatemer strand or its complement (1068), there are two copies of the sequence of the second region of the first primer or its complement (1062) and two copies of the sequence of the sequence of the second region of the second primer or its complement (1064), which are both present in two copies as part of the two copies of the polynucleotide template sequence or its complement. Blocks 1060, 1062, 1064, 1066, and 1068 are not to be interpreted to as to dictate any particular required size relationship between the elements of Figure 10; rather blocks 1060, 1062, 1064, 1066, and 1068 are simply to help indicate the general position of the elements of Figure 10 relative to each other in certain embodiments provided herein.

[0200] A primer provided herein may be prepared by any suitable method. For example, a primer may be chemically synthesized. In another example, a naturally occurring nucleic acid may be isolated, cleaved (e.g. with restriction enzymes), and/or modified to generate or to become part of a primer described herein.

[0201] In some embodiments, a label may be attached to a primer. Labels include, for example, binding ligands (e.g. digoxin or biotin), enzymes, fluorescent molecules / fluorophores, luminescent molecules, quencher molecules, or radioisotopes. In other embodiments, a base of an oligonucleotide may be replaced with a fluorescent analog, such as 2-aminopurine (see, for example, Proc.Acad. Sci. USA, 91, 6644—6648 (1994), which is herein incorporated by reference in its entirety).

[0202] In some embodiments, conditions such that: i) a template-binding region of a first copy of a first primer anneals to a strand of a nucleic acid template, ii) a template-binding region of a second primer anneals to an extension product of a first copy of a first primer, iii) a template-binding region of a second copy of a first primer anneals to an extension product of a second primer, or iv) a 3' terminal region of an extension product of a second copy of a first primer of a first copy of a secondary nucleic acid anneals to a 3' terminal region of an extension product of a second primer of a second copy of a secondary nucleic acid, to

produce a cross-over structure comprising these strands, may each include (i.e. any of i), ii), iii), or iv) may include) incubating the nucleic acids at a temperature such that the strands of double-stranded nucleic acid molecules "breathe" (i.e. undergo brief periods of localized rupture of hydrogen bonds connecting base pairs) to a degree sufficient to facilitate the entry of a primer or different nucleic acid strand between the strands of a double-stranded molecule, and sufficient to permit the annealing of the primer or different nucleic acid strand to one of the strands of the opened double-stranded nucleic acid molecule. In some embodiments, methods or steps thereof may be performed or incubated at a temperature of at least 10, 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 90, or 95 C. In some embodiments, methods or steps thereof may be performed or incubated at a temperature of no greater than 10, 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 90, or 95 C. In some embodiments, methods or steps thereof may be performed or incubated at a temperature between 10, 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, or 90 C and 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 90 or 95 C.

[0203] In some embodiments, for a method or steps thereof provided herein, the step or method is performed at a temperature below the melting temperature (Tm) of the relevant potentially paired nucleotide strands, or regions thereof (e.g. the template-binding region of the first primer to the first strand of a nucleic acid template, the template-binding region of the second primer to the extension product of the first copy of the first primer, the first primer to the extension product of the second primer, the 3' terminal region of the extension product of the second primer to its complement, the 3' terminal region of the extension product of the first copy of the first primer to its complement, etc.). In some embodiments, for a method or steps thereof provided herein, the step or method is performed at a temperature above the Tm of the relevant potentially paired nucleotide strands, or regions thereof (e.g. the template-binding region of the first primer to the first strand of a nucleic acid template, the template-

binding region of the second primer to the extension product of the first copy of the first primer, the first primer to the extension product of the second primer, the 3' terminal region of the extension product of the second primer to its complement, the 3' terminal region of the extension product of the first copy of the first primer to its complement, etc.). In some embodiments, for a method or steps thereof provided herein, the step or method is performed at a temperature at or within +/- 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, or 25 C of the Tm of the relevant potentially paired nucleotide strands, or regions thereof (e.g. the template-binding region of the first primer to the first strand of a nucleic acid template, the template-binding region of the second primer to the extension product of the first copy of the first primer, the first primer to the extension product of the second primer, the 3' terminal region of the extension product of the second primer to its complement, the 3' terminal region of the extension product of the first copy of the first primer to its complement, etc.).

[0204] In some embodiments, a nucleic acid polymerase is included with a method or composition provided herein. A polymerase may generate an extension product of a primer. The primer and extension product thereof may be complementary to a template nucleic acid strand. Generally, a nucleic acid polymerase will initiate synthesis of an extension product of a primer at the 3' end of the primer. In some embodiments, a DNA polymerase is included with a method or composition provided herein. As used herein, a "DNA polymerase" refers to a nucleic acid polymerase which has primary or exclusive polymerase activity on DNA templates. In some embodiments, a reverse transcriptase is included with a method or composition provided herein. As used herein, a "reverse transcriptase" refers to a nucleic acid polymerase which can synthesize a DNA strand from an RNA template. In some embodiments, an RNA polymerase may be included with a method or composition provided herein. As used herein, a "RNA polymerase" refers to a nucleic acid polymerase which can synthesize an RNA strand from a DNA or RNA template.

[0205] In some embodiments, a polymerase provided herein may have strand displacement activity. Polymerases having strand displacement activity include, for example, exo-Bca DNA polymerase, phi29 DNA polymerase, Klenow Fragment of *E. coli* DNA Polymerase I, $Vent_R$ DNA polymerase, Deep $Vent_R$ DNA polymerase, $9°N_m$ DNA polymerase, Bst 2.0 DNA polymerase, and Large Fragment of Bst DNA Polymerase. Other polymerases having strand displacement activity may also be used.

[0206] Modified versions of polymerases may also be used with the methods and compositions provided herein, provided that the modified polymerase has sequence-dependent nucleic acid synthesis activity. A modified version of a polymerase ("modified polymerase") may have, for example, 100 or fewer, 70 or fewer, 50 or fewer, 40 or fewer, 30 or fewer, 20 or fewer, 10 or fewer, 5 or fewer, 4 or fewer, 3 or fewer, 2 or fewer, or 1 different amino acid from the sequence of the parent version of the polymerase. In some embodiments, a modified polymerase may contain no more than 1000, 700, 500, 400, 300, 200, 100, 50, 40, 30, 20, 10, or 5 greater or fewer amino acids than the parent polymerase. In some embodiments, a modified polymerase may comprise a fragment of a parent polymerase. In some embodiments, a modified polymerase may comprise a chimeric polypeptide with a portion derived from a polymerase and a portion derived from a non-polymerase protein. In some embodiments, a modified polymerase may have, for example, increased catalytic activity, increased stability, or increased thermostability as compared to the parent polymerase.

[0207] In some embodiments, a polymerase provided herein is thermostable. A thermostable polymerase may have, for example, a half-life of at least 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, or 180 minutes at a temperature of up to 25, 30, 35 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, or 95 C. In some embodiments, a modified polymerase may be thermostable.

[0208] In some embodiments, methods and steps thereof provided herein include or are performed under conditions sufficient to support polymerase-based nucleic acid synthesis. Example conditions for polymerase-based nucleic acid synthesis are known in the art and are provided, for example, in Green and Sambrook, *supra*. Non-limiting components for a polymerase-based nucleic acid synthesis reaction may include one or more of: polymerase enzyme (at a concentration between, for example, 0.01 and 10 units enzyme per 50 microliters reaction volume, or any range therein including, for example, between 0.01-1, 0.1-10, 0.1-5, 0.5-10, 0.5-5, 0.5-2, 1-10, or 1-5 units enzyme per 50 microliters reaction volume, where 1 unit of enzyme will incorporate 15 nmol of dNTPs into polymerization product in 30 minutes at 75 C); template (at a concentration of at least, for example, 1, 10, 100, 1,000, 10,000, or 100,000 copies per reaction); primer (at a concentration between, for example, 0.01 and 10 micromolar, or any range therein including, for example, between 0.01-1, 0.1-10, 0.1-5, 0.5-5, or 0.5-2 micromolar); dNTPs (e.g. dATP, dTTP, dGTP, and dCTP, at a concentration between, for example, 50 and 500 micromolar each of dATP, dTTP, dGTP,

and dCTP, or any range therein including, for example, between 50-350, 100-500, 100-300, 200-500, or 300-400 micromolar each of dATP, dTTP, dGTP, and dCTP); salt (e.g. KCl or potassium acetate, at a concentration between, for example, 1 and 200 millimolar, or any range therein including, for example, between 1-100, 1-50, 1-20, 1-10, 10-20, 10-50, or 10-200 millimolar); buffer (e.g. Tris-HCl or Tris-acetate, pH 7.8 - 8.5, at a concentration between, for example, 1 and 100 millimolar, or any range therein including, for example, between 1-50, 1-20, 1-10, 1-5, 10-100, 20-100, or 50-100 millimolar); and magnesium ions (at a concentration between, for example 0.1 and 10 millimolar, or any range therein, including, for example, between 0.1-5, 0.1-1, 0.5-10, 0.5-5, or 0.5-2.5 millimolar). Additional non-limiting components for a polymerase-based nucleic acid synthesis reaction may increase the speed of the reaction, increase the fidelity of the reaction, or increase the stability of enzymes or DNA in the reaction, and may include one or more of: gelatin (at a concentration between, for example, 0.0001% and 0.1% w/v), BSA (at a concentration between, for example, 0.01 and 1 microgram per microliter), sucrose (at a concentration between, for example 0.01 molar and 0.8 molar), trehalose (at a concentration between, for example 0.01 molar and 0.8 molar), DMSO (at a concentration between, for example, 0.01 and 10% v/v), betaine (at a concentration between, for example, 0.1 and 10 molar), formamide (at a concentration between, for example, 0.1 and 10% v/v), glycerol (at a concentration between, for example, 0.1 and 20% v/v), polyethylene glycol (at a concentration between, for example, 0.1 and 20% v/v), non-ionic detergents [e.g. NP-40 (at a concentration between, for example, 0.01 and 1% v/v), Tween-20 (at a concentration between, for example, 0.01 and 1% v/v), or Triton X-100 (at a concentration between, for example, 0.01 and 1% v/v)], ammonium ions [e.g. ammonium sulfate (at a concentration between, for example, 1 and 100 millimolar)], and EDTA (at a concentration between, for example, 0.001 and 0.1 millimolar). Other reagents may also be present in a polymerase-based nucleic acid synthesis reaction provided herein. For example, reagents sufficient to synthesize RNA reaction products or reaction products containing non-standard nucleotides may be used. Conditions sufficient to support polymerase-based nucleic acid synthesis may include a variety of temperatures and pH values. For example, the pH of a polymerase-based nucleic acid synthesis reaction may be between, for example pH 6.0 and pH 10.0, such as 6.5,7, 7.5, 7.8, 7.9, 8, 8.1, 8.2, 8.5, 9, or 9.5. The temperature of a polymerase-based nucleic acid synthesis reaction may be constant or varied. A constant temperature may be between, for example, 10 C and 95 C, such as 20, 25, 30, 35, 37, 40, 42, 45, 50, 55, 60, 65, 70, 75, 80,

or 85 C. A varied temperature may be two or more different temperatures between, for example, 10 C and 95 C, such as two or more temperatures selected from 20, 25, 30, 35, 37, 40, 42, 45, 50, 55, 60, 65, 70, 75, 80, or 85 C.

[0209] Methods provided herein may be performed at a variety of temperatures. In some embodiments, all steps of a method are performed at the same temperature. Thus, temperature cycling such as in PCR is not necessary with methods disclosed herein. In some embodiments, methods provided herein may be performed at two or more different temperatures. In some embodiments, a reaction mixture containing reagents for a method provided herein is incubated at two or more different temperatures. In some examples, different temperatures may be selected to optimize the rate, accuracy, or other feature of different steps of a method provided herein. For example, a temperature may be selected to increase the enzymatic activity of a polymerase. In some examples, different temperatures may be selected to increase the binding specificity of a primer to a template or to increase the accessibility of a template to a primer (e.g. higher temperatures may promote the separation of duplex template nucleic acids). In some embodiments, all of the steps of a method provided herein are performed at a temperature of no greater than 80, 70, 60, 50, 40, 30, 20 or 10 °C. In some embodiments, a method provided herein is performed at a temperature between 20-60, 30-70, 40-80, 20-40, 30-50, 40-60, 50-70, 60-80, 30-40, 35-45, 40-50, 45-55, 50-60, 55-65 °C. In certain embodiments, a sample containing a target nucleic acid may be heated to a temperature greater than 40, 50, 60, 70, 80, 90, or 95 C before the initiation of a method provided herein. In certain embodiments, a reaction mixture provided herein may be heated one time to an elevated temperature greater than 40, 50, 60, 70, 75, 80, 85, 90, or 95 C before or after the initiation of a method provided herein. After heating the reaction mixture to the elevated temperature, it may be maintained at a lower temperature as provided elsewhere herein (e.g. at a temperature between 40-70 C) for the remainder of the performance of the method. In embodiments, if a reaction mixture or sample is heated to an elevated temperature before the initiation of a method provided herein, a nucleic acid polymerase may be added to the reaction mixture or sample after the reaction mixture or sample has been heated to the elevated temperature, and the reaction mixture or sample has been returned to a lower temperature as provided herein. Methods disclosed herein may be performed with or without a thermocycler.

[0210] As one consideration, the temperature used for a method or step thereof provided herein may be selected to be appropriate for the enzyme(s) being used in the step of the method. In some embodiments, for methods in which a polymerase is used, the temperature(s) of the reaction is selected such that it does not significantly impair the activity of the polymerase (e.g. the temperature of the reaction may be selected such that polymerase has a half-life of at least 24, 12, 6, 4, 3, 2, 1, 0.75, 0.5, 0.25, or 0.1 hours). Alternatively, methods may be performed at a temperature that impairs the activity of the enzyme(s) being used in the method (e.g. the temperature of the reaction may be selected such that an enzyme in the reaction has a half-life of no more than 24, 12, 6, 4, 3, 2, 1, 0.75, 0.5, 0.25, or 0.1 hours). In some embodiments, if a method is performed at a temperature or other condition (e.g. pH) that impairs the activity of one or more enzyme(s), additional enzyme may be added to the reaction at one or more intervals after the initiation of the method to supplement the activity of the impaired enzyme(s).

[0211] In some embodiments, one or more steps of a method provided herein occur in the same reaction vessel (e.g. tube, tip, container, etc.). In some embodiments, all of the steps of a method occur in the same reaction vessel.

[0212] Reagents for methods provided herein can all be provided together at the start of a reaction, or they may be added sequentially, where after one, two, or more steps new reagents are added to a reaction. In some circumstances, new reagents (e.g. enzymes, primers) may be added to a reaction vessel during the course of the reaction, to increase the amount of reagents available to act on substrates or to replace the function of reagents that have become inactivated (e.g. enzymes). New reagents may be added to a reaction at one or more selected time intervals after the initiation of a reaction of a method provided herein (for example, at 1, 3, 5, 7, 10, 15, 20, 30, 45, or 60 minutes after the initiation of a reaction).

[0213] In some embodiments, one or more steps of a method provided herein may occur simultaneously. For example, after the generation of a single copy of an extension product of a first copy of the first primer, multiple copies of an extension product of a second primer may be sequentially generated from that single copy of an extension product of a first copy of the first primer. As copies of the extension product of a second primer are sequentially generated from the single copy of the extension product of a first copy of the first primer, the individual copies of the extension product of a second primer may, for example, serve as a template for the formation of an extension product of a second copy of the first primer, be a

component of a secondary nucleic acid, be a component of a cross-over structure, or may be an initiation point for the formation of an extension product of an extension product of a second primer / first concatemer strand. In another example, two copies of a secondary nucleic acid may form a cross-over structure at the same time that an extension product of a first copy of a first primer is generated from a first strand of a nucleic acid template. In another example, one copy of an extension product of a first copy of a first primer is generated from a first strand of a nucleic acid template at the same time that an extension product of the second primer is generated from a different copy of the extension product of a first copy of a first primer. Other steps provided herein may also occur simultaneously. In addition, in methods and compositions provided herein involving a double-stranded nucleic acid template, either strand of the nucleic acid template may be considered a "first strand", and either primer of a primer pair provided herein may be considered a "first primer". Accordingly, in some examples, both strands of a double-stranded nucleic acid may be simultaneously used as "first strand" of a nucleic acid according to a method provided herein, with opposite primers of the primer pair serving as the "first primer" for the different "first strands". Also, various structures generated according to methods provided herein may optionally enter different pathways of methods provided herein. For example, in embodiments, a first secondary nucleic acid may pair with a second secondary nucleic acid to form a cross-over structure as described elsewhere herein. In other embodiments, a first secondary nucleic acid may be invaded by a first primer, and the extension product of the second primer may serve as a template for the generation of a new extension product of the first primer. In order to generate the new extension product of the first primer, the extension product of the first primer which is part of the secondary nucleic acid is displaced. In another example, in embodiments, a first concatemer may pair with another concatemer to form a cross-over structure. In other embodiments, a first concatemer may be invaded by a first primer, and the first concatemer strand may serve as a template for the generation of a new second concatemer strand. In order to generate the new first concatemer strand, the first concatemer strand which is part of the concatemer is displaced. Other similar events may also occur during methods provided herein.

[0214] Reactions and compositions provided herein may contain multiple copies of first primers, second primers, primary nucleic acids, secondary nucleic acids, extension products of a first copy of the first primer, extension products of a second copy of the first primer, extension products of the second primer, cross-over structures, concatemers, and the like.

Accordingly, methods provided herein may include processes wherein multiple steps provided herein occur simultaneously, and such steps may occur with multiple copies of the relevant molecules.

[0215] In some embodiments, two or more sets of first and second primers are provided in a method or composition provided herein, where each set contains a first primer and a second primer, and where different primer sets are complementary to different nucleic acid templates. The template-binding region of both the first and second primers in a set may be complementary to different strands of the same double-stranded nucleic acid template. Typically, the tail regions of the first and second primers in a given set have different, non-complementary sequences from the tail regions of the first and second primer of other primer sets to be used in the same method or composition, so that the primer tails from the different primer sets are not complementary. This may be desirable in order to prevent the formation of hybrid cross-over structures containing strands derived from different nucleic acid templates. Alternatively, tail regions of the first and second primers in two or more different primer sets may have complementary sequences, in order to create hybrid cross-over structures and concatemers containing strands derived from different nucleic acid templates. Inclusion of two or more primer sets in a method or composition provided herein may support the simultaneous amplification of multiple different nucleic acid templates in the same reaction vessel. This may be useful, for example, for amplifying multiple templates of interest in a sample, or for assaying for the presence of multiple different templates in a sample. In some embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60, 70, 100, 200, 500 or more sets of first and second primers are provided in a method provided herein, in order to amplify or assay for the presence of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 60, 70, 100, 200, 500 or more different nucleic acid templates.

[0216] In some embodiments, in a method provided herein, a nucleic acid template may be amplified rapidly. For example, in some embodiments, a nucleic acid template may be amplified at least 500-fold within 0.1, 0.5, 1, 3, 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, or 180 minutes of starting the method. In another example, in some embodiments, a nucleic acid template may be amplified at least 10,000-fold within 0.1, 0.5, 1, 3, 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, or 180 minutes of starting the method. In another example, in some embodiments, a nucleic acid template may be amplified at least 5, 10, 25, 50, 100, 250, 500, 1,000, 5,000, 10,000, 50,000, 100,000, 500,000, or 1,000,000-fold over the original amount

of the nucleic acid template present in a reaction mixture at the start of the method within 0.1 minute, 0.5 minute, 1 minute, 3 minutes, 5 minutes, 10 minutes, 15 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 60 minutes, 90 minutes, 2 hours, 3 hours, 4 hours, 6 hours, 8 hours, 12 hours, 16 hours, or 24 hours of initiation of the method. In some embodiments, when a method is initiated, all of the reagents for the first step of the method are in a vessel containing the reaction mixture for the method. In some embodiments, when a method is initiated, all of the reagents for all of the steps of the method are in a vessel containing the reaction mixture for the method.

[0217] In some embodiments, in a method provided herein, a nucleic acid template may be amplified at greater than a linear rate. In some embodiments, in a method provided herein, a nucleic acid template may be amplified exponentially. In some embodiments, in a method provided herein, a nucleic acid template may at least double in number every 1, 2, 3, 5, 10, 15, 20, 25, 30, 45, 60, 90, 120, 180, or 240 minutes after the initiation of the method. In some embodiments, a nucleic acid template may amplified at least 5, 10, 25, 50, 100, 250, 500, 1,000, 5,000, 10,000, 50,000, 100,000, 500,000, 1,000,000, or 10,000,000-fold over the original amount of the nucleic acid template present in the reaction at the start of the method.

[0218] The presence of multiple copies of a nucleic acid template in a concatemer generated according to a method provided herein may contribute to the rapid amplification of nucleic acid templates according to methods provided herein. In particular, since multiple copies of a strand of a nucleic acid template may be present in a single concatemer strand, the loading of a single polymerase onto a single concatemer strand may result in the generation of multiple copies of a strand of the nucleic acid template as the polymerase moves along the concatemer strand. In some situations, the time required for nucleic acid polymerases to encounter and load onto nucleic acid strands may significantly impact the overall speed of an amplification reaction. For example, if each nucleic acid strand that a polymerase encounters during a replication reaction only contains a single copy of a strand of a nucleic acid template, a polymerase may need to encounter and load onto a new template strand after each copy of the strand of the template is generated. In contrast, with a concatemer, after the polymerase encounters and loads on a concatemer strand, it may synthesize multiple copies of a strand of the template without needing to leave the concatemer strand or encounter and load onto another strand.

[0219] In some embodiments, provided herein are methods and compositions for the generation of a double-stranded DNA concatemer from a single-stranded or double-stranded RNA template. The method may be performed as described herein, except that a reverse transcriptase enzyme (e.g. AMV reverse transcriptase, M-MLV reverse transcriptase, Superscript II ™ reverse transcriptase, Superscript III ™ reverse transcriptase, or ThermoScript™ reverse transcriptase) is also included with the methods provided herein. The first copy of the first primer may anneal to the RNA template, and the reverse transcriptase enzyme may generate the extension product of the first copy of the first primer, which is formed as a DNA strand by the process of reverse transcription. Methods and conditions for reverse transcription of RNA are known in the art and are disclosed, for example, in RNA: A Laboratory Manual, D. Rio et al., Cold Spring Harbor Laboratory Press (2011), which is herein incorporated by reference in its entirety. After the generation (from an RNA strand) of the extension product of the first copy of the first primer (which is a DNA strand) by a reverse transcriptase enzyme, the rest of the steps for the generation of a concatemer may be the same as described elsewhere herein.

[0220] In some embodiments, methods and compositions provided herein may include one or more "bumping primers". Bumping primers may be used with methods and compositions provided herein to, for example, increase the rate or specificity of generation of reaction products by, for example, increasing the rate at which a second strand of nucleic acid template is displaced from a first strand of nucleic acid template, increasing the rate at which a first strand of a nucleic acid template is displaced from a first primer extension product, or increasing the rate at which a first primer extension product is displaced from a second primer extension product. As used herein a "bumping primer" refers to a primer which is complementary to a sequence on a first strand or second strand of a nucleic acid template which is downstream from a sequence on the same strand to which the template-binding region of a first primer or second primer binds. Thus, when a bumping primer is annealed to the same nucleic acid strand that a first primer or second primer is annealed to, the 3' terminus of the bumping primer is oriented towards the 5' terminus of the first primer or second primer (i.e. the 5' terminal nucleotide and tail region of the first primer or second primer). When incubated with a nucleic acid polymerase and under conditions to support the generation of primer extension products, an extension product of a bumping primer may be formed by the polymerase, from the 3' terminus of the bumping primer. Since the 3' terminus of the bumping primer is oriented toward the 5' terminus of the first primer or

second primer, as the extension product of the bumping primer increases in length, it may eventually encounter the 5' terminus of the first primer or second primer as the extension product. The polymerase may then displace the first primer or second primer from the strand, as well as any extension product of the first primer or second primer. Accordingly, bumping primers may accelerate reactions provided herein.

[0221] In some embodiments, provided herein is a vessel containing one or more enzymes, primers, or other reagents provided herein. Vessels may include any structure capable of supporting or containing a liquid or solid material and may include, tubes, containers, tips, etc. In some embodiments, a wall of a vessel may permit the transmission of light through the wall. A vessel may be optically clear. A vessel may contain, for example, any one or more of an isolated nucleic acid polymerase, an isolated DNA polymerase, an isolated reverse transcriptase, a first primer, a second primer, a nucleic acid dye, or a nucleic acid probe, as described elsewhere herein. Any number of copies of any of the contents of a vessel may be provided (e.g. a first copy, a second copy, a third copy, etc.) The contents of a vessel may be in fluid communication. In some embodiments, a vessel may further contain a nucleic acid template. In some embodiments, a vessel may further contain nucleotides, buffers, salts, water, or other reagents provided herein for the amplification of nucleic acids. In some embodiments, a vessel may contain two or more sets of primers, wherein each primer set comprises a first and second primer, and the different primer sets are complementary to different nucleic acid templates.

[0222] Two or more reagents useful for a method provided herein may be packaged and provided as a kit. For example, a kit may include any two or more of: a nucleic acid template, a first primer, a second primer, a nucleic acid polymerase, a DNA polymerase, a reverse transcriptase, buffers, a nucleic acid dyes, a nucleic acid probe, or dNTPs, as described elsewhere herein. Within the kit, the two or more reagents may be packaged in separate vessels or the same vessel. In some embodiments, a kit may further contain nucleotides, buffers, salts, water, or other reagents provided herein for the amplification of nucleic acids.

[0223] In embodiments, a first primer and second primer as provided herein may be provided together as a primer set. The primer set may be provided as a stand-alone kit or composition, or the primer set may be provided in a kit with one or more other reagents for performing a method provided herein.

[0224] In some embodiments, a nucleic acid ligase may be included with a method or composition provided herein. Ligases catalyze the formation of phosphodiester bonds between nucleotides, typically between the 5' phosphate of one nucleotide, and the 3' hydroxyl group of another nucleotide. A reaction provided herein may amplify a target nucleic acid at a greater rate with the inclusion of a ligase in the reaction, as compared to the reaction without the inclusion of a ligase. A ligase may, for example, increase the size or number of concatemers present in a reaction provided herein.

[0225] Nucleic acid ligases include *E. coli* DNA ligase, Taq DNA ligase, T3 DNA ligase, T4 DNA ligase, T7 DNA ligase, Ampligase ™, T4 RNA ligase 1, and T4 RNA ligase 2.

[0226] In order to catalyze the ligation reaction, certain ligases require ATP (e.g. T4 DNA ligase) or NAD+ (E. coli DNA ligase). In some embodiments, a ligase may ligate nucleic acids having blunt ends. In some embodiments, a ligase may ligate nucleic acids having sticky ends. In some embodiments, a ligase may ligate nucleic acids having both blunt and sticky ends.

[0227] Modified versions ligases may also be used with the methods and compositions provided herein, provided that the modified ligase has the ability to catalyze the formation of phosphodiester bonds between nucleotides. A modified version of a ligase ("modified ligase") may have, for example, 100 or fewer, 70 or fewer, 50 or fewer, 40 or fewer, 30 or fewer, 20 or fewer, 10 or fewer, 5 or fewer, 4 or fewer, 3 or fewer, 2 or fewer, or 1 different amino acid from the sequence of the parent, naturally occurring version of the ligase. In some embodiments, a modified ligase may contain no more than 1000, 700, 500, 400, 300, 200, 100, 50, 40, 30, 20, 10, or 5 greater or fewer amino acids than the parent ligase. In some embodiments, a modified ligase may comprise a fragment of a parent ligase. In some embodiments, a modified ligase may comprise a chimeric polypeptide with a portion derived from a ligase and a portion derived from a non-ligase protein. In some embodiments, a modified ligase may have, for example, increased catalytic activity, increased stability, or increased thermostability as compared to the parent ligase.

[0228] In some embodiments, a ligase provided herein is thermostable. A thermostable ligase may have, for example, a half-life of at least 5, 10, 15, 20, 30, 40, 50, 60, 90, 120, or 180 minutes at a temperature of at up to 25, 30, 35 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, or 95 C. In some embodiments, a modified ligase may be thermostable.

[0229] In some embodiments, a ligase included with methods and compositions provided herein may be a modified ligase referred to herein as "p50-Tth", which has the amino acid sequence:

MGHHHHHHHHHHSSGHIEGRAS*ADGPYLQILEQPKQRGFRFRYVCEGPSHGGLPGASS*
EKNKKSYPQVKICNYVGPAKVIVQLVTNGKNIHLHAHSLVGKHCEDGICTVTAGPKDMVV
GFANLGILHVTKKKVFETLEARMTEACIRGYNPGLLVHPDLAYLQAEGGGDRQLGDREK
ELIRQAALQQTKEMDLSVVRLMFTAFLPDSTGSFTRRLEPVVSDAIYDSKAPNASNLKIVRM
DRTAGCVTGGEEIYLLCDKVQKDDIQIRFYEEEENGGVWEGFGDFSPTDVHRQFAIVFKT
*PKYKDINITKPASVFVQLRRKSDLETSEPKPFLYYPEIKDKEEVQRKRQK*GSSGTSGGGSG
GGMTLEEARKRVNELRDLIRYHNYRYYVLADPEISDAEYDRLLRELKELEERFPELK
SPDSPTLQVGARPLEATFRPVRHPTRMYSLDNAFNLDELKAFEERIERALGRKGPFAY
TVEHKVDGLSVNLYYEEGVLVYGATRGDGEVGEEVTQNLLTIPTIPRRLKGVPERLE
VRGEVYMPIEAFLRLNEELEERGERIFKNPRNAAAGSLRQKDPRITAKRGLRATFYAL
GLGLEEVEREGVATQFALLHWLKEKGFPVEHGYARAVGAEGVEAVYQDWLKKRR
ALPFEADGVVVKLDELALWRELGYTARAPRFAIAYKFPAEEKETRLLDVVFQVGRT
GRVTPVGILEPVFLEGSEVSRVTLHNESYIEELDIRIGDWVLVHKAGGVIPEVLRVLKE
RRTGEERPIRWPETCPECGHRLLKEGKVHRCPNPLCPAKRFEAIRHFASRKAMDIQGL
GEKLIERLLEKGLVKDVADLYRLRKEDLVGLERMGEKSAQNLLRQIEESKKRGLERL
LYALGLPGVGEVLARNLAARFGNMDRLLEASLEELLEVEEVGELTARAILETLKDPA
FRDLVRRLKEAGVEMEAKEKGGEALKGLTFVITGELSRPREEVKALLRRLGAKVTDS
VSRKTSYLVVGENPGSKLEKARALGVPTLTEEELYRLLEARTGKKAEELV (SEQ ID NO: 79). Ligase p50-Tth has thermostable blunt-end ligation activity at temperatures of at least 60 C. Ligase p50-Tth is a chimeric protein which comprises a His10-containing leader sequence, a p50 sequence from the human NF-kappa-B protein accession number NP_003989 amino acids 40-366 (indicated in italics), a flexible glycine rich sequence, and a Tth DNA ligase sequence, from *Thermus Thermophilus* HB8, accession YP_144363 (indicated with underlining). In some embodiments, a modified version of p50-Tth ligase may be used with methods and compositions provided herein (e.g. with 100 or fewer, 70 or fewer, 50 or fewer, 40 or fewer, 30 or fewer, 20 or fewer, 10 or fewer, 5 or fewer, 4 or fewer, 3 or fewer, 2 or fewer, or 1 different amino acids from p50-Tth ligase). In embodiments, a ligase used with a composition or method provided herein may be a ligase described in U.S. Provisional Patent Application No. 61/802,124, filed March 15, 2013 or PCT Application No.

PCT/US14/30003, filed March 15, 2014, both of which are herein incorporated by reference in their entirety for all purposes.

[0230] The various methods and compositions provided herein for the amplification of a nucleic acid template / the generation of a concatemer containing at least two copies of the template can fulfill many of the functions that have previously been carried out by other methods and compositions for isothermal and thermocycler-dependent nucleic acid amplification. A nucleic acid template for amplification according to methods provided herein may also be referred to herein as a "target nucleic acid" or the like. Methods and compositions provided herein may be used, for example, for isolation and cloning of nucleic acids of interest, gene expression analysis, diagnostic identification of nucleic acids, synthesis of novel nucleic acids, nucleic acid probe synthesis and labeling, forensic identification of a subject, allele identification from a subject, genetic screening, nucleic acid sequencing, and related applications. A target nucleic acid molecule may be of any type, including single-stranded or double stranded and DNA or RNA (e.g. mRNA). A target nucleic acid may be of any type or function (e.g. a protein-coding sequence, a regulatory sequence, an intron, etc.). A target nucleic acid may be the entirety of a gene, or a portion thereof.

[0231] In some embodiments, a method or composition provided herein may be used to detect the amount of a target nucleic acid in a sample (including the presence or absence of the target), to measure the amount of an amplification product of a target formed from a sample in a selected period of time, or to determine the amount of time necessary to generate a certain number of copies of a template from a sample. Samples which may be used with methods and compositions provided herein are described elsewhere herein, and may include, for example, a bodily fluid, a secretion, or a tissue of a subject. In embodiments, a sample may be processed prior to use of the sample in an assay to amplify a target nucleic acid in the sample according to a method provided herein. Processing of the sample may include any processing step as described elsewhere herein, and may include, for example, sonication or chemical lysing steps.

[0232] In some embodiments, a method provided herein may be performed to simultaneously assay for at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, or more different target nucleic acids in the same reaction vessel. Typically, for each target nucleic acid of interest, a first primer and a second primer are provided, each being complementary to a strand of the nucleic acid target, or a complement thereof. The amplification of the

different target nucleic acids in the same vessel may be monitored, for example, by the use of nucleic acid probes having sequence specificity for detection sequences in the different target nucleic acids, and different fluorophores.

[0233] In some embodiments, a method or composition provided herein may be used to detect the presence or absence of a particular nucleotide of interest in a target nucleic acid (e.g. in the case of a mutation or SNP). For example, a first or second primer may be selected which selectively binds to a region in a target nucleic acid which includes or is adjacent to the nucleotide of interest. The primer may be designed such that it selectively either: i) binds to the region when the region contains the nucleotide of interest, or ii) does not bind to the region when the region contains the nucleotide of interest. A method as described herein may be performed with the selected primer, and the outcome of the amplification reaction may provide information regarding the presence or absence of the nucleotide of interest in the target nucleic acid. For example, if the template-binding region of a first primer is designed to have a nucleotide sequence which is complementary to a sequence in the target nucleic acid which includes a particular nucleotide of interest (e.g. a mutation), successful amplification of the target nucleic acid with the selected primer from a sample may indicate that the sample contains a target nucleic acid having the particular nucleotide of interest. In some embodiments, a primer used for analysis of a nucleotide of interest in a target nucleic acid may contain a critical nucleotide (i.e. a nucleotide which corresponds to the same position of a nucleotide of interest in the target nucleic acid) at the 3' terminus of the primer. In such a case, the annealing of the 3' terminal nucleotide of the primer may be dependent on the presence of the nucleotide of interest in the target nucleic acid. If the 3' terminal nucleotide of the primer does not anneal with a nucleotide in the target nucleic acid (e.g. due to a mismatch between the nucleotides), the mismatch may significantly impair a nucleic acid polymerase from synthesizing an extension product from the primer. Accordingly, in some embodiments, a primer having a 3' terminal nucleotide which corresponds to a nucleotide of interest may be useful for determining the presence or absence of a particular nucleotide in a target nucleic acid. In such embodiments, in some situations the critical nucleotide at the 3' terminus of the primer may be selected to be complementary the nucleotide of interest in the target nucleic acid, and in some other situations the critical nucleotide at the 3' terminus of the primer may be selected to be non-complementary the nucleotide of interest in the target nucleic acid. The nucleotide of interest may represent, for example, a wild-type form, a mutant form, or a polymorphism of a target nucleic acid.

[0234] In other embodiments, a particular nucleotide of interest in a target nucleic acid (e.g. a mutation or SNP) may be detected by selecting primers such that the nucleotide of interest is present in the target nucleic acid in a region which is not complementary to a template-binding region of a first or second primer. For example, the nucleotide of interest may be approximately in the middle of a target nucleic acid sequence. In embodiments, the nucleotide of interest may be in an "internal motif" described elsewhere herein. When a nucleotide of interest is in an internal motif, in embodiments, a primer pair may be prepared to contain a nucleotide sequence in the tail region of the primers which is complementary to an internal motif or the complement thereof, and which may be used to assay for the presence or absence of the nucleotide of interest in the internal motif in the target sequence. As explained elsewhere herein, the temporary annealing of a nucleotide sequence in the tail region of a primer to an internal motif in an extension product of that primer may increase the rate of a reaction provided herein. In some circumstances, the greater the affinity of a nucleotide sequence in the tail region of a primer to the internal motif in an extension product of that primer, the faster the reaction may occur. Also, typically, the greater the number of nucleotides in the nucleotide sequence in the tail region of the primer which can bind to nucleotides in the internal motif in the extension product of the primer, the greater the affinity of the nucleotide sequence in the tail region of the extension product for the internal motif in the extension product. Thus, with compositions and methods provided herein, in embodiments, the presence or absence of a nucleotide of interest in a target sequence may be determined through the use of primers which have a nucleotide sequence in the tail region of the primer which can bind to the internal motif in the target sequence or a complement thereof, and which, within the tail region, do or do not have a nucleotide which specifically binds with the particular nucleotide of interest in the internal motif or its complement. Typically, the reaction will occur faster when the nucleotide sequence in the tail region of a primer contains a nucleotide which is complementary to the nucleotide in the extension product of that primer which corresponds to the nucleotide of interest in the target, than when the relevant nucleotide in the tail region of the primer is not complementary to the nucleotide in the extension product of that primer which corresponds to the nucleotide of interest in the target. For example, the internal motif of a wild-type version of a target nucleic acid strand may have the nucleotide sequence: 5' TATTGCAT 3'. However, the "G" in the sequence may frequently be mutated to an "A" in many individuals in the population. In order to determine whether a particular target nucleic acid contains a "G" or other nucleotide in the

sequence, a primer may be prepared which contain a nucleotide sequence in its tail region having the sequence: 5'ATGCAATA 3'. If this primer (and an appropriate second primer) is used to amplify the target nucleic acid according to a method provided herein, the amplification reaction may have a certain reaction rate if the "G" is present in the internal motif, versus if a different nucleotide is present at the position of the "G" in the internal motif. This is because the nucleotide sequence in the tail region of the primer will be perfectly complementary to the internal motif if the "G" is present in the internal motif, but it will not be if a nucleotide other than a "G" is present at the G's normal position. Generally, in this example, if a "G" is present in the internal motif, the tail region of the primer will anneal to the internal motif more frequently than if the "G" is not present, and this may in turn lead to a faster reaction rate if the "G" is present than not present. With systems and methods provided herein, primers may be prepared to assay for either the presence or absence of a nucleotide of interest (e.g. primers can be prepared such that the reaction occurs more quickly if a mutated version of a nucleotide is present than if a wild-type version of the nucleotide is present, or vice-versa). Also, in embodiments, primers may be prepared so as to determine the identity of an unknown nucleotide in a position of interest in an internal motif in a target. For example, four different primer pairs may be prepared, each containing either an A, T, G, or C as appropriate corresponding to a nucleotide of interest in a target nucleic acid. In embodiments, the primer pair which yields the fastest reaction results may indicate which nucleotide is present in the position of interest in the internal motif in the target nucleic acid. For instance, a target nucleic acid may have an internal motif which commonly has the sequence, in the 5' to 3' direction of: GTAACGAG. However, different variants of the target nucleic acid may have different nucleotides in the 5th position in the internal motif (i.e. the position of the "C"). Continuing with the example, if a sample containing the target nucleic acid is provided, wherein it is unknown what nucleotide is in the 5th position in the of the internal motif, the sample can be divided into at least 4 portions, and the four portions can be used for a first reaction mixture, a second reaction mixture, a third reaction mixture, and a fourth reaction mixture. To the first reaction mixture, a first primer pair is provided, where the primers have tail regions with the sequences, in the 5' to 3' direction: CTCGTTAC and GTAACGAG, respectively. These tail regions will be perfectly complementary to the internal motif or complement thereof, if a "C" is present at 5th position in internal motif. Thus, if the target is amplified most quickly in this reaction mixture, it indicates that a "C" is present in the position of the nucleotide of interest in the internal motif in the target. To the

second reaction mixture, a second primer pair is provided, where the primers have tail regions with the sequences, in the 5' to 3' direction: CTCATTAC and GTAATGAG, respectively. These tail regions will be perfectly complementary to the internal motif or complement thereof, if a "T" is present at 5th position in internal motif. Thus, if the target is amplified most quickly in this reaction mixture, it indicates that a "T" is present in the position of the nucleotide of interest in the internal motif in the target. To the third reaction mixture, a third primer pair is provided, where the primers have tail regions with the sequences, in the 5' to 3' direction: CTCCTTAC and GTAAGGAG, respectively. These tail regions will be perfectly complementary to the internal motif or complement thereof, if a "G" is present at 5th position in internal motif. Thus, if the target is amplified most quickly in this reaction mixture, it indicates that a "G" is present in the position of the nucleotide of interest in the internal motif in the target. To the fourth reaction mixture, a fourth primer pair is provided, where the primers have tail regions with the sequences, in the 5' to 3' direction: CTCTTTAC and GTAAAGAG, respectively. These tail regions will be perfectly complementary to the internal motif or complement thereof, if an "A" is present at 5th position in internal motif. Thus, if the target is amplified most quickly in this reaction mixture, it indicates that an "A" is present in the position of the nucleotide of interest in the internal motif in the target. In embodiments, methods or compositions as generally described above may optionally be prepared with only 2 or 3 reaction mixtures with primer pairs corresponding to only 2 or 3 options for a nucleotide at a position of interest, if for example, it is not desired to screen all possible nucleotide variants (for instance if only 2 different nucleotides are typically found in a position of interest).

[0235] In embodiments, a sample containing a target nucleic acid may be divided into at least a first and a second portion, where the first portion is incubated in a first reaction mixture with a first primer pair, and the second portion is incubated in a second reaction mixture with a second primer pair, according to methods provided herein. In embodiments, the first primer pair and the second primer pair differ from each other by only a single nucleotide in the respective tail / first regions of the primer (e.g. the sequence of the first region of the first primer of the first pair differs from the sequence of the first region of the first primer of the second pair by only a single nucleotide). The single nucleotide which is different in the tail region of the primers may correspond to the position of a nucleotide of interest in the target nucleic acid. By comparing the rate or quantity of amplification of the target nucleic acid in the first reaction mixture to that in the second reaction mixture, the identity of a nucleotide of

interest in the target nucleic acid may be determined. In embodiments, the method outlined above may be performed with a first, second, third, and fourth portion of the sample, and with a first primer pair, second primer pair, third primer pair, and fourth primer pair, wherein the first primer pair, second primer pair, third primer pair, and fourth primer pair differ from each other by only a single nucleotide in the respective tail / first region of the primers, as described above.

[0236] In embodiments, at least a first target nucleic acid and a second target nucleic acid may be provided, wherein the first target nucleic acid and the second target nucleic acid differ by a single nucleotide at a position of interest in an internal motif as described elsewhere herein. In embodiments, the first target nucleic acid may be incubated in a first reaction mixture containing a primer pair described herein and the second target nucleic acid may be incubated in a second reaction mixture containing the same primer pair, according to methods provided herein. By comparing the rate or quantity of amplification of the first target nucleic acid in the first reaction mixture to that of the second target nucleic acid in second reaction mixture or to an absolute value, the identity of the nucleotide in the position of interest in one or both of the first target nucleic acid and the second target nucleic may be determined, according to principles described elsewhere herein.

[0237] In addition, in embodiments, compositions and methods described herein for detecting a single nucleotide of interest may also be used to detect multi-nucleotide mutations or polymorphisms.

[0238] Methods and compositions provided herein may be used to amplify a nucleic acid from any sample which may contain nucleic acids. Examples of samples may include various fluid samples. In some instances, the sample may be a bodily fluid sample from a subject. The sample may include one or more fluid component. In some instances, solid or semi-solid samples may be provided. The sample may include tissue collected from the subject. The sample may include a bodily fluid, secretion, or tissue of a subject. The sample may be a biological sample. The biological sample may be a bodily fluid, a secretion, or a tissue sample. Examples of biological samples may include but are not limited to, blood, serum, saliva, urine, gastric and digestive fluid, tears, stool, semen, vaginal fluid, interstitial fluids derived from tumorous tissue, ocular fluids, sweat, mucus, earwax, oil, glandular secretions, breath, spinal fluid, hair, fingernails, skin cells, plasma, nasal swab or nasopharyngeal wash, spinal fluid, cerebral spinal fluid, tissue, throat swab, biopsy, placental

fluid, amniotic fluid, cord blood, emphatic fluids, cavity fluids, sputum, pus, microbiota, meconium, breast milk or other excretions. The sample may be provided from a human or animal. Samples may be from a plant, microorganism (e.g. virus, bacteria), or other biological material.

[0239] In some embodiments, methods and compositions provided herein may be performed at or used at point of service locations (e.g. a subject's home or work, grocery stores, drug stores, clinics, schools, etc.). Methods and compositions provided herein may permit the rapid amplification of nucleic acids in a sample from a subject, in order to aid in the diagnosis or treatment of a subject. For example, methods and compositions provided here may be used test a sample from a subject for the presence of nucleic acid from a pathogen, such as a virus (e.g. influenza) or bacteria (e.g. streptococcus).

[0240] In embodiments, methods and compositions provided herein may be used to detect a pathogen, or a feature of a pathogen (e.g. a gene or gene variant within the pathogen). Pathogens or features thereof which may be detected with a method or composition provided herein may include, for example, *Acinetobacter baumannii*, blaROB resistance gene, blaTEM resistance gene, *Bordetalla holmesii, Burkholderia cepacia, Candida albicans, Clostridium difficile, Clostridium sordellii, Enterobacter aerogenes, Enterobacter cloacae, Enterococcus faecaelis, Enterococcus faecium, Escherichia coli, Klebsiella pneumoniae*, KPC resistance gene, *Legionella pneumophila*, mecA resistance gene, mecC resistance gene, *Moraxella catarrhalis, Mycobacterium abscessus, Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus, Staphylococcus aureus (Vancomycin-resistant) VRSA, Streptococcus agalactiae (Strep B), Streptococcus pneumonia, Streptococcus pneumonia (penicillin-sensitive), Streptococcus pyogenes*, vanA resistance gene, vanB resistance gene, *Adenovirus B, Adenovirus C, Adenovirus E, Bocavirus 1+3, Bocavirus 2+4, Bordetella parapertussis, Bordetella pertussis, Chlamydia pneumonia, Clostridium sordellii, Coronavirus 229E, Coronavirus HKU1, Coronavirus MERS, Coronavirus NL63, Coronavirus OC43, Human metapneumovirus A, Human metapneumovirus B, Influenza A – MP, Influenza B – MP, Influenza H1N1 (2009), Influenza H1N1 (seasonal), Influenza H3N2, Influenza H5N1, Influenza H7N9 (HA gene), Influenza H7N9 (NA gene), measles virus, mumps virus, Mycobacterium tuberculosis, Mycoplasma pneumonia, Parainfluenza 1, Parainfluenza 2, Parainfluenza 3, Parainfluenza 4a, Parainfluenza 4b, Respiratory syncytial virus A (RSV A), Respiratory syncytial virus B (RSV B), Rhinovirus A, Rhinovirus B, Rhinovirus C, rubella*

virus, Streptococcus pyogenes, Candida albicans, Chlamydia trachomatis, human papilloma virus, HPV 16, HPV 18, HSV-1/2, mycoplasma genitalium, Neisseria gonorrhoeae, Treponema pallidum (syphilis), Trichomonas vaginalis, Varicella-zoster virus, Epstein-Barr Virus, Hepatitis A, Hepatitis B, Hepatitis C, Hepatitis D, HIV, HIV-1 group M, HIV-1 group O1, HIV-1 group O3, HIV-2 group A, HIV-2 group B, Mycoplasma hominis, Proteus mirabilis, Proteus penneri, Proteus vulgaris, S. saprophyticus, Ureaplasma urealyticum, Bacillus anthracis, Yersina pestis, Dengue virus, Plasmodium, Tryponosoma cruzi (chagas), West Nile Virus 1, West Nile Virus 2, Norovirus lineage 1, Norovirus lineage 2, Ebola virus. In embodiments, 1, 2, 3, 5, 10, 20, 30, 50, or more of such pathogens or features thereof may be assayed for from a single sample from a subject using a method provided herein. In embodiments, any of the nucleic acid amplification-based assays or assay panels as described in International Application No. PCT/US2014/54424, filed September 5, 2014 or U.S. Provisional Patent Application No. 62/001,050, filed May 20, 2014, which are hereby incorporated by reference in its entirety for all purposes, may be performed using a method for nucleic acid amplification described herein. Similarly, any of the pathogens or features thereof described in International Application No. PCT/US2014/54424 or U.S. Provisional Patent Application No. 62/001,050 may be assayed for with a nucleic acid amplification method provided herein.

[0241] In embodiments, an influenza panel may be performed using methods provided herein for amplification of nucleic acids from one or more strains of influenza. For example, using methods provided herein, one or more nucleic acids from one or more of Influenza A, Influenza A H3N2, Influenza A H5N1, Influenza A H7N9 – HA gene, Influenza A H7N9 – NA gene, or Influenza B may be amplified. Other panels that may be performed using methods provided herein include, for example, a respiratory panel, a sexually transmitted disease panel, a blood-borne pathogen panel, or a hospital acquired infections panel. Any of these panels may include assays for at least 2, 3, 4, 5, 10, 15, or 20 different pathogens or genes.

[0242] In embodiments, methods and compositions provided herein may be used for Influenza A virus detection and sub-typing. Influenza A subtypes are defined by the type of two viral genes, hemagglutin (HA) and neuraminidase (NA). Presently, there are 18 known HA genes (H1-H18) and 11 known NA genes (N1-N11). These genes are encoded on different molecules in the Influenza A virus, and thus it is possible for an Influenza A subtype

to carry any combination of different HA and NA gene. In order to accurately detect and type an Influenza A virus, it may be desirable to test a sample which may or is known to contain Influenza A virus for at least two and up to all of the 18 known HA genes and at least two and up to all the 11 known NA genes. In embodiments, a single sample from a subject may be divided into two or more portions, and the portions may be incubated under reaction conditions provided herein with primer pairs having template-binding regions specific for each of the 18 known HA genes and 11 known NA genes (i.e. 29 different primer pairs). In embodiments, a single sample from a subject may be divided into at least 29 different portions, wherein the first portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H1, wherein the second portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H2, wherein the third portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H3, wherein the fourth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H4, wherein the fifth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H5, wherein the sixth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H6, wherein the seventh portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H7, wherein the eighth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H8, wherein the ninth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H9, wherein the tenth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H10, wherein the eleventh portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H11, wherein the twelfth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H12, wherein the thirteen portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H13, wherein the fourteenth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H14, wherein the fifteenth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions

specific for H15, wherein the sixteenth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H16, wherein the seventeenth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H17, wherein the eighteenth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for H18, wherein the nineteenth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for N1, wherein the twentieth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for N2, wherein the twenty-first portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for N3, wherein the twenty-second portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for N4, wherein the twenty-third portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for N5, wherein the twenty-fourth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for N6, wherein the twenty-fifth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for N7, wherein the twenty-sixth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for N8, wherein the twenty-seventh portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for N9, wherein the twenty-eighth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for N10, and wherein the twenty-ninth portion is incubated under reaction conditions provided herein with a primer pair having template-binding regions specific for N11. In some embodiments, primer pairs for 2, 3, 4, 5, or more different HA or NA subtypes may be incubated with a single portion of a sample from a subject (e.g. in a common vessel), and the amplification reaction products may be identified using a sequence-specific probe (e.g. DNA probe or PNA probe), as described elsewhere herein. In embodiments, a panel may be performed wherein a single sample is used for amplification reactions with primer pairs for at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, or all 29 of the HA and NA subtypes described above. In embodiments, a panel may include reactions to amplify one or more of H1, H2, H3, H5, H7, or N9. In embodiments, a panel or method described herein for the detection of Influenza A or sub-

types thereof may further include reagents or reactions to detect, for example, Influenza A matrix protein gene (this is present in all Influenza A subtypes, and thus may be used to identify Influenza A in a sample), human RNaseP (to ensure adequate sample collection from a human subject; for non-human subjects, other appropriate species-specific control genes may be used), or a spike-in nucleic acid control, which may be added at one or more points during a sample collection or processing step, in order to ensure adequate sample collection or processing. In embodiments, kits may be provided to perform any of the methods or for any of the panels provided above.

[0243] In embodiments, using methods provided herein, a single pair of primers may be used to amplify a greater variety of target nucleic acid sequences than may be amplified with a single pair of primers using other nucleic acid amplification methods (e.g. PCR or LAMP). For example, in embodiments, the template-binding regions of primers provided herein may sufficiently anneal to templates to amplify the templates according to methods provided herein even when there are 1, 2, 3, 4, 5 or more nucleotide mismatches between the template-binding region of the primer and the template. In embodiments, methods provided herein may be performed when at least 6, 8, 10, 12, 14, 16, 18, 20, 22, or 24 % of the nucleotides in the template binding region of at least a first primer and optionally also the second primer used in a method provided are not complementary to corresponding nucleotides in the template sequence. Use of primers and methods provided herein, therefore, may facilitate the amplification of multiple similar but different sequences by the same pair of primers. For instance, primers and methods provided herein may be useful for the detection of multiple different strains of influenza A, where the strains have similar but different nucleotide sequences. For example, there may be two different strains of Influenza A, where both strains are defined as H1N1 sub-type, but the strains have slightly different H1 or N1 nucleotide sequences. Using compositions and methods provided herein, in embodiments, both the first strain and the second strain may be amplified using the same primer pair.

[0244] In addition, primers used for the amplification of nucleic acids from pathogens may be selected based on highly conserved regions in nucleic acids of pathogens, to further aid in the detection multiple different strains of a pathogen with the same primer pair according to methods provided herein. Such highly conserved regions may be identified, for example, by sequence alignment of a gene of interest across multiple strains of a pathogen. For instance, HA3 sequences with Genbank ID numbers: AEK98400, ABG47862, AEO00689, ABF17954,

AFG99237, AFG99027, ABO33069, ABE12623, AFG72790, AAA87553, AAZ38605, AFN19700, ABB04371, ABC39805 are from different isolates of H3N2 Influenza A virus. These HA3 genes each have slightly different nucleotide sequences. Nonetheless, through careful selection of primers, all such HA3 genes may be amplified with the same set of primers according to a method provided herein. For example, a portion of the HA3 gene having the sequence, in 5'-3' order:
AACCTCACTACGTAAGTCTTGACGTAAACGGTTACCCACGTAGACTAGAGTAATA ACTCGAAAAGGGT (SEQ ID NO: 91) may be selected as a target for amplification according to a method provided herein (the sequence is provided in DNA, rather than RNA form). Describing this sequence, in the 5' to 3' direction, the first underlined sequence the second portion of the template, the second underlined sequence is the internal motif, and the third underlined sequence is the first portion of the template, as these terms are used, for example in Figure 10. In this and other such examples, both the total target sequence for amplification, as well as the particular sequences to function as the first portion of the template, second portion of the template, and internal motif may be selected such that primers as described herein may be used to successfully amplify a wide range of related sequences according to methods provided herein. In embodiments, a first primer as described herein may be provided wherein the template binding region of the primer is complementary to the first portion of the above template, and a second primer as described herein may be provided wherein the template binding region of the primer is complementary to a partner sequence which is complementary to the second portion of the template. Optionally, the primers may further have tail regions which are complementary to the internal motif or a complementary sequence thereof. Such primers may be provided, for example, in compositions or methods provided herein.

[0245] With methods provided herein pathogens or genes may be positively identified at concentrations as low as, for example, less than 1000, 500, 100, 50, 10, 5, 2, or 1 copy per microliter in a sample or a reaction mixture. In embodiments, methods provided herein may specifically amplify nucleic acid from one type of pathogen or gene and not amplify nucleic acid from a related pathogen or gene. For example, assays provided herein to amplify Influenza A matrix protein gene may amplify this gene from multiple different strains of Influenza A, but not amplify genetic material from Influenza B.

[0246] In embodiments, methods provided herein may be successfully performed in the presence of one or more potentially interfering substances. Examples of potentially interfering substances include BSA, glucose, bilirubin, nitrites, beta-hCG, acetone, low pH conditions, high pH conditions, acetaminophen, aspirin, progestin, ethinyl estradiol, urine preservatives, seminal fluid, personal lubricants, contraceptive jellies, spermicides, feminine powders, hemorrhoid creams, miconzole, human genomic DNA, lotions, universal transport media (viral), amies transport media (bacteria), blood, mucin, acyclovir, cold sore treatments, urine, feces, hemoglobin, triglycerides, EDTA, heparin, cholesterol, gamma-globulin, ampicillin, nicotine, cotinine, nasal sprays, nasal drops, or any combination thereof. In embodiments, methods provided herein may be performed in the presence of a potentially interfering substance which is at a concentration of up to at least as great as provided in U.S. Provisional Patent Application No. 62/001,050, filed May 20, 2014, which is hereby incorporated by reference for all purposes. In embodiments, methods provided herein may be performed in the presence of a potentially interfering substance which is at a concentration of up to at least 10%, 25%, 50%, or 100% greater than provided in U.S. Provisional Patent Application No. U.S. Provisional Patent Application No. 62/001,050.

[0247] The assays and methods disclosed herein may be performed on a device, or on a system, for processing a sample. The assays and methods disclosed herein can be readily incorporated into and used in a device for processing a sample, or a system for processing a sample, which may be an automated assay device, or may be an automated assay system. Such a device, and such a system, may be useful for the practice of the methods disclosed herein. For example, a device may be useful for receiving a sample. A device may be useful for preparing, or for processing a sample. A device may be useful for performing an assay on a sample. A device may be useful for obtaining data from a sample. A device may be useful for transmitting data obtained from a sample. A device may be useful for disposing of a sample following processing or assaying of a sample.

[0248] A device may be part of a system, a component of which may be a sample processing device. A device may be a sample processing device. A sample processing device may be configured to facilitate collection of a sample, prepare a sample for a clinical test, or perform a method with one or more reagents, as disclosed herein. A sample processing device may be configured to obtain data from a sample. A sample processing device may be configured to transmit data obtained from a sample. A sample processing device may be configured to

analyze data from a sample. A sample processing device may be configured to communicate with another device, or a laboratory, or an individual affiliated with a laboratory, to analyze data obtained from a sample.

[0249] A sample processing device may be configured to be placed in or on a subject. A sample processing device may be configured to accept a sample from a subject, either directly or indirectly. A sample may be, for example, a blood sample (e.g., a sample obtained from a fingerstick, or from venipuncture, or an arterial blood sample), a urine sample, a biopsy sample, a tissue slice, stool sample, or other biological sample; a water sample, a soil sample, a food sample, an air sample; or other sample. A blood sample may comprise, e.g., whole blood, plasma, or serum. A sample processing device may receive a sample from the subject through a housing of the device. The sample collection may occur at a sample collection site, or elsewhere. The sample may be provided to the device at a sample collection site.

[0250] In some embodiments, a sample processing device may be configured to accept or hold a cartridge. In some embodiments, a sample processing device may comprise a cartridge. The cartridge may be removable from the sample processing device. In some embodiments, a sample may be provided to the cartridge of the sample processing device. Alternatively, a sample may be provided to another portion of a sample processing device. The cartridge and/or device may comprise a sample collection unit that may be configured to accept a sample.

[0251] A cartridge may include a sample, and may include reagents for use in processing or testing a sample, disposables for use in processing or testing a sample, or other materials. A cartridge may contain reagents disclosed herein for the performing a method disclosed herein. Following placement of a cartridge on, or insertion of a cartridge into, a sample processing device, one or more components of the cartridge may be brought into fluid communication with other components of the sample processing device. For example, if a sample is collected at a cartridge, the sample may be transferred to other portions of the sample processing device. Similarly, if one or more reagents are provided on a cartridge, the reagents may be transferred to other portions of the sample processing device, or other components of the sample processing device may be brought to the reagents. In some embodiments, the reagents or components of a cartridge may remain on-board the cartridge.

In some embodiments, no fluidics are included that require tubing or that require maintenance (e.g., manual or automated maintenance).

[0252] A sample or reagent may be transferred to a device, such as a sample processing device. A sample or reagent may be transferred within a device. Such transfer of sample or reagent may be accomplished without providing a continuous fluid pathway from cartridge to device. Such transfer of sample or reagent may be accomplished without providing a continuous fluid pathway within a device. In embodiments, such transfer of sample or reagent may be accomplished by a sample handling system (e.g., a pipette); for example, a sample, reagent, or aliquot thereof may be aspirated into an open-tipped transfer component, such as a pipette tip, which may be operably connected to a sample handling system which transfers the tip, with the sample, reagent, or aliquot thereof contained within the tip, to a location on or within the sample processing device. The sample, reagent, or aliquot thereof can be deposited at a location on or within the sample processing device. Sample and reagent, or multiple reagents, may be mixed using a sample handling system in a similar manner. One or more components of the cartridge may be transferred in an automated fashion to other portions of the sample processing device, and vice versa.

[0253] A device, such as a sample processing device, may have a fluid handling system. A fluid handling system may perform, or may aid in performing, transport, dilution, extraction, aliquotting, mixing, and other actions with a fluid, such as a sample. In some embodiments, a fluid handling system may be contained within a device housing. A fluid handling system may permit the collection, delivery, processing and/or transport of a fluid, dissolution of dry reagents, mixing of liquid and/or dry reagents with a liquid, as well as collection, delivery, processing and/or transport of non-fluidic components, samples, or materials. The fluid may be a sample, a reagent, diluent, wash, dye, or any other fluid that may be used by the device, and may include, but not limited to, homogenous fluids, different liquids, emulsions, suspensions, and other fluids. A fluid handling system, including without limitation a pipette, may also be used to transport vessels (with or without fluid contained therein) around the device. The fluid handling system may dispense or aspirate a fluid. The sample may include one or more particulate or solid matter floating within a fluid.

[0254] In embodiments, a fluid handling system may comprise a pipette, pipette tip, syringe, capillary, or other component. The fluid handling system may have a portion with an interior surface and an exterior surface and an open end. The fluid handling system may comprise a

pipette, which may include a pipette body and a pipette nozzle, and may comprise a pipette tip. A pipette tip may or may not be removable from a pipette nozzle. In embodiments, a fluid handling system may use a pipette mated with a pipette tip; a pipette tip may be disposable. A tip may form a fluid-tight seal when mated with a pipette. A pipette tip may be used once, twice, or more times. In embodiments, a fluid handling system may use a pipette or similar device, with or without a pipette tip, to aspirate, dispense, mix, transport, or otherwise handle the fluid. The fluid may be dispensed from the fluid handling system when desired. The fluid may be contained within a pipette tip prior to being dispensed, e.g., from an orifice in the pipette tip. In embodiments, or instances during use, all of the fluid may be dispensed; in other embodiments, or instances during use, a portion of the fluid within a tip may be dispensed. A pipette may selectively aspirate a fluid. The pipette may aspirate a selected amount of fluid. The pipette may be capable of actuating stirring mechanisms to mix the fluid within the tip or within a vessel. The pipette may incorporate tips or vessels creating continuous flow loops for mixing, including of materials or reagents that are in non-liquid form. A pipette tip may also facilitate mixture by metered delivery of multiple fluids simultaneously or in sequence, such as in 2-part substrate reactions.

[0255] The fluid handling system may include one or more fluidically isolated or hydraulically independent units. For example, the fluid handling system may include one, two, or more pipette tips. The pipette tips may be configured to accept and confine a fluid. The tips may be fluidically isolated from or hydraulically independent of one another. The fluid contained within each tip may be fluidically isolated or hydraulically independent from fluids in other tips and from other fluids within the device. The fluidically isolated or hydraulically independent units may be movable relative to other portions of the device and/or one another. The fluidically isolated or hydraulically independent units may be individually movable. A fluid handling system may comprise one or more base or support. A base or support may support one or more pipette or pipette units. A base or support may connect one or more pipettes of the fluid handling system to one another.

[0256] A sample processing device may be configured to perform processing steps or actions on a sample obtained from a subject. Sample processing may include sample preparation, including, e.g., sample dilution, division of a sample into aliquots, extraction, contact with a reagent, filtration, separation, centrifugation, or other preparatory or processing action or step. A sample processing device may be configured to perform one or more sample

preparation action or step on the sample. Optionally, a sample may be prepared for a chemical reaction and/or physical processing step. A sample preparation action or step may include one or more of the following: centrifugation, separation, filtration, dilution, enriching, purification, precipitation, incubation, pipetting, transport, chromatography, cell lysis, cytometry, pulverization, grinding, activation, ultrasonication, micro column processing, processing with magnetic beads, processing with nanoparticles, or other sample preparation action or steps. For example, sample preparation may include one or more step to separate blood into serum and/or particulate fractions, or to separate any other sample into various components. Sample preparation may include one or more step to dilute and/or concentrate a sample, such as a blood sample, or other biological samples. Sample preparation may include adding an anti-coagulant or other ingredients to a sample. Sample preparation may also include purification of a sample. In embodiments, all sample processing, preparation, or assay actions or steps are performed by a single device. In embodiments, all sample processing, preparation, or assay actions or steps are performed within a housing of a single device. In embodiments, most sample processing, preparation, or assay actions or steps are performed by a single device, and may be performed within a housing of a single device. In embodiments, many sample processing, preparation, or assay actions or steps are performed by a single device, and may be performed within a housing of a single device. In embodiments, sample processing, preparation, or assay actions or steps may be performed by more than one device.

[0257] A sample processing device may be configured to run one or more assays on a sample, and to obtain data from the sample. A sample processing device may perform methods provided herein, as well as additional assays. An assay may include one or more physical or chemical treatments, and may include running one or more chemical or physical reactions. A sample processing device may be configured to perform one, two or more assays on a small sample of bodily fluid. One or more chemical reaction may take place on a sample having a volume, as described elsewhere herein. For example, one or more chemical reaction may take place in a pill having less than femtoliter volumes. In an instance, the sample collection unit is configured to receive a volume of the bodily fluid sample equivalent to a single drop or less of blood or interstitial fluid. In embodiments, the volume of a sample may be a small volume, where a small volume may be a volume that is less than about 1000 μL, or less than about 500 μL, or less than about 250 μL, or less than about 150 μL, or less than about 100 μL, or less than about 75 μL, or less than about 50 μL, or less than about 40

μL, or less than about 20 μL, or less than about 10 μL, less than about 5 μL, less than about 1 μL, less than about 0.5 μL, less than about 0.1 μL, or other small volume. In embodiments, all sample assay actions or steps are performed on a single sample. In embodiments, all sample assay actions or steps are performed by a single device. In embodiments, all sample assay actions or steps are performed within a housing of a single device. In embodiments, most sample assay actions or steps are performed by a single device, and may be performed within a housing of a single device. In embodiments, many sample assay actions or steps are performed by a single device, and may be performed within a housing of a single device. In embodiments, sample processing, preparation, or assay actions or steps may be performed by more than one device.

[0258] A sample processing device may be configured to perform a plurality of assays on a sample. In some embodiments, a sample processing device may be configured to perform a method provided herein and one, two, or more additional assays. In embodiments, a sample processing device may be configured to perform a plurality of assays on a single sample. In embodiments, a sample processing device may be configured to perform a plurality of assays on a single sample, where the sample is a small sample. For example, a small sample may have a sample volume that is a small volume of less than about 1000 μL, or less than about 500 μL, or less than about 250 μL, or less than about 150 μL, or less than about 100 μL, or less than about 75 μL, or less than about 50 μL, or less than about 40 μL, or less than about 20 μL, or less than about 10 μL, less than about 5 μL, less than about 1 μL, less than about 0.5 μL, less than about 0.1 μL, or other small volume. A sample processing device may be capable of performing multiplexed assays on a single sample. A plurality of assays may be run simultaneously; may be run sequentially; or some assays may be run simultaneously while others are run sequentially. One or more control assays and/or calibrators (e.g., including a configuration with a control of a calibrator for the assay/tests) can also be incorporated into the device; control assays and assay on calibrators may be performed simultaneously with assays performed on a sample, or may be performed before or after assays performed on a sample, or any combination thereof. In embodiments, all sample assay actions or steps are performed by a single device. In embodiments, all of a plurality of assay actions or steps are performed within a housing of a single device. In embodiments, most sample assay actions or steps, of a plurality of assays, are performed by a single device, and may be performed within a housing of a single device. In embodiments, many sample assay actions or steps, of a plurality of assays, are performed by a single device, and may be

performed within a housing of a single device. In embodiments, sample processing, preparation, or assay actions or steps may be performed by more than one device.

[0259] In embodiments, all of a plurality of assays may be performed in a short time period. In embodiments, such a short time period comprises less than about three hours, or less than about two hours, or less than about one hour, or less than about 40 minutes, or less than about 30 minutes, or less than about 25 minutes, or less than about 20 minutes, or less than about 15 minutes, or less than about 10 minutes, or less than about 5 minutes, or less than about 4 minutes, or less than about 3 minutes, or less than about 2 minutes, or less than about 1 minute, or other short time period.

[0260] A sample processing device may be configured to detect one or more signals relating to the sample. A sample processing device may be configured to identify one or more properties of the sample. For instance, the sample processing device may be configured to detect the presence or concentration of one analyte (e.g. a target nucleic acid) or a plurality of analytes or a disease condition in the sample (e.g., in or through a bodily fluid, secretion, tissue, or other sample). Alternatively, the sample processing device may be configured to detect a signal or signals that may be analyzed to detect the presence or concentration of one or more analytes (which may be indicative of a disease condition) or a disease condition in the sample. The signals may be analyzed on board the device, or at another location. Running a clinical test may or may not include any analysis or comparison of data collected.

[0261] A chemical reaction or other processing steps may be performed, with or without the sample. Examples of steps, tests, or assays that may be prepared or run by the device may include, but are not limited to immunoassay, nucleic acid assay (e.g. methods provided herein), receptor-based assay, cytometric assay, colorimetric assay, enzymatic assay, electrophoretic assay, electrochemical assay, spectroscopic assay, chromatographic assay, microscopic assay, topographic assay, calorimetric assay, turbidimetric assay, agglutination assay, radioisotope assay, viscometric assay, coagulation assay, clotting time assay, protein synthesis assay, histological assay, culture assay, osmolarity assay, and/or other types of assays, centrifugation, separation, filtration, dilution, enriching, purification, precipitation, pulverization, incubation, pipetting, transport, cell lysis, or other sample preparation action or steps, or combinations thereof. Steps, tests, or assays that may be prepared or run by the device may include imaging, including microscopy, cytometry, and other techniques preparing or utilizing images. Steps, tests, or assays that may be prepared or run by the device

may further include an assessment of histology, morphology, kinematics, dynamics, and/or state of a sample, which may include such assessment for cells.

[0262] A device may be capable of performing all on-board steps (e.g., steps or actions performed by a single device) in a short amount of time. A device may be capable of performing all on-board steps on a single sample in a short amount of time. For example, from sample collection from a subject to transmitting data and/or to analysis may take about 3 hours or less, 2 hours or less, 1 hour or less, 50 minutes or less, 45 minutes or less, 40 minutes or less, 30 minutes or less, 20 minutes or less, 15 minutes or less, 10 minutes or less, 5 minutes or less, 4 minutes or less, 3 minutes or less, 2 minutes or less, or 1 minute or less. The amount of time from accepting a sample within the device to transmitting data and/or to analysis from the device regarding such a sample may depend on the type or number of steps, tests, or assays performed on the sample. The amount of time from accepting a sample within the device to transmitting data and/or to analysis from the device regarding such a sample may take about 3 hours or less, 2 hours or less, 1 hour or less, 50 minutes or less, 45 minutes or less, 40 minutes or less, 30 minutes or less, 20 minutes or less, 15 minutes or less, 10 minutes or less, 5 minutes or less, 4 minutes or less, 3 minutes or less, 2 minutes or less, or 1 minute or less.

[0263] A device may be configured to prepare a sample for disposal, or to dispose of a sample, such as a biological sample, following processing or assaying of a sample.

[0264] In embodiments, a sample processing device may be configured to transmit data obtained from a sample. In embodiments, a sample processing device may be configured to communicate over a network. A sample processing device may include a communication module that may interface with the network. A sample processing device may be connected to the network via a wired connection or wirelessly. The network may be a local area network (LAN) or a wide area network (WAN) such as the Internet. In some embodiments, the network may be a personal area network. The network may include the cloud. The sample processing device may be connected to the network without requiring an intermediary device, or an intermediary device may be required to connect a sample processing device to a network. A sample processing device may communicate over a network with another device, which may be any type of networked device, including but not limited to a personal computer, server computer, or laptop computer; personal digital assistants (PDAs) such as a Windows CE device; phones such as cellular phones, smartphones (e.g., iPhone, Android,

Blackberry, etc.), or location-aware portable phones (such as GPS); a roaming device, such as a network-connected roaming device; a wireless device such as a wireless email device or other device capable of communicating wireless with a computer network; or any other type of network device that may communicate possibly over a network and handle electronic transactions. Such communication may include providing data to a cloud computing infrastructure or any other type of data storage infrastructure which may be accessed by other devices.

[0265] A sample processing device may provide data regarding a sample to, e.g., a health care professional, a health care professional location, such as a laboratory, or an affiliate thereof. One or more of a laboratory, health care professional, or subject may have a network device able to receive or access data provided by the sample processing device. A sample processing device may be configured to provide data regarding a sample to a database. A sample processing device may be configured to provide data regarding a sample to an electronic medical records system, to a laboratory information system, to a laboratory automation system, or other system or software. A sample processing device may provide data in the form of a report.

[0266] A laboratory, device, or other entity or software may perform analysis on data regarding a sample in real-time. A software system may perform chemical analysis and/or pathological analysis, or these could be distributed amongst combinations of lab, clinical, and specialty or expert personnel. Analysis may include qualitative and/or quantitative evaluation of a sample. Data analysis may include a subsequent qualitative and/or quantitative evaluation of a sample. Optionally, a report may be generated based on raw data, pre-processed data, or analyzed data. Such a report may be prepared so as to maintain confidentiality of the data obtained from the sample, the identity and other information regarding the subject from whom a sample was obtained, analysis of the data, and other confidential information. The report and/or the data may be transmitted to a health care professional. Data obtained by a sample processing device, or analysis of such data, or reports, may be provided to a database, an electronic medical records system, to a laboratory information system, to a laboratory automation system, or other system or software.

[0267] Description and disclosure of examples of reagents, assays, methods, kits, devices, and systems which may use, or be used with, methods, compositions, or other reagents disclosed herein may be found, for example, in U.S. Patent 8,088,593; U.S. Patent 8,380,541;

U.S. Pat. App. Ser. No. 13/769,798, filed February 18, 2013; U.S. Pat. App. Ser. No. 13/769,779, filed February 18, 2013; U.S. Pat. App. Ser. No. 13/244,947 filed Sept. 26, 2011; International Application No. PCT/US2012/57155, filed September 25, 2012; U.S. Application Serial No. 13/244,946, filed September 26, 2011; U.S. Patent Application 13/244,949, filed September 26, 2011; and U.S. Application Serial No. 61/673,245, filed September 26, 2011, International Application No. PCT/US14/30034, filed March 15, 2014, and U.S. Patent Application No. 14/214,850, filed March 15, 2014, the disclosures of which patents and patent applications are all hereby incorporated by reference in their entireties.

[0268] This application claims the benefit of, and priority to U.S. Provisional Patent Application No. 61/908,027, filed November 22, 2013; U.S. Provisional Patent Application No. 62/001,050, filed May 20, 2014; U.S. Non-Provisional Patent Application No. 14/214,850, filed March 15, 2014; and International Patent Application No. PCT/US14/30034, filed March 15, 2014, the disclosure of each of which is hereby incorporated by reference in its entirety for all purposes. The disclosure of U.S. Provisional Patent Application No. 61/800,606, filed March 15, 2013 is also hereby incorporated by reference in its entirety for all purposes.

[0269] **Examples**

[0270] The following examples are offered for illustrative purposes only, and are not intended to limit the present disclosure in any way.

[0271] Example 1 – Amplification of a template nucleic acid with primers of variable tail region length

[0272] A method as provided herein was used to amplify a target nucleic acid. Reactions were prepared to assay for about an 102 nucleotide portion of target nucleic acid T124A1, which is a 464 nucleotide portion of an influenza A virus hemagglutinin (HA3) gene (an RNA molecule). The nucleotide sequence of T124A1 is provided in SEQ ID NO: 89. 12 variants of a first primer ("P1") and 12 variants of a second primer ("P2") were prepared. The sequences of all of the primer variants are provided in Figure 2A. All of the primers contained a template-binding region 15 nucleotides in length. The sequence of the template-binding region of all of the variants of the first primer was: CAAACCGTACCAACC (SEQ ID NO: 80) (in the 5'-3' direction). The sequence of the template-binding region of all of the variants of the second primer was: ATGCGGAATGTACC (SEQ ID NO: 81) (in the 5'-3'

direction). The different primer variants contained tail regions ranging from 8 to 22 nucleotides in length, specifically 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, or 22 nucleotides in length. The tail region of the first primer had a base sequence of: CGCCGGATGGCTCTTGGGAAAC (SEQ ID NO: 82) (in the 5'-3' direction). The base sequence is 22 nucleotides in length; the shorter tail regions of the first primer have the same sequence, minus the appropriate number of nucleotides from the 5' end of the tail region (e.g. the primer with the 18 nucleotide length tail region has a tail region with the same sequence as the base sequence, minus the first 4 nucleotides (CGCC) of the base sequence). The tail region of the second primer had a base sequence of: GTTTCCCAAGAGCCATCCGGCG (SEQ ID NO: 83) (in the 5'-3' direction). The base sequence is 22 nucleotides in length; the shorter tail regions of the first primer have the same sequence, minus the appropriate number of nucleotides from the 3' end of the tail region (e.g. the primer with the 18 nucleotide length tail region has a tail region with the same sequence as the base sequence, minus the last 4 nucleotides (GGCG) of the base sequence).

[0273] 150 microliter reaction mixtures were prepared, each containing: 50 mM potassium acetate, 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 1 mM DTT, 30 μg bovine serum albumin (BSA), 0.8 M betaine, 1.4 mM each of dATP, dTTP, dGTP, and dCTP, 2 uM SYTO ® 59 (Life Technologies), 0.8 units / μl Bst DNA polymerase (New England BioLabs), 0.016 units / μl AMV reverse trancriptase enzyme (New England Biolabs), 1 unit / μl murine RNase inhibitor (New England Biolabs), 0.8 μM of a first primer variant, 0.8 μM of a second primer variant, and 100,000 copies T124A1 template per microliter, and incubated at 59 C for 100 minutes in a CFX 96 Touch instrument (Bio-Rad). The inflection points for the assays were determined using a single-threshold method with CFX Manager software (Bio-Rad), and are shown in Figure 2B. The X-axis provides the length in nucleotides of the tail region of the first and second primer used in the reaction, and the Y-axis provides the inflection time (in minutes) of the assay. For each tail region nucleotide length, two adjacent bars are shown: the left bar is the inflection time for the reaction containing template, and the right bar is the inflection time for the reaction lacking template ["no template control" ("NTC")]. For inflection times over 90 minutes, no bar is shown. As shown in Fig 2B, under these reaction conditions, primers with tail regions of 8-15 nucleotides, and, more particularly, 8-11 nucleotides supported the fastest inflection times.

The no template control reactions eventually show an inflection time; this is due to background non-specific products that are formed over time.

[0274] Example 2 – Amplification of a template nucleic acid with primers of variable tail region length and variable template-binding region length

[0275] A method as provided herein was used to amplify a target nucleic acid. Reactions were prepared to assay for target nucleic acid T124A1, which is described above in Example 1. Two different groups of first primer and second primer pair sets were prepared. In the first group of primer pairs, each of the primers had a template binding region 20 nucleotides in length (the "20 nucleotide template binding region" group). In the second group of primer pairs, each of the primers had a template binding region 16 nucleotides in length (the "16 nucleotide template binding region" group). Within each group, first and second primer pair sets with 8 different tail region lengths were prepared: 7, 8, 9, 10, 11, 12, 14, and 16 nucleotides. Figure 3 provides the nucleotide sequences of the different primer pair sets for the 16 nucleotide template binding region group (Fig. 3A) and the 20 nucleotide template binding region group (Fig. 3B). In Figs. 3A and 3B, the "tail" sequence refers to the tail region, and the "primer" region refers to the template-binding region of the primer. The sequences of the primers are shown in the 5'-3' direction.

[0276] 110 microliter reaction mixtures were prepared, each containing: 50 mM potassium acetate, 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 1 mM DTT, 30 μg bovine serum albumin (BSA), 0.8 M betaine, 1.4 mM each of dATP, dTTP, dGTP, and dCTP, 2 uM SYTO ® 59 (Life Technologies), 0.8 units / μl Bst DNA polymerase (New England BioLabs), 0.016 units / μl AMV reverse trancriptase enzyme (New England Biolabs), 1 unit / μl murine RNase inhibitor (New England Biolabs), 0.8 μM of a first primer variant, 0.8 μM of a second primer variant, and 100,000 copies T124A1 template per microliter, and incubated at 56 C for 100 minutes in a CFX 96 Touch instrument (Bio-Rad). The inflection points for the assays are shown in Figures 3C (20 nucleotide template binding region group) and 3D (16 nucleotide template binding region group). In both Figures 3C and 3D, the X-axis provides the length in nucleotides of the tail region of the first and second primer used in the reaction, and the Y-axis provides the inflection time (in minutes) of the assay. For each tail region nucleotide length, two adjacent bars are shown: the left bar is the inflection time for the reaction containing template, and the right bar is the inflection time for the reaction lacking template. As shown in Figure 3, under these reaction conditions, reactions containing

primers having relatively shorter tail regions showed faster inflection times and greater separation between the inflection time of reactions containing template versus reactions without template. For example, for both the 16bp and 20 bp primers, reactions containing primers having a tail region length of 7, 8, or 9 nucleotides showed faster inflection times than reactions containing primers having a tail region length of 12, 14, or 16 nucleotides.

[0277] Example 3 – Amplification of a template nucleic acid at variable temperatures

[0278] A method as provided herein was used to amplify a target nucleic acid. Reactions were prepared to assay for target nucleic acid T124A1, described above in Example 1. First primer "RLX0892" (sequence: 5' TTGGGAAACCAAACCGTACCAACC 3')(SEQ ID NO: 20) and second primer "RLX0893" (sequence: 5' GTTTCCCAAATGCGGAATGTACC 3')(SEQ ID NO: 32) were used to amplify T124A1. Both of these primers have a 9 nucleotide tail region, RLX0892 has a 15 nucleotide template-binding region and RLX0893 has a 14 nucleotide template-binding region. The tail region of RLX0892 is: 5' TTGGGAAAC 3' (SEQ ID NO: 84) and the tail region of RLX0893 is: 5' GTTTCCCAA 3' (SEQ ID NO: 85).

[0279] 80 microliter reaction mixtures were prepared, each containing: 50 mM potassium acetate, 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 1 mM DTT, 20 μg bovine serum albumin (BSA), 0.8 M betaine, 1.4 mM each of dATP, dTTP, dGTP, and dCTP, 2 uM SYTO ® 59 (Life Technologies), 0.8 units / μl Bst DNA polymerase (New England BioLabs), 0.016 units / μl AMV reverse trancriptase enzyme (New England Biolabs), 1 unit / μl murine RNase inhibitor (New England Biolabs), 0.8 μM of first primer RLX0892, 0.8 μM of second primer RLX0893, and 10,000, 1,000, 100 or 0 copies T124A1 template per microliter, and incubated in triplicate at 52, 52.7, 54, 55.9, 58.4, 60.3, 61.4 or 62 C for 100 minutes in a CFX 96 Touch instrument (Bio-Rad). The inflection points for the assays are shown in Figure 4. The X-axis provides the incubation temperature of the reaction, and the Y-axis provides the inflection time (in minutes) of the assay. For each temperature, 4 adjacent bars are shown, from left to right: 10,000 copies template / microliter, 1000 copies template / microliter, 100 copies template / microliter, or no template control. As shown in Figure 4, under these reaction conditions, the assays effectively amplified the template at 1000 copies template / microliter across the full range of temperatures from 52-62 C, and at some temperatures, at concentrations of template at least as low as 100 copies template / microliter.

[0280] Example 4 – Amplification of a template nucleic acid in the presence of human genomic DNA

[0281] A method as provided herein was used to amplify a target nucleic acid. Reactions were prepared to assay for target nucleic acid T124A1 (described above) in the presence of human DNA. First primer "RLX0892" (described above) and second primer "RLX0893" (described above) were used to amplify T124A1.

[0282] 160 microliter reaction mixtures were prepared, each containing: 50 mM potassium acetate, 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 1 mM DTT, 20 μg bovine serum albumin (BSA), 0.8 M betaine, 1.4 mM each of dATP, dTTP, dGTP, and dCTP, 0.4 x SYTO ® 59 (Life Technologies), 0.8 units / μl Bst DNA polymerase (New England BioLabs), 0.016 units / μl AMV reverse trancriptase enzyme (New England Biolabs), 1 unit / μl murine RNase inhibitor (New England Biolabs), 0.8 μM of first primer RLX0892, 0.8 μM of second primer RLX0893, and 1,000, 100 or 0 copies T124A1 template per microliter, and 0, 1, 2.5, 5, or 10 nanograms human genomic DNA, and incubated at 56 C for 100 minutes in a CFX 96 Touch instrument (Bio-Rad). The inflection points for the assays are shown in Figure 5. The X-axis provides the quantity of human genomic DNA ("hDNA") in the reaction, and the Y-axis provides the inflection time (in minutes) of the assay. For each quantity of hDNA, 3 adjacent bars are shown, from left to right: 1000 copies template / microliter, 100 copies template / microliter, or no template. As shown in Figure 5, under these reaction conditions, the assays effectively amplified the template at 1000 copies template / microliter in the presence of at least 10 ng hDNA. Also, the addition of hDNA the reactions caused only a relatively small decrease in NTC inflection times, even at concentrations of 5 or 10 ng hDNA.

[0283] Example 5 – Amplification of a template nucleic acid from a viral lysate eluate

[0284] A method as provided herein was used to amplify a target nucleic acid from two different samples containing the target. Target nucleic acid T124A1 was prepared in two samples: 1) a sample containing isolated T124A1 RNA molecule (as in Example 1), and 2) a sample containing isolated nucleic acids from an influenza A H3N2 viral sample. T124A1 is a portion of the HA3 gene, and therefore is expected to be present in a sample of isolated nucleic acids from influenza A H3N2 virus. Influenza A viral lysate was prepared with a Chemagic viral DNA/RNA kit (PerkinElmer), according to the manufacturer's instructions.

The concentration of T124A1 in the two different samples was quantified by qPCR, and the samples were diluted to normalize the concentration of T124A1 in the samples. First primer "RLX0892" and second primer "RLX0893" were used to amplify T124A1.

[0285] 25 microliter reaction mixtures were prepared, each containing: 50 mM potassium acetate, 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 1 mM DTT, 20 μg bovine serum albumin (BSA), 0.8 M betaine, 1.4 mM each of dATP, dTTP, dGTP, and dCTP, 2 uM SYTO ® 59 (Life Technologies), 0.8 units / μl Bst DNA polymerase (New England BioLabs), 0.016 units / μl AMV reverse trancriptase enzyme (New England Biolabs), 1 unit / μl murine RNase inhibitor (New England Biolabs), 0.8 μM of first primer RLX0892, 0.8 μM of second primer RLX0893, and 10,000, 1000 or 0 copies T124A1 template per microliter, and incubated at 56 C for 100 minutes in a CFX 96 Touch instrument (Bio-Rad). The inflection points for the assays are shown in Figure 6. The X-axis provides the copies of template / microliter in the reaction, and the Y-axis provides the inflection time (in minutes) of the assay. For each quantity of copies of template / microliter, 2 adjacent bars are shown, from left to right: isolated T124A1, and influenza A viral lysate. The inflection point for the NTC is also shown. As shown in Figure 6, under these reaction conditions, the assays effectively amplified both isolated T124A1 template, as well as T124A1 template in an influenza A viral lysate.

[0286] Example 6 – Amplification of a template nucleic acid

[0287] A method as provided herein was used to amplify a target nucleic acid. Reactions were prepared to assay for a 54 nucleotide portion of T129D1, which is a 298 nucleotide portion of an influenza B virus hemagglutinin (HA) gene (an RNA molecule). The nucleotide sequence of T129D1 is provided in SEQ ID NO: 90. First primer "A8" (nucleotide sequence: 5' TCTTGAGAGAACCCACTAAC 3') (SEQ ID NO: 86) and second primer "B8" (nucleotide sequence: 5' TCTCAAGAATTTGGTCTTCC 3') (SEQ ID NO: 87) were used to amplify T129D1. In both of these primers, the first 8 nucleotides (from the 5' terminus) are the tail region, and the last 12 nucleotides are the template-binding region. Together, these primers target a 54 nucleotide portion of T129D1.

[0288] A 100 microliter reaction mixture was prepared, containing: 50 mM potassium acetate, 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 1 mM DTT, 20 μg bovine serum albumin (BSA), 0.8 M betaine, 1.4 mM each of dATP, dTTP, dGTP, and dCTP, 0.4 x

SYTO ® 59 (Life Technologies), 0.8 units / µl Bst DNA polymerase (New England BioLabs), 0.016 units / µl AMV reverse trancriptase enzyme (New England Biolabs), 1 unit / µl murine RNase inhibitor (New England Biolabs), 0.8 µM of first primer A8, 0.8 µM of second primer B8, and 100,000 T129D1 template per microliter, and incubated at 58 C for 100 minutes in a CFX 96 Touch instrument (Bio-Rad). After the 100 minutes, a sample was taken from the reaction, and ligated into cloning vectors. Vectors containing reaction products were sequenced using vector-specific primers, directed to toward the cloning site. A portion of the results from multiple example sequencing reactions are shown in Figure 7. The sequencing results show that concatemers having the expected structure are formed. Specifically, in these examples, in a single strand of the reaction product, multiple copies of the targeted 54 nucleotide sequence from the T129D1 gene are present, separated by the sequence of the tail region of the A8 primer (5' TCTTGAGA 3') (SEQ ID NO: 88). In Figure 7, only a portion of the second (from left to right) occurrence of the T129D1 is shown; the sequences continue beyond the nucleotides shown in the figure.

[0289] Example 7 – Primers with Tails Complementary to an Internal Motif

[0290] To further describe embodiments provided herein in which a primer tail contains a sequence which is complementary to an internal motif within a target nucleic acid, an example with exemplary nucleic acid sequences will now be provided. A polynucleotide template of 54 nucleotides total length may be provided ("Example 7 Template"). Example 7 Template corresponds to element 1000 of Figure 10. The sequence of the Example 7 Template is as follows: 5' TCGCTTGAACTGAGGACTTAGCGCCTAGAAACTTGGCTCAGGTAACCAACTGGT 3' (SEQ ID NO: 92). Describing the Example 7 Template, in the 5' to the 3' direction, the first underlined sequence is the second portion of the polynucleotide template (e.g. similar to element 1002 as provided in Figure 10), the second underlined sequence is the internal motif (e.g. corresponding to element 1003 provided in Figure 10), and the third underlined sequence is the first portion of the polynucleotide template (e.g. corresponding to element 1001 provided in Figure 10). Specific nucleotides of the Example 7 Template and other sequences provided herein may be referred to in regards to their position in the respective sequence read in the 5' to 3' direction. Thus, for example, the "T" at the 5' end of the Example 7 Template is in the 1st position, the "C" adjacent to the "T" at the 5' end is in the 2nd position, and so on. A first primer of 24 nucleotides total length may be provided

("Example 7 First Primer"). Example 7 First Primer corresponds to element 1010 of Figure 10. The sequence of the Example 7 First Primer is as follows: 5' CTAGAAACACCAGTTGGTTACCTG 3' (SEQ ID NO: 93). The underlined sequence is the first / tail region of the Example 7 First Primer (corresponding to element 1011 of Figure 10), and the non-underlined sequence is the second / template-binding region of the Example 7 First Primer (corresponding to element 1012 of Figure 10). Also of note is the fact that the first region of the Example 7 First Primer has the same nucleotide sequence as the internal motif of the Example 7 Template. Example 7 First Primer can anneal to the Example 7 Template as follows: When the Example 7 Template and Example 7 First Primer are oriented so that the 3' end of the Example 7 Template and the 5' end of the Example 7 First Primer face the same direction, the non-underlined sequence of the Example 7 First Primer (read in the 5' to 3' direction, as in ACCAG...) is annealed to the first portion of the Example 7 Template (read in the 3' to 5' direction, as in TGGTC..). More specifically, the "A" in the 9th position of the Example 7 First Primer pairs with the "T" in the 54th position of the Example 7 Template, the "C" in the 10th position of the Example 7 First Primer pairs with the "G" in the 53rd position of the Example 7 Template, the "C" in the 11th position of the Example 7 First Primer pairs with the "G" in the 52nd position of the Example 7 Template, etc. When the Example 7 First Primer is annealed to the Example 7 Template, a polymerase having strand displacement activity may extend the Example 7 First Primer, as described elsewhere herein, to generate an Example 7 First Primer Extension Product. The Example 7 First Primer Extension Product will have the nucleotide sequence in the 5' to 3' direction of: 5' CTAGAAACACCAGTTGGTTACCTGAGCCAAGTTTCTAGGCGCTAAGTCCTCAGTTCAAGCGA 3' (SEQ ID NO: 94). Describing the Example 7 First Primer Extension Product , in the 5' to 3' direction, the first non-underlined sequence is the sequence of the first region of the Example 7 First Primer, the first underlined sequence is a sequence which is complementary to the first portion of the Example 7 Template, the second underlined sequence is the sequence of the internal motif of the Example 7 First Primer Extension Product, and the third underlined sequence is a sequence which is complementary to the second portion of the Example 7 Template. In embodiments, when the Example 7 First Primer Extension Product is annealed to the Example 7 Template, in some circumstances, the portion of the Example 7 First Primer Extension Product which is the sequence of the first region of the Example 7 First Primer may temporarily anneal to the internal motif of the

Example 7 First Primer Extension Product, such that the "C" in the 1st position anneals to the "G" in the 38th position of the Example 7 First Primer Extension Product, the "T" in the 2nd position anneals to the "A" in the 37th position, the "A" in the 3rd position anneals to the "T" in the 36th position, etc., up to the "C" in the 8th position annealing to the "G" in the 31st position. When the portion of the Example 7 First Primer Extension Product which is the sequence of the first region of the Example 7 First Primer temporarily anneals to the portion of the internal motif of the Example 7 First Primer Extension Product, the portion of the Example 7 First Primer Extension Product between these portions (including, for example, the sequence which is complementary to the first portion of the Example 7 Template) may become un-annealed from the Example 7 Template, thus facilitating, for example, the binding of another copy of the Example 7 First Primer to the Example 7 Template, and the subsequent generation of additional Example 7 First Primer Extension Products.

[0291] A second primer of 24 nucleotides total length may also be provided ("Example 7 Second Primer"). Example 7 Second Primer corresponds to element 1020 of Figure 10. The sequence of the Example 7 Second Primer is as follows: 5' GTTTCTAGTCGCTTGAACTGAGGA 3' (SEQ ID NO: 95). The underlined sequence is the first region of the Example 7 Second Primer (corresponding to element 1021 of Figure 10), and the non-underlined sequence is the second region of the Example 7 Second Primer (corresponding to element 1022 of Figure 10). The Example 7 Second Primer may anneal to the Example 7 First Primer Extension Product as follows: The "T" in the 9th position of the Example 7 Second Primer pairs with the "A" in the 62nd position of the Example 7 First Primer Extension Product, the "C" in the 10th position of the Example 7 Second Primer pairs with the "G" in the 61st position of the Example 7 First Primer Extension Product, the "G" in the 11th position of the Example 7 Second Primer pairs with the "C" in the 60th position of the Example 7 First Primer Extension Product, etc. The Example 7 Second Primer may be extended by a polymerase having strand displacement activity as described elsewhere herein, to yield an Example 7 Second Primer Extension Product. The Example 7 Second Primer Extension Product will have the nucleotide sequence in the 5' to 3' direction of: 5' GTTTCTAGTCGCTTGAACTGAGGACTTAGCGCCTAGAAACTTGGCTCAGGTAACC AACTGGTGTTTCTAG 3' (SEQ ID NO: 96). Describing the Example 7 Second Primer Extension Product, in the 5' to 3' direction, the first non-underlined sequence is the sequence of the first region of the Example 7 Second Primer, the first underlined sequence the sequence of the second portion of the Example 7 Template, the second underlined sequence

is the sequence of the internal motif of the Example 7 Second Primer Extension Product, the third underlined sequence is the sequence of the first portion of the Example 7 Template, and the fourth non-underlined sequence is the sequence of the first region of the Example 7 Second Primer and the sequence of a sequence which is complementary to the first region of the Example 7 First Primer. In embodiments, when the Example 7 Second Primer Extension Product is annealed to the Example 7 First Primer Extension Product, in some circumstances, the portion of the Example 7 Second Primer Extension Product which is the sequence of the first region of the Example 7 Second Primer may temporarily anneal to the internal motif of the Example 7 Second Primer Extension Product, such that the "G" in the 1st position anneals to the "C" in the 40th position of the Example 7 Second Primer Extension Product, the "T" in the 2nd position anneals to the "A" in the 39th position, the "T" in the 3rd position anneals to the "A" in the 38th position, etc., up to the "G" in the 8th position annealing to the "C" in the 33rd position. When the portion of the Example 7 Second Primer Extension Product which is the sequence of the first region of the Example 7 Second Primer temporarily anneals to the internal motif of the Example 7 Second Primer Extension Product, the portion of the Example 7 Second Primer Extension Product between these portions (including, for example, the sequence of the second portion of the Example 7 Template) may become un-annealed from the Example 7 First Primer Extension Product, thus facilitating, for example, the binding of another copy of the Example 7 Second Primer to the Example 7 First Primer Extension, and the subsequent generation of additional Example 7 Second Primer Extension Products.

[0292] In embodiments, methods provided elsewhere herein may proceed according to the general mechanisms outlined above. Any of the descriptions and details provided elsewhere herein for primers, target nucleic acids, reaction conditions, reaction products, etc. may apply to reactions involving the general mechanism described above in Example 7.

[0293] Example 8 – SNP Detection

[0294] A method provided herein was used for identifying a SNP / mutation in a target nucleic acid. Two versions of the target nucleic acid were provided, which differed by a single nucleotide. The first strand of Version 1 of the nucleic acid template ("Version 1 Template"; also referred to as the "wild-type" sequence) has the sequence, in the 5' to 3' direction:

GCATTCATCACGTTTTTGCGAGTCCTTTCC*A*TCCCACCAACAGCAGGGATTCTGA AGAGATGGGGACAGTT (SEQ ID NO: 97). Describing the Version 1 Template, in the 5'

to 3' direction, the first underlined sequence is the second portion of the Version 1 Template, the second underlined sequence is the internal motif of the Version 1 Template, and the third underlined sequence is the first portion of the Version 1 Template. The first strand of Version 2 of the nucleic acid template ("Version 2 Template"; also referred to as the "SNP" sequence) has the sequence, in the 5' to 3' direction: GCATTCATCACGTTTTTGCGAGTCCTTTCC***T***TCCCACCAACAGCAGGGATTCTGAAGAGATGGGGACAGTT (SEQ ID NO: 98). Describing the Version 2 Template, in the 5' to 3' direction, the first underlined sequence is the second portion of the Version 2 Template, the second underlined sequence is the internal motif of the Version 2 Template, and the third underlined sequence is the first portion of the Version 2 Template. The Version 1 Template and the Version 2 Template sequences differ only in a single nucleotide in the internal motif sequence of the respective Templates. Specifically, the Version 1 Template has an "A" in the 31st position in the sequence (emphasized with bold and italicized font), whereas the Version 2 Template has a "T" in the 31st position in the sequence (emphasized with bold and italicized font). A first primer and a second primer were also provided to amplify the Template sequences. The first primer ("Example 8 Primer 1") has a sequence of: 5' TCCATCCCAACTGTCCCCATCT 3' (SEQ ID NO: 99). The underlined sequence is the first / tail region of the Example 8 Primer 1, and the non-underlined sequence is the second / template-binding region of the Example 8 Primer 1. The second primer ("Example 8 Primer 2") has a sequence of: 5' GGGATGGAGCATTCATCACGTTT 3' (SEQ ID NO: 100). The underlined sequence is the first / tail region of the Example 8 Primer 2, and the non-underlined sequence is the second / template-binding region of the Example 8 Primer 2.

[0295] Separate amplification reactions as described elsewhere herein were performed for either the Version 1 Template or the Version 2 Template, with the reactions for both the Version 1 Template and the Version 2 Template using Example 8 Primer 1 and Example 8 Primer 2 as the primers. Multiple reactions under the same conditions were performed for each Template.

[0296] In the Version 1 Template reactions, upon the annealing of the Example 8 Primer 1 to the Version 1 Template and the generation by a polymerase of an extension product of the Example 8 Primer 1 ("Version 1 Template Primer 1 Extension Product"), the Version 1 Template Primer 1 Extension Product will have a sequence as follows: 5' TCCATCCCAACTGTCCCCATCTCTTCAGAATCCCTGCTGTTGGTGGGA***T***GGAAAG

GACTCGCAAAAACGTGATGAATGC (SEQ ID NO: 101). Describing the Version 1 Template Primer 1 Extension Product , in the 5' to 3' direction, the first non-underlined sequence is the sequence of the first / tail region of the Example 8 Primer 1, the first underlined sequence is a sequence which is complementary to the first portion of the Version 1 Template, the second underlined sequence is the sequence of the internal motif of the Version 1 Template Primer 1 Extension Product (where the bold, italicized "T" is the nucleotide of interest), and the third underlined sequence is a sequence which is complementary to the second portion of the Version 1 Template. According to methods described herein, in embodiments, the portion of the Version 1 Template Primer 1 Extension Product corresponding to the sequence of the first / tail region of the Example 8 Primer 1 sequence may temporarily anneal to the internal motif of the Version 1 Template Primer 1 Extension Product, such that the "T" in the 1st position anneals to the "A" in the 52nd position, the "C" in the 2nd position anneals to the "G" in the 51st position, the "C" in the 3rd position anneals to the "G" in the 50th position, etc., up to the "C" in the 8th position annealing to the "G" in the 45th position. Of note, the portion of the Version 1 Template Primer 1 Extension Product corresponding to the sequence of the first / tail region of the Example 8 Primer 1 sequence is perfectly complementary to the internal motif sequence of the Version 1 Template Primer 1 Extension Product (i.e. there are no mis-matches between any of the 8 sets of paired nucleotides).

[0297] In the Version 2 Template reactions, upon the annealing of the Example 8 Primer 1 to the Version 2 Template and the generation by a polymerase of an extension product of the Example 8 Primer 1 ("Version 2 Template Primer 1 Extension Product"), the Version 2 Template Primer 1 Extension Product will have a sequence as follows: 5' TCCATCCCAACTGTCCCCATCTCTTCAGAATCCCTGCTGTTGGTGGGA*A*GGAAAG GACTCGCAAAAACGTGATGAATGC (SEQ ID NO: 102). Describing the Version 2 Template Extension Product , in the 5' to 3' direction, the first non-underlined sequence is the sequence of the first / tail region of the Example 8 Primer 1, the first underlined sequence is a sequence which is complementary to the first portion of the Version 2 Template, the second underlined sequence is the sequence of the internal motif of the Version 2 Template Primer 1 Extension Product (where the bold, italicized "A" is the nucleotide of interest), and the third underlined sequence is a sequence which is complementary to the second portion of the Version 2 Template. According to methods described herein, in embodiments, the portion of the Version 2 Template Primer 1 Extension Product corresponding to the sequence

of the first / tail region of the Example 8 Primer 1 sequence may temporarily anneal to the internal motif of the Version 2 Template Primer 1 Extension Product, such that the "T" in the 1st position anneals to the "A" in the 52nd position, the "C" in the 2nd position anneals to the "G" in the 51st position, the "C" in the 3rd position anneals to the "G" in the 50th position, etc., up to the "C" in the 8th position annealing to the "G" in the 45th position. Of note, the portion of the Version 2 Template Primer 1 Extension Product corresponding to the sequence of the first / tail region of the Example 8 Primer 1 sequence is not perfectly complementary to the internal motif sequence of the Version 2 Template Primer 1 Extension Product, as there is a mis-match between the "A" in the 4th position and the "A" in the 49th position. Thus, comparing the Version 1 Template Primer 1 Extension Product to the Version 2 Template Primer 1 Extension Product, the portion of the respective Extension Product corresponding to the first / tail region of the Example 8 Primer 1 will have greater complementation to and will more stably anneal to the internal motif in the Version 1 Template Primer 1 Extension Product than in Version 2 Template Primer 1 Extension Product. In particular, the sequence "TCCATCCC" in the Version 1 Template Primer 1 Extension Product is perfectly complementary to the internal motif in the Version 1 Template Primer 1 Extension Product (8 out of 8 nucleotides can properly pair), whereas the sequence "TCCATCCC" in the Version 2 Template Primer 1 Extension Product is not perfectly complementary to the internal motif in the Version 2 Template Primer 1 Extension Product (only 7 out of 8 nucleotides can properly pair). Typically, greater complementarity between the portion of an Extension Product corresponding to a primer tail and an internal motif in the Extension Product results in a more stable annealing between these two portions. When an Extension Product is configured such that these two portions are annealed, it may accelerate the rate of a reaction provided herein, for example, by permitting the binding of a new primer to un-annealed nucleotides in a complementary strand exposed by the internal annealing of regions of an Extension Product or by the increased rate of concatemer formation according to methods provided herein.

[0298] A similar mechanism as described above also occurs for the Version 1 Template and Version 2 Template Primer 2 Extension Products, where the portion of the tail region of the Extension Products is perfectly complementary for the Version 1 Template Primer 2 Extension Product, but is not perfectly complementary for the Version 2 Template Primer 2 Extension Product.

[0299] Figure 11 shows the results of the amplification reactions with these templates and primers, where the X-axis shows time and the Y-axis shows relative fluorescence units (RFU) (indicating amplified DNA). As shown in Figure 11, the amplification reactions with the Version 1 Template ("wild-type" sequence) occurred more quickly than reactions with the Version 2 Template ("SNP" sequence). Thus, these results show that methods provided herein can be used, for example, to distinguish between sequences having single-nucleotide variations.

[0300] Example 9 – SNP Detection

[0301] A method provided herein was used for identifying a SNP / mutation in a human target nucleic acid. The Q356R variant of the BRCA1 protein may be associated with an increased risk of cancer. The Q356R variant may occur, for example, when the codon (nucleotide sequence CAG) encoding the glutamine at the 356 amino acid position of the wild-type BRCA1 protein instead has the nucleotide sequence CGG, so that the codon encodes an arginine instead of glutamine.

[0302] Four different versions of a portion of the BRCA1 genomic sequence which contains, among other nucleotides, the codon for the 356 amino acid position were obtained. The first version ("Wild-Type Target") contains the wild-type codon encoding the glutamine at the 356 amino acid position (sequence CAG), and has the nucleotide sequence, in the 5' to 3' direction:
AAGATGTTCCTTGGATAACACTAAATAGCAGCATTC*A*GAAAGTTAATGAGTGGTT TTCCAGA (SEQ ID NO: 103). The second version ("Q356R Target") contains a codon encoding an arginine at the 356 amino acid position (sequence CGG), and has the nucleotide sequence, in the 5' to 3' direction:
AAGATGTTCCTTGGATAACACTAAATAGCAGCATTC*G*GAAAGTTAATGAGTGGTT TTCCAGA (SEQ ID NO: 104). The third version ("Q356P Target") contains a codon encoding a proline at the 356 amino acid position (sequence CCG), and has the nucleotide sequence, in the 5' to 3' direction:
AAGATGTTCCTTGGATAACACTAAATAGCAGCATTC*C*GAAAGTTAATGAGTGGTT TTCCAGA (SEQ ID NO: 105). The fourth version ("Q356L Target") contains a codon encoding a leucine at the 356 amino acid position (sequence CTG), and has the nucleotide sequence, in the 5' to 3' direction:
AAGATGTTCCTTGGATAACACTAAATAGCAGCATTC*T*GAAAGTTAATGAGTGGTT

TTCCAGA (SEQ ID NO: 106). In each of the above four Targets, in the 5' to 3' direction, the first underlined sequence is the second portion of the Target, the second underlined sequence is the internal motif of the Target, and the third underlined sequence is the first portion of the Target.

[0303] The above four Target sequences differ by only a single nucleotide in the internal motif sequence of the respective Targets. Specifically, the Wild-Type Target has an "A" in the 37th position in the sequence (emphasized with bold and italicized font), whereas the Q356R Target has a "G" in the 37st position in the sequence, the Q356P Target has a "C" in the 37st position in the sequence, and the Q356L Target has a "T" in the 37st position in the sequence. A first primer and a second primer were also provided to amplify the Target sequences. The first primer ("Example 9 Primer 1") has a sequence of: 5' ATTCAGAATCTGGAAAACCACTC 3' (SEQ ID NO: 107). The underlined sequence is the first / tail region of the Example 9 Primer 1, and the non-underlined sequence is the second / template-binding region of the Example 9 Primer 1. The second primer ("Example 9 Primer 2") has a sequence of: 5' TTCTGAATAAGATGTTCCTTGGAT 3' (SEQ ID NO: 108). The underlined sequence is the first / tail region of the Example 9 Primer 2, and the non-underlined sequence is the second / template-binding region of the Example 9 Primer 2.

[0304] Separate amplification reactions as described herein were performed for each of the above four Targets, with the reactions for all four Targets using Example 9 Primer 1 and Example 9 Primer 2 as the primers. The conditions for the reactions were as follows: 50 mM potassium acetate, 20 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 1 mM DTT, 0.8 M betaine, 2 uM SYTO ® 59 (Life Technologies), 0.8 units / µl Bst DNA polymerase (New England BioLabs), 0.8 µM of Example 9 Primer 1, 0.8 µM of Example 9 Primer 2, and 10 copies Target molecule / µl. The reactions were held at 56 C for 90 minutes, and the inflection point of each reaction determined using a single-threshold method with CFX Manager software (Bio-Rad). Multiple reactions under the same conditions were performed for each Target.

[0305] The amplification reactions occurred by mechanisms described elsewhere herein, for example, as in Example 8. For example, Example 9 Primer 1 and Example 9 Primer 2 extension products were formed from each of the above Target nucleic acids, in each respective reaction, to form, for example a Wild-Type Target Example 9 Primer 1 Extension Product, a Q356R Target Example 9 Primer 1 Extension Product, a Q356P Target Example 9

Primer 1 Extension Product, and a Q356L Target Example 9 Primer 1 Extension Product, and corresponding extension products from each target from Example 9 Primer 2.

[0306] Of note, whereas the portion of, for example, the Wild-Type Target Example 9 Primer 1 Extension Product corresponding to the first / tail region of the Example 9 Primer 1 is perfectly complementary to the internal motif in the Wild-Type Target Example 9 Primer 1 Extension Product, the portion of the Extension Product corresponding to the first / tail region of the Example 9 Primer 1 for each of the Q356R Target, Q356P Target, and Q356L Target Primer 1 Extension Products is not perfectly complementary to the internal motif in the respective Extension Product. Specifically, there is a single-nucleotide mis-match at the position involving the nucleotide in the 37th position of the target sequence, as described above. This mismatch may affect the rate of a reaction provided herein.

[0307] The average inflection time of the assays with each of the different Targets was as follows: Wild-Type Target: 24.5 minutes; Q356R Target: 49.5 minutes, Q356P Target: 55.7 minutes, and Q356L Target: 59.8 minutes. Thus, as indicated by the inflection times of the assays, the reactions can readily differentiate between targets which vary by a single nucleotide in an internal motif. The significantly faster inflection time of the assay with the Wild-Type Target as compared to the other Targets may be a result of, for example, the perfect complementation between the portion of the Wild-Type Target Example 9 Primer 1 Extension Product corresponding to the first / tail region of the Example 9 Primer 1 and the internal motif in the Wild-Type Target Example 9 Primer 1 Extension Product, in contrast to the imperfect complementation between the portion of the Q356R Target, Q356P Target, and Q356L Target Example 9 Primer 1 Extension Product which corresponds to the first / tail region of the Example 9 Primer 1 and the internal motif in the respective Example 9 Primer 1 Extension Product. A similar reasoning also applies to the extension products of the Primer 2 Extension Products.

[0308] Example 10 – Influenza N9 Gene Amplification

[0309] A method provided herein was used to amplify a portion of the neuraminidase gene (N9) from a sample containing Influenza A virus, subtype H7N9. The target sequence from N9 was, in the 5' to 3' direction (in DNA form):
AACTTTAAGGACGAACAAGAGAGAAGGTAAACCTACAACCACTACAAACAAAGT
AATATTATCAACAAATAATAACAAACCGAACACAACCAACAAAGT (SEQ ID NO: 109). The first primer used to amplify the template had the sequence, in the 5' to 3'

direction: CTTCCATTTGAAACAACCAACAC (SEQ ID NO: 110). Describing the first primer, the underlined region is the tail / first region of the primer and the non-underlined region is the second / template-binding region. The second primer used to amplify the template had the sequence, in the 5' to 3' direction: AATGGAAGTTGAAATTCCTGCTTG (SEQ ID NO: 111). Describing the first primer, the underlined region is the tail / first region of the primer and the non-underlined region is the second / template-binding region. Multiple reactions as described herein were performed using the first primer and second primer, where the reactions either contained template sequence or did not contain template sequence. The reactions containing the template had 250,000 copies template sequence per reaction. The results of the reactions are provided in Figure 12. The numbers on the X-axis are minutes, and the Y-axis values are relative fluorescence units (RFU). As shown in the graph, in the templated reactions, the RFU rapidly rises at about 20 minutes, indicating the amplification of the template sequence. The increase in RFU in the no template reactions at about 60 minutes and later is a result of non-specific background products.

[0310] Nucleotide and amino acid sequences provided herein are artificial sequences, unless otherwise noted.

[0311] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. The foregoing description is not intended to be exhaustive or to limit the invention to the precise embodiments disclosed, and other modifications and variations may be possible in light of the above teachings without departing from the invention. Any feature, whether preferred or not, may be combined with any other feature, whether preferred or not. It should also be understood that while the invention provided herein has been described herein using a limited number of terms and phrases for purposes of expediency, the invention could also be described using other terms and phrases not provided herein which also accurately describe the invention. The appended claims are not to be interpreted as including means-plus-function limitations, unless such a limitation is explicitly recited in a given claim using the phrase "means for." It should be understood that as used in the description herein and throughout the claims that follow, the meaning of "a," "an," and "the" includes plural reference unless the context clearly dictates otherwise. For example, a reference to "an assay" may refer to a single assay or multiple assays. Also, as used in the description herein and throughout the claims that follow, the meaning of "in" includes "in"

and "on" unless the context clearly dictates otherwise. As used in the description herein and through the claims that follow, a first object described as containing "at least a portion" of a second object may contain the full amount of / the complete second object. As used in the description herein and throughout the claims that follow, the terms "comprise", "include", and "contain" and related tenses are inclusive and open-ended, and do not exclude additional, unrecited elements or method steps. Also, the presence of broadening words and phrases such as "one or more," "at least," "but not limited to" or other like phrases in some instances shall not be read to mean that the narrower case is intended or required in instances where such broadening phrases may be absent. Finally, as used in the description herein and throughout the claims that follow, the meaning of "or" includes both the conjunctive and disjunctive unless the context expressly dictates otherwise. Thus, the term "or" includes "and/or" unless the context expressly dictates otherwise.

We claim:

1. A method of copying a polynucleotide template, the method comprising:

incubating the polynucleotide template in a reaction mixture comprising a first primer and a second primer, wherein:

the first primer comprises a first region and a second region, wherein the second region of the first primer comprises a nucleotide sequence which is complementary to a first portion of the polynucleotide template;

the second primer comprises a first region and a second region, wherein the second region of the second primer comprises a nucleotide sequence which is complementary to a partner nucleotide sequence, wherein the partner nucleotide sequence is complementary to a second portion of the polynucleotide template; and

upon incubation of the polynucleotide template with the first primer and the second primer, at least one concatemer strand is formed, wherein the concatemer strand comprises a 5' end and a 3' end, and comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', wherein: C' represents the nucleotide sequence of the first region of the second primer, T represents the nucleotide sequence of the polynucleotide template or an analogous sequence thereof, and X represents any number and sequence of nucleotides.

2. The method of claim 1, wherein the concatemer strand is a first concatemer strand, and wherein upon incubation of the polynucleotide template with the first primer and the second primer, a second concatemer strand is also formed, wherein the second concatemer strand comprises a 5' end and a 3' end, and comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C-X'-T'-C-T'-C, wherein: C represents the nucleotide sequence of the first region of the first primer, T' represents a nucleotide sequence which is complementary to the polynucleotide template, and X' represents a nucleotide sequence which is complementary to the nucleotide sequence of X.

3. The method of claim 1, wherein X contains a sequence having the general structure in the 5' to 3' direction of $[(C'-T)_N]$ wherein C' represents the nucleotide sequence of the first

region of the second primer, T represents the nucleotide sequence of the polynucleotide template or an analogous sequence thereof, and N is any integer between 0 and 1000.

4. The method of claim 1, wherein X represents zero nucleotides.

5. The method of claim 1, wherein the reaction mixture further comprises a DNA polymerase having strand-displacement activity.

6. The method of claim 5, wherein the reaction mixture further comprises a reverse transcriptase.

7. The method of claim 1, wherein the polynucleotide template is an RNA molecule

8. The method of claim 7, wherein the T represents the nucleotide sequence of a DNA sequence which is analogous to the RNA sequence of the polynucleotide template.

9. The method of claim 1, wherein the polynucleotide template comprises one strand of double-stranded nucleic acid template.

10. The method claim 1, wherein during the incubation of the reaction mixture, the temperature of the reaction mixture does not exceed 80 C.

11. The method of claim 1, wherein the first region of the first primer contains between 4 and 25 nucleotides.

12. The method of claim 1, wherein the first region of the first primer and the first region of the second primer contain the same number of nucleotides.

13. The method of claim 1, wherein the polynucleotide template contains between 10 and 1000 nucleotides.

14. The method of claim 1, wherein the reaction mixture further comprises a nucleic acid dye.

15. The method of claim 1, wherein the number of copies of the polynucleotide template in the reaction mixture is increased at least 10-fold within 60 minutes of initiation of the method.

16. A method of assaying for a target polynucleotide template in a biological sample, the method comprising:

A) incubating the biological sample or portion thereof in a reaction mixture comprising a first primer and a second primer, wherein:

the first primer comprises a first region and a second region, wherein the second region of the first primer comprises a nucleotide sequence which is complementary to a first portion of the polynucleotide template;

the second primer comprises a first region and a second region, wherein the second region of the second primer comprises a nucleotide sequence which is complementary to a partner nucleotide sequence, wherein the partner nucleotide sequence is complementary to a second portion of the polynucleotide template; and

upon incubation of the polynucleotide template with the first primer and the second primer, at least one concatemer strand is formed, wherein the concatemer strand comprises a 5' end and a 3' end, and comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', wherein: C' represents the nucleotide sequence of the first region of the second primer, T represents the nucleotide sequence of the polynucleotide template or an analogous sequence thereof, and X represents any number and sequence of nucleotides; and

B) measuring an amount of amplified nucleic acid in the reaction mixture of A) at one or more points after the initiation of the incubating step of A).

17. The method of claim 16, wherein the measuring an amount of amplified nucleic acid in the reaction mixture of comprises determining a level of fluorescence in the reaction mixture.

18. The method of claim 16, further comprising determine an inflection time for nucleic acid amplification in the reaction mixture.

19. The method of claim 16, wherein the biological sample or portion thereof is from a human.

20. A method of generating a concatemer comprising at least two copies of a double stranded nucleic acid template, the method comprising:

incubating in a reaction mixture at least a first template molecule and a second template molecule, wherein:

the first template molecule comprises a first nucleic acid strand and a second nucleic acid strand, wherein:

the first nucleic acid strand of the first template molecule comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: H'-S-Y_1-H', wherein: H' represents the nucleotide sequence of a first homology sequence, S represents the nucleotide sequence of a first strand of the double stranded nucleic acid template, and Y_1 represents any number and sequence of nucleotides; and

the second nucleic acid strand of the first template molecule comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: H-Y_1'-S'-H, wherein: H represents the nucleotide sequence of a second homology sequence, wherein the first homology sequence and second homology sequence are complementary to each other, Y_1' represents a nucleotide sequence which is complementary to the nucleotide sequence of Y_1, and S' represents the nucleotide

sequence of a second strand of the double stranded nucleic acid template, wherein the first strand and second strand of the double stranded nucleic acid template are complementary to each other; and

the second template molecule comprises a first nucleic acid strand and a second nucleic acid strand, wherein:

the first nucleic acid strand of the second template molecule comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: H'-S-Y_2-H', wherein: H' represents the nucleotide sequence of the first homology sequence, S represents the nucleotide sequence of the first strand of the double stranded nucleic acid template, and Y_2 represents any number and sequence of nucleotides; and

the second nucleic acid strand of the first template molecule comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: H-Y_2'-S'-H, wherein: H represents the nucleotide sequence of the second homology sequence, Y_2' represents a nucleotide sequence which is complementary to the nucleotide sequence of Y_2, and S' represents the nucleotide sequence of the second strand of the double stranded nucleic acid template; and

upon incubation of the first template molecule with the second template molecule in the reaction mixture, at least one concatemer comprising at least two copies of the double stranded nucleic acid template is formed, wherein the concatemer comprises a first concatemer strand and a second concatemer strand, wherein the first concatemer strand comprises a 5' end and a 3' end, and comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: H'-S-Y_2-H'-S-Y_1-H', wherein each of H', Y_1, S, and Y_2 represent nucleotide sequences as described above; and wherein the second concatemer strand comprises a 5' end and a 3' end, and comprises a sequence having the general structure in the 5' to 3' direction of: H-Y_1'-S'-H-Y_2'-S'-H, wherein each of H', Y_1, S, and Y_2 represent nucleotide sequences as described above.

21. The method of claim 20, wherein at least one of Y_1 and Y_2 represents 0 nucleotides.

22. The method of claim 21, wherein both of Y_1 and Y_2 represent 0 nucleotides.

23. The method of claim 20, wherein Y_1 contains a sequence having the general structure in the 5' to 3' direction of $[(H'\text{-}S)_{N1}]$ wherein H' represents the nucleotide sequence of a first homology sequence, S represents the nucleotide sequence of a first strand of the double stranded nucleic acid template, and N1 is any integer between 0 and 2000.

24. The method of claim 20, wherein Y_1 contains a sequence having the general structure in the 5' to 3' direction of $[(H'\text{-}S)_{N1}]$ wherein H' represents the nucleotide sequence of a first homology sequence, S represents the nucleotide sequence of a first strand of the double stranded nucleic acid template, and N1 is any integer between 0 and 2000, and wherein Y_2 contains a sequence having the general structure in the 5' to 3' direction of $[(H'\text{-}S)_{N2}]$ wherein H' represents the nucleotide sequence of a first homology sequence, S represents the nucleotide sequence of a first strand of the double stranded nucleic acid template, and N2 is any integer between 0 and 2000.

25. The method of claim 24, wherein N1 and N2 are different integers.

26. The method of claim 25, wherein N1 is 0, and N2 is an integer between 1 and 2000.

27. The method of claim 20, wherein the first template molecule and second template molecule are both double-stranded DNA molecules.

28. The method of claim 20, wherein the first homology sequence contains between 4 and 25 nucleotides.

29. A method for generating a concatemer comprising two or more copies of a double-stranded nucleic acid template, the method comprising:

(A) treating a primary double-stranded nucleic acid comprising the double-stranded nucleic acid template with a first copy of a first primer and a polymerase under conditions such that

an extension product of the first copy of the first primer is synthesized which is annealed to a first strand of the double-stranded nucleic acid template,

wherein

the first primer comprises a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions:

(i) a tail region comprising

(a) the 5' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide

(c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and

(ii) a template-binding region comprising

(a) the 3' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide

(c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and

the template-binding region of the first copy of the first primer anneals to the first strand of the double-stranded nucleic acid template,

(B) treating the extension product of the first copy of the first primer of step (A) with a second primer and a polymerase under conditions such that an extension product of the second primer is synthesized which is annealed to the extension product of the first copy of the first primer of step (A),

wherein

the second primer comprises a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions:

(i) a tail region comprising

(a) the 5' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide

(c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and

(ii) a template-binding region comprising

(a) the 3' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide

(c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides,

the tail region of the second primer contains a nucleotide sequence which is complementary to the nucleotide sequence of the tail region of the first primer,

the template-binding region of the second primer anneals to the extension product of the first copy of the first primer of step (A), and

the extension product of the second primer contains a 5' terminal nucleotide, a 3' terminal nucleotide, and a 3' terminal region comprising the 3' terminal nucleotide, wherein the 3' terminal region contains the same nucleotide sequence as the nucleotide sequence of the tail region of the second primer read in the 5' to 3' direction, and the final nucleotide of the 3' terminal region is the 3' terminal nucleotide of the extension product of the second primer,

(C) treating the extension product of the second primer of step (B) with a second copy of the first primer and a polymerase under conditions such that an extension product of the second copy of the first primer is synthesized which is annealed to the extension product of the second primer of step (B), to produce a first copy of a secondary nucleic acid comprising the

extension product of the second primer of step (B) and the extension product of the second copy of the first primer,

wherein

the extension product of the second copy of the first primer contains a 5' terminal nucleotide, a 3' terminal nucleotide, and a 3' terminal region comprising the 3' terminal nucleotide, wherein the 3' terminal region contains the same nucleotide sequence as the nucleotide sequence of the tail region of the first primer read in the 5' to 3' direction, and the final nucleotide of the 3' terminal region is the 3' terminal nucleotide of the extension product of the second primer,

(D) repeating at least step (C) one or more additional times to generate at least a second copy of the secondary nucleic acid of step (C),

(E) treating the first copy of the secondary nucleic acid of step (C) and the second copy of the secondary nucleic acid of step (D) under conditions such that the 3' terminal region of the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid anneals to the 3' terminal region of the extension product of the second primer of the second copy of the secondary nucleic acid, to produce a cross-over structure comprising the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid and the extension product of the second primer of the second copy of the secondary nucleic acid,

(F) treating the cross-over structure of step (E) with a polymerase under conditions such that an extension product of the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid is synthesized and an extension product of the extension product of the second primer of the second copy of the secondary nucleic acid is synthesized, to produce a concatemer comprising two copies of the double-stranded nucleic acid template of step (A), wherein the concatemer comprises the extension product of the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid and the extension product of the extension product of the second primer of the second copy of the secondary nucleic acid.

30. The method claim 29, wherein the nucleic acid polymerase of step (A) is a DNA polymerase having strand-displacement activity.

31. The method of claim 29, wherein all steps of the method are performed at a temperature of no greater than 80 C.

32. The method of claim 29, wherein two or more of the steps occur simultaneously.

33. A method for generating a concatemer comprising two or more copies of a polynucleotide template or an analogous sequence thereof, the method comprising,

(A) treating a primary nucleic acid comprising the polynucleotide template with a first copy of a first primer and a polymerase under conditions such that an extension product of the first copy of the first primer is synthesized which is annealed to the polynucleotide template,

wherein

the first primer comprises a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions:

(i) a tail region comprising

(a) the 5' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide

(c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and

(ii) a template-binding region comprising

(a) the 3' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide

(c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and the template-binding region of the first copy of the first primer anneals to the polynucleotide template,

(B) treating the extension product of the first copy of the first primer of step (A) with a second primer and a polymerase under conditions such that an extension product of the second primer is synthesized which is annealed to the extension product of the first copy of the first primer of step (A),

wherein

the second primer comprises a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions:

(i) a tail region comprising

(a) the 5' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide

(c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides

(ii) a template-binding region comprising

(a) the 3' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide

(c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides,

the tail region of the second primer contains a nucleotide sequence which is complementary to the nucleotide sequence of the tail region of the first primer,

the template-binding region of the second primer anneals to the extension product of the first copy of the first primer of step (A), and

the extension product of the second primer contains a 5' terminal nucleotide, a 3' terminal nucleotide, and a 3' terminal region comprising the 3' terminal nucleotide, wherein the 3' terminal region contains the same nucleotide sequence as the nucleotide sequence of the tail region of the second primer read in the 5' to 3' direction, and the final nucleotide of the 3' terminal region is the 3' terminal nucleotide of the extension product of the second primer,

(C) treating the extension product of the second primer of step (B) with a second copy of the first primer and a polymerase under conditions such that an extension product of the second copy of the first primer is synthesized which is annealed to the extension product of the second primer of step (B), to produce a first copy of a secondary nucleic acid comprising the extension product of the second primer of step (B) and the extension product of the second copy of the first primer,

wherein

the extension product of the second copy of the first primer contains a 5' terminal nucleotide, a 3' terminal nucleotide, and a 3' terminal region comprising the 3' terminal nucleotide, wherein the 3' terminal region contains the same nucleotide sequence as the nucleotide sequence of the tail region of the first primer read in the 5' to 3' direction, and the final nucleotide of the 3' terminal region is the 3' terminal nucleotide of the extension product of the second primer

(D) repeating at least step (C) one or more addition times to generate at least a second copy of the secondary nucleic acid comprising the extension product of the second primer of step (B) and the extension product of the second copy of the first primer of step (C),

(E) treating the first copy of the secondary nucleic acid of step (C) and the second copy of the secondary nucleic acid of step (D) under conditions such that the 3' terminal region of the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid anneals to the 3' terminal region of the extension product of the second primer of the second copy of the secondary nucleic acid, to produce a cross-over structure comprising the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid and the extension product of the second primer of the second copy of the secondary nucleic acid,

(F) treating the cross-over structure of step (E) with a polymerase under conditions such that an extension product of the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid is synthesized and an extension product of the extension product of the second primer of the second copy of the secondary nucleic acid is synthesized, to produce a concatemer comprising two copies of the polynucleotide template of step (A), wherein the concatemer comprises the extension product of the extension product of the second copy of the first primer of the first copy of the secondary nucleic acid and the

extension product of the extension product of the second primer of the second copy of the secondary nucleic acid.

34. The method claim 33, wherein the nucleic acid polymerase of step (A) is a DNA polymerase.

35. The method of claim 33, wherein the nucleic acid polymerase of step (A) is a reverse transcriptase.

36. The method of claim 33, wherein all steps of the method are performed at a temperature of no greater than 80 C.

37. The method of claim 33, wherein two or more of the steps occur simultaneously.

38. A method for generating a concatemer comprising two or more copies of a double-stranded nucleic acid template, the method comprising,

(A) preparing a reaction mixture comprising:

(i) a primary nucleic acid comprising the double-stranded nucleic acid template,

(ii) an isolated nucleic acid polymerase,

(iii) a first primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions:

(a) a tail region comprising

(1) the 5' terminal nucleotide of the primer

(2) an innermost nucleotide, wherein the innermost nucleotide is downstream

from the 5' terminal nucleotide

(3) a middle section between the 5' terminal nucleotide and the

innermost nucleotide, comprising one or more nucleotides, and

(b) a template-binding region comprising

(1) the 3' terminal nucleotide of the primer

(2) an innermost nucleotide, wherein the innermost nucleotide is upstream

from the 3' terminal nucleotide

(3) a middle section between the 3' terminal nucleotide and the

innermost nucleotide, comprising one or more nucleotides,

wherein the template-binding region is complementary to a first strand of the nucleic acid template,

(iv) a second primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions:

(a) a tail region comprising

(1) the 5' terminal nucleotide of the primer

(2) an innermost nucleotide, wherein the innermost nucleotide is downstream

from the 5' terminal nucleotide

(3) a middle section between the 5' terminal nucleotide and the

innermost nucleotide, comprising one or more nucleotides, and

(b) a template-binding region comprising

(1) the 3' terminal nucleotide of the primer

(2) an innermost nucleotide, wherein the innermost nucleotide is upstream

from the 3' terminal nucleotide

(3) a middle section between the 3' terminal nucleotide and the

innermost nucleotide, comprising one or more nucleotides, and

wherein the template-binding region is complementary to a second strand of the nucleic acid template, and

wherein the tail region of the second primer contains a nucleotide sequence which is complementary to the nucleotide sequence of the tail region of the first primer, and

(B) incubating the reaction mixture at a temperature of no greater than 80 C for at least 3 minutes.

39. A method for generating a concatemer comprising two or more copies of a polynucleotide template, the method comprising,

(A) preparing a reaction mixture comprising:

(i) a nucleic acid comprising the polynucleotide template

(ii) an isolated nucleic acid polymerase,

(iii) a first primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions:

(a) a tail region comprising

(1) the 5' terminal nucleotide of the primer

(2) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide

(3) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and

(b) a template-binding region comprising

(1) the 3' terminal nucleotide of the primer

(2) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide

(3) a middle section between the 3' terminal nucleotide and the

innermost nucleotide, comprising one or more nucleotides, and

wherein the template-binding region is complementary to the polynucleotide template,

(iv) a second primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions:

(a) a tail region comprising

(1) the 5' terminal nucleotide of the primer

(2) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide

(3) a middle section between the 5' terminal nucleotide and the

innermost nucleotide, comprising one or more nucleotides, and

(b) a template-binding region comprising

(1) the 3' terminal nucleotide of the primer

(2) an innermost nucleotide, wherein the innermost nucleotide is upstream

from the 3' terminal nucleotide

(3) a middle section between the 3' terminal nucleotide and the

innermost nucleotide, comprising one or more nucleotides, and

wherein the template-binding region is complementary to a nucleotide sequence complementary to the polynucleotide template, and

wherein the tail region of the second primer contains a nucleotide sequence which is complementary to the nucleotide sequence of the tail region of the first primer, and

(B) incubating the reaction mixture at a temperature of no greater than 80 C for at least 3 minutes.

40. The method of any of claims 29-39, wherein the double-stranded nucleic acid template or polynucleotide template is amplified at least 10-fold within 60 minutes of initiation of the method.

41. The method of any of claims 29-39, wherein the tail region of the first primer contains between 4 and 25 nucleotides.

42. A vessel, comprising in fluid communication therein:

a first primer, wherein the first primer comprises a first region and a second region, and wherein the second region of the first primer comprises a nucleotide sequence which is complementary to a first portion of a polynucleotide template;

a second primer, wherein the second primer comprises a first region and a second region, and wherein the second region of the second primer comprises a nucleotide sequence which is complementary to a partner nucleotide sequence, wherein the partner nucleotide sequence is complementary to a second portion of the polynucleotide template; and

at least one concatemer strand, wherein the concatemer strand comprises a 5' end and a 3' end, and comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C'-T-C'-T -X-C', wherein: C' represents the nucleotide sequence of the first region of the second primer, T represents the nucleotide sequence of the polynucleotide template or an analogous sequence thereof, and X represents any number and sequence of nucleotides.

43. The vessel of claim 42, wherein the concatemer strand is a first concatemer strand, and the vessel further comprises a second concatemer strand, wherein the second concatemer strand comprises a 5' end and a 3' end, and comprises a nucleotide sequence having the general structure in the 5' to 3' direction of: C-X'-T'-C-T'-C, wherein: C represents the nucleotide sequence of the first region of the first primer, T' represents a nucleotide sequence which is complementary to the polynucleotide template, and X' represents a nucleotide sequence which is complementary to the nucleotide sequence of X.

44. The vessel of claim 42, wherein X contains a sequence having the general structure in the 5' to 3' direction of $[(C'-T)_N]$ wherein C' represents the nucleotide sequence of the first region of the second primer, T represents the nucleotide sequence of the polynucleotide template or an analogous sequence thereof, and N is any integer between 0 and 1000.

45. The vessel of claim 42, wherein X represents zero nucleotides.

46. A vessel, comprising in fluid communication therein:

(A) an isolated nucleic acid polymerase,

(B) a nucleic acid template comprising at least a first strand,

(C) a first primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions:

(i) a tail region comprising

(a) the 5' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is downstream

from the 5' terminal nucleotide

(c) a middle section between the 5' terminal nucleotide and the

innermost nucleotide, comprising one or more nucleotides, and

(ii) a template-binding region comprising

(a) the 3' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is upstream

from the 3' terminal nucleotide

(c) a middle section between the 3' terminal nucleotide and the

innermost nucleotide, comprising one or more nucleotides,

wherein the template-binding region is complementary to a first strand of the nucleic acid template, and

(D) a second primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions:

(i) a tail region comprising

(a) the 5' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide

(c) a middle section between the 5' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and

(ii) a template-binding region comprising

(a) the 3' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is upstream from the 3' terminal nucleotide

(c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and

wherein the template-binding region is complementary to a nucleotide sequence complementary to first strand of the nucleic acid template, and

wherein the tail region of the second primer contains a nucleotide sequence which is complementary to the nucleotide sequence of the tail region of the first primer.

47. The vessel of any of claims 42-46, wherein the vessel comprises a DNA polymerase having strand-displacement activity.

48. The vessel of claim 47, wherein the vessel further comprises a reverse transcriptase.

49. The vessel of claim 42, wherein the polynucleotide template is an RNA molecule.

50. The vessel of claim 49, wherein the T represents the nucleotide sequence of a DNA sequence which is analogous to the RNA sequence of the polynucleotide template.

51. The vessel of claim 42, wherein the polynucleotide template is a DNA molecule.

52. The vessel of claim 51, wherein the polynucleotide template comprises one strand of double-stranded nucleic acid template.

53. The vessel of claim 42, wherein the first region of the first primer contains between 4 and 25 nucleotides.

54. The vessel of claim 42, wherein the first region of the first primer and the first region of the second primer contain the same number of nucleotides.

55. The vessel of claim 46, wherein the tail region of the first primer contains between 4 and 25 nucleotides.

56. The vessel of claim 46, wherein the tail region of the first primer and the first region of the second primer contain the same number of nucleotides.

57. The vessel of any of claims 42-56, wherein the vessel further comprises a nucleic acid dye.

58. The vessel of any of claims 42-57, wherein the vessel further comprises nucleotides and buffer.

59. The vessel of any of claims 42-58, wherein the vessel further comprises at least a portion of a biological sample from a subject.

60. The vessel of claim 59, wherein the subject is a human.

61. A kit comprising two or more fluidically isolated containers, the containers collectively comprising:

a first primer, wherein the first primer comprises a first region and a second region, and wherein the second region of the first primer comprises a nucleotide sequence which is complementary to a first portion of a polynucleotide template;

a second primer, wherein the second primer comprises a first region and a second region, and wherein the second region of the second primer comprises a nucleotide sequence which is complementary to a partner nucleotide sequence, wherein the partner nucleotide sequence is complementary to a second portion of the polynucleotide template; and

an isolated DNA polymerase having strand-displacement activity; wherein:

the first region of the first primer and the first region of the second primer are complementary.

62. A kit for detecting a target nucleic acid of interest comprising at least a first strand, the kit comprising two or more fluidically isolated containers, the containers collectively comprising:

(A) an isolated DNA polymerase having strand-displacement activity,

(B) a first primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions:

(i) a tail region comprising

(a) the 5' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is downstream

from the 5' terminal nucleotide

(c) a middle section between the 5' terminal nucleotide and the

innermost nucleotide, comprising one or more nucleotides, and

(ii) a template-binding region comprising

(a) the 3' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is upstream

from the 3' terminal nucleotide

(c) a middle section between the 3' terminal nucleotide and the

innermost nucleotide, comprising one or more nucleotides,

wherein the template-binding region is complementary to the first strand of the target nucleic acid, and

(C) a second primer comprising a 5' terminal nucleotide, a 3' terminal nucleotide, and two regions:

(i) a tail region comprising

(a) the 5' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is downstream from the 5' terminal nucleotide

(c) a middle section between the 5' terminal nucleotide and the

innermost nucleotide, comprising one or more nucleotides, and

(ii) a template-binding region comprising

(a) the 3' terminal nucleotide of the primer

(b) an innermost nucleotide, wherein the innermost nucleotide is upstream

from the 3' terminal nucleotide

(c) a middle section between the 3' terminal nucleotide and the innermost nucleotide, comprising one or more nucleotides, and

wherein the template-binding region is complementary to a nucleotide sequence complementary to the first strand of the target nucleic acid, and

wherein the tail region of the second primer contains a nucleotide sequence which is complementary to the nucleotide sequence of the tail region of the first primer.

63. The kit of claim 61 or 62, further comprising a reverse transcriptase.

64. The kit of claim 61, wherein the first region of the first primer contains between 4 and 25 nucleotides.

65. The kit of claim 62, wherein the tail region of the first primer contains between 4 and 25 nucleotides.

66. The kit of any of claims 61-65, further comprising a nucleic acid containing the nucleotide sequence of the target nucleic acid of interest.

67. A method for copying a polynucleotide template, the method comprising:

incubating a reaction mixture comprising the polynucleotide template, a first primer, and a second primer under conditions without thermocycling, wherein:

the polynucleotide template comprises a first portion and a second portion;

the first primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to the first portion of the polynucleotide template;

the second primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to the second portion of the polynucleotide template;

the first region of the first primer is complementary to the first region of the second primer; and

multiple copies of the polynucleotide template are generated.

68. The method of claim 67, wherein the polynucleotide template further comprises a third portion, wherein the third portion is situated in the polynucleotide template between the first portion and the second portion, and wherein the first region of the second primer is complementary to the third portion of the polynucleotide template.

69. A method for amplifying a polynucleotide template, the method comprising incubating the polynucleotide template in a reaction mixture comprising a first primer and a second primer, wherein:

the polynucleotide template comprises a first portion, a second portion and a third portion, wherein the third portion is situated in the polynucleotide template between the first portion and the second portion;

the first primer comprises a first region and a second region, wherein the second region of the first primer is complementary to the first portion of the polynucleotide template; and

the second primer comprises a first region and a second region, wherein the second region of the second primer is complementary to a sequence which is complementary to the second portion of the polynucleotide template, the first region of the second primer is complementary to the first region of the first primer, and the first region of the second primer is complementary to the third portion of the polynucleotide template.

70. A method for assessing the identity of a nucleotide at a position of interest in a nucleotide sequence in a polynucleotide template, the method comprising:

A) providing copies of the polynucleotide template in each of at least a first reaction mixture and a second reaction mixture, wherein:

the polynucleotide template comprises a first portion, a second portion and a third portion, wherein the third portion is situated in the polynucleotide template between the first portion and the second portion and wherein the position of interest is in the third portion;

the first reaction mixture comprises copies of the polynucleotide template, a first primer, and a second primer, wherein:

the first primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a first portion of the polynucleotide template;

the second primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to a second portion of the polynucleotide template;

the first region of the first primer is complementary to the first region of the second primer; and

the first region of the second primer is complementary to the third portion of the polynucleotide template;

the second reaction mixture comprises copies of the polynucleotide template, a third primer, and a fourth primer, wherein:

the third primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a first portion of the polynucleotide template;

the fourth primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to a second portion of the polynucleotide template;

the first region of the third primer is complementary to the first region of the fourth primer; and

the first region of the fourth primer is complementary to the third portion of the polynucleotide template; and

the nucleotide sequence of the first region of the second primer differs from the nucleotide sequence of first region of the fourth primer by a single nucleotide, wherein the position of the different nucleotide in the second and fourth primers corresponds to the position of the nucleotide of interest in the polynucleotide template if the nucleotide sequence of the first region of the second primer or fourth primer is oriented with the nucleotide sequence of the third portion of the polynucleotide template for maximum complementation of the sequences;

B) incubating the first reaction mixture and second reaction mixture under conditions without thermocycling; and

C) comparing the rate or amount of amplification of the polynucleotide template in the first reaction mixture to the rate or amount of amplification of the polynucleotide template in the second reaction mixture, wherein the rate or amount of amplification of the polynucleotide template is indicative of the degree of complementation between first region of the second or fourth primer and the nucleotide sequence of the third portion of the polynucleotide template.

71. The method of any of claims 67-70, wherein the polynucleotide template is a DNA strand.

72. The method of any of claims 67-70, wherein the polynucleotide template is an RNA strand.

73. The method of any of claims 67-70, wherein the polynucleotide template is one strand of a duplex DNA molecule.

74. The method of any of claims 67-73, wherein the first portion and second portion of the polynucleotide template are each between 6 and 30 nucleotides in length.

75. The method of any of claims 67-74, wherein the third portion of the polynucleotide template is between 4 and 14 nucleotides in length.

76. The method of any of claims 67-75, wherein the number of copies of the polynucleotide template in the reaction mixture is increased at least 10-fold within 60 minutes of initiation of the method.

77. The method of any of claims 67-76, wherein a concatemer strand comprising at least three copies of the polynucleotide template is generated during the incubation of the reaction mixture.

78. A method for amplifying a double stranded nucleic acid molecule, the method comprising incubating the double stranded nucleic acid molecule in a reaction mixture comprising a first primer and a second primer, wherein:

the double stranded nucleic acid molecule comprises a first strand and a second strand, wherein the first strand comprises a first portion and a third portion and the second strand comprises a second portion;

the first primer comprises a first region and a second region, wherein the second region of the first primer is complementary to the first portion of the first strand; and

the second primer comprises a first region and a second region, wherein the second region of the second primer is complementary to the second portion of the second strand, the first region of the second primer is complementary to the third portion of the first strand, and the first region of the second primer is complementary to the first region of the first primer.

79. The method of any of claims 69-78, wherein the reaction mixture is incubated without thermocycling.

80. The method of any of claims 67-79, wherein the reaction mixture further comprises a nucleic acid dye.

81. The method of any of claims 67-80, wherein the reaction mixture further comprises a reverse transcriptase.

82. A reaction mixture comprising:

a polynucleotide template, a first primer, and a second primer, wherein:

the polynucleotide template comprises a first portion, a second portion and a third portion, wherein the third portion is situated in the polynucleotide template between the first portion and the second portion;

the first primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to the first portion of the polynucleotide template;

the second primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to the second portion of the polynucleotide template;

the first region of the first primer is complementary to the first region of the second primer; and

the first region of the second primer is complementary to the third portion of the polynucleotide template.

83. The reaction mixture of claim 82, wherein the reaction mixture further comprises a DNA polymerase having strand-displacement activity.

84. The reaction mixture of any of claims 82-83, wherein the reaction mixture further comprises a reverse transcriptase.

85. The reaction mixture of any of claims 82-84, wherein the reaction mixture further comprises a nucleic acid dye.

86. The reaction mixture of any of claims 82-85, wherein the reaction mixture further comprises nucleotides and buffer.

87. The reaction mixture of any of claims 82-86, wherein the polynucleotide template is a DNA strand.

88. The reaction mixture of any of claims 82-87, wherein the polynucleotide template is an RNA strand.

89. The reaction mixture of any of claims 82-88, wherein the polynucleotide template is part of a duplex DNA molecule.

90. The reaction mixture of any of claims 82-89, wherein the first portion and second portion of the polynucleotide template are each between 6 and 30 nucleotides in length.

91. The reaction mixture of any of claims 82-90, wherein the third portion of the polynucleotide template is between 4 and 14 nucleotides in length.

92. A kit for the amplification of a polynucleotide template, the kit comprising:

a first primer and a second primer, wherein:

the first primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a first portion of the polynucleotide template;

the second primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer,

and the second region is complementary to a sequence which is complementary to a second portion of the polynucleotide template;

the first region of the first primer is complementary to the first region of the second primer; and

the first region of the second primer is complementary to a third portion of the polynucleotide template, wherein the third portion is situated in the polynucleotide template between the first portion and the second portion.

93. The kit of claim 92, wherein the first primer and the second primer are in separate fluidically isolated containers.

94. A kit comprising two or more fluidically isolated containers, the containers collectively comprising:

a first primer, a second primer, a third primer, and a fourth primer, wherein:

the first primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a first portion of a polynucleotide template;

the second primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to a second portion of the polynucleotide template;

the third primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to the first portion of the polynucleotide template;

the fourth primer comprises a first region and a second region, wherein the first region comprises a 5' end of the primer, the second region comprises a 3' end of the primer, and the second region is complementary to a sequence which is complementary to a second portion of the polynucleotide template;

the first region of the first primer is complementary to the first region of the second primer;

the first region of the third primer is complementary to the first region of the fourth primer; and

the nucleotide sequence of the first region of the first primer differs from the nucleotide sequence of first region of the third primer by a single nucleotide.

95. The kit of claim 94, wherein the first region of the second primer and the first region of the fourth primer are both complementary to a third portion of the polynucleotide template, wherein the third portion is situated in the polynucleotide template between the first portion and the second portion.

96. The kit of any of claims 92-95, wherein the kit further comprises a control nucleic acid strand comprising the nucleotide sequence of the polynucleotide template.

97. The kit of any of claims 92-96, wherein the kit further comprises a DNA polymerase having strand-displacement activity.

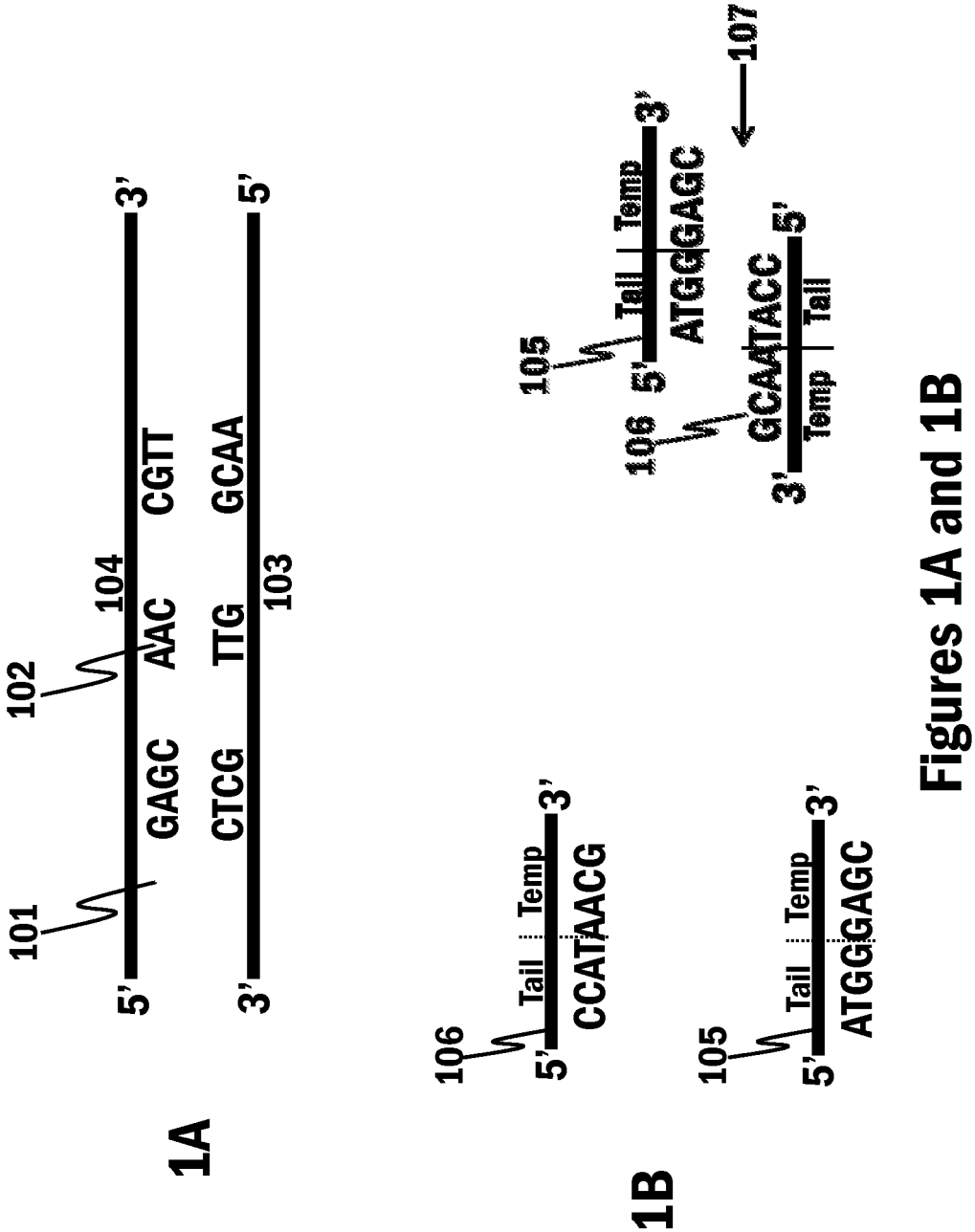
98. The kit of any of claims 92-97, wherein the kit further comprises a reverse transcriptase.

99. The kit of any of claims 92-98, wherein the kit further comprises a nucleic acid dye.

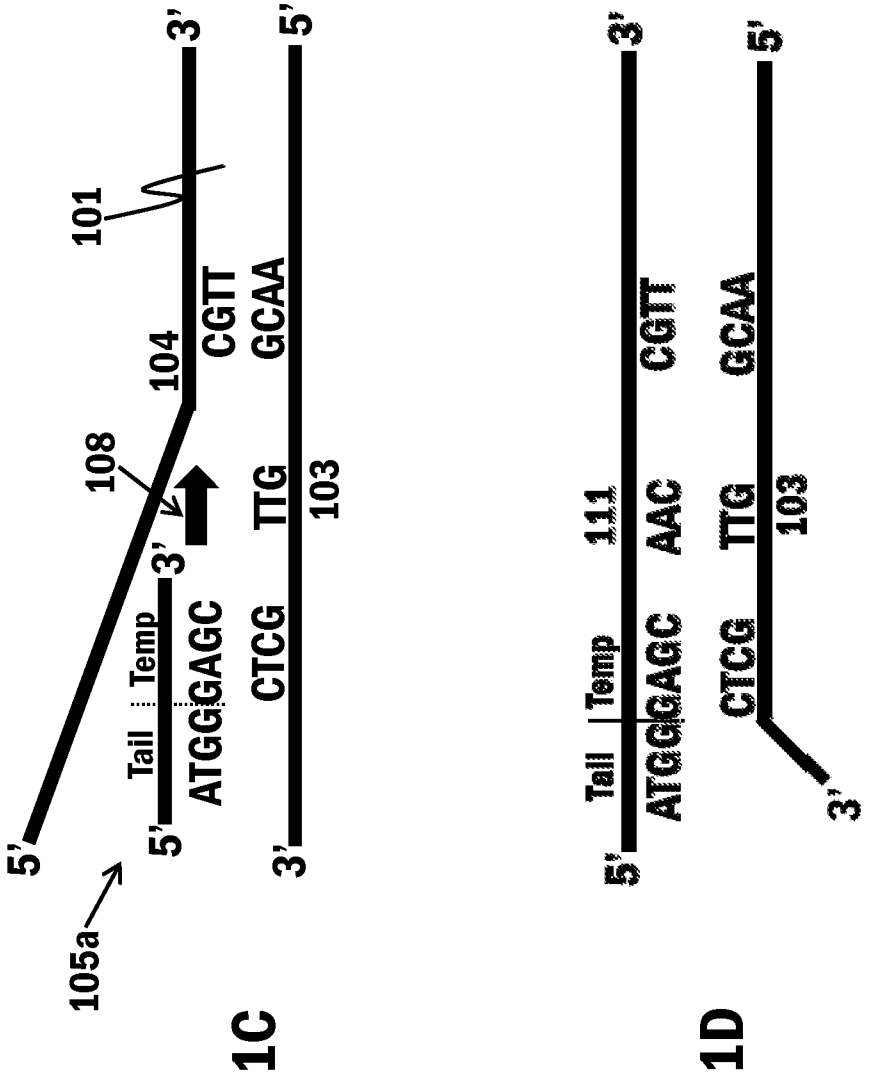
100. The kit of any of claims 92-99, wherein the kit further comprises nucleotides and buffer.

101. The kit of any of claims 92-100, wherein the second region of the each of the first primer, second primer, third primer, and fourth primers is between 6 and 30 nucleotides in length.

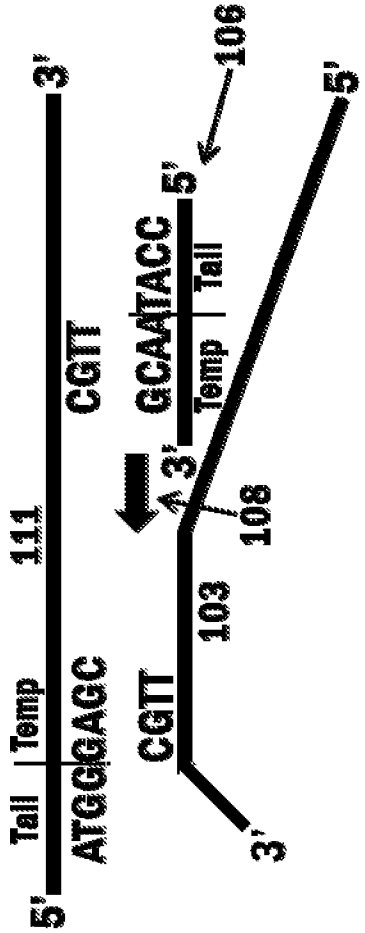
102. The kit of any of claims 92-101, wherein the first region of each of the first primer, second primer, third primer, and fourth primers is between 4 and 14 nucleotides in length.



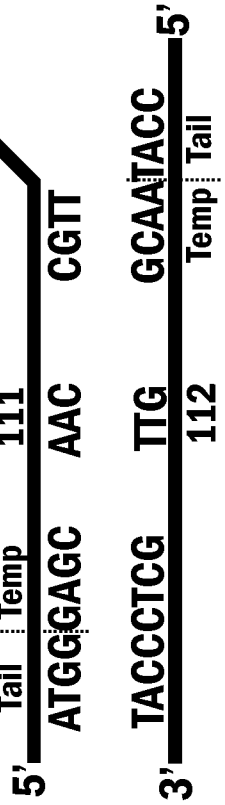
Figures 1A and 1B



Figures 1C and 1D

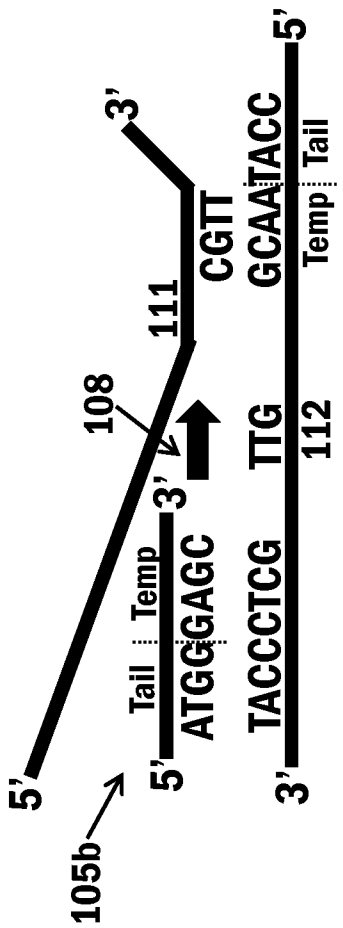


1E

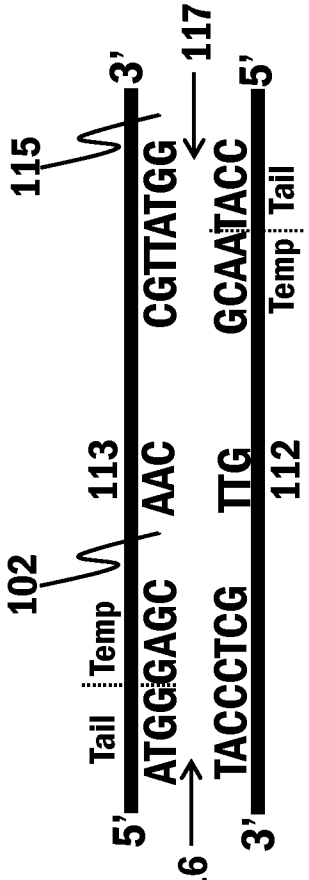


1F

Figures 1E and 1F

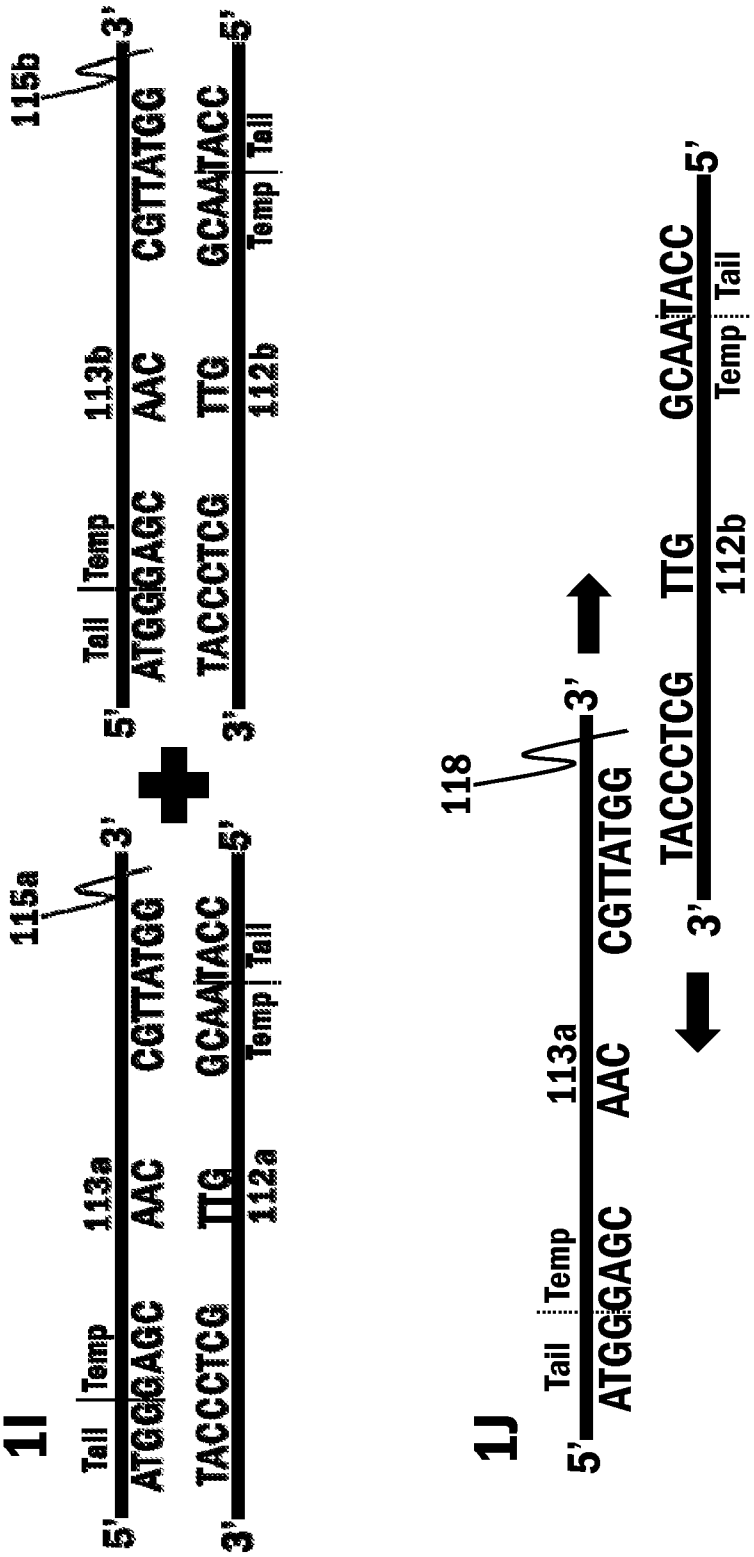


1G



1H

Figures 1G and 1H



Figures 1I and 1J

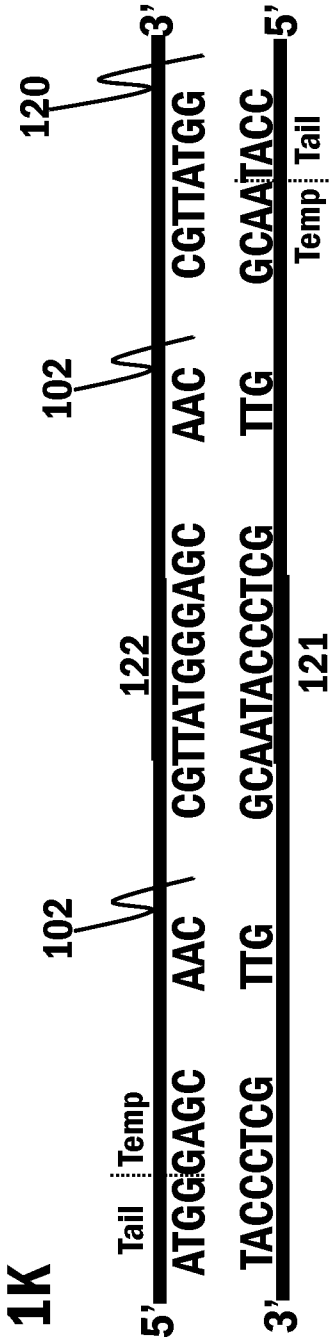

1K
5'
3'
Tail
Temp
ATGGGAGC
TACCCTCG
102
AAC
TTG
CGTTATGGGAGC
GCAATACCCTCG
122
121
102
AAC
TTG
120
3'
5'
CGTTATGG
GCAATACC
Temp
Tail

Figure 1K

2A

T-Primer 1 (P1)	Length	Tail Length	T*-Primer 2 (P2)	Length	Tail Length
CGCCGGATGGCTCTTGGGAAACCAAACCGTACCAACC	37	22	GTTTCCCAAGAGCCATCCGGCGATGCGGAATGTACC	36	22
CCGGATGGCTCTTGGGAAACCAAACCGTACCAACC	35	20	GTTTCCCAAGAGCCATCCGGATGCGGAATGTACC	34	20
GGATGGCTCTTGGGAAACCAAACCGTACCAACC	33	18	GTTTCCCAAGAGCCATCCATGCGGAATGTACC	32	18
ATGGCTCTTGGGAAACCAAACCGTACCAACC	31	16	GTTTCCCAAGAGCCATATGCGGAATGTACC	30	16
TGGCTCTTGGGAAACCAAACCGTACCAACC	30	15	GTTTCCCAAGAGCCAATGCGGAATGTACC	29	15
GGCTCTTGGGAAACCAAACCGTACCAACC	29	14	GTTTCCCAAGAGCCATGCGGAATGTACC	28	14
GCTCTTGGGAAACCAAACCGTACCAACC	28	13	GTTTCCCAAGAGCATGCGGAATGTACC	27	13
CTCTTGGGAAACCAAACCGTACCAACC	27	12	GTTTCCCAAGAGATGCGGAATGTACC	26	12
TCTTGGGAAACCAAACCGTACCAACC	26	11	GTTTCCCAAGAATGCGGAATGTACC	25	11
CTTGGGAAACCAAACCGTACCAACC	25	10	GTTTCCCAAGATGCGGAATGTACC	24	10
TTGGGAAACCAAACCGTACCAACC	24	9	GTTTCCCAAATGCGGAATGTACC	23	9
TGGGAAACCAAACCGTACCAACC	23	8	GTTTCCCAATGCGGAATGTACC	22	8

Figure 2A

2B

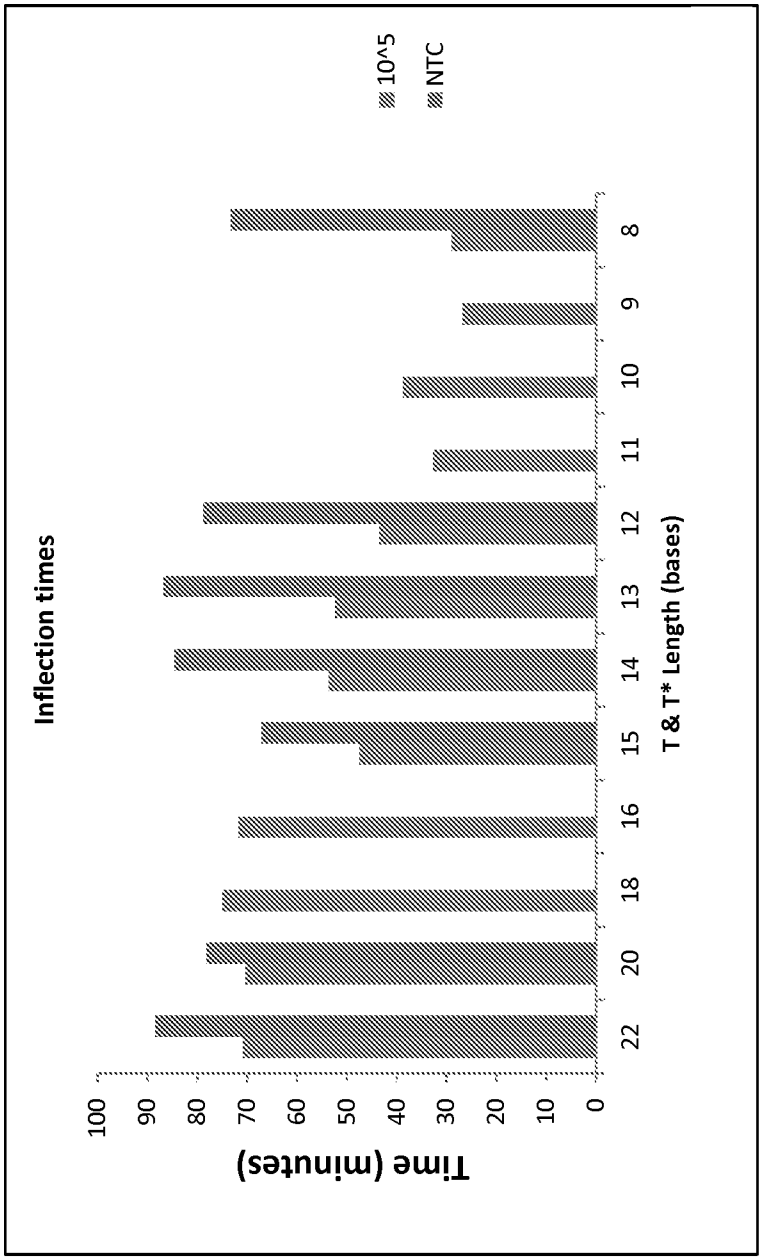

Inflection times
Time (minutes)
100
90
80
70
60
50
40
30
20
10
0
22
20
18
16
15
14
13
12
11
10
9
8
T & T* Length (bases)
10^5
NTC

Figure 2B

3A

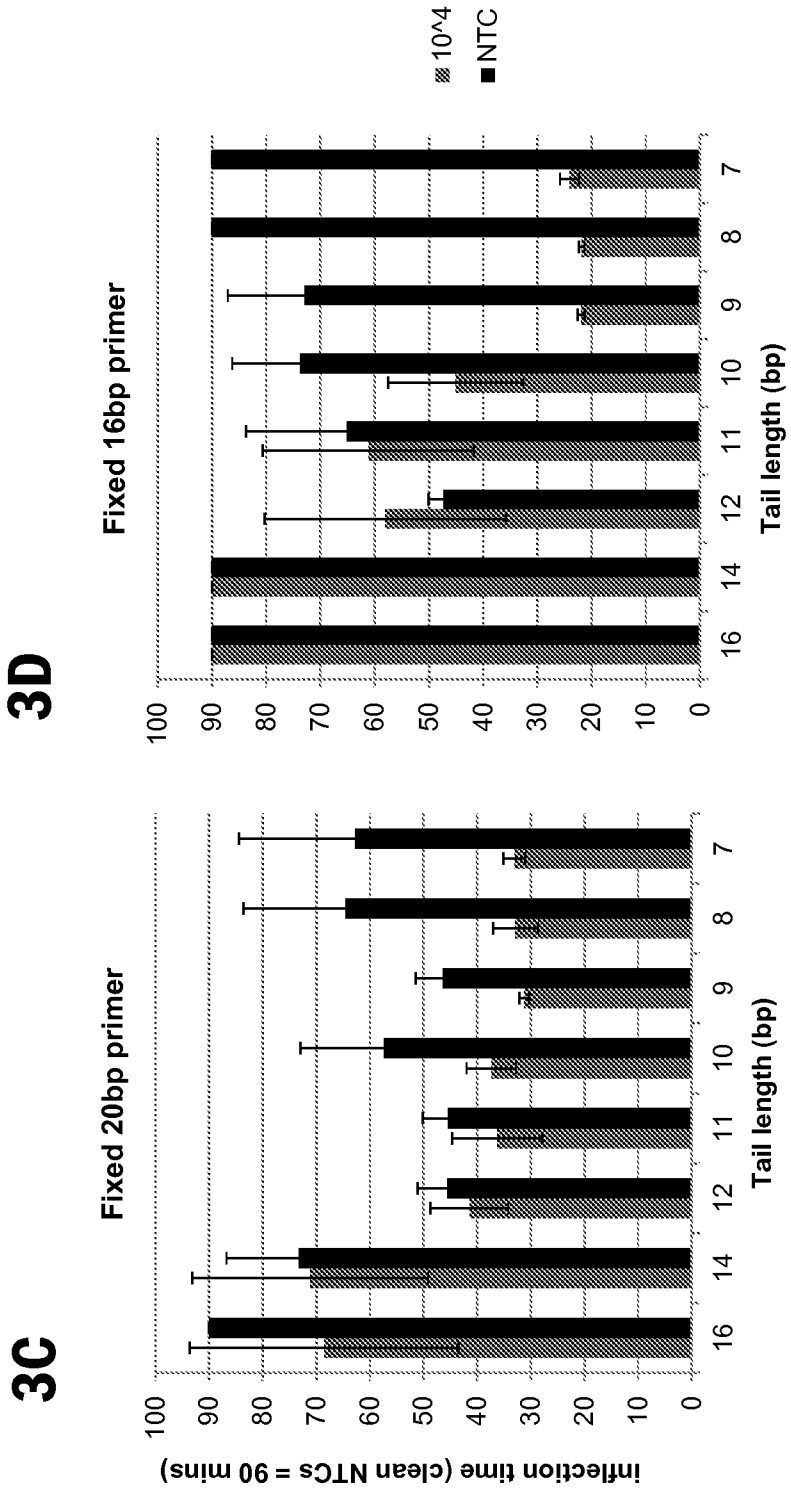
	Tm	Primer (15m)	
C5	AIGGCTTIGGAAC	[illegible]	Forward Primer
D5	GIIICCCAGAGCCAI	CATCCCAATCTAAC	Reverse Primer
C6	GGTCTIGGGAAC	[illegible]	Forward Primer
D6	GIIICCCAGAGCC	CATCCCAATCTAAC	Reverse Primer
C7	CICTIGGGAAC	[illegible]	Forward Primer
D7	GIIICCCAGAG	CATCCCAATCTAAC	Reverse Primer
C8	ICTIGGGAAC	[illegible]	Forward Primer
D8	GIIICCCAGA	CATCCCAATCTAAC	Reverse Primer
C9	CTIGGGAAC	[illegible]	Forward Primer
D9	GIIICCCAG	CATCCCAATCTAAC	Reverse Primer
C10	TIGGGAAC	[illegible]	Forward Primer
D10	GIIICCCAA	CATCCCAATCTAAC	Reverse Primer
C11	TGGGAAC	[illegible]	Forward Primer
D11	GIIICCCA	CATCCCAATCTAAC	Reverse Primer
C12	GGGAAC	[illegible]	Forward Primer
D12	GIIICCC	CATCCCAATCTAAC	Reverse Primer

Figure 3A

3B

	Tm	Primer (20nt)	
A1	AGGCTTGGAAC	[illegible]	Forward Primer
B1	GTTCCAAGCCT	ACGCGATCGAATGTAC	Reverse Primer
A2	GGCTTGGAAC	[illegible]	Forward Primer
B2	GTTCCAAGCC	ACGCGATCGAATGTAC	Reverse Primer
A3	CTCTGGAAC	[illegible]	Forward Primer
B3	GTTCCAGAG	ACGCGATCGAATGTAC	Reverse Primer
A4	TCTGGAAC	[illegible]	Forward Primer
B4	GTTCCAGA	ACGCGATCGAATGTAC	Reverse Primer
A5	CTGGAAC	[illegible]	Forward Primer
B5	GTTCCAG	ACGCGATCGAATGTAC	Reverse Primer
A6	TGGAAC	[illegible]	Forward Primer
B6	GTTCCA	ACGCGATCGAATGTAC	Reverse Primer
A7	GGAAC	[illegible]	Forward Primer
B7	GTTCC	ACGCGATCGAATGTAC	Reverse Primer
A8	GAAC	[illegible]	Forward Primer
B8	GTTC	ACGCGATCGAATGTAC	Reverse Primer

Figure 3B



Figures 3C and 3D

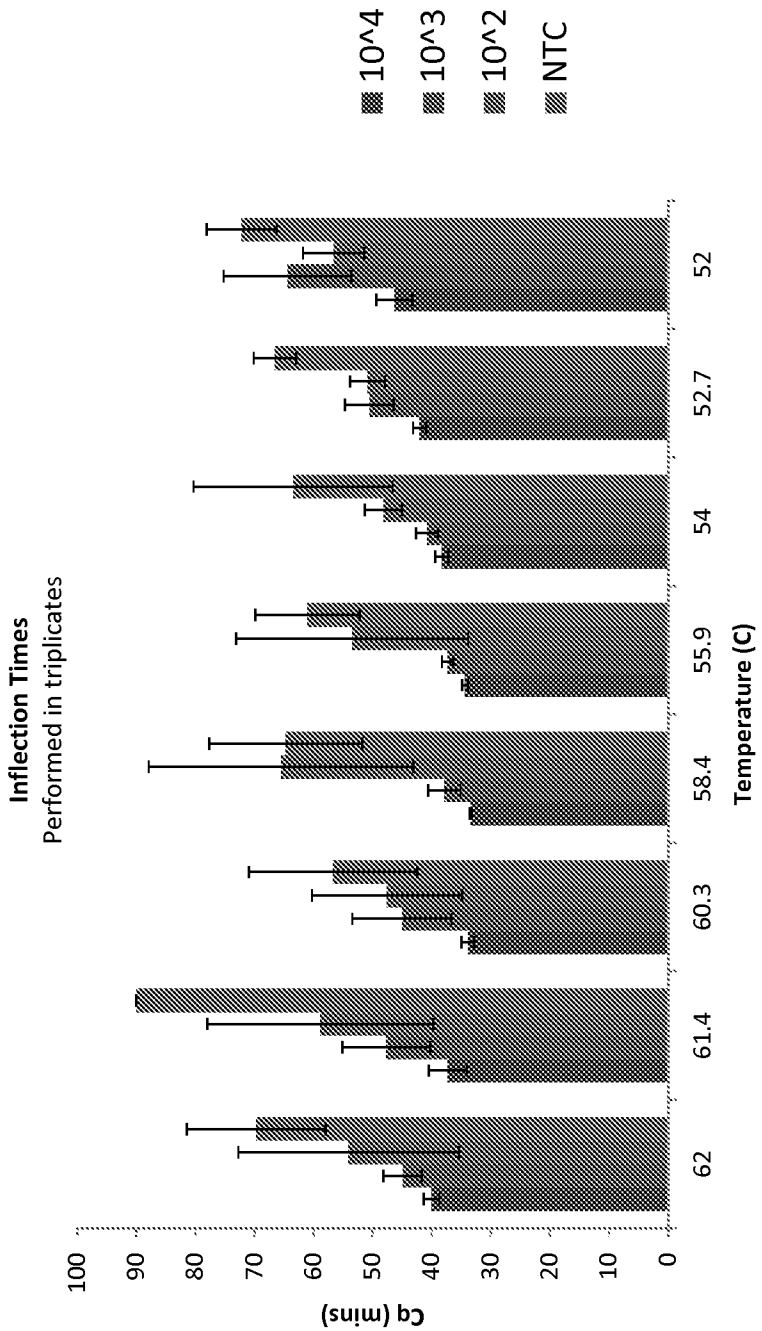

Inflection Times
Performed in triplicates
Cq (mins)
100
90
80
70
60
50
40
30
20
10
0
62
61.4
60.3
58.4
55.9
54
52.7
52
Temperature (C)
10^4
10^3
10^2
NTC

Figure 4

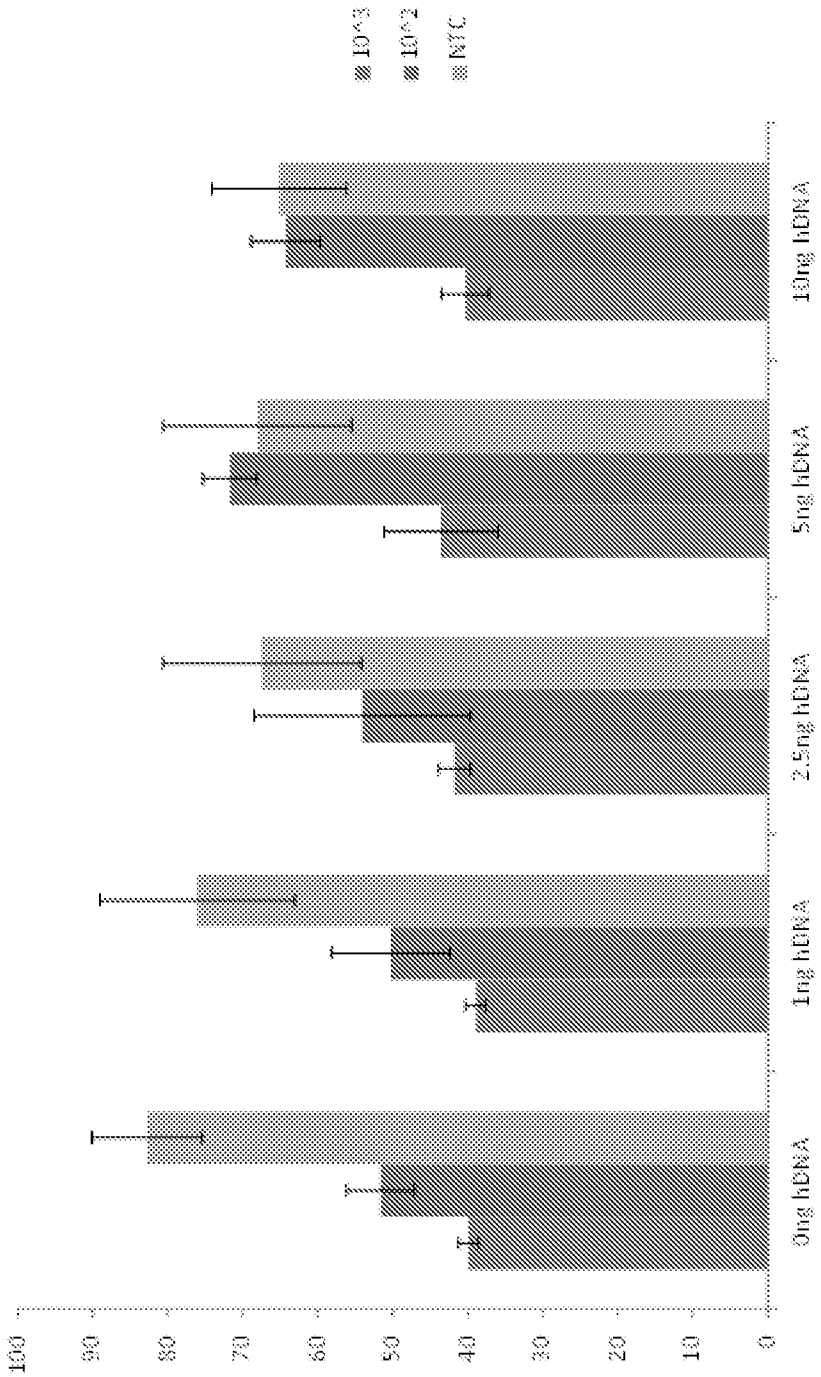

10^3
10^2
NTC
100
90
80
70
60
50
40
30
20
10
0
0ng hDNA
1ng hDNA
2.5ng hDNA
5ng hDNA
10ng hDNA

Figure 5

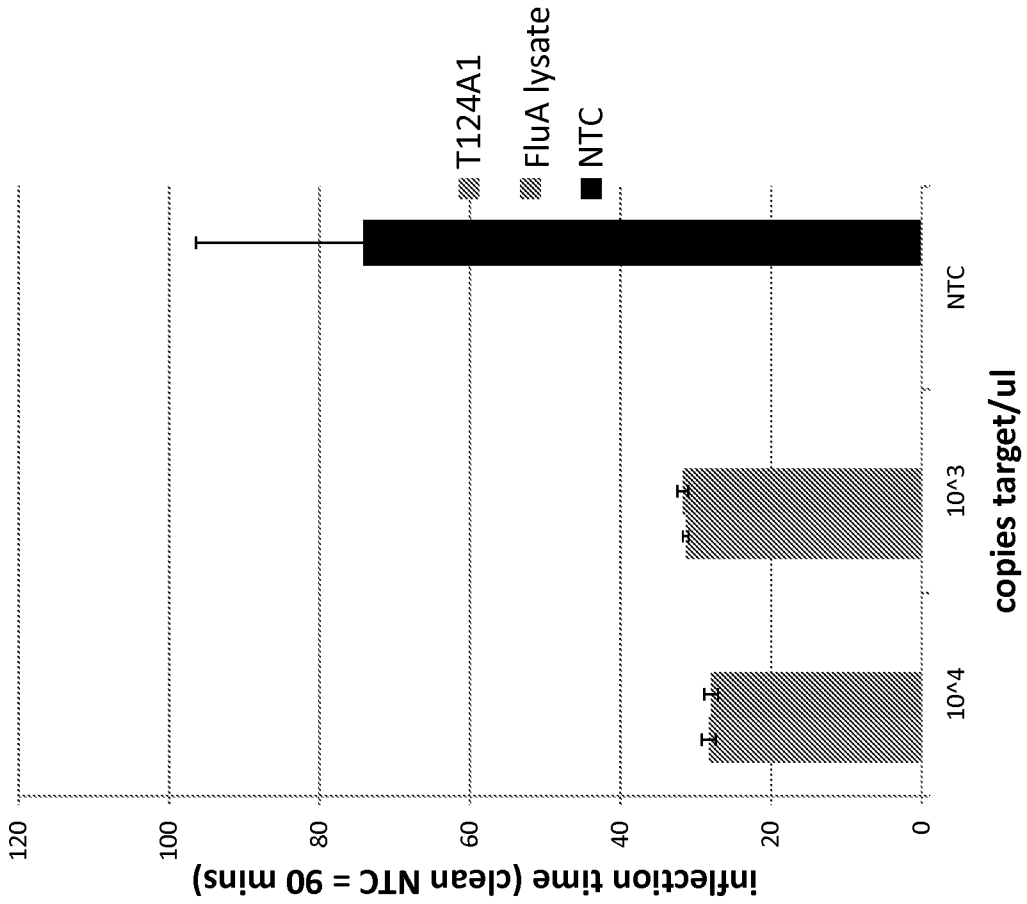

T124A1
FluA lysate
NTC
120
100
80
60
40
20
0
infilection time (clean NTC = 90 mins)
10^4
10^3
NTC
copies target/ul

Figure 6

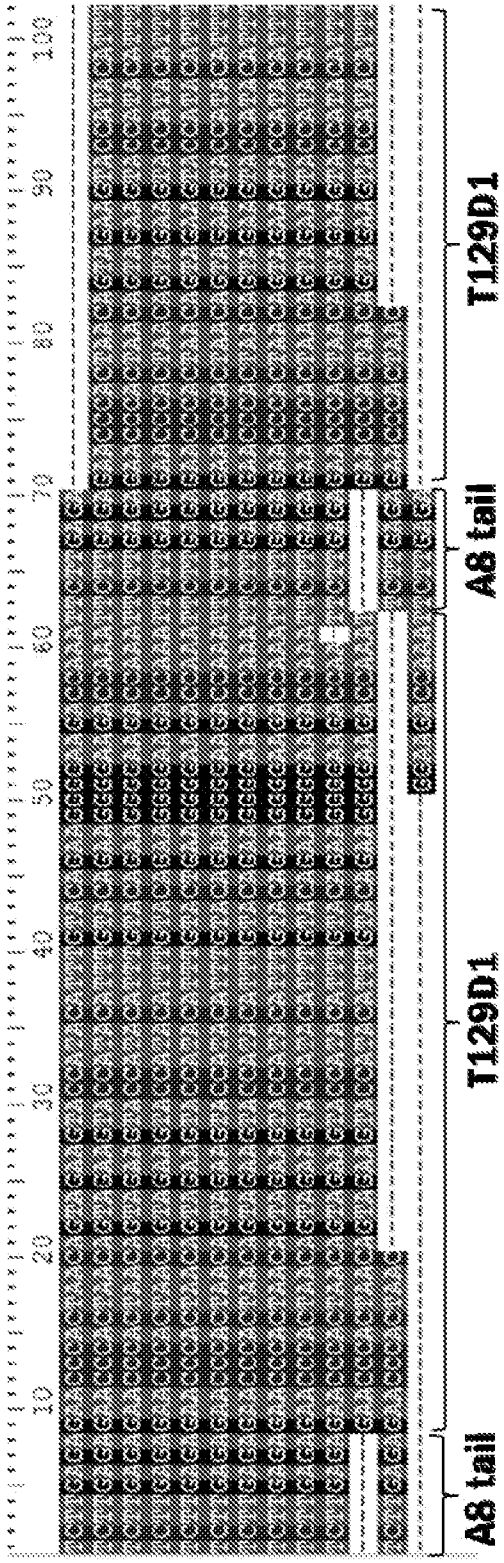
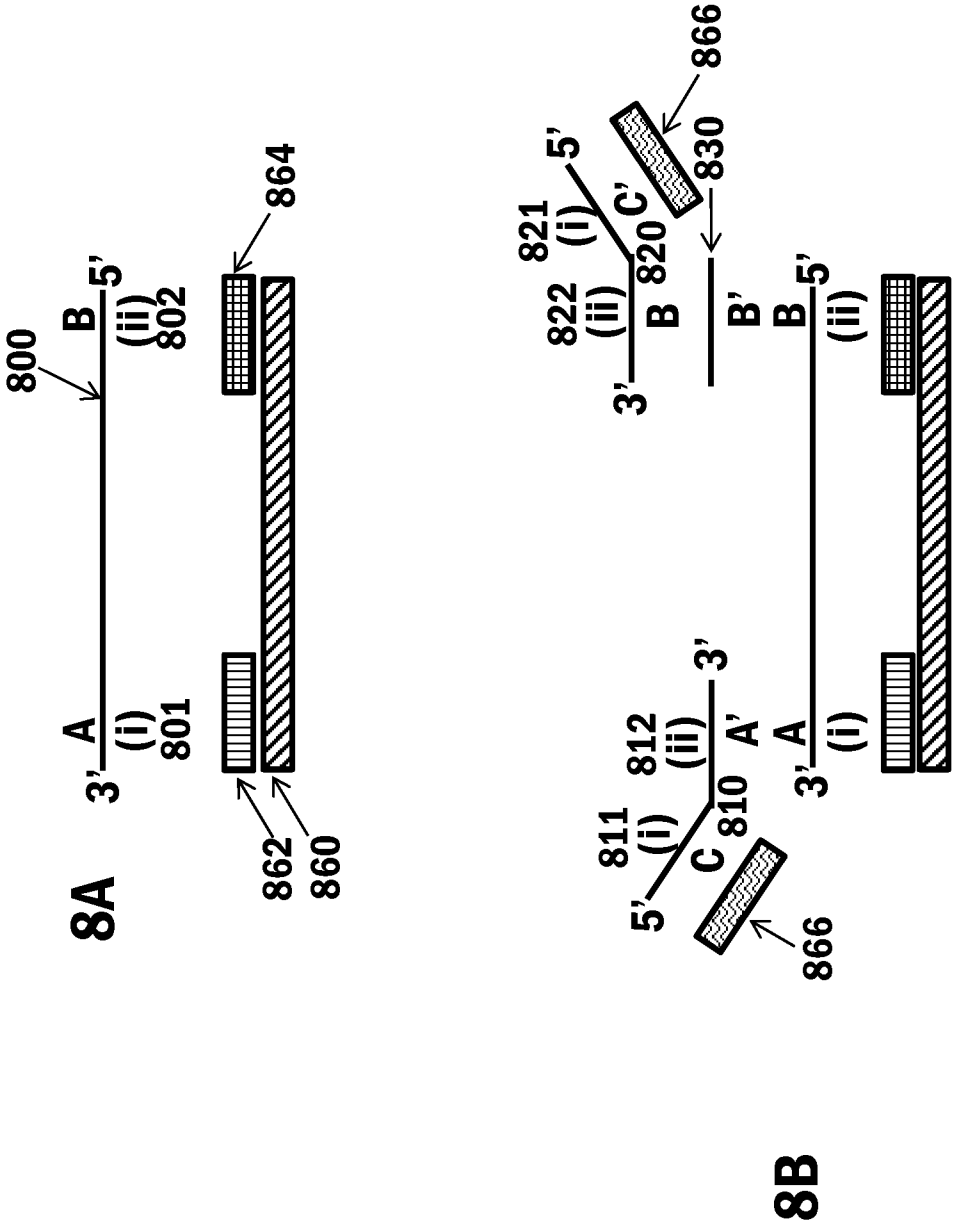


Figure 7



Figures 8A and 8B

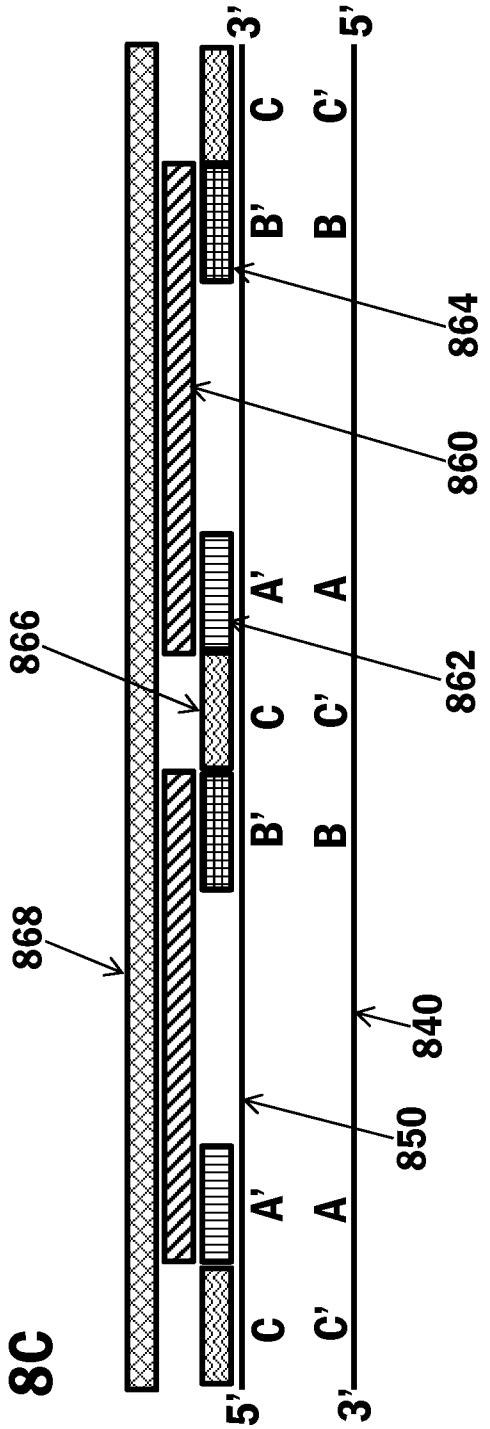


Figure 8C

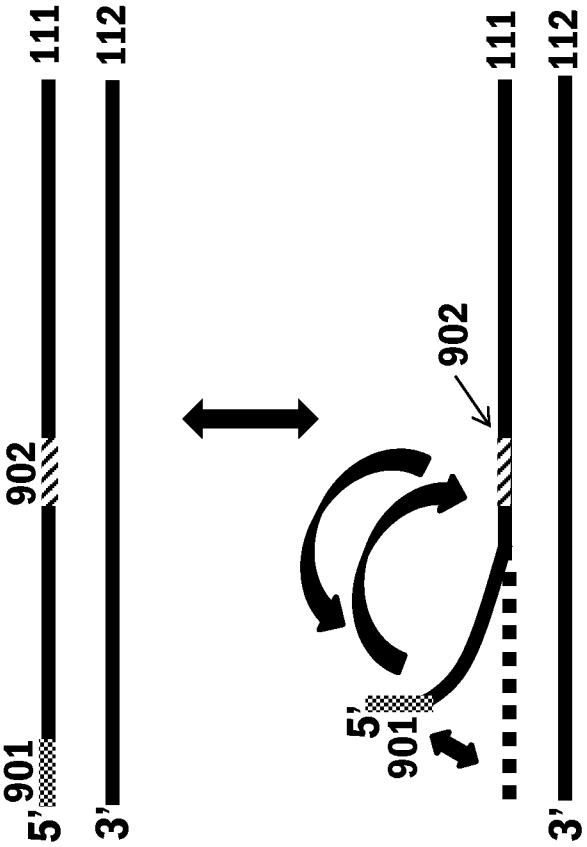
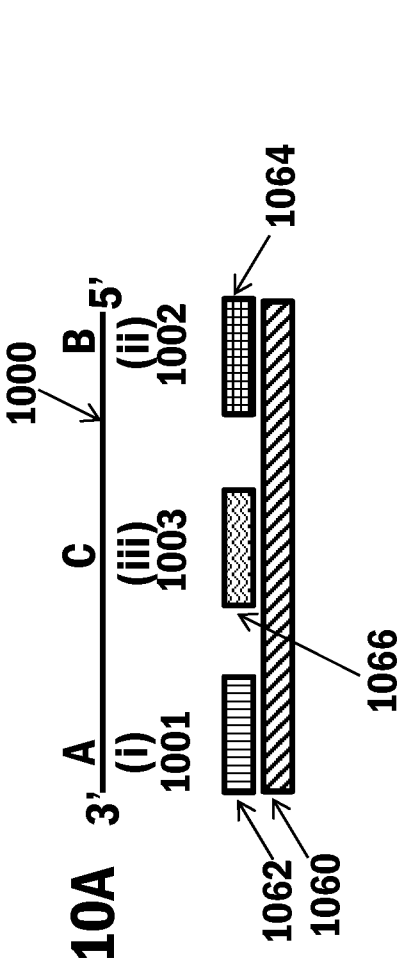
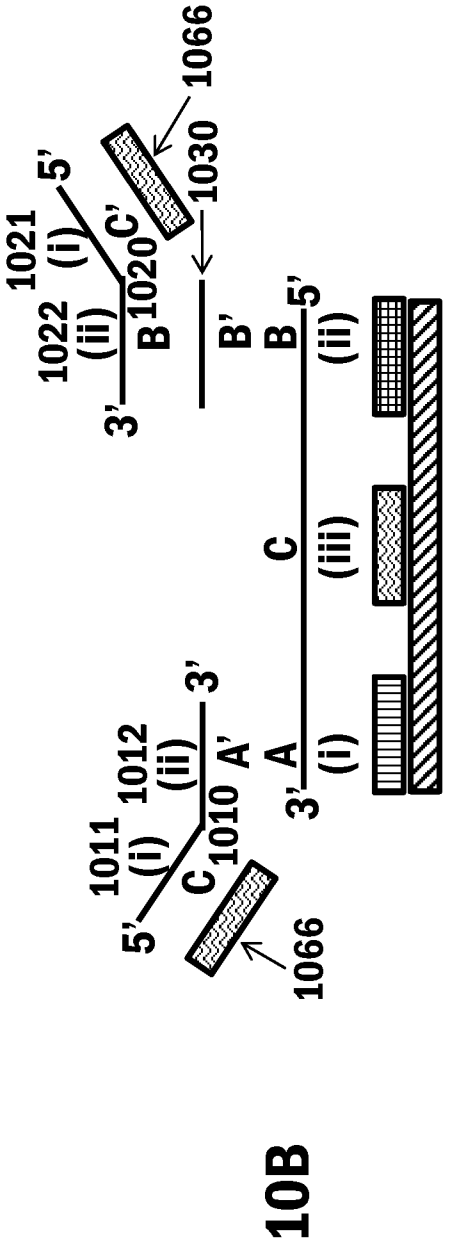


Figure 9





Figures 10A and 10B

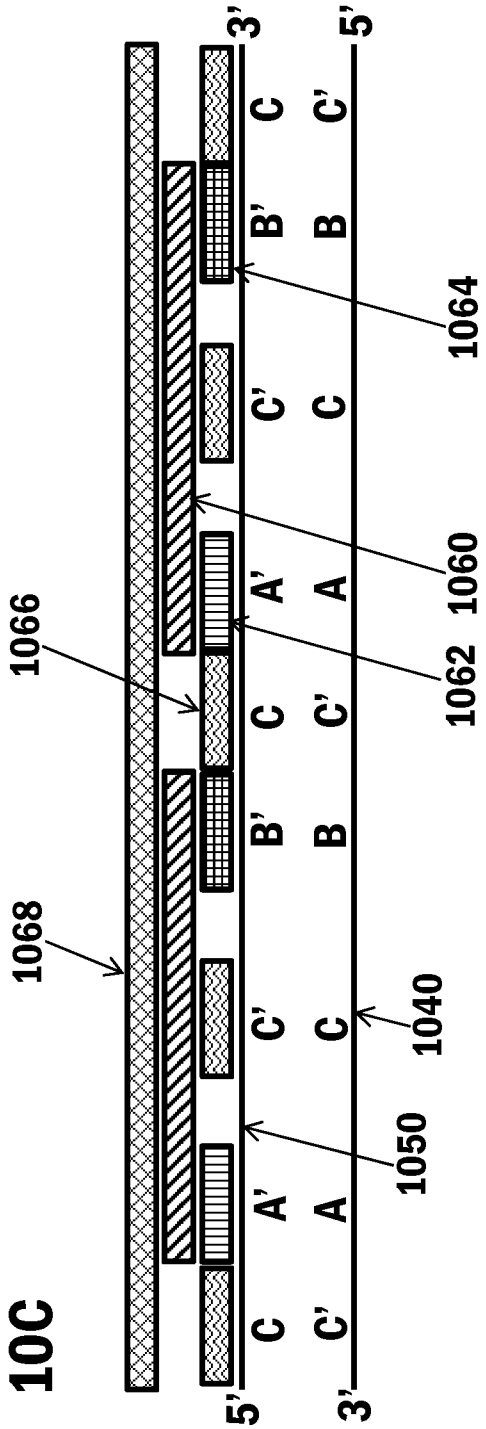

10C
1066
1068
1064
1060
1062
1040
1050
5'
3'
3'
5'
C
C'
A'
A
C'
C
B'
B
C
C'
A'
A
C'
C
B'
B
C
C'

Figure 10C

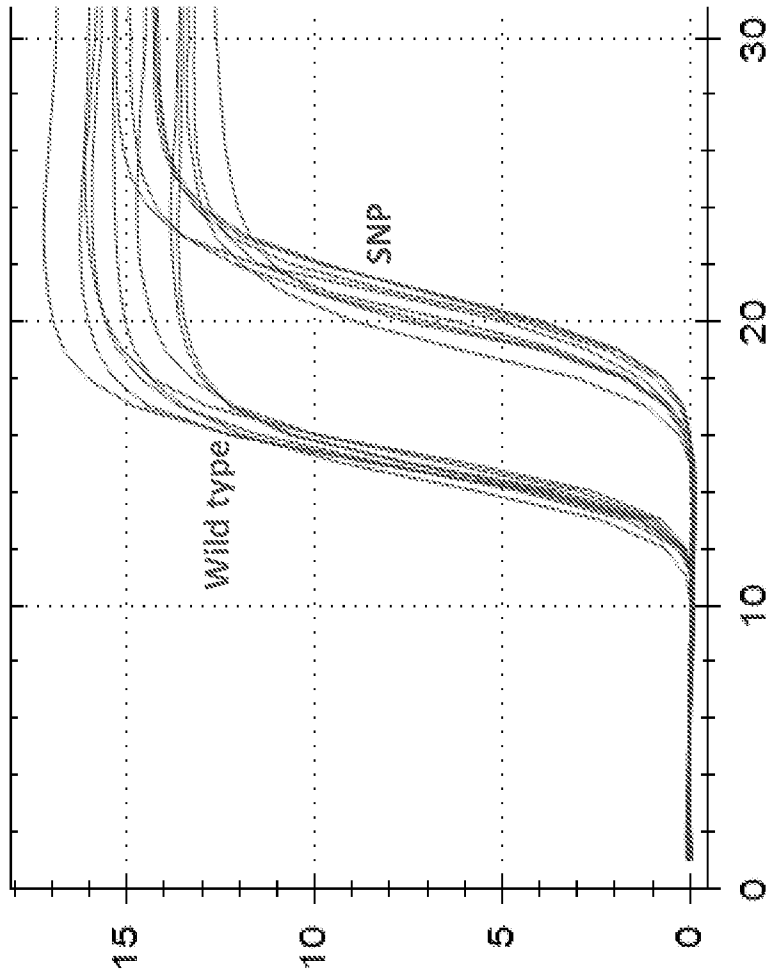


Figure 11

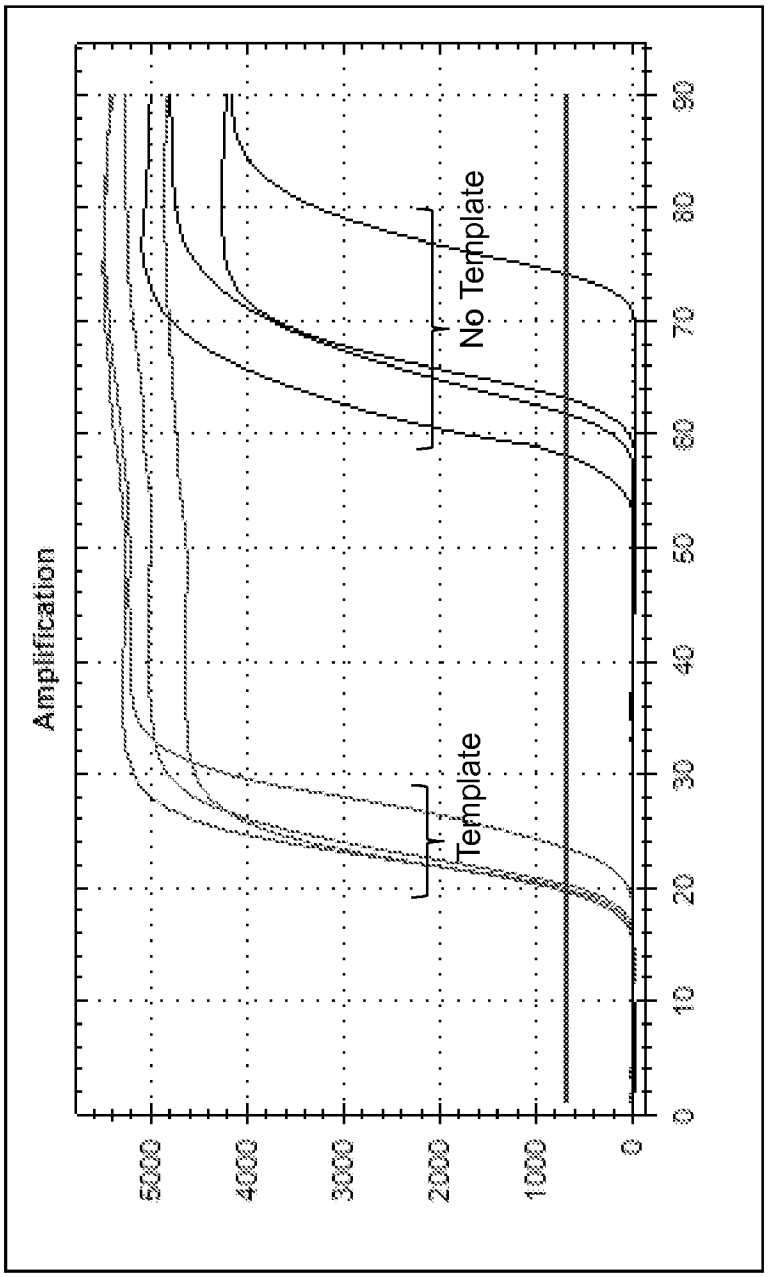

Amplification
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80
90
Template
No Template

Figure 12

INTERNATIONAL SEARCH REPORT	International application No. PCT/US 2014/056151

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68 *(2006.01)*
C12N 15/11 *(2006.01)*
C12M 1/00 *(2006.01)*

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 1/68, C12N 15/11, C12M 1/00

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)

PubMed, USPTO, PatSearch (RUPRO internal), Esp@cenet, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2009/0143235 A1 (COMPLETE GENOMICS, INC.) 04.06.2009, claims	1-102
A	WO 2006/095169 A1 (LINGVITAE AS) 14.09.2006, claims	1-102
A	WO 2008/032058 A2 (LINGVITAE AS) 20.03.2008, claims	1-102

☐ 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

"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
23 December 2014 (23.12.2014)	25 December 2014 (25.12.2014)
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37	Authorized officer M.Hudyuev Telephone No. 495 531 65 15

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 106255763 A
(43)申请公布日 2016.12.21

(21)申请号 201480073710.5

(22)申请日 2014.09.17

(30)优先权数据
61/908,027 2013.11.22 US
14/214,850 2014.03.15 US
PCT/US2014/030034 2014.03.15 US
62/001,050 2014.05.20 US

(85)PCT国际申请进入国家阶段日
2016.07.20

(86)PCT国际申请的申请数据
PCT/US2014/056151 2014.09.17

(87)PCT国际申请的公布数据
WO2015/076919 EN 2015.05.28

(71)申请人 赛拉诺斯股份有限公司
地址 美国加利福尼亚州

(72)发明人 K·贝尔霍钦 J·李 P·帕特尔 A·理查德森 S·塔巴克曼

(74)专利代理机构 上海专利商标事务所有限公司 31100
代理人 杨昀 余颖

(51)Int.Cl.
C12Q 1/68(2006.01)
C12N 15/11(2006.01)
C12M 1/00(2006.01)

权利要求书14页 说明书68页
序列表8页 附图20页

(54)发明名称

核酸扩增

(57)摘要

本文公开了用于核酸扩增和多联体生成的方法和组合物。本文提供的扩增方法可以在等温条件下进行。方法和组合物可以包括诸如核酸聚合酶和引物等试剂。

CN 106255763 A

1.一种拷贝多核苷酸模板的方法,该方法包括:

在包含第一引物和第二引物的反应混合物中温育该多核苷酸模板,其中:

第一引物包含第一区域和第二区域,其中第一引物的第二区域包含与该多核苷酸模板的第一部分互补的核苷酸序列;

第二引物包含第一区域和第二区域,其中第二引物的第二区域包含与配偶体核苷酸序列互补的核苷酸序列,其中该配偶体核苷酸序列与该多核苷酸模板的第二部分互补;并且

在该多核苷酸模板与第一引物和第二引物一起温育时,形成至少一条多联体链,其中该多联体链包含5'端和3'端,并且包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列,其中:C'表示第二引物的第一区域的核苷酸序列,T表示该多核苷酸模板的核苷酸序列或其类似序列,且X表示任何数目和序列的核苷酸。

2.如权利要求1所述的方法,其中所述多联体链是第一多联体链,并且其中在所述多核苷酸模板与第一引物和第二引物一起温育时,还形成了第二多联体链,其中第二多联体链包含5'端和3'端,并且包含具有5'至3'方向的通用结构C-X'-T'-C-T'-C的核苷酸序列,其中:C表示第一引物的第一区域的核苷酸序列,T'表示与多核苷酸模板互补的核苷酸序列,且X'表示与X的核苷酸序列互补的核苷酸序列。

3.如权利要求1所述的方法,其中X含有具有5'至3'方向的通用结构$[(C'-T)_N]$的序列,其中C'表示第二引物的第一区域的核苷酸序列,T表示多核苷酸模板的核苷酸序列或其类似序列,且N为0至1000之间的任何整数。

4.如权利要求1所述的方法,其中X表示0个核苷酸。

5.如权利要求1所述的方法,其中所述反应混合物进一步包含具有链置换活性的DNA聚合酶。

6.如权利要求5所述的方法,其中所述反应混合物进一步包含逆转录酶。

7.如权利要求1所述的方法,其中所述多核苷酸模板是RNA分子。

8.如权利要求7所述的方法,其中T表示与所述多核苷酸模板的RNA序列类似的DNA序列的核苷酸序列。

9.如权利要求1所述的方法,其中所述多核苷酸模板包含双链核酸模板的一条链。

10.如权利要求1所述的方法,其中在所述反应混合物的温育过程中,反应混合物的温度不超过80℃。

11.如权利要求1所述的方法,其中所述第一引物的第一区域含有4至25个核苷酸。

12.如权利要求1所述的方法,其中所述第一引物的第一区域和所述第二引物的第一区域含有相同数目的核苷酸。

13.如权利要求1所述的方法,其中所述多核苷酸模板含有10至1000个核苷酸。

14.如权利要求1所述的方法,其中所述反应混合物进一步包含核酸染料。

15.如权利要求1所述的方法,其中在该方法开始后60分钟内,所述反应混合物中的多核苷酸模板的拷贝数增加至少10倍。

16.一种测定生物样品中的靶多核苷酸模板的方法,该方法包括:

A)在包含第一引物和第二引物的反应混合物中温育该生物样品或其部分,其中:

第一引物包含第一区域和第二区域,其中第一引物的第二区域包含与该多核苷酸模板的第一部分互补的核苷酸序列;

第二引物包含第一区域和第二区域，其中第二引物的第二区域包含与配偶体核苷酸序列互补的核苷酸序列，其中该配偶体核苷酸序列与多核苷酸模板的第二部分互补；并且

在多核苷酸模板与第一引物和第二引物一起温育时，形成至少一条多联体链，其中该多联体链包含5'端和3'端，并且包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列，其中：C'表示第二引物的第一区域的核苷酸序列，T表示多核苷酸模板的核苷酸序列或其类似序列，且X表示任何数目和序列的核苷酸；以及

B)在A)的温育步骤开始后在一个或多个点测量A)的反应混合物中扩增的核酸的量。

17.如权利要求16所述的方法，其中测量反应混合物中扩增的核酸的量包括确定反应混合物中的荧光的水平。

18.如权利要求16所述的方法，其进一步包括确定反应混合物中的核酸扩增的转变时间。

19.如权利要求16所述的方法，其中所述生物样品或其部分来自人。

20.一种生成包含双链核酸模板的至少两个拷贝的多联体的方法，该方法包括：

在反应混合物中温育至少第一模板分子和第二模板分子，其中：

第一模板分子包含第一核酸链和第二核酸链，其中：

第一模板分子的第一核酸链包含具有5'至3'方向的通用结构H'-S-Y_1-H'的核苷酸序列，其中：H'表示第一同源序列的核苷酸序列，S表示双链核酸模板的第一链的核苷酸序列，且Y_1表示任何数目和序列的核苷酸；并且

第一模板分子的第二核酸链包含具有5'至3'方向的通用结构H-Y_1'-S'-H的核苷酸序列，其中：H表示第二同源序列的核苷酸序列，其中第一同源序列与第二同源序列彼此互补，Y_1'表示与Y_1的核苷酸序列互补的核苷酸序列，且S'表示双链核酸模板的第二链的核苷酸序列，其中双链核酸模板的第一链和第二链彼此互补；并且

第二模板分子包含第一核酸链和第二核酸链，其中：

第二模板分子的第一核酸链包含具有5'至3'方向的通用结构H'-S-Y_2-H'的核苷酸序列，其中：H'表示第一同源序列的核苷酸序列，S表示双链核酸模板的第一链的核苷酸序列，且Y_2表示任何数目和序列的核苷酸；且

第一模板分子的第二核酸链包含具有5'至3'方向的通用结构H-Y_2'-S'-H的核苷酸序列，其中：H表示第二同源序列的核苷酸序列，Y_2'表示与Y_2的核苷酸序列互补的核苷酸序列，且S'表示双链核酸模板的第二链的核酸序列；并且

在反应混合物中的第一模板分子与第二模板分子一起温育时，形成包含双链核酸模板的至少两个拷贝的至少一个多联体，其中该多联体包含第一多联体链和第二多联体链，其中第一多联体链包含5'端和3'端，并包含具有5'至3'方向的通用结构H'-S-Y_2-H'-S-Y_1-H'的核苷酸序列，其中H'、Y_1、S和Y_2中的每一个均表示如上所述的核苷酸序列；并且其中第二多联体链包含5'端和3'端，并包含具有5'至3'方向的通用结构H-Y_1'-S'-H-Y_2'-S'-H的序列，其中，H'、Y_1、S和Y_2中的每一个均表示如上所述的核苷酸序列。

21.如权利要求20所述的方法，其中Y_1和Y_2中的至少一个表示0个核苷酸。

22.如权利要求21所述的方法，其中Y_1和Y_2均表示0个核苷酸。

23.如权利要求20所述的方法，其中Y_1含有具有5'至3'方向的通用结构[$(H'-S)_{N1}$]的序列，其中H'表示第一同源序列的核苷酸序列，S表示双链核酸模板的第一链的核苷酸序列，

且N1是0至2000之间的任何整数。

24.如权利要求20所述的方法，其中Y_1含有具有5'至3'方向的通用结构[(H'-S)$_{N1}$]的序列，其中H'表示第一同源序列的核苷酸序列，S表示双链核酸模板的第一链的核苷酸序列，且N1是0至2000之间的任何整数，并且其中Y_2含有具有5'至3'方向的通用结构[(H'-S)$_{N2}$]的序列，其中H'表示第一同源序列的核苷酸序列，S表示双链核酸模板的第一链的核苷酸序列，且N2是0至2000之间的任何整数。

25.如权利要求24所述的方法，其中N1和N2是不同的整数。

26.如权利要求25所述的方法，其中N1为0，且N2是1至2000之间的整数。

27.如权利要求20所述的方法，其中第一模板分子和第二模板分子均为双链DNA分子。

28.如权利要求20所述的方法，其中第一同源序列含有4至25个核苷酸。

29.一种生成包含双链核酸模板的两个或更多个拷贝的多联体的方法，该方法包括：

(A)在使得合成与双链核酸模板的第一链退火的第一引物的第一拷贝的延伸产物的条件下，用第一引物的第一拷贝和聚合酶处理包含双链核酸模板的一级双链核酸，

其中

第一引物包含5'末端核苷酸、3'末端核苷酸以及两个区域：

(i)尾区，其包含：

(a)引物的5'末端核苷酸

(b)最内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游

(c)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及

(ii)模板结合区，其包括

(a)引物的3'末端核苷酸

(b)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游

(c)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，并且

第一引物的第一拷贝的模板结合区与双链核酸模板的第一链退火，

(B)在使得合成与步骤(A)的第一引物的第一拷贝的延伸产物退火的第二引物的延伸产物的条件下，用第二引物和聚合酶处理步骤(A)的第一引物的第一拷贝的延伸产物，

其中

第二引物包含5'末端核苷酸、3'末端核苷酸以及两个区域：

(i)尾区，其包含

(a)引物的5'末端核苷酸

(b)最内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游

(c)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及

(ii)模板结合区，其包含

(a)引物的3'末端核苷酸

(b)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游

(c)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，

第二引物的尾区包含与第一引物的尾区的核苷酸序列互补的核苷酸序列

第二引物的模板结合区与步骤(A)的第一引物的第一拷贝的延伸产物退火,并且

第二引物的延伸产物含有5'末端核苷酸、3'末端核苷酸以及包含3'末端核苷酸的3'末端区,其中该3'末端区含有与在5'至3'方向读取的第二引物的尾区的核苷酸序列相同的核苷酸序列,且该3'末端区的最后的核苷酸是第二引物的延伸产物的3'末端核苷酸,

(C)在使得合成与步骤(B)的第二引物的延伸产物退火的第一引物的第二拷贝的延伸产物的条件下,用第一引物的第二拷贝和聚合酶处理步骤(B)的第二引物的延伸产物,以产生包含步骤(B)的第二引物的延伸产物和第一引物的第二拷贝的延伸产物的二级核酸的第一拷贝,

其中

第一引物的第二拷贝的延伸产物含有5'末端核苷酸、3'末端核苷酸以及包含3'末端核苷酸的3'末端区,其中该3'末端区含有与在5'至3'方向读取的第一引物的尾区的核苷酸序列相同的核苷酸序列,并且该3'末端区的最后的核苷酸是第二引物的延伸产物的3'末端核苷酸,

(D)将至少步骤(C)重复另外一次或多次以生成步骤(C)的二级核酸的至少第二拷贝,

(E)在使得二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物的3'末端区与二级核酸的第二拷贝的第二引物的延伸产物的3'末端区退火的条件下,处理步骤(C)的二级核酸的第一拷贝以及步骤(D)的二级核酸的第二拷贝,以产生包含二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物以及二级核酸的第二拷贝的第二引物的延伸产物的交换结构,

(F)在使得合成二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物的延伸产物以及合成二级核酸的第二拷贝的第二引物的延伸产物的延伸产物的条件下,用聚合酶处理步骤(E)的交换结构,以产生包含步骤(A)的双链核酸模板的两个拷贝的多联体,其中该多联体包含二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物的延伸产物以及二级核酸的第二拷贝的第二引物的延伸产物的延伸产物。

30.如权利要求29所述的方法,其中步骤(A)的核酸聚合酶是具有链置换活性的DNA聚合酶。

31.如权利要求29所述的方法,其中该方法的所有步骤均在不高于80℃的温度下进行。

32.如权利要求29所述的方法,其中两个或更多个步骤同时进行。

33.一种生成包含多核苷酸模板或其类似序列的两个或更多个拷贝的多联体的方法,该方法包括,

(A)在使得合成与多核苷酸模板退火的第一引物的第一拷贝的延伸产物的条件下,用第一引物的第一拷贝和聚合酶处理包含多核苷酸模板的一级核酸,

其中

第一引物包含5'末端核苷酸、3'末端核苷酸以及两个区域:

(i)尾区,其包含

(a)引物的5'末端核苷酸

(b)最内核苷酸,其中该最内核苷酸在5'末端核苷酸的下游

(c)5'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,

以及

(ii)模板结合区,其包括

(a)引物的3'末端核苷酸

(b)最内核苷酸,其中该最内核苷酸在3'末端核苷酸的上游

(c)3'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,并且第一引物的第一拷贝的模板结合区与多核苷酸模板退火,

(B)在使得合成与步骤(A)的第一引物的第一拷贝的延伸产物退火的第二引物的延伸产物的条件下,用第二引物和聚合酶处理步骤(A)的第一引物的第一拷贝的延伸产物,

其中

第二引物包含5'末端核苷酸、3'末端核苷酸以及两个区域:

(i)尾区,其包含

(a)引物的5'末端核苷酸

(b)最内核苷酸,其中该最内核苷酸在5'末端核苷酸的下游

(c)5'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸

(ii)模板结合区,其包含

(a)引物的3'末端核苷酸

(b)最内核苷酸,其中该最内核苷酸在3'末端核苷酸的上游

(c)3'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,

第二引物的尾区包含与第一引物的尾区的核苷酸序列互补的核苷酸序列,

第二引物的模板结合区与步骤(A)的第一引物的第一拷贝的延伸产物退火,并且

第二引物的延伸产物含有5'末端核苷酸、3'末端核苷酸以及包含3'末端核苷酸的3'末端区,其中该3'末端区含有与在5'至3'方向读取的第二引物的尾区的核苷酸序列相同的核苷酸序列,且该3'末端区的最后的核苷酸是第二引物的延伸产物的3'末端核苷酸,

(C)在使得合成与步骤(B)的第二引物的延伸产物退火的第一引物的第二拷贝的延伸产物的条件下,用第一引物的第二拷贝和聚合酶处理步骤(B)的第二引物的延伸产物,以产生包含步骤(B)的第二引物的延伸产物和第一引物的第二拷贝的延伸产物的二级核酸的第一拷贝,

其中

第一引物的第二拷贝的延伸产物含有5'末端核苷酸、3'末端核苷酸以及包含3'末端核苷酸的3'末端区,其中该3'末端区含有与在5'至3'方向读取的第一引物的尾区的核苷酸序列相同的核苷酸序列,并且该3'末端区的最后的核苷酸是第二引物的延伸产物的3'末端核苷酸,

(D)将至少步骤(C)重复另外一次或多次以生成包含步骤(B)的第二引物的延伸产物和步骤(C)的第一引物的第二拷贝的延伸产物的二级核酸的至少第二拷贝,

(E)在使得二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物的3'末端区与二级核酸的第二拷贝的第二引物的延伸产物的3'末端区退火的条件下,处理步骤(C)的二级核酸的第一拷贝以及步骤(D)的二级核酸的第二拷贝,以产生包含二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物以及二级核酸的第二拷贝的第二引物的延伸产物的交换结构,

(F)在使得合成二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物的延伸产物以及合成二级核酸的第二拷贝的第二引物的延伸产物的延伸产物的条件下,用聚合酶处理步骤(E)的交换结构,以产生包含步骤(A)的多核苷酸模板的两个拷贝的多联体,其中该多联体包含二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物的延伸产物以及二级核酸的第二拷贝的第二引物的延伸产物的延伸产物。

34.如权利要求33所述的方法,其中步骤(A)的核酸聚合酶是DNA聚合酶。

35.如权利要求33所述的方法,其中步骤(A)的核酸聚合酶是逆转录酶。

36.如权利要求33所述的方法,其中该方法的所有步骤均在不高于80℃的温度下进行。

37.如权利要求33所述的方法,其中两个或更多个步骤同时进行。

38.一种用于生成包含双链核酸模板的两个或更多个拷贝的多联体的方法,该方法包括,

(A)制备反应混合物,其包含:

(i)包含双链核酸模板的一级核酸,

(ii)分离的核酸聚合酶,

(iii)第一引物,其包含5'末端核苷酸、3'末端核苷酸以及两个区域:

(a)尾区,其包含

(1)引物的5'末端核苷酸

(2)最内核苷酸,其中该最内核苷酸在5'末端核苷酸的下游

(3)5'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,以及

(b)模板结合区,其包括

(1)引物的3'末端核苷酸

(2)最内核苷酸,其中该最内核苷酸在3'末端核苷酸的上游

(3)3'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,

其中模板结合区与核酸模板的第一链互补

(iv)第二引物,其包含5'末端核苷酸、3'末端核苷酸以及两个区域:

(a)尾区,其包含

(1)引物的5'末端核苷酸

(2)最内核苷酸,其中该最内核苷酸在5'末端核苷酸的下游

(3)5'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,以及

(b)模板结合区,其包含

(1)引物的3'末端核苷酸

(2)最内核苷酸,其中该最内核苷酸在3'末端核苷酸的上游

(3)3'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,并且

其中模板结合区与核酸模板的第二链互补,并且

其中第二引物的尾区含有与第一引物的尾区的核苷酸序列互补的核苷酸序列,以及

(B)在不高于80℃的温度下将反应混合物温育至少3分钟。

39.一种用于生成包含多核苷酸模板的两个或更多个拷贝的多联体的方法，该方法包括，

(A)制备反应混合物，其包含：

(i)包含多核苷酸模板的核酸

(ii)分离的核酸聚合酶，

(iii)第一引物，其包含5'末端核苷酸、3'末端核苷酸以及两个区域：

(a)尾区，其包含

(1)引物的5'末端核苷酸

(2)最内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游

(3)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及

(b)模板结合区，其包括

(1)引物的3'末端核苷酸

(2)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游

(3)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，并且

其中模板结合区与多核苷酸模板互补，

(iv)第二引物，其包含5'末端核苷酸、3'末端核苷酸以及两个区域：

(a)尾区，其包含

(1)引物的5'末端核苷酸

(2)最内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游

(3)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及

(b)模板结合区，其包含

(1)引物的3'末端核苷酸

(2)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游

(3)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，并且

其中模板结合区与互补于多核苷酸模板的核苷酸序列互补，并且

其中第二引物的尾区含有与第一引物的尾区的核苷酸序列互补的核苷酸序列，以及

(B)在不高于80℃的温度下将反应混合物温育至少3分钟。

40.如权利要求29-39中任一项所述的方法，其中在该方法开始后60分钟内，所述双链核酸模板或多核苷酸模板扩增至少10倍。

41.如权利要求29-39中任一项所述的方法，其中所述第一引物的尾区含有4至25个核苷酸。

42.一种容器，其包含在其中流体连通的：

第一引物，其中第一引物包含第一区域和第二区域，并且其中第一引物的第二区域包含与多核苷酸模板的第一部分互补的核苷酸序列；

第二引物，其中第二引物包含第一区域和第二区域，并且其中第二引物的第二区域包

含与配偶体核苷酸序列互补的核苷酸序列，其中该配偶体核苷酸序列与核苷酸模板的第二部分互补；以及

至少一条多联体链，其中该多联体链包含5'端和3'端，并且包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列，其中：C'表示第二引物的第一区域的核苷酸序列，T表示多核苷酸模板的核苷酸序列或其类似序列，且X表示任何数目和序列的核苷酸。

43.如权利要求42所述的容器，其中所述多联体链是第一多联体链，并且该容器进一步包含第二多联体链，其中第二多联体链包含5'端和3'端，并且包含具有5'至3'方向的通用结构C-X'-T'-C-T'-C的核苷酸序列，其中：C表示第一引物的第一区域的核苷酸序列，T'表示与多核苷酸模板互补的核苷酸序列，且X'表示与X的核苷酸序列互补的核苷酸序列。

44.如权利要求42所述的容器，其中X含有具有5'至3'方向的通用结构$[(C'-T)_N]$的序列，其中C'表示第二引物的第一区域的核苷酸序列，T表示多核苷酸模板的核苷酸序列或其类似序列，且N为0至1000之间的任何整数。

45.如权利要求42所述的容器，其中X表示0个核苷酸。

46.一种容器，其包含在其中流体连通的：

(A)分离的核酸聚合酶，

(B)包含至少第一链的核酸模板，

(C)第一引物，其包含5'末端核苷酸、3'末端核苷酸以及两个区域：

(i)尾区，其包含

(a)引物的5'末端核苷酸

(b)最内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游

(c)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及

(ii)模板结合区，其包括：

(a)引物的3'末端核苷酸

(b)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游

(c)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，

其中模板结合区与核酸模板的第一链互补，以及

(D)第二引物，其包含5'末端核苷酸、3'末端核苷酸以及两个区域：

(i)尾区，其包含

(a)引物的5'末端核苷酸

(b)最内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游

(c)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及

(ii)模板结合区，其包含

(a)引物的3'末端核苷酸

(b)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游

(c)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，并且

其中模板结合区与互补于核酸模板的第一链的核苷酸序列互补，并且

其中第二引物的尾区含有与第一引物的尾区的核苷酸序列互补的核苷酸序列。

47.如权利要求42-46中任一项所述的容器，其中该容器包含具有链置换活性的DNA聚合酶。

48.如权利要求47所述的容器，其中该容器进一步包含逆转录酶。

49.如权利要求42所述的容器，其中所述多核苷酸模板是RNA分子。

50.如权利要求49所述的容器，其中T表示与所述多核苷酸模板的RNA序列类似的DNA序列的核苷酸序列。

51.如权利要求42所述的容器，其中所述多核苷酸模板是DNA分子。

52.如权利要求51所述的容器，其中所述多核苷酸模板包含双链核酸模板的一条链。

53.如权利要求42所述的容器，其中所述第一引物的第一区域含有4至25个核苷酸。

54.如权利要求42所述的容器，其中所述第一引物的第一区域和所述第二引物的第一区域含有相同数目的核苷酸。

55.如权利要求46所述的容器，其中所述第一引物的尾区含有4至25个核苷酸。

56.如权利要求46所述的容器，其中所述第一引物的尾区和所述第二引物的第一区域含有相同数目的核苷酸。

57.如权利要求42-56中任一项所述的容器，其中该容器进一步包含核酸染料。

58.如权利要求42-57中任一项所述的容器，其中该容器进一步包含核苷酸和缓冲液。

59.如权利要求42-58中任一项所述的容器，其中该容器进一步包含来自受试者的生物样品的至少一部分。

60.如权利要求59所述的容器，其中所述受试者是人。

61.一种试剂盒，其包含两个或更多个流体隔离的器皿，该器皿共同包含：

第一引物，其中第一引物包含第一区域和第二区域，并且其中第一引物的第二区域包含与多核苷酸模板的第一部分互补的核苷酸序列；

第二引物，其中第二引物包含第一区域和第二区域，并且其中第二引物的第二区域包含与配偶体核苷酸序列互补的核苷酸序列，其中该配偶体核苷酸序列与核苷酸模板的第二部分互补；以及

分离的具有链置换活性的DNA聚合酶；其中：

第一引物的第一区域和第二引物的第一区域互补。

62.一种用于检测包含至少第一链的感兴趣的靶核酸的试剂盒，该试剂盒包含两个或更多个流体隔离的器皿，该器皿共同包含：

(A)分离的具有链置换活性的DNA聚合酶，

(B)第一引物，其包含5’末端核苷酸、3’末端核苷酸以及两个区域：

(i)尾区，其包含

(a)引物的5’末端核苷酸

(b)最内核苷酸，其中该最内核苷酸在5’末端核苷酸的下游

(c)5’末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及

(ii)模板结合区，其包括

(a)引物的3’末端核苷酸

(b)最内核苷酸,其中该最内核苷酸在3'末端核苷酸的上游

(c)3'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,

其中模板结合区与靶核酸的第一链互补,以及

(C)第二引物,其包含5'末端核苷酸、3'末端核苷酸以及两个区域:

(i)尾区,其包含

(a)引物的5'末端核苷酸

(b)最内核苷酸,其中该最内核苷酸在5'末端核苷酸的下游

(c)5'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,以及

(ii)模板结合区,其包含

(a)引物的3'末端核苷酸

(b)最内核苷酸,其中该最内核苷酸在3'末端核苷酸的上游

(c)3'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,并且

其中模板结合区与互补于靶核酸的第一链的核苷酸序列互补,并且

其中第二引物的尾区含有与第一引物的尾区的核苷酸序列互补的核苷酸序列。

63.如权利要求61或62所述的试剂盒,其进一步包含逆转录酶。

64.如权利要求61所述的试剂盒,其中所述第一引物的第一区域含有4至25个核苷酸。

65.如权利要求62所述的试剂盒,其中所述第一引物的尾区含有4至25个核苷酸。

66.如权利要求61-65中任一项所述的试剂盒,其进一步包含含有感兴趣的靶核酸的核苷酸序列的核酸。

67.一种用于拷贝多核苷酸模板的方法,该方法包括:

在没有热循环的条件下温育包含所述多核苷酸模板、第一引物和第二引物的反应混合物,其中:

所述多核苷酸模板包含第一部分和第二部分;

所述第一引物包含第一区域和第二区域,其中所述第一区域包含该引物的5'端,所述第二区域包含该引物的3'端,并且所述第二区域与所述多核苷酸模板的第一部分互补;

所述第二引物包含第一区域和第二区域,其中所述第一区域包含该引物的5'端,所述第二区域包含该引物的3'端,并且所述第二区域与同所述多核苷酸模板的第二部分互补的序列互补;

所述第一引物的第一区域与所述第二引物的第一区域互补;并且

生成所述多核苷酸模板的多个拷贝。

68.如权利要求67所述的方法,其中所述多核苷酸模板进一步包含第三部分,其中所述第三部分位于该多核苷酸模板中第一部分与第二部分之间,并且其中所述第二引物的第一区域与所述多核苷酸模板的第三部分互补。

69.一种用于扩增多核苷酸模板的方法,该方法包括在包含第一引物和第二引物的反应混合物中温育该多核苷酸模板,其中:

所述多核苷酸模板包含第一部分、第二部分和第三部分,其中所述第三部分位于该多核苷酸模板中所述第一部分与所述第二部分之间;

所述第一引物包含第一区域和第二区域，其中所述第一引物的第二区域与所述多核苷酸模板的第一部分互补；并且

所述第二引物包含第一区域和第二区域，其中所述第二引物的第二区域与同所述多核苷酸模板的第二部分互补的序列互补，所述第二引物的第一区域与所述第一引物的第一区域互补，并且所述第二引物的第一区域与所述多核苷酸模板的第三部分互补。

70.一种用于评估在多核苷酸模板中核苷酸序列的感兴趣位置处的核苷酸的身份的方法，该方法包括：

A)在至少第一反应混合物和第二反应混合物的每一个中提供所述多核苷酸模板的拷贝，其中：

所述多核苷酸模板包含第一部分、第二部分和第三部分，其中所述第三部分位于该多核苷酸模板中所述第一部分与所述第二部分之间，并且其中所述感兴趣位置是在第三位置；

所述第一反应混合物包含所述多核苷酸模板的拷贝、第一引物和第二引物，其中：

所述第一引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与所述多核苷酸模板的第一部分互补；

所述第二引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与同所述多核苷酸模板的第二部分互补的序列互补；

所述第一引物的第一区域与所述第二引物的第一区域互补；并且

所述第二引物的第一区域与所述多核苷酸模板的第三部分互补；

所述第二反应混合物包含所述多核苷酸模板的拷贝、第三引物和第四引物，其中：

所述第三引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与所述多核苷酸模板的第一部分互补；

所述第四引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与同所述多核苷酸模板的第二部分互补的序列互补；

所述第三引物的第一区域与所述第四引物的第一区域互补；并且

所述第四引物的第一区域与所述多核苷酸模板的第三部分互补；并且

所述第二引物的第一区域的核苷酸序列与所述第四引物的第一区域的核苷酸序列相差一个核苷酸，其中如果为了序列的最大互补而用所述多核苷酸模板的第三部分的核苷酸序列定位所述第二引物或第四引物的第一区域的核苷酸序列，则在所述第二引物与第四引物中不同的核苷酸的位置对应于所述多核苷酸模板中感兴趣的核苷酸的位置；

B)在没有热循环的条件下温育所述第一反应混合物和第二反应混合物；以及

C)比较所述第一反应混合物中多核苷酸模板的扩增速率或量与所述第二反应混合物中多核苷酸模板的扩增速率或量，其中所述多核苷酸模板的扩增速率或量指示所述第二或第四引物的第一区域与所述多核苷酸模板的第三部分的核苷酸序列之间的互补程度。

71.如权利要求67-70中任一项所述的方法，其中所述多核苷酸模板为DNA链。

72.如权利要求67-70中任一项所述的方法，其中所述多核苷酸模板为RNA链。

73.如权利要求67-70中任一项所述的方法，其中所述多核苷酸模板是双链体DNA分子

的一条链。

74.如权利要求67-73中任一项所述的方法，其中所述多核苷酸模板的第一部分和第二部分各自为6至30个核苷酸的长度。

75.如权利要求67-74中任一项所述的方法，其中所述多核苷酸模板的第三部分为4至14个核苷酸的长度。

76.如权利要求67-75中任一项所述的方法，其中所述反应混合物中多核苷酸模板的拷贝数在该方法开始的60分钟内增加至少10倍。

77.如权利要求67-76中任一项所述的方法，其中在所述反应混合物的温育过程中生成包含所述多核苷酸模板的至少三个拷贝的多联体链。

78.一种用于扩增双链核酸分子的方法，该方法包括在包含第一引物和第二引物的反应混合物中温育该双链核酸分子，其中：

所述双链核酸分子包含第一链和第二链，其中所述第一链包含第一部分和第三部分，并且所述第二链包含第二部分；

所述第一引物包含第一区域和第二区域，其中所述第一引物的第二区域与所述第一链的第一部分互补；并且

所述第二引物包含第一区域和第二区域，其中所述第二引物的第二区域与所述第二链的第二部分互补，所述第二引物的第一区域与所述第一链的第三部分互补，并且所述第二引物的第一区域与所述第一引物的第一区域互补。

79.如权利要求69-78中任一项所述的方法，其中所述反应混合物在没有热循环的情况下温育。

80.如权利要求67-79中任一项所述的方法，其中所述反应混合物进一步包含核酸染料。

81.如权利要求67-80中任一项所述的方法，其中所述反应混合物进一步包含逆转录酶。

82.一种反应混合物，其包含：

多核苷酸模板、第一引物和第二引物，其中：

所述多核苷酸模板包含第一部分、第二部分和第三部分，其中所述第三部分位于该多核苷酸模板中所述第一部分与所述第二部分之间；

所述第一引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与所述多核苷酸模板的第一部分互补；

所述第二引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与同所述多核苷酸模板的第二部分互补的序列互补；

所述第一引物的第一区域与所述第二引物的第一区域互补；并且

所述第二引物的第一区域与所述多核苷酸模板的第三部分互补。

83.如权利要求82所述的反应混合物，其中所述反应混合物进一步包含具有链置换活性的DNA聚合酶。

84.如权利要求82-83中任一项所述的反应混合物，其中所述反应混合物进一步包含逆转录酶。

85.如权利要求82-84中任一项所述的反应混合物,其中所述反应混合物进一步包含核酸染料。

86.如权利要求82-85中任一项所述的反应混合物,其中所述反应混合物进一步包含核苷酸和缓冲液。

87.如权利要求82-86中任一项所述的反应混合物,其中所述多核苷酸模板为DNA链。

88.如权利要求82-87中任一项所述的反应混合物,其中所述多核苷酸模板为RNA链。

89.如权利要求82-88中任一项所述的反应混合物,其中所述多核苷酸模板是双链体DNA分子的一部分。

90.如权利要求82-89中任一项所述的反应混合物,其中所述多核苷酸模板的第一部分和第二部分各自为6至30个核苷酸的长度。

91.如权利要求82-90中任一项所述的反应混合物,其中所述多核苷酸模板的第三部分为4至14个核苷酸的长度。

92.一种用于扩增多核苷酸模板的试剂盒,该试剂盒包含:

第一引物和第二引物,其中:

所述第一引物包含第一区域和第二区域,其中所述第一区域包含该引物的5'端,所述第二区域包含该引物的3'端,并且所述第二区域与所述多核苷酸模板的第一部分互补;

所述第二引物包含第一区域和第二区域,其中所述第一区域包含该引物的5'端,所述第二区域包含该引物的3'端,并且所述第二区域与同所述多核苷酸模板的第二部分互补的序列互补;

所述第一引物的第一区域与所述第二引物的第一区域互补;并且

所述第二引物的第一区域与所述多核苷酸模板的第三部分互补,其中所述第三部分位于该多核苷酸模板中第一部分与第二部分之间。

93.如权利要求92所述的试剂盒,其中所述第一引物和所述第二引物在单独的流体隔离的器皿中。

94.一种包含两个或更多个流体隔离的器皿的试剂盒,所述器皿共同包含:

第一引物、第二引物、第三引物和第四引物,其中:

所述第一引物包含第一区域和第二区域,其中所述第一区域包含该引物的5'端,所述第二区域包含该引物的3'端,并且所述第二区域与多核苷酸模板的第一部分互补;

所述第二引物包含第一区域和第二区域,其中所述第一区域包含该引物的5'端,所述第二区域包含该引物的3'端,并且所述第二区域与同所述多核苷酸模板的第二部分互补的序列互补;

所述第三引物包含第一区域和第二区域,其中所述第一区域包含该引物的5'端,所述第二区域包含该引物的3'端,并且所述第二区域与所述多核苷酸模板的第一部分互补;

所述第四引物包含第一区域和第二区域,其中所述第一区域包含该引物的5'端,所述第二区域包含该引物的3'端,并且所述第二区域与同所述多核苷酸模板的第二部分互补的序列互补;

所述第一引物的第一区域与所述第二引物的第一区域互补;

所述第三引物的第一区域与所述第四引物的第一区域互补;并且

所述第一引物的第一区域的核苷酸序列与所述第三引物的第一区域的核苷酸序列相

差一个核苷酸。

95.如权利要求94所述的试剂盒，其中所述第二引物的第一区域和所述第四引物的第一区域均与所述多核苷酸模板的第三部分互补，其中所述第三部分位于该多核苷酸模板中第一部分与第二部分之间。

96.如权利要求92-95中任一项所述的试剂盒，其中该试剂盒进一步包含对照核酸链，该对照核酸链包含所述多核苷酸模板的核苷酸序列。

97.如权利要求92-96所述的试剂盒，其中该试剂盒进一步包含具有链置换活性的DNA聚合酶。

98.如权利要求92-97中任一项所述的试剂盒，其中该试剂盒进一步包含逆转录酶。

99.如权利要求92-98中任一项所述的试剂盒，其中该试剂盒进一步包含核酸染料。

100.如权利要求92-99中任一项所述的试剂盒，其中该试剂盒进一步包含核苷酸和缓冲液。

101.如权利要求92-100中任一项所述的试剂盒，其中所述第一引物、第二引物、第三引物和第四引物中的每一个的第二区域为6至30个核苷酸的长度。

102.如权利要求92-101中任一项所述的试剂盒，其中所述第一引物、第二引物、第三引物和第四引物中的每一个的第一区域为4至14个核苷酸的长度。

核酸扩增

背景技术

[0001] 对于用于核酸扩增的方法和试剂的需求日益增加。生成特定核酸的多个拷贝通常是必要的或有用的，以便核酸可用于给定的应用。例如，为了分析感兴趣的核酸的核苷酸序列，在分析该序列之前经常复制核酸以增加它的拷贝数。在另一个实例中，为了确定样品中特定核酸的存在或不存在，可在一定条件下处理样品以使得如果特定核酸存在于该样品中则它可被扩增。在另一个实例中，用作探针的核酸可进行重复拷贝以生成大量含有与原始核酸模板相同的序列的核酸，从而生成核酸的许多拷贝，该拷贝可用作探针。

[0002] 已知多种用于核酸扩增的方法。例如，聚合酶链反应(“PCR”)(参见，例如，美国专利号4,683,202)是一种用于核酸扩增的普遍方法。为了成功地进行PCR反应，该反应必须在重复多个循环的多个不同温度下进行。这需要用于重复地改变PCR反应的温度的硬件或其他机构。用于核酸扩增的另一种方法被称为环介导等温扩增(“LAMP”)(参见，例如，美国专利号6,410,278)。LAMP反应可在等温下进行，但通常包括四种不同引物的使用，这些引物识别靶核酸上总计六种不同的序列。

[0003] 为促进生成扩增的核酸以用于使用扩增的核酸的多个和增加数量的应用，需要用于核酸扩增的新的方法和试剂。

[0004] 援引并入

[0005] 本说明书中提到的所有出版物、专利和专利申请均通过引用而以相同程度并入本文，犹如特别地且单独地指出每个单独的出版物、专利或专利申请通过引用而并入。然而，在本公开的内容与本文通过引用并入的文献的内容之间冲突的情况下，以本公开的内容为准。

发明内容

[0006] 本文提供了涉及核酸扩增和多联体生成的方法和组合物。

[0007] 在一些实施方式中，本文提供了用于生成包含双链核酸模板的两个或更多个拷贝的多联体的方法，该方法包括：(A)在使得合成与双链核酸模板的第一链退火的第一引物的第一拷贝的延伸产物的条件下，用第一引物的第一拷贝和聚合酶处理包含双链核酸模板的一级双链核酸，其中第一引物包含5’末端核苷酸、3’末端核苷酸以及两个区域：(i)尾区，其包含：(a)引物的5’末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在5’末端核苷酸的下游，(c)5’末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及(ii)模板结合区，其包括：(a)引物的3’末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在3’末端核苷酸的上游，(c)3’末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，并且第一引物的第一拷贝的模板结合区与双链核酸模板的第一链退火，(B)在使得合成与步骤(A)的第一引物的第一拷贝的延伸产物退火的第二引物的延伸产物的条件下，用第二引物和聚合酶处理步骤(A)的第一引物的第一拷贝的延伸产物，其中第二引物包含5’末端核苷酸、3’末端核苷酸以及两个区域：(i)尾区，其包含：(a)引物的5’末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在5’末端核苷酸的下游，(c)5’末端核苷酸与

最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及(ii)模板结合区，其包含：(a)引物的3'末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游，(c)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，第二引物的尾区包含与第一引物的尾区的核苷酸序列互补的核苷酸序列，如果将所述序列对齐使得第二引物的5'末端核苷酸与第一引物的尾区的最内核苷酸对齐并且第一引物的5'末端核苷酸与第二引物的尾区的最内核苷酸对齐，第二引物的模板结合区与步骤(A)的第一引物的第一拷贝的延伸产物退火，并且第二引物的延伸产物含有5'末端核苷酸、3'末端核苷酸以及包含3'末端核苷酸的3'末端区，其中该3'末端区含有与在5'至3'方向读取的第二引物的尾区的核苷酸序列相同的核苷酸序列，且3'末端区的最后的核苷酸是第二引物的延伸产物的3'末端核苷酸，(C)在使得合成与步骤(B)的第二引物的延伸产物退火的第一引物的第二拷贝的延伸产物的条件下，用第一引物的第二拷贝和聚合酶处理步骤(B)的第二引物的延伸产物，以产生包含步骤(B)的第二引物的延伸产物和第一引物的第二拷贝的延伸产物的二级核酸的第一拷贝，其中第一引物的第二拷贝的延伸产物含有5'末端核苷酸、3'末端核苷酸以及包含3'末端核苷酸的3'末端区，其中该3'末端区含有与在5'至3'方向读取的第一引物的尾区的核苷酸序列相同的核苷酸序列，并且该3'末端区的最后的核苷酸是第二引物的延伸产物的3'末端核苷酸，(D)将至少步骤(C)重复另外一次或多次以生成步骤(C)的二级核酸的至少第二拷贝，(E)在使得二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物的3'末端区与二级核酸的第二拷贝的第二引物的延伸产物的3'末端区退火的条件下，处理步骤(C)的二级核酸的第一拷贝以及步骤(D)的二级核酸的第二拷贝，以产生包含二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物以及二级核酸的第二拷贝的第二引物的延伸产物的交换结构，(F)在使得合成二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物的延伸产物以及合成二级核酸的第二拷贝的第二引物的延伸产物的延伸产物的条件下，用聚合酶处理步骤(E)的交换结构，以产生包含步骤(A)的双链核酸模板的两个拷贝的多联体，其中该多联体包含二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物的延伸产物以及二级核酸的第二拷贝的第二引物的延伸产物的延伸产物。

[0008] 在一些实施方式中，本文提供了用于生成包含多核苷酸模板或其类似序列的两个或更多个拷贝的多联体的方法，该方法包括：(A)在使得合成与多核苷酸模板退火的第一引物的第一拷贝的延伸产物的条件下，用第一引物的第一拷贝和聚合酶处理包含多核苷酸模板的一级核酸，其中第一引物包含5'末端核苷酸、3'末端核苷酸以及两个区域：(i)尾区，其包含：(a)引物的5'末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游，(c)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及(ii)模板结合区，其包括：(a)引物的3'末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游，(c)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，并且第一引物的第一拷贝的模板结合区与多核苷酸模板退火，(B)在使得合成与步骤(A)的第一引物的第一拷贝的延伸产物退火的第二引物的延伸产物的条件下，用第二引物和聚合酶处理步骤(A)的第一引物的第一拷贝的延伸产物，其中第二引物包含5'末端核苷酸、3'末端核苷酸以及两个区域：(i)尾区，其包含：(a)引物的5'末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游，(c)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，(ii)模板结合区，其包含：(a)引物

的3'末端核苷酸,(b)最内核苷酸,其中该最内核苷酸在3'末端核苷酸的上游,(c)3'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,第二引物的尾区包含与第一引物的尾区的核苷酸序列互补的核苷酸序列,如果将所述序列对齐使得第二引物的5'末端核苷酸与第一引物的尾区的最内核苷酸对齐以及第一引物的5'末端核苷酸与第二引物的尾区的最内核苷酸对齐,第二引物的模板结合区与步骤(A)的第一引物的第一拷贝的延伸产物退火,并且第二引物的延伸产物含有5'末端核苷酸、3'末端核苷酸以及包含3'末端核苷酸的3'末端区,其中该3'末端区含有与在5'至3'方向读取的第二引物的尾区的核苷酸序列相同的核苷酸序列,且3'末端区的最后的核苷酸是第二引物的延伸产物的3'末端核苷酸,(C)在使得合成与步骤(B)的第二引物的延伸产物退火的第一引物的第二拷贝的延伸产物的条件下,用第一引物的第二拷贝和聚合酶处理步骤(B)的第二引物的延伸产物,以产生包含步骤(B)的第二引物的延伸产物和第一引物的第二拷贝的延伸产物的二级核酸的第一拷贝,其中第一引物的第二拷贝的延伸产物含有5'末端核苷酸、3'末端核苷酸以及包含3'末端核苷酸的3'末端区,其中该3'末端区含有与在5'至3'方向读取的第一引物的尾区的核苷酸序列相同的核苷酸序列,并且该3'末端区的最后的核苷酸是第二引物的延伸产物的3'末端核苷酸,(D)将至少步骤(C)重复另外一次或多次以生成包含步骤(B)的第二引物的延伸产物和步骤(C)的第一引物的第二拷贝的延伸产物的二级核酸的至少第二拷贝,(E)在使得二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物的3'末端区与二级核酸的第二拷贝的第二引物的延伸产物的3'末端区退火的条件下,处理步骤(C)的二级核酸的第一拷贝以及步骤(D)的二级核酸的第二拷贝,以产生包含二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物以及二级核酸的第二拷贝的第二引物的延伸产物的交换结构,(F)在使得合成二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物的延伸产物以及合成二级核酸的第二拷贝的第二引物的延伸产物的延伸产物的条件下,用聚合酶处理步骤(E)的交换结构,以产生包含步骤(A)的多核苷酸模板的两个拷贝的多联体,其中该多联体包含二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物的延伸产物以及二级核酸的第二拷贝的第二引物的延伸产物的延伸产物。在一些实施方式中,步骤(A)的核酸聚合酶是DNA聚合酶。在一些实施方式中,步骤(A)的核酸聚合酶是逆转录酶。

[0009] 在一些实施方式中,本文提供了用于生成包含双链核酸模板的两个或更多个拷贝的多联体的方法,该方法包括:(A)制备反应混合物,其包含:(i)包含双链核酸模板的一级核酸,(ii)分离的核酸聚合酶,(iii)第一引物,其包含5'末端核苷酸、3'末端核苷酸以及两个区域:(a)尾区,其包含:(1)引物的5'末端核苷酸,(2)最内核苷酸,其中该最内核苷酸在5'末端核苷酸的下游,(3)5'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,以及(b)模板结合区,其包括:(1)引物的3'末端核苷酸,(2)最内核苷酸,其中该最内核苷酸在3'末端核苷酸的上游,(3)3'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,其中模板结合区与核酸模板的第一链互补,(iv)第二引物,其包含5'末端核苷酸、3'末端核苷酸以及两个区域:(a)尾区,其包含:(1)引物的5'末端核苷酸,(2)最内核苷酸,其中该最内核苷酸在5'末端核苷酸的下游,(3)5'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,以及(b)模板结合区,其包含:(1)引物的3'末端核苷酸,(2)最内核苷酸,其中该最内核苷酸在3'末端核苷酸的上游,(3)3'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸,

并且其中模板结合区与核酸模板的第二链互补，并且其中如果将所述序列对齐使得第二引物的5'末端核苷酸与第一引物的尾区的最内核苷酸对齐并且第一引物的5'末端核苷酸与第二引物的尾区的最内核苷酸对齐，第二引物的尾区含有与第一引物的尾区的核苷酸序列互补的核苷酸序列，以及(B)在没有热循环的情况下将反应混合物温育至少3分钟。

[0010] 在一些实施方式中，本文提供了用于生成包含多核苷酸模板的两个或更多个拷贝的多联体的方法，该方法包括：(A)制备反应混合物，其包含：(i)包含多核苷酸模板的核酸，(ii)分离的核酸聚合酶，(iii)第一引物，其包含5'末端核苷酸、3'末端核苷酸以及两个区域：(a)尾区，其包含：(1)引物的5'末端核苷酸，(2)最内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游，(3)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及(b)模板结合区，其包括：(1)引物的3'末端核苷酸，(2)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游，(3)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，并且其中模板结合区与多核苷酸模板互补，(iv)第二引物，其包含5'末端核苷酸、3'末端核苷酸以及两个区域：(a)尾区，其包含：(1)引物的5'末端核苷酸，(2)最内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游，(3)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及(b)模板结合区，其包含：(1)引物的3'末端核苷酸，(2)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游，(3)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，并且其中模板结合区与互补于多核苷酸模板的核苷酸序列互补，并且其中如果将所述引物的序列对齐使得第二引物的5'末端核苷酸与第一引物的尾区的最内核苷酸对齐并且第一引物的5'末端核苷酸与第二引物的尾区的最内核苷酸对齐，第二引物的尾区含有与第一引物的尾区的核苷酸序列互补的核苷酸序列，以及(B)在不高于80℃的温度下将反应混合物温育至少3分钟。

[0011] 在一些实施方式中，本文提供了用于生成包含双链核酸模板的两个或更多个拷贝的多联体的方法，该方法包括：将包含双链核酸模板的双链核酸分子的第一拷贝和第二拷贝与聚合酶一起温育，以产生包含与交换结构的第二链的延伸产物退火的交换结构的第一链的延伸产物的多联体，其中该多联体包含双链核酸模板的两个拷贝，其中双链核酸分子包含各自含有多个核苷酸的第一链和第二链，第一链包含5'末端核苷酸和3'末端核苷酸，并含有5'至3'方向的通用格式的区域：A1-B-A2，第二链包含5'末端核苷酸和3'末端核苷酸，并含有5'至3'方向的通用格式的区域：C1-D-C2，区域B包含双链核酸模板的第一链的核苷酸序列，区域D包含双链核酸模板的第二链的核苷酸序列，在双链核酸分子中，区域A1与C2退火，B与D退火，且A2与C1退火，生成了包含双链核酸分子的第一拷贝的第一链以及双链核酸分子的第二拷贝的第二链的交换结构，其中第一链的A2区域与第二链的C2区域退火，合成了交换结构的第一链的延伸产物，并合成了交换结构的第二链的延伸产物。

[0012] 在实施方式中，本文提供了拷贝多核苷酸模板的方法，该方法包括：在包含第一引物的多个拷贝和第二引物的多个拷贝的反应混合物中温育多核苷酸模板，其中：第一引物包含第一区域和第二区域，其中第一引物的第二区域包含与多核苷酸模板的第一部分互补的核苷酸序列；第二引物包含第一区域和第二区域，其中第二引物的第二区域包含与配偶体核苷酸序列互补的核苷酸序列，其中该配偶体核苷酸序列与多核苷酸模板的第二部分互补；并且在多核苷酸模板与第一引物的多个拷贝和第二引物的多个拷贝一起温育时，形成

至少一条多联体链，其中该多联体链包含5'端和3'端，并且包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列，其中：C'表示第二引物的第一区域的核苷酸序列，T表示多核苷酸模板的核苷酸序列或其类似序列，且X表示任何数目和序列的核苷酸。

[0013] 在一些实施方式中，在本文提供的包括形成包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列的多联体链的方法中，该多联体链是第一多联体链，并且还形成了第二多联体链，其中该第二多联体链包含5'端和3'端，并且包含具有5'至3'方向的通用结构C-X'-T'-C-T'-C的核苷酸序列，其中：C表示第一引物的第一区域的核苷酸序列，T'表示与多核苷酸模板互补的核苷酸序列，且X'表示与X的核苷酸序列互补的核苷酸序列。

[0014] 在实施方式中，本文提供了测定生物样品中的靶多核苷酸模板的方法，该方法包括：A)在包含第一引物的多个拷贝和第二引物的多个拷贝的反应混合物中温育生物样品或其部分，其中：第一引物包含第一区域和第二区域，其中第一引物的第二区域包含与多核苷酸模板的第一部分互补的核苷酸序列；第二引物包含第一区域和第二区域，其中第二引物的第二区域包含与配偶体核苷酸序列互补的核苷酸序列，其中该配偶体核苷酸序列与多核苷酸模板的第二部分互补；并且在多核苷酸模板与第一引物的多个拷贝和第二引物的多个拷贝一起温育时，形成至少一条多联体链，其中该多联体链包含5'端和3'端，并且包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列，其中：C'表示第二引物的第一区域的核苷酸序列，T表示多核苷酸模板的核苷酸序列或其类似序列，且X表示任何数目和序列的核苷酸；以及B)在A)温育步骤开始后在一个或多个点测量A)的反应混合物中扩增的核酸的量。在实施方式中，测量反应混合物中扩增的核酸的量可包括确定反应混合物中的荧光的水平。在实施方式中，该方法可进一步包括确定反应混合物中的核酸扩增的转变时间。

[0015] 在实施方式中，在本文提供的包含多联体链(其包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列)的方法、反应混合物、容器(vessel)或试剂盒中，X可含有具有5'至3'方向的通用结构[$(C'-T)_N$]的序列，其中C'表示第二引物的第一区域的核苷酸序列，T表示多核苷酸模板的核苷酸序列或其类似序列，且N为0至2000之间的任何整数。在实施方式中，N可以是0至10、0至100、0至1000、0至5000、0至10,000、1至10、1至100、1至1000、1至2000、1至5000或1至10,000之间的任何整数。

[0016] 在实施方式中，在本文提供的包含多联体链(其包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列)的方法、反应混合物、容器或试剂盒中，X可含有不多于0、1、2、3、4、5、6、7、8、9、10、15、20、25、50、100、500、1000、10,000、50,000、100,000或500,000个核苷酸。在实施方式中，在本文提供的包含多联体链(其包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列)的方法、容器或试剂盒中，X可含有至少1、2、3、4、5、6、7、8、9、10、15、20、25、50、100、500、1000、10,000、50,000、100,000或500,000个核苷酸。在实施方式中，在本文提供的包含多联体链(其包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列)的方法、容器或试剂盒中，X可含有至少1、2、3、4、5、6、7、8、9、10、15、20、25、50、100、500、1000、10,000、50,000、100,000个核苷酸，并且不多于2、3、4、5、6、7、8、9、10、15、20、25、50、100、500、1000、10,000、50,000、100,000或500,000个核苷酸。

[0017] 在实施方式中，在本文提供的包含多联体链(其包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列)的方法、反应混合物、容器或试剂盒中，在至少一个C'与T之间，存在并非C'或T序列的一部分的一个或多个额外的核苷酸。所述一个或多个额外的核苷

酸可以是，例如，1至10、1至20、1至100或1至1000个核苷酸。

[0018] 在实施方式中，在本文提供的包含多联体链(其包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列)的方法、反应混合物、容器或试剂盒中，至少一个C'或T序列可缺失一个或多个核苷酸。在2个或更多个核苷酸缺失的情况下，缺失的核苷酸可以是邻近的，或者可以在分开的位置。所述一个或多个缺失的核苷酸可以是，例如，1至10、1至20、1至100或1至1000个核苷酸。

[0019] 在实施方式中，在本文提供的包含多联体链(其包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列)的方法、反应混合物、容器或试剂盒中，至少一个C'或T序列可具有一个或多个点突变。在存在两个或更多个点突变的情况下，点突变可以是邻近的，或者可以在分开的位置。所述一个或多个点突变可以是，例如，1至10、1至20、1至100或1至1000个点突变。

[0020] 在实施方式中，在本文提供的包含多联体链(其包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列)的方法、反应混合物、容器或试剂盒中，核苷酸序列具有以下特点中的两个或全部三个：i)在至少一个C'与T之间，存在并非C'或T序列的一部分的一个或多个额外的核苷酸；ii)至少一个C'或T序列缺失一个或多个核苷酸；以及iii)至少一个C'或T序列含有一个或多个点突变。

[0021] 在实施方式中，在本文提供的包含多联体链(其包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列)的方法、反应混合物、容器或试剂盒中，在多核苷酸模板是RNA分子的实施方式中，T可表示与多核苷酸模板的RNA序列类似的DNA序列的核苷酸序列。

[0022] 在实施方式中，在本文提供的包含多联体链(其包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列)的方法、反应混合物、容器或试剂盒中，该多联体链进一步包含位于最5'的C'序列的5'的一个或多个核苷酸。所述一个或多个核苷酸可以是，例如，1至10、1至20、1至100或1至1000个核苷酸。

[0023] 在实施方式中，在本文提供的包含多联体链(其包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列)的方法、反应混合物、容器或试剂盒中，该多联体链进一步包含位于最3'的C'序列的3'的一个或多个核苷酸。所述一个或多个核苷酸可以是，例如，1至10、1至20、1至100或1至1000个核苷酸。

[0024] 在实施方式中，本文提供了生成包含双链核酸模板的至少两个拷贝的多联体的方法，该方法包括：在反应混合物中温育至少第一模板分子和第二模板分子，其中：第一模板分子包含第一核酸链和第二核酸链，其中：第一模板分子的第一核酸链包含具有5'至3'方向的通用结构H'-S-Y_1-H'的核苷酸序列，其中：H'表示第一同源序列的核苷酸序列，S表示双链核酸模板的第一链的核苷酸序列，且Y_1表示任何数目和序列的核苷酸；并且第一模板分子的第二核酸链包含具有5'至3'方向的通用结构H-Y_1'-S'-H的核苷酸序列，其中：H表示第二同源序列的核苷酸序列，其中第一同源序列与第二同源序列彼此互补，Y_1'表示与Y_1的核苷酸序列互补的核苷酸序列，且S'表示双链核酸模板的第二链的核苷酸序列，其中双链核酸模板的第一链和第二链彼此互补；并且第二模板分子包含第一核酸链和第二核酸链，其中：第二模板分子的第一核酸链包含具有5'至3'方向的通用结构H'-S-Y_2-H'的核苷酸序列，其中：H'表示第一同源序列的核苷酸序列，S表示双链核酸模板的第一链的核苷酸序列，且Y_2表示任何数目和序列的核苷酸；且第一模板分子的第二核酸链包含具有5'至3'方向的

通用结构H-Y_2'-S'-H的核苷酸序列，其中：H表示第二同源序列的核苷酸序列，Y_2'表示与Y_2的核苷酸序列互补的核苷酸序列，且S'表示双链核酸模板的第二链的核酸序列；并且在反应混合物中的第一模板分子与第二模板分子一起温育时，形成包含双链核酸模板的至少两个拷贝的至少一个多联体，其中多联体包含第一多联体链和第二多联体链，其中第一多联体链包含5'端和3'端，并包含具有5'至3'方向的通用结构H'-S-Y_2-H'-S-Y_1-H'的核苷酸序列，其中H'、Y_1、S和Y_2中的每一个均表示如上所述的核苷酸序列；并且其中第二多联体链包含5'端和3'端，并包含具有5'至3'方向的通用结构H-Y_1'-S'-H-Y_2'-S'-H的序列，其中，H'、Y_1、S和Y_2中的每一个均表示如上所述的核苷酸序列。

[0025] 在实施方式中，在本文提供的包含第一多联体链(其包含具有5'至3'方向的通用结构H'-S-Y_2-H'-S-Y_1-H'的核苷酸序列)的方法、反应混合物、容器或试剂盒中，Y_1和Y_2中的至少一个或两者可表示0个核苷酸。

[0026] 在实施方式中，在本文提供的包含第一多联体链(其包含具有5'至3'方向的通用结构H'-S-Y_2-H'-S-Y_1-H'的核苷酸序列)的方法、反应混合物、容器或试剂盒中，Y_1可含有具有5'至3'方向的通用结构[$(H'-S)_{N1}$]的序列，其中H'和S表示如上所述的核苷酸序列，且N1是0至2000之间的任何整数。

[0027] 在实施方式中，在本文提供的包含第一多联体链(其包含具有5'至3'方向的通用结构H'-S-Y_2-H'-S-Y_1-H'的核苷酸序列)的方法、反应混合物、容器或试剂盒中，Y_2可含有具有5'至3'方向的通用结构[$(H'-S)_{N2}$]的序列，其中H'和S表示如上所述的核苷酸序列，且N2是0至2000之间的任何整数。

[0028] 在实施方式中，在本文提供的包含第一模板分子和第二模板分子的方法、反应混合物、容器或试剂盒中，第一模板分子和第二模板分子两者均为双链DNA分子。

[0029] 在实施方式中，在本文提供的包含第一模板分子的第一核酸链的方法、反应混合物、容器或试剂盒中，其中第一核酸链包含具有5'至3'方向的通用结构H'-S-Y_1-H'的核苷酸序列，其中H'表示第一同源序列的核苷酸序列，第一同源序列可含有不多于4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、40、50、60、70、80、90或100个核苷酸。在实施方式中，第一同源序列可含有至少4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、40、50、60、70、80、90或100个核苷酸。在实施方式中，第一同源序列可含有至少4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、40、50、60、70、80、90或100个且不多于5、6、7、8、9、10、11、12、13、14、15、20、25、30、40、50、60、70、80、90、100或200个核苷酸。

[0030] 在实施方式中，本文提供的反应混合物、容器或试剂盒包含核酸聚合酶。在实施方式中，核酸聚合酶是具有链置换活性的DNA聚合酶。在实施方式中，核酸聚合酶是RNA聚合酶。在实施方式中，核酸聚合酶是逆转录酶。在实施方式中，反应混合物、容器或试剂盒包含多于一种核酸聚合酶，诸如具有链置换活性的DNA聚合酶和逆转录酶两种酶。在实施方式中，本文提供的反应混合物、容器或试剂盒包含核苷酸和缓冲液。

[0031] 在实施方式中，在本文提供的包含多核苷酸模板的方法、反应混合物、容器或试剂盒中，多核苷酸模板是单链分子。在实施方式中，在本文提供的包含多核苷酸模板的方法、反应混合物、容器或试剂盒中，多核苷酸模板包含双链核酸模板的一条链。在实施方式中，在本文提供的包含多核苷酸模板的方法、反应混合物、容器或试剂盒中，多核苷酸模板是DNA或RNA分子。

[0032] 在实施方式中，在本文提供的包含核酸模板的方法、反应混合物、容器或试剂盒中，核酸模板是RNA或DNA分子。在实施方式中，核酸模板可以是单链或双链分子。

[0033] 在实施方式中，在本文提供的包括温育反应混合物的方法中，在温育反应混合物的过程中，反应混合物的温度不超过90、85、80、75、70、65、60、55、50、45、40、37、35、30、25或20℃。在实施方式中，在本文提供的方法中，该方法的所有步骤均在不高于90、85、80、75、70、65、60、55、50、45、40、37、35、30、25或20℃的温度下进行。在实施方式中，本文提供的反应混合物、容器或试剂盒保持不高于90、85、80、75、70、65、60、55、50、45、40、37、35、30、25、或20℃的温度。在实施方式中，本文提供的方法在没有热循环的情况下进行。

[0034] 在实施方式中，在本文提供的包含含有第一部分的多核苷酸模板的方法、反应混合物、容器或试剂盒中，第一部分含有不多于5、10、15、20、25、30、35、40、50、60、70、80、90、100或200个核苷酸。在实施方式中，在本文提供的包含含有第一部分的多核苷酸模板的方法、反应混合物、容器或试剂盒中，第一部分含有至少5、10、15、20、25、30、35、40、50、60、70、80、90、100或200个核苷酸。在实施方式中，在本文提供的包含含有第一部分的多核苷酸模板的方法、反应混合物、容器或试剂盒中，第一部分含有至少5、10、15、20、25、30、35、40、50、60、70、80、90或100个且不多于10、15、20、25、30、35、40、50、60、70、80、90、100或200个核苷酸。

[0035] 在实施方式中，在本文提供的包含含有第一区域的引物的方法、反应混合物、容器或试剂盒中，第一区域含有至少4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90、或100个核苷酸。在实施方式中，在本文提供的包含含有第一区域的引物的方法、反应混合物、容器或试剂盒中，第一区域含有不多于4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90或100个核苷酸。在实施方式中，在本文提供的包含含有第一区域的引物的方法、反应混合物、容器或试剂盒中，第一区域含有至少4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80或90个且不多于5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90或100个核苷酸。在实施方式中，在本文提供的包含含有第一区域的第一引物和含有第一区域的第二引物的方法、反应混合物、容器或试剂盒中，第一引物和第二引物两者均可具有以上所述的任何特征。在实施方式中，在本文提供的包含含有第一区域的第一引物和含有第一区域的第二引物的方法、反应混合物、容器或试剂盒中，第一引物的第一区域和第二引物的第一区域可含有相同数目的核苷酸。在实施方式中，在本文提供的包含含有第一区域的第一引物和含有第一区域的第二引物的方法、反应混合物、容器或试剂盒中，第一引物的第一区域和第二引物的第一区域可含有不同数目的核苷酸。

[0036] 在实施方式中，在本文提供的包含含有第二区域的引物的方法、反应混合物、容器或试剂盒中，第二区域含有至少4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90、或100个核苷酸。在实施方式中，在本文提供的包含含有第二区域的引物的方法、反应混合物、容器或试剂盒中，第二区域含有不多于4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90或100个核苷酸。在实施方式中，在本文提供的包含含有第二区域的引物的方法、反应混合物、容器或试剂盒中，第二区域含有至少4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80或90个且不多于5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90或100个核苷酸。在实施方式中，在本文提供的

包含含有第二区域的第一引物和含有第二区域的第二引物的方法、反应混合物、容器或试剂盒中，第一引物和第二引物两者均可具有以上所述的任何特征。在实施方式中，在本文提供的包含含有第二区域的第一引物和含有第二区域的第二引物的方法、反应混合物、容器或试剂盒中，第一引物的第二区域和第二引物的第二区域可含有相同数目的核苷酸。在实施方式中，在本文提供的包含含有第二区域的第一引物和含有第二区域的第二引物的方法、反应混合物、容器或试剂盒中，第一引物的第二区域和第二引物的第二区域可含有不同数目的核苷酸。

[0037] 在实施方式中，在本文提供的包含含有第二区域的第二引物和包含第二部分的多核苷酸模板的方法、反应混合物、容器或试剂盒中，第二引物的第二区域的核苷酸序列与多核苷酸模板的第二部分的核苷酸序列相同。

[0038] 在实施方式中，在本文提供的包含含有尾区的引物的方法、反应混合物、容器或试剂盒中，尾区含有至少4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90或100个核苷酸。在实施方式中，在本文提供的包含含有尾区的引物的方法、反应混合物、容器或试剂盒中，尾区含有不多于4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90或100个核苷酸。在实施方式中，在本文提供的包含含有尾区的引物的方法、反应混合物、容器或试剂盒中，尾区含有至少4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80或90个且不多于5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90或100个核苷酸。在实施方式中，在本文提供的包含含有尾区的第一引物和含有尾区的第二引物的方法、反应混合物、容器或试剂盒中，第一引物和第二引物两者均可具有以上所述的任何特征。在实施方式中，在本文提供的包含含有尾区的第一引物和含有尾区的第二引物的方法、反应混合物、容器或试剂盒中，第一引物的尾区和第二引物的尾区可含有相同数目的核苷酸。在实施方式中，在本文提供的包含含有尾区的第一引物和含有尾区的第二引物的方法、反应混合物、容器或试剂盒中，第一引物的尾区和第二引物的尾区可含有不同数目的核苷酸。

[0039] 在实施方式中，在本文提供的包含含有模板结合区的引物的方法、反应混合物、容器或试剂盒中，该模板结合区含有至少4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90或100个核苷酸。在实施方式中，在本文提供的包含含有模板结合区的引物的方法、反应混合物、容器或试剂盒中，模板结合区含有不多于4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90或100个核苷酸。在实施方式中，在本文提供的包含含有模板结合区的引物的方法、反应混合物、容器或试剂盒中，模板结合区含有至少4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80或90个且不多于5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90或100个核苷酸。在实施方式中，在本文提供的包含含有模板结合区的第一引物和含有模板结合区的第二引物的方法、反应混合物、容器或试剂盒中，第一引物和第二引物两者均可具有以上所述的任何特征。在实施方式中，在本文提供的包含含有模板结合区的第一引物和含有模板结合区的第二引物的方法、反应混合物、容器或试剂盒中，第一引物的模板结合区和第二引物的模板结合区可含有相同数目的核苷酸。在实施方式中，在本文提供的包含含有模板结合区的第一引物和含有模板结合区的第二引物的方法、反应混合物、容器或试剂盒中，第一引物的模板结合区和第二引物的模板结合区可含有不同数目的核苷酸。

[0040] 在实施方式中，在本文提供的包含多核苷酸模板的方法、反应混合物、容器或试剂盒中，多核苷酸模板可含有至少8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90、100、200、500、1000、2000或5000个核苷酸。在实施方式中，在本文提供的包含多核苷酸模板的方法、反应混合物、容器或试剂盒中，多核苷酸模板可含有不多于8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90、100、200、500、1000、2000、5000或10,000个核苷酸。在实施方式中，在本文提供的包含多核苷酸模板的方法、反应混合物、容器或试剂盒中，多核苷酸模板可含有至少8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90、100、200、500、1000、2000或5000个且不多于9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90、100、200、500、1000、2000、5000或10,000个核苷酸。

[0041] 在实施方式中，在本文提供的包含双链核酸模板的方法、反应混合物、容器或试剂盒中，双链核酸模板的每一条链可含有至少8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90、100、200、500、1000、2000或5000个核苷酸。在实施方式中，在本文提供的包含双链核酸模板的方法、反应混合物、容器或试剂盒中，双链核酸模板的每一条链可含有不多于8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90、100、200、500、1000、2000、5000或10,000个核苷酸。在实施方式中，在本文提供的包含双链核酸模板的方法、反应混合物、容器或试剂盒中，双链核酸模板的每一条链可含有至少8、9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90、100、200、500、1000、2000或5000个且不多于9、10、11、12、13、14、15、20、25、30、35、40、50、60、70、80、90、100、200、500、1000、2000、5000或10,000个核苷酸。

[0042] 在实施方式中，本文提供的反应混合物、容器或试剂盒不含有重组酶。

[0043] 在实施方式中，本文提供的方法、反应混合物、容器或试剂盒可含有或包含引物的多个拷贝。所述多个拷贝可以是，例如，引物的至少5、10、15、20、50、100、500、1000、10,000、100,000或1,000,000个拷贝。

[0044] 在实施方式中，本文提供的反应混合物或容器可包含来自受试者的生物样品的至少一部分。该生物样品可以是，例如，唾液、血液、尿液、脸颊拭子或鼻拭子。该受试者可以是人。

[0045] 在一些实施方式中，本文提供的方法的所有步骤均在不高于70、65、60、65、60、55、50、45、40、35、30、25、20、15或10℃的温度下进行。在一些实施方式中，本文提供的方法的一些步骤在不高于70、65、60、65、60、55、50、45、40、35、30、25、20、15或10℃的温度下进行。

[0046] 在一些实施方式中，本文提供的方法的两个或更多个步骤在相同的反应混合物中同时进行。在一些实施方式中，本文提供的方法的所有步骤均在相同的反应混合物中同时进行。

[0047] 在一些实施方式中，在本文提供的方法中，在该方法开始后的5、10、15、20、30、40、50、60、70、80、90、120或180分钟内核酸模板扩增了至少10、100、1000、10,000、100,000或1,000,000倍。在一些实施方式中，在本文提供的方法中，在该方法开始后的5、10、15、20、30、40、50、60、70、80、90、120或180分钟内反应混合物中核酸模板的拷贝数增加了至少10、100、1000、10,000、100,000或1,000,000倍。

[0048] 在实施方式中，本文提供的核酸模板可以是单链或双链核酸模板。

[0049] 在实施方式中，本文提供了一种容器，该容器包含在其中流体连通的：第一引物，

其中该第一引物包含第一区域和第二区域，并且其中第一引物的第二区域包含与多核苷酸模板的第一部分互补的核苷酸序列；第二引物，其中该第二引物包含第一区域和第二区域，并且其中第二引物的第二区域包含与配偶体核苷酸序列互补的核苷酸序列，其中该配偶体核苷酸序列与多核苷酸模板的第二部分互补；以及至少一条多联体链，其中该多联体链包含5'端和3'端，并且包含具有5'至3'方向的通用结构C'-T-C'-T-X-C'的核苷酸序列，其中：C'表示第二引物的第一区域的核苷酸序列，T表示多核苷酸模板的核苷酸序列或其类似序列，且X表示任何数目和序列的核苷酸。

[0050] 在一些实施方式中，本文提供了一种容器，该容器包含在其中流体流通的：(A)分离的核酸聚合酶，(B)包含至少第一链的核酸模板，(C)第一引物，其包含5'末端核苷酸、3'末端核苷酸以及两个区域：(i)尾区，其包含：(a)引物的5'末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游，(c)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及(ii)模板结合区，其包括：(a)引物的3'末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游，(c)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，其中模板结合区与核酸模板的第一链互补，以及(D)第二引物，其包含5'末端核苷酸、3'末端核苷酸以及两个区域：(i)尾区，其包含：(a)引物的5'末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游，(c)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及(ii)模板结合区，其包含：(a)引物的3'末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游，(c)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，并且其中模板结合区与互补于核酸模板的第一链的核苷酸序列互补，并且其中如果将引物的序列对齐使得第二引物的5'末端核苷酸与第一引物的尾区的最内核苷酸对齐并且第一引物的5'末端核苷酸与第二引物的尾区的最内核苷酸对齐，第二引物的尾区含有与第一引物的尾区的核苷酸序列互补的核苷酸序列。

[0051] 在实施方式中，本文提供了一种试剂盒，其包含两个或更多个流体隔离的器皿(container)，该器皿共同包含：第一引物，其中第一引物包含第一区域和第二区域，并且其中第一引物的第二区域包含与多核苷酸模板的第一部分互补的核苷酸序列；第二引物，其中第二引物包含第一区域和第二区域，并且其中第二引物的第二区域包含与配偶体核苷酸序列互补的核苷酸序列，其中该配偶体核苷酸序列与核苷酸模板的第二部分互补；以及分离的具有链置换活性的DNA聚合酶；其中：第一引物的第一区域和第二引物的第一区域互补。

[0052] 在一些实施方式中，本文提供了一种用于检测包含至少第一链的感兴趣的靶核酸的试剂盒，该试剂盒包含两个或更多个流体隔离的器皿，该器皿共同包含：(A)分离的核酸聚合酶，(B)第一引物，其包含5'末端核苷酸、3'末端核苷酸以及两个区域：(i)尾区，其包含：(a)引物的5'末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游，(c)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及(ii)模板结合区，其包括：(a)引物的3'末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游，(c)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，其中模板结合区与靶核酸的第一链互补，以及(C)第二引物，其包含5'末端核苷酸、3'末端核苷酸以及两个区域：(i)尾区，其包含：(a)引物的5'末端核苷酸，(b)最

内核苷酸，其中该最内核苷酸在5'末端核苷酸的下游，(c)5'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，以及(ii)模板结合区，其包含：(a)引物的3'末端核苷酸，(b)最内核苷酸，其中该最内核苷酸在3'末端核苷酸的上游，(c)3'末端核苷酸与最内核苷酸之间的中间部分，该中间部分包含一个或多个核苷酸，并且其中模板结合区与互补于靶核酸的第一链的核苷酸序列互补，并且其中如果将引物的序列对齐使得第二引物的5'末端核苷酸与第一引物的尾区的最内核苷酸对齐并且第一引物的5'末端核苷酸与第二引物的尾区的最内核苷酸对齐，第二引物的尾区含有与第一引物的尾区的核苷酸序列互补的核苷酸序列。

[0053] 在一些实施方式中，本文提供的试剂盒包含具有感兴趣的靶核酸的核苷酸序列的核酸。

[0054] 在一些实施方式中，本文提供的反应混合物、容器或试剂盒包含核酸染料。

[0055] 在一些实施方式中，在本文提供的包含分离的核酸聚合酶的容器或试剂盒中，分离的核酸聚合酶是DNA聚合酶。在一些实施方式中，在本文提供的包含分离的核酸聚合酶的容器或试剂盒中，分离的核酸聚合酶是逆转录酶。在一些实施方式中，在本文提供的包含分离的核酸聚合酶的容器或试剂盒中，该容器或试剂盒包含DNA聚合酶和逆转录酶两者。

[0056] 在一些实施方式中，在本文提供的包含核酸聚合酶的方法、容器或试剂盒中，该核酸聚合酶具有链置换活性。

[0057] 在一些实施方式中，本文提供的方法包括用核酸染料处理一个或多个反应组分或该方法的步骤。

[0058] 在一些实施方式中，在本文提供的包含核酸模板的方法、容器或试剂盒中，模板是DNA分子。在一些实施方式中，在本文提供的包含核酸模板的方法、容器或试剂盒中，模板是RNA分子。

[0059] 在一些实施方式中，在本文提供的包含第一引物的方法、容器或试剂盒中，第一引物的尾区包含至少4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19或20个核苷酸。在一些实施方式中，在本文提供的包含第一引物的方法、容器或试剂盒中，第一引物的尾区包含不多于4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、30、40、50或60个核苷酸。

[0060] 在一些实施方式中，在本文提供的包含第二引物的方法、容器或试剂盒中，第二引物的尾区包含至少4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19或20个核苷酸。在一些实施方式中，在本文提供的包含第二引物的方法、容器或试剂盒中，第二引物的尾区包含不多于4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、30、40、50或60个核苷酸。

[0061] 在一些实施方式中，在本文提供的包含第一引物的方法、容器或试剂盒中，第一引物的模板结合区包含至少4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19或20个核苷酸。在一些实施方式中，在本文提供的包含第一引物的方法、容器或试剂盒中，第一引物的模板结合区包含不多于4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、30、40、50或60个核苷酸。

[0062] 在一些实施方式中，在本文提供的包含第二引物的方法、容器或试剂盒中，第二引物的模板结合区包含至少4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19或20个核苷酸。在一些实施方式中，在本文提供的包含第二引物的方法、容器或试剂盒中，第二引物的模板结合区包含不多于4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、30、40、50或60个核

苷酸。

[0063] 在一些实施方式中，在本文提供的其中RNA分子是模板分子或一级核酸的方法和组合物中，模板的扩增可以指对应于RNA分子的DNA链的拷贝的生成。

[0064] 在一些实施方式中，本文提供的方法包括测量来自包括本方法的测定的荧光信号。

[0065] 在一些实施方式中，核酸连接酶可包含在本文提供的方法或组合物中。在一些实施方式中，相比于如果核酸连接酶不包含在反应中，当连接酶包含在反应混合物中用于本文提供的方法时，采用本文提供的方法可更快速地扩增核酸模板。在实施方式中，本文提供的反应混合物、容器或试剂盒可含有具有连接酶活性的酶。

[0066] 在实施方式中，本文提供了测定样品中的病原体的方法，该方法包括进行如本文提供的方法以从病原体中扩增核酸。在实施方式中，在本文提供的组合物或方法中使用的靶核酸可以是来自病原体的核酸。在实施方式中，在本文提供的方法中使用的第一和第二引物可各自含有与病原体的核酸中的序列互补的区域，或者与互补于病原体核酸中的序列的序列互补的区域。在实施方式中，病原体的核酸可以是DNA或RNA。病原体可包括但不限于病毒、细菌、真菌和原生生物。样品可来自受试者，并可具有本文其他地方所述的任何样品特征。

[0067] 在实施方式中，本文提供的用于核酸扩增的方法可用于人体或动物体外部的诊断方法。例如，样品可从人或动物中获得，并且可用本文提供的用于核酸扩增的方法测定样品中感兴趣的靶核酸。

[0068] 在实施方式中，本文提供的方法可包括：a)在反应混合物中提供一种或多种用于进行如本文所述的方法的试剂(例如，第一引物、第二引物、核酸模板、核酸聚合酶、核苷酸、缓冲液、水等中的一种或多种)，以及b)在基本等温的温度下温育反应混合物，其中在温育期间反应混合物的温度偏离中心温度不多于或少于20、15、10、9、8、7、6、5、4、3、2或1摄氏度。在实施方式中，中心温度可以是，例如，30、35、40、45、50、55、60、65、70、75或80摄氏度。

[0069] 在实施方式中，本文提供的方法可以在基本等温的温度下进行。在实施方式中，基本等温的温度可以是30、35、40、45、50、55、60、65、70、75或80摄氏度加或减20、15、10、9、8、7、6、5、4、3、2或1摄氏度中的任何温度。

[0070] 在实施方式中，本文提供的方法可在一个或多个温度下进行，该温度均不超过90、85、80、75、70、65、60、55、50、45、40、35、30、或25摄氏度。

[0071] 在实施方式中，本文提供的方法可在没有热循环的情况下进行/反应混合物可在没有热循环(即没有升高和降低温育温度以分离链或使引物杂交的循环，如在例如基于PCR的方法中使用的)的条件下温育。

[0072] 在本文提供的包含含有第一区域的第一引物和含有第一区域的第二引物的组合物和方法中，其中第一引物的第一区域与第二引物的第一区域互补，在实施方式中，第一引物的第一区域和第二引物的第一区域含有核苷酸序列，使得双链结构不会形成限制酶识别序列，该双链结构根据沃森-克里克(Watson-Crick)碱基配对原则通过第一引物的第一区域与第二引物的第一区域退火而形成。

[0073] 在本文提供的包含核酸聚合酶的组合物和方法中，在实施方式中，该核酸聚合酶具有3'至5'外切核酸酶活性。

[0074] 在实施方式中，本文提供了用于扩增靶核酸链的方法，该方法包括：在基本上等温的条件下温育包含靶核酸链、第一引物和第二引物的反应混合物，其中：所述靶核酸链包含第一部分和第二部分；所述第一引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与该核酸链的第一部分互补；所述第二引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与同该核酸链的第二部分互补的序列互补；所述第一引物的第一区域与所述第二引物的第一区域互补；并扩增靶核酸链。任选地，该靶核酸链可进一步包含第三部分，其中所述第三部分位于该靶核酸链中第一部分与第二部分之间，并且其中所述第二引物的第一区域与所述靶核酸链的第三部分互补。

[0075] 在实施方式中，在本文提供的任何方法中，多核苷酸模板、靶核酸链等可含有内部基序，其中本文提供的用于扩增靶核酸链的引物的尾区与该内部基序互补。

[0076] 在实施方式中，本文提供的方法可进一步包括向本文提供的反应混合物中添加肽-核酸(PNA)探针和与DNA-PNA杂合体结合的染料，其中该PNA探针与用于在反应混合物中扩增的靶核酸或其互补序列互补。

[0077] 在实施方式中，本文提供的反应混合物可包含具有链置换活性的DNA聚合酶。

[0078] 当核酸在本文被描述为被“扩增”等时，该核酸还可被描述为被“拷贝”等。

[0079] 在实施方式中，在本文描述为具有第一区域和第二区域的引物中，所述第一区域可含有该引物的5'端，并且所述第二区域可含有该引物的3'端。

[0080] 在实施方式中，在本文描述为具有尾区和模板结合区的引物中，所述第一区域可含有该引物的5'端，并且所述第二区域可含有该引物的3'端。

[0081] 在实施方式中，本文提供了用于拷贝多核苷酸模板的方法，该方法包括：在没有热循环的条件下温育包含多核苷酸模板、第一引物和第二引物的反应混合物，其中：所述多核苷酸模板包含第一部分和第二部分；所述第一引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与该多核苷酸模板的第一部分互补；所述第二引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与同该多核苷酸模板的第二部分互补的序列互补；所述第一引物的第一区域与所述第二引物的第一区域互补；并生成该多核苷酸模板的多个拷贝。任选地，该多核苷酸模板可进一步包含第三部分，其中所述第三部分位于该多核苷酸模板中第一部分与第二部分之间，并且其中所述第二引物的第一区域与所述多核苷酸模板的第三部分互补。

[0082] 在实施方式中，本文提供了用于扩增多核苷酸模板的方法，该方法包括在包含第一引物和第二引物的反应混合物中温育所述多核苷酸模板，其中：所述多核苷酸模板包含第一部分、第二部分和第三部分，其中所述第三部分位于该多核苷酸模板中第一部分与第二部分之间；所述第一引物包含第一区域和第二区域，其中所述第一引物的第二区域与所述多核苷酸模板的第一部分互补；并且所述第二引物包含第一区域和第二区域，其中所述第二引物的第二区域与同所述多核苷酸模板的第二部分互补的序列互补，所述第二引物的第一区域与所述第一引物的第一区域互补，并且所述第二引物的第一区域与所述多核苷酸模板的第三部分互补。

[0083] 在实施方式中，本文提供了用于评估在多核苷酸模板中核苷酸序列的感兴趣位置

处的核苷酸的身份的方法，该方法包括：A)在至少第一反应混合物和第二反应混合物的每一个中提供所述多核苷酸模板的拷贝，其中：该多核苷酸模板包含第一部分、第二部分和第三部分，其中所述第三部分位于该多核苷酸模板中所述第一部分与所述第二部分之间，并且其中所述感兴趣的位置在第三部分中；所述第一反应混合物包含所述多核苷酸模板的拷贝、第一引物和第二引物，其中：所述第一引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与所述多核苷酸模板的第一部分互补；所述第二引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与同所述多核苷酸模板的第二部分互补的序列互补；所述第一引物的第一区域与所述第二引物的第一区域互补；并且所述第二引物的第一区域与所述多核苷酸模板的第三部分互补；所述第二反应混合物包含所述多核苷酸模板的拷贝、第三引物和第四引物，其中：所述第三引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与所述多核苷酸模板的第一部分互补；所述第四引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与同所述多核苷酸模板的第二部分互补的序列互补；所述第三引物的第一区域与所述第四引物的第一区域互补；并且所述第四引物的第一区域与所述多核苷酸模板的第三部分互补；并且所述第二引物的第一区域的核苷酸序列与所述第四引物的第一区域的核苷酸序列相差一个核苷酸，其中如果为了序列的最大互补而用所述多核苷酸模板的第三部分的核苷酸序列定位所述第二引物或第四引物的第一区域的核苷酸序列，则在所述第二引物与第四引物中不同的核苷酸的位置对应于所述多核苷酸模板中感兴趣的核苷酸的位置；B)在没有热循环的条件下温育所述第一反应混合物和第二反应混合物；以及C)比较所述第一反应混合物中多核苷酸模板的扩增速率或量与所述第二反应混合物中多核苷酸模板的扩增速率或量，其中所述多核苷酸模板的扩增速率或量指示所述第二或第四引物的第一区域与所述多核苷酸模板的第三部分的核苷酸序列之间的互补程度。

[0084] 任选地，在本文提供的涉及多核苷酸模板的实施方式中，该多核苷酸模板是DNA链。

[0085] 任选地，在本文提供的涉及多核苷酸模板的实施方式中，该多核苷酸模板是RNA链。

[0086] 任选地，在本文提供的涉及多核苷酸模板的实施方式中，该多核苷酸模板是双链体核酸分子的一条链。在实施方式中，该双链体核酸分子是双链体DNA分子或双链体RNA分子。

[0087] 任选地，在本文提供的涉及反应混合物、容器或试剂盒的实施方式中，该反应混合物、容器或试剂盒包含具有链置换活性的DNA聚合酶。

[0088] 任选地，在本文提供的涉及反应混合物、容器或试剂盒的实施方式中，该反应混合物、容器或试剂盒包含逆转录酶。

[0089] 任选地，在本文提供的涉及反应混合物、容器或试剂盒的实施方式中，该反应混合物、容器或试剂盒包含核酸染料、核苷酸或缓冲液。

[0090] 任选地，在本文提供的涉及含有第一部分、第二部分和第三部分的多核苷酸模板的实施方式中，该多核苷酸模板可具有3'至5'方向的元件的通用结构1P-1S-3P-2S-2P，其

中“1P”是所述第一部分，“1S”是第一间隔区(space)，“3P”是所述第三部分，“2S”是第二间隔区，并且“2P”是所述第二部分。所述“第一间隔区”和“第二间隔区”是所述多核苷酸模板的部分，它们含有不是所述第一部分、第二部分或第三部分的一部分的核苷酸。在实施方式中，所述第一部分可具有，例如，6至30个核苷酸或如本文其他地方所述的任何其他数目的核苷酸。在实施方式中，所述第一间隔区可具有，例如，2至30个核苷酸或如本文其他地方所述的任何其他数目的核苷酸。在实施方式中，所述第三部分可具有，例如，4至14个核苷酸或如本文其他地方所述的任何其他数目的核苷酸。在实施方式中，所述第一间隔区可具有，例如，2至30个核苷酸或如本文其他地方所述的任何其他数目的核苷酸。在实施方式中，所述第一部分可具有，例如，6至30个核苷酸或如本文其他地方所述的任何其他数目的核苷酸。

[0091] 在实施方式中，在本文提供的涉及在反应混合物中温育多核苷酸模板的方法中，可以在该反应混合物的温育过程中生成包含所述多核苷酸模板的至少2、3、4、5或10个拷贝的多联体链。

[0092] 在实施方式中，本文提供了用于扩增双链核酸分子的方法，该方法包括在包含第一引物和第二引物的反应混合物中温育所述双链核酸分子，其中：所述双链核酸分子包含第一链和第二链，其中所述第一链包含第一部分和第三部分，并且所述第二链包含第二部分；所述第一引物包含第一区域和第二区域，其中所述第一引物的第二区域与所述第一链的第一部分互补；并且所述第二引物包含第一区域和第二区域，其中所述第二引物的第二区域与所述第二链的第二部分互补，所述第二引物的第一区域与所述第一链的第三部分互补，并且所述第二引物的第一区域与所述第一引物的第一区域互补。

[0093] 在实施方式中，本文提供了一种反应混合物，其包含：多核苷酸模板、第一引物和第二引物，其中：所述多核苷酸模板包含第一部分、第二部分和第三部分，其中所述第三部分位于该多核苷酸模板中第一部分与第二部分之间；所述第一引物包含第一区域和第二区域，其中所述第一区域包含该引物的5’端，所述第二区域包含该引物的3’端，并且所述第二区域与所述多核苷酸模板的第一部分互补；所述第二引物包含第一区域和第二区域，其中所述第一区域包含该引物的5’端，所述第二区域包含该引物的3’端，并且所述第二区域与同所述多核苷酸模板的第二部分互补的序列互补；所述第一引物的第一区域与所述第二引物的第一区域互补；并且所述第二引物的第一区域与所述多核苷酸模板的第三部分互补。

[0094] 在实施方式中，本文提供了用于扩增多核苷酸模板的试剂盒，该试剂盒包含：第一引物和第二引物，其中：所述第一引物包含第一区域和第二区域，其中所述第一区域包含该引物的5’端，所述第二区域包含该引物的3’端，并且所述第二区域与所述多核苷酸模板的第一部分互补；所述第二引物包含第一区域和第二区域，其中所述第一区域包含该引物的5’端，所述第二区域包含该引物的3’端，并且所述第二区域与同所述多核苷酸模板的第二部分互补的序列互补；所述第一引物的第一区域与所述第二引物的第一区域互补；并且所述第二引物的第一区域与所述多核苷酸模板的第三部分互补，其中所述第三部分位于该多核苷酸模板中第一部分与第二部分之间。

[0095] 任选地，在本文提供的涉及试剂盒的实施方式中，该试剂盒的组分可以在至少两个单独的流体隔离的器皿之间分配。

[0096] 在实施方式中，本文提供了包含两个或更多个流体隔离的器皿的试剂盒，所述器皿共同包含：第一引物、第二引物、第三引物和第四引物，其中：所述第一引物包含第一区域

和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与多核苷酸模板的第一部分互补；所述第二引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与同所述多核苷酸模板的第二部分互补的序列互补；所述第三引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与所述多核苷酸模板的第一部分互补；所述第四引物包含第一区域和第二区域，其中所述第一区域包含该引物的5'端，所述第二区域包含该引物的3'端，并且所述第二区域与同所述多核苷酸模板的第二部分互补的序列互补；所述第一引物的第一区域与所述第二引物的第一区域互补；所述第三引物的第一区域与所述第四引物的第一区域互补；并且所述第一引物的第一区域的核苷酸序列与所述第三引物的第一区域的核苷酸序列相差一个核苷酸。任选地，所述第二引物的第一区域和所述第四引物的第一区域均与所述多核苷酸模板的第三部分互补，其中所述第三部分位于该多核苷酸模板中第一部分与第二部分之间。任选地，所述第一引物、第二引物、第三引物和第四引物中的每一个的第二区域为6至30个核苷酸的长度。任选地，所述第一引物、第二引物、第三引物和第四引物中的每一个的第二区域为6至30个核苷酸的长度。

[0097] 在本文提供的涉及试剂盒的实施方式中，该试剂盒可包含对照核酸链，该对照核酸链包含有待用该试剂盒的试剂检测的多核苷酸模板的核苷酸序列。

[0098] 在本文提供的方法和组合物中，任选地，可包括PNA探针和染料$DiSc_2(5)$。该PNA探针可以与用于在相关反应中扩增的靶序列及其拷贝特异性地退火，从而形成DNA-PNA双链体。本文提供的方法可包括检测在染料$DiSc_2(5)$与DNA-PNA双链体结合时该染料的颜色变化。在实施方式中，可以在反应开始之后或反应结束之后，向本文提供的反应混合物中添加PNA探针和染料。在实施方式中，可以在反应开始之后至少5、10、15、20、30、40、50、60、70、80、90或100分钟，向本文提供的反应混合物中添加PNA探针和染料。在实施方式中，可以在反应开始之后不超过5、10、15、20、30、40、50、60、70、80、90或100分钟，向本文提供的反应混合物中添加PNA探针和染料。

[0099] 除非上下文另外明确指出，本文中对生成多核苷酸模板或核酸模板的拷贝或扩增多核苷酸模板或核酸模板的提及包括生成含有多核苷酸模板/核酸模板的序列的拷贝，以及生成含有多核苷酸模板/核酸模板的类似序列的拷贝。例如，如果多核苷酸模板是RNA，则生成模板的拷贝可包括生成这样的拷贝，该拷贝是含有多核苷酸模板的RNA序列的DNA形式的DNA分子(即，在DNA序列中，含有“T”而非“U”)。

附图说明

[0100] 在附图中，

[0101] 图1为本文提供的方法的总的示意图；图1A-1K示出了本方法的步骤和特征。图1包含以下的核苷酸序列：103：AACGGTTGCTC(SEQ ID NO:1)；104：GAGCAACCGTT(SEQ ID NO:2)；105：ATGGGAGC(SEQ ID NO:3)；106：CCATAACG(SEQ ID NO:4)；111：ATGGGAGCAACCGTT(SEQ ID NO:5)；112：CCATAACGGTTGCTCCCAT(SEQ ID NO:6)；113：ATGGGAGCAACCGTTATGG(SEQ ID NO:7)；121：CCATAACGGTTGCTCCCATAACGGTTGCTCCCAT(SEQ ID NO:8)；122：ATGGGAGCAACCGTTATGGGAGCAACCGTTATGG(SEQ ID NO:9)。

[0102] 图2A示出了与本文提供的方法一起使用的某些引物的核苷酸序列。图2A包含以下的核苷酸序列:P1引物,37个核苷酸:CGCCGGATGGCTCTTGGGAAACCAAACCGTACCAACC(SEQ ID NO:10);P1引物,35个核苷酸:CCGGATGGCTCTTGGGAAACCAAACCGTACCAACC(SEQ ID NO:11);P1引物,33个核苷酸:GGATGGCTCTTGGGAAACCAAACCGTACCAACC(SEQ ID NO:12);P1引物,31个核苷酸:ATGGCTCTTGGGAAACCAAACCGTACCAACC(SEQ ID NO:13);P1引物,30个核苷酸:TGGCTCTTGGGAAACCAAACCGTACCAACC(SEQ ID NO:14);P1引物,29个核苷酸:GGCTCTTGGGAAACCAAACCGTACCAACC(SEQ ID NO:15);P1引物,28个核苷酸:GCTCTTGGGAAACCAAACCGTACCAACC(SEQ ID NO:16);P1引物,27个核苷酸:CTCTTGGGAAACCAAACCGTACCAACC(SEQ ID NO:17);P1引物,26个核苷酸:TCTTGGGAAACCAAACCGTACCAACC(SEQ ID NO:18);P1引物,25个核苷酸:CTTGGGAAACCAAACCGTACCAACC(SEQ ID NO:19);P1引物,24个核苷酸:TTGGGAAACCAAACCGTACCAACC(SEQ ID NO:20);P1引物,23个核苷酸:TGGGAAACCAAACCGTACCAACC(SEQ ID NO:21);P2引物,36个核苷酸:GTTTCCCAAGAGCCATCCGGCGATGCGGAATGTACC(SEQ ID NO:22);P2引物,34个核苷酸:GTTTCCCAAGAGCCATCCGGATGCGGAATGTACC(SEQ ID NO:23);P2引物,32个核苷酸:GTTTCCCAAGAGCCATCCATGCGGAATGTACC(SEQ ID NO:24);P2引物,30个核苷酸:GTTTCCCAAGAGCCATATGCGGAATGTACC(SEQ ID NO:25);P2引物,29个核苷酸:GTTTCCCAAGAGCCAATGCGGAATGTACC(SEQ ID NO:26);P2引物,28个核苷酸:GTTTCCCAAGAGCCATGCGGAATGTACC(SEQ ID NO:27);P2引物,27个核苷酸:GTTTCCCAAGAGCATGCGGAATGTACC(SEQ ID NO:28);P2引物,26个核苷酸:GTTTCCCAAGAGATGCGGAATGTACC(SEQ ID NO:29);P2引物,25个核苷酸:GTTTCCCAAGAATGCGGAATGTACC(SEQ ID NO:30);P2引物,24个核苷酸:GTTTCCCAAGATGCGGAATGTACC(SEQ ID NO:31);P2引物,23个核苷酸:GTTTCCCAAATGCGGAATGTACC(SEQ ID NO:32);以及P2引物,22个核苷酸:GTTTCCCAATGCGGAATGTACC(SEQ ID NO:33)。

[0103] 图2B为示出了根据本文提供的方法进行的反应的结果的图。

[0104] 图3A和3B示出了与本文提供的方法一起使用的某些引物的核苷酸序列。图3A包含以下的核苷酸序列:C5:ATGGCTCTTGGGAAACTGAAACCGTACCAACC(SEQ ID NO:34);D5:GTTTCCCAAGAGCCATGGATGCGGAATGTACC(SEQ ID NO:35);C6:GGCTCTTGGGAAACTGAAACCGTACCAACC(SEQ ID NO:36);D6:GTTTCCCAAGAGCCGGATGCGGAATGTACC(SEQ ID NO:37);C7:CTCTTGGGAAACTGAAACCGTACCAACC(SEQ ID NO:38);D7:GTTTCCCAAGAGGGATGCGGAATGTACC(SEQ ID NO:39);C8:TCTTGGGAAACTGAAACCGTACCAACC(SEQ ID NO:40);D8:GTTTCCCAAGAGGATGCGGAATGTACC(SEQ ID NO:41);C9:CTTGGGAAACTGAAACCGTACCAACC(SEQ ID NO:42);D9:GTTTCCCAAGGGATGCGGAATGTACC(SEQ ID NO:43);C10:TTGGGAAACTGAAACCGTACCAACC(SEQ ID NO:44);D10:GTTTCCCAAGGATGCGGAATGTACC(SEQ ID NO:45);C11:TGGGAAACTGAAACCGTACCAACC(SEQ ID NO:46);D11:GTTTCCCAGGATGCGGAATGTACC(SEQ ID NO:47);C12:GGGAAACTGAAACCGTACCAACC(SEQ ID NO:48);以及D12:GTTTCCCGGATGCGGAATGTACC(SEQ ID NO:49)。图3B包含以下的核苷酸序列:A1:

ATGGCTCTTGGGAAACTGCCTGAAACCGTACCAACC(SEQ ID NO:50);B1:GTTTCCCAAGAGCCATACAGGGATGCGGAATGTACC(SEQ ID NO:51);A2:GGCTCTTGGGAAACTGCCTGAAACCGTACCAACC(SEQ ID NO:52);B2:GTTTCCCAAGAGCCACAGGGATGCGGAATGTACC(SEQ ID NO:53);A3:CTCTTGGGAAACTGCCTGAAACCGTACCAACC(SEQ ID NO:54);B3:GTTTCCCAAGAGACAGGGATGCGGAATGTACC(SEQ ID NO:55);A4:TCTTGGGAAACTGCCTGAAACCGTACCAACC(SEQ ID NO:56);B4:GTTTCCCAAGAACAGGGATGCGGAATGTACC(SEQ ID NO:57);A5:CTTGGGAAACTGCCTGAAACCGTACCAACC(SEQ ID NO:58);B5:GTTTCCCAAGACAGGGATGCGGAATGTACC(SEQ ID NO:59);A6:TTGGGAAACTGCCTGAAACCGTACCAACC(SEQ ID NO:60);B6:GTTTCCCAAACAGGGATGCGGAATGTACC(SEQ ID NO:61);A7:TGGGAAACTGCCTGAAACCGTACCAACC(SEQ ID NO:62);B7:GTTTCCCAACAGGGATGCGGAATGTACC(SEQ ID NO:63);A8:GGGAAACTGCCTGAAACCGTACCAACC(SEQ ID NO:64);以及B8:GTTTCCCACAGGGATGCGGAATGTACC(SEQ ID NO:65)。

[0105] 图3C和3D为示出了根据本文提供的方法进行的反应的结果的图。

[0106] 图4为示出了根据本文提供的方法进行的反应的结果的图。

[0107] 图5为示出了根据本文提供的方法进行的反应的结果的图。

[0108] 图6为示出了根据本文提供的方法进行的反应的结果的图。

[0109] 图7为示出了根据本文提供的方法进行的反应中生成的产物的核苷酸序列的序列比对。图7包含以下的核苷酸序列(按从比对中的顶部序列到比对中的底部序列的顺序):SEQ ID NO:66:TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAAGACCAAATTCTTGAGA;SEQ ID NO:67:TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT;SEQ ID NO:68:TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT;SEQ ID NO:69:TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT;SEQ ID NO:70:TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACTGAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT;SEQ ID NO:71:TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT;SEQ ID NO:72:TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT;SEQ ID NO:73:TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT;SEQ ID NO:74:TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT;SEQ ID NO:75:TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAAGACCAATTCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTT;SEQ ID NO:76:GAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGGAAGACCAAATGAACCCACTAACAGTAGAAGTACCATACATTT;SEQ ID NO:77:TCTTGAGAGAACCCACTAACTCTTGAGAGAACCCACTAAC;以及SEQ ID NO:78:GGAAGACCAAATTCTTGAGA。

[0110] 图8A-8C是本文提供的分子的实施方式的特征的一般示意图。

[0111] 图9是本文提供的分子的实施方式的特征的一般示意图。

[0112] 图10A-10C是本文提供的分子的实施方式的特征的一般示意图。

[0113] 图11是描绘根据本文提供的方法进行的反应的结果的图。

[0114] 图12是描绘根据本文提供的方法进行的反应的结果的图。

[0115] 需要注意的是,附图和其中的元件不必要按形状或比例绘制。例如,为便于呈现或使呈现清楚,附图中元件的形状或比例可进行简化或修改。应进一步理解,附图和其中的元件仅用于示例性说明目的,不应理解为以任何方式进行限制。

具体实施方式

[0116] 尽管本发明包括各种修改和替代形式,但其具体实施方式通过附图中的示例示出并将在本文中详细描述。然而,应理解,并非意在将本发明限于所公开的特定形式,而是相反,本发明覆盖如由权利要求所限定的本发明的精神和范围内的所有修改、等同项和替代方案。

[0117] 在一些实施方式中,本文提供了涉及核酸扩增和多联体生成的方法和组合物。

[0118] 在本文提供的方法中,核酸多联体的生成还增加了该多联体中的核酸模板/特定核酸的拷贝数。因此,本文中对用于生成多联体的方法和组合物的提及还适用于核酸的扩增。

[0119] 如本文所用的,“多核苷酸”指的是含有两个或更多个核苷酸的聚合链。“多核苷酸”包括引物、寡核苷酸、核酸链等。多核苷酸可含有标准或非标准核苷酸。通常,多核苷酸在链的一个末端(“5’末端”)含有5’磷酸并在链的另一末端(“3’末端”)含有3’羟基基团。多核苷酸的最5’核苷酸在本文中可被称为多核苷酸的“5’末端核苷酸”。多核苷酸的最3’核苷酸在本文中可被称为多核苷酸的“3’末端核苷酸”。

[0120] 在含有5’末端核苷酸和3’末端核苷酸的多核苷酸的上下文中如本文所用的术语“下游”指的是比多核苷酸中的参考位置更接近3’末端核苷酸的该多核苷酸中的位置。例如,在具有序列5’ATAAGC 3’的引物中,“G”在“T”和全部“A”的下游。

[0121] 在含有5’末端核苷酸和3’末端核苷酸的多核苷酸的上下文中,如本文所用的术语“上游”指的是比多核苷酸中的参考位置更接近5’末端核苷酸的该多核苷酸中的位置。例如,在具有序列5’ATAAGC 3’的引物中,“T”在“G”、“C”以及最接近“G”的两个“A”的上游。

[0122] 如本文所用的,“核酸”包括DNA和RNA两者,包括含有非标准核苷酸的DNA和RNA。“核酸”含有至少一个多核苷酸(“核酸链”)。核酸可以是单链或双链的。

[0123] 如本文所用的,“多联体”指的是在其内含有特定核酸的两个或更多个拷贝的核酸分子,其中这些拷贝串联连接。在该多联体内,特定核酸的拷贝可彼此直接连接,或者它们可间接地连接(例如,在特定核酸的拷贝之间可具有核苷酸)。在一个实例中,该特定核酸可以是双链核酸模板的核酸,以使得多联体可含有该双链核酸模板的两个或更多个拷贝。在另一个实例中,该特定核酸可以是多核苷酸模板的核酸,以使得多联体可含有该多核苷酸模板的两个或更多个拷贝。

[0124] 如本文所用的,“靶”核酸或分子指的是感兴趣的核酸。靶核酸/分子可以是任何类型,包括单链或双链DNA或RNA(例如,mRNA)。

[0125] 如本文所用的,“互补”序列指的是这样的两个核苷酸序列,这两个核苷酸序列在

彼此反平行对齐时，含有按照标准碱基配对原则(例如，A-T、G-C或A-U)可彼此配对的多个单独的核苷酸碱基，使得含有该序列的分子可彼此特异性地退火。对于被认为“互补”的序列，不必要两个序列中的每一个核苷酸碱基均能够彼此配对。例如，如果当两个序列对于互补而最佳对齐时，该两个序列中至少30％、40％、50％、55％、60％、65％、70％、75％、80％、85％、90％、95％、98％、99％或100％的核苷酸碱基可彼此配对，则该序列可被认为是互补的。此外，当两个序列的总长度彼此明显不同时，该序列仍可被认为“互补”。例如，如果当15个核苷酸的引物与含有数百个核苷酸的更长的多核苷酸的特定区域反向平行对齐时，该引物的多个单独的核苷酸碱基可与更长的多核苷酸中的核苷酸碱基配对，则该15个核苷酸的引物可被认为与该含有数百个核苷酸的更长的多核苷酸“互补”。此外，“互补”序列可含有一种或多种核苷酸类似物或核碱基类似物。如本文所用的，“完全互补的”或“完全互补”等是指彼此100％互补的两个序列(即当所述序列为了最大互补而配对时，所述序列的核苷酸之间没有错配)。

[0126] 如本文所用的，当应用于蛋白质、核酸或其他生物分子时，术语“分离的”指的是已从其天然存在的环境的组分中纯化或分离的分子(例如，从其天然产生的细胞中纯化的蛋白质)。“分离的”分子可与其他分子相接触(例如，作为反应混合物的一部分)。如本文所用的，“分离的”分子还包括具有天然存在的氨基酸或核苷酸序列的重组产生的蛋白质或核酸。“分离的”核酸包括在细胞中包含的编码多肽的核酸分子，该细胞通常在例如该核酸分子在与天然细胞不同的染色体位置时表达多肽。在一些实施方式中，“分离的”多肽被纯化为至少50％、60％、70％、80％、90％、95％、98％或100％的同质性，如通过多肽的SDS-PAGE以及随后的考马斯蓝、银或其他蛋白质染色方法所显示的。

[0127] 除非上下文中另外明确指出，如本文所用的，描述为含有模板或其他核酸的“序列”的核酸分子也可被认为含有该模板或其他核酸本身(例如，被描述为含有模板的序列的分子也可以被描述为含有该模板)。

[0128] 如本文所用的，当第一多核苷酸被描述为与第二多核苷酸“退火”等时，第一多核苷酸全部或其任何部分可与第二多核苷酸退火，反之亦然。

[0129] 如本文所用的，提及在某一条件下或采用其他对象等“处理”给定对象指的是直接或间接地将给定对象暴露于所提及的条件或其他对象。因此，尽管“处理”步骤可包括不同的相关操作(例如，将酶加入到容器中，振动容器等)，但并非每一个“处理”步骤都需要不同的相关操作。例如，包括一种或多种试剂的反应可设置在容器中，并且一旦反应已经开始，则多个事件或步骤可以在容器中发生而不进一步人为或机械干预容器中的内容物。在容器中进行的这些多个事件或步骤中的一个或多个可以被描述为“处理”容器中的对象，即使在反应开始后不单独地干预容器中的内容物。

[0130] 可参考图1对本文提供的方法和组合物的实施方式进行描述。可提供一级核酸101(图1A)。一级核酸101可含有核酸模板102的序列。核酸模板102可以是含有第一链103和第二链104的双链核酸。在图1中，双链核酸模板的第一链103具有5’至3’方向的示例性核苷酸序列AACGGTTGCTC(SEQ ID NO:1)。第一链103还包含5’末端核苷酸(最5’的A)和3’末端核苷酸(最3’的C)。第二链104具有5’至3’方向的示例性核苷酸序列GAGCAACCGTT(SEQ ID NO:2)。第二链104还包含5’末端核苷酸(最5’的G)和3’末端核苷酸(最3’的T)。

[0131] 还可提供第一引物105和第二引物106(图1B)。在图1中，第一引物105具有5’至3’

方向的示例性核苷酸序列ATGGGAGC(SEQ ID NO:3)。第一引物还包含5'末端核苷酸(最5'的A)和3'末端核苷酸(最3'的C)。第二引物106具有5'至3'方向的示例性核苷酸序列CCATAACG(SEQ ID NO:4)。第二引物还包含5'末端核苷酸(最5'的C)和3'末端核苷酸(最3'的G)。虽然图1的核酸模板102是11个核苷酸的长度并具有示例性核苷酸序列,但这些是示例性的,并不被认为是限制。

[0132] 第一引物105可包含两个区域:i)尾区以及ii)模板结合区("模板"区)。第一引物105的尾区可包含三个部分:a)引物的5'末端核苷酸,b)最内核苷酸,其中该最内核苷酸在5'末端核苷酸的下游,以及c)5'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸。在图1中,第一引物105的尾区具有5'至3'方向的示例性核苷酸序列:ATGG,在该序列中,引物的5'末端核苷酸为A,中间部分为中间的TG,且最内核苷酸为最3'的G。第一引物105的模板结合区可包含三个部分:a)引物的3'末端核苷酸,b)最内核苷酸,其中该最内核苷酸在3'末端核苷酸的上游,以及c)3'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸。在图1中,第一引物105的模板结合区具有5'至3'方向的示例性核苷酸序列:GAGC,在该序列中,引物的3'末端核苷酸为C,中间部分为中间的AG,且最内核苷酸为最5'的G。

[0133] 第二引物106可包含两个区域:i)尾区以及ii)模板结合区。第一引物106的尾区可包含三个部分:a)引物的5'末端核苷酸,b)最内核苷酸,其中该最内核苷酸在5'末端核苷酸的下游,以及c)5'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸。在图1中,第二引物106的尾区具有5'至3'方向的示例性核苷酸序列:CCAT,在该序列中,引物的5'末端核苷酸为最5'的C,中间部分为中间的CA,且最内核苷酸为T。第二引物106的模板结合区可包含三个部分:a)引物的3'末端核苷酸,b)最内核苷酸,其中该最内核苷酸在3'末端核苷酸的上游,以及c)3'末端核苷酸与最内核苷酸之间的中间部分,该中间部分包含一个或多个核苷酸。在图1中,第二引物106的模板结合区具有5'至3'方向的示例性核苷酸序列:AACG,在该序列中,引物的3'末端核苷酸为G,中间部分为中间的AC,且最内核苷酸为最5'的A。

[0134] 在一些实施方式中,如果将引物的序列对齐使得第二引物的5'末端核苷酸与第一引物的尾区的最内核苷酸对齐并且第一引物的5'末端核苷酸与第二引物的尾区的最内核苷酸对齐,则第二引物的尾区可含有与第一引物的尾区的核苷酸序列互补的核苷酸序列。例如,在图1中,第二引物106的尾区具有5'至3'方向的核苷酸序列CCAT,并且第一引物105的尾区具有5'至3'方向的核苷酸序列ATGG。当第二引物的5'末端核苷酸(C)与第一引物的尾区的最内核苷酸(G)对齐并且第一引物的5'末端核苷酸(A)与第二引物的尾区的最内核苷酸(T)对齐107时,这些序列是互补的(图1B)。

[0135] 应注意,虽然第二引物的尾区可含有与第一引物的尾区的核苷酸序列互补的核苷酸序列,但通常由第一引物与第二引物退火而形成的产物对于本文提供的方法或组合物不是期望的或有用的。因此,在一些实施方式中,可以采取步骤来最小化第一引物-第二引物退火产物的形成。这样的步骤可包括,例如,在开始本文提供的方法之前不在引物可相互作用持续延长的时间段的条件下对第一引物和第二引物进行预温育。

[0136] 可在使得第一引物的第一拷贝105a的模板结合区与核酸模板的第一链103退火(图1C)并且形成第一引物的第一拷贝的延伸产物111(图1D)的条件下,用第一引物的第一

拷贝105a和聚合酶108处理一级核酸101。聚合酶108可催化第一引物的第一拷贝的延伸产物111的形成。该聚合酶可具有链置换活性。在合成第一引物的第一拷贝的延伸产物111的过程中，聚合酶可从核酸模板的第一链103置换核酸模板的第二链104。通常，由第一引物的第一拷贝105a的3'端生成延伸产物。在生成第一引物的第一拷贝的延伸产物111的过程中，第一引物的第一拷贝105a可与合成的延伸产物共价连接，使得第一引物的第一拷贝变为本文描述为"第一引物的第一拷贝的延伸产物"的分子的一部分。第一引物的第一拷贝的延伸产物111与核酸模板的第一链103互补。在一些实施方式中，当第一引物的第一拷贝105a与核酸模板的第一链103退火时，模板结合区而非第一引物的第一拷贝105a的尾区与核酸模板的第一链退火。

[0137] 在一些实施方式中，使得第一引物的第一拷贝105a的模板结合区与核酸模板的第一链103退火并形成第一引物的第一拷贝的延伸产物111的条件可包括足以支持基于聚合酶的核酸合成的任何条件。针对基于聚合酶的核酸合成的示例性条件是本领域已知的并提供于例如，Molecular Cloning:A Laboratory Manual，M.R.Green and J.Sambrook，Cold Spring Harbor Laboratory Press(2012)中，该文献通过引用全文并入本文。在实施方式中，第一引物或第二引物的模板结合区可支持由核酸聚合酶进行的引物的模板依赖性延伸，这可从引物的3'端延伸引物。

[0138] 可在使得第二引物106的模板结合区与第一引物的第一拷贝的延伸产物111退火(图1E)并形成第二引物的延伸产物112(图1F)的条件下，用第二引物106和聚合酶108处理第一引物的第一拷贝的延伸产物111。聚合酶108可催化第二引物106的延伸产物的形成。该聚合酶可具有链置换活性。聚合酶可以是与用于生成第一引物的第一拷贝的延伸产物111的聚合酶相同类型的聚合酶，或者它可以是不同类型的聚合酶。在合成第二引物的延伸产物112的过程中，聚合酶可从第一引物的第一拷贝的延伸产物111置换核酸模板的第一链103。通常，由第二引物106的3'端生成延伸产物。在生成第二引物的延伸产物112的过程中，第二引物106可与合成的延伸产物共价连接，使得第二引物变为本文描述为"第二引物的延伸产物"的分子的一部分。第二引物的延伸产物112与第一引物的第一拷贝的延伸产物111互补。在一些实施方式中，当第二引物106与第一引物的第一拷贝的延伸产物111退火时，模板结合区而非第二引物106的尾区与第一引物的第一拷贝的延伸产物111退火。第二引物的延伸产物112含有5'末端核苷酸、3'末端核苷酸以及3'末端区。3'末端区的最后的核苷酸(T)是第二引物的延伸产物的3'末端核苷酸(T)，并且该3'末端区含有与在5'至3'方向读取的第二引物的尾区的核苷酸序列(CCAT)相同的核苷酸序列。

[0139] 在一些实施方式中，使得第二引物106的模板结合区与第一引物的第一拷贝的延伸产物111退火并形成第二引物的延伸产物112的条件可包括足以支持基于聚合酶的核酸合成的任何条件，包括本文其他地方讨论的任何条件。该条件可与用于生成第一引物的第一拷贝的延伸产物111的条件相同，或者它们可以是不同的条件。

[0140] 可在使得第一引物的第二拷贝105b的模板结合区与第二引物的延伸产物112退火(图1G)并形成第一引物的第二拷贝的延伸产物113(图1H)的条件下，用第一引物的第二拷贝105b和聚合酶108处理第二引物的延伸产物112。聚合酶108可催化第一引物的第二拷贝的延伸产物113的形成。该聚合酶可具有链置换活性。聚合酶可以是与用于生成第一引物的第一拷贝的延伸产物111或第二引物的延伸产物112的聚合酶相同类型的聚合酶，或者它可

以是不同类型的聚合酶。在合成第一引物的第二拷贝的延伸产物113的过程中，聚合酶可从第二引物的延伸产物112置换第一引物的第一拷贝的延伸产物111。通常，由第一引物的第二拷贝105b的3'端生成延伸产物。在生成第一引物的第二拷贝的延伸产物113的过程中，第一引物的第二拷贝105b可与合成的延伸产物共价连接，使得第一引物的第二拷贝变为本文描述为"第一引物的第二拷贝的延伸产物"的分子的一部分。第一引物的第二拷贝的延伸产物113与第二引物的延伸产物112互补。在一些实施方式中，当第一引物的第二拷贝105b与第二引物的延伸产物112退火时，第一引物的第二拷贝105b的模板结合区和尾区均与第二引物的延伸产物112退火。第一引物的第二拷贝的延伸产物113含有5'末端核苷酸、3'末端核苷酸以及3'末端区。3'末端区的最后的核苷酸(G)是第二引物的延伸产物的3'末端核苷酸(G)，并且该3'末端区含有与在5'至3'方向读取的第一引物的尾区的核苷酸序列(ATGG)相同的核苷酸序列。

[0141] 在一些实施方式中，使得第一引物的第二拷贝105b的模板结合区与第二引物的延伸产物112退火并形成第一引物的第二拷贝的延伸产物113的条件可包括足以支持基于聚合酶的核酸合成的任何条件，包括本文其他地方讨论的任何条件。该条件可与用于生成第一引物的第一拷贝的延伸产物111或第二引物的延伸产物112的条件相同，或者它们可以是不同的条件。

[0142] 第一引物的第二拷贝的延伸产物113的生成可导致生成包含第一引物的第二拷贝的延伸产物113和第二引物的延伸产物112的分子，该分子在本文中可被称为"二级核酸"115。在二级核酸115内，第一引物的第二拷贝的延伸产物113可与第二引物的延伸产物112退火。此外，二级核酸115含有核酸模板102的序列。该二级核酸115具有比单独核酸模板102长的核苷酸长度，因为除核酸模板102的序列之外，它还可包含第一引物和第二引物的尾区的序列及其互补序列。具体而言，二级核酸115可具有第一端区域116和第二端区域117。第一端区域116可包含第二引物的延伸产物112的3'末端区及其互补序列。第二端区域117可包含第一引物的第二拷贝的延伸产物113的3'末端区及其互补序列。

[0143] 可形成或提供二级核酸115的第一拷贝115a和第二拷贝115b(图1I)。二级核酸115的第一拷贝115a和第二拷贝115b可采用通过其可生成具有二级核酸115的通用结构的核酸的任何方法生成，包括本文其他地方讨论的任何方法。例如，如上文所述的用于由一级核酸101生成二级核酸115的全部过程可重复两次，以便生成该二级核酸的第一拷贝115a和第二拷贝115b。在另一个实例中，二级核酸的第一拷贝115a和第二拷贝115b可由第一引物的第一拷贝的延伸产物111的单一拷贝生成。在这一实例中，第二引物的延伸产物的两个拷贝112a、112b可由第一引物的第一拷贝的延伸产物111的单一拷贝生成，这在从第一引物的第一拷贝的延伸产物111的单一拷贝置换第二引物的延伸产物的第一拷贝112a时，可能发生，从而允许由第一引物的第一拷贝的延伸产物111的单一拷贝生成第二引物的延伸产物的第二拷贝112b。然后，例如，第一引物的第二拷贝的延伸产物113a、113b可分别由第二引物的延伸产物的各个拷贝112a、112b生成。也可进行用于生成二级核酸的第一拷贝115a和第二拷贝115b的另外的程序并将该程序与本文提供的方法一起使用。

[0144] 可在使得二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物113a的3'末端区与二级核酸的第二拷贝的第二引物的延伸产物112b的3'末端区退火的条件下，处理二级核酸的第一拷贝115a和第二拷贝115b，以产生包含这些链的交换结构118(图1J)(为简单起

见，未示出链113b和112a)。可在使得形成两个组分链112b、113a的延伸产物(该延伸产物在本文中可分别称为“第一多联体链”121和“第二多联体链”122)的条件下，用聚合酶进一步处理交换结构118。第一多联体链121和第二多联体链122可退火在一起并可共同被称为多联体120(图1K)。多联体120可含有核酸模板102的两个或更多个拷贝。在多联体120内，核酸模板102的两个或更多个拷贝中的一些或全部可通过第一引物105和第二引物106的尾区的序列彼此分隔开，其中尾区的序列彼此退火。例如，在图1K的多联体120中，双链核酸模板的第一链的第一拷贝通过5’-3’顺序的序列CCAT与双链核酸模板的第一链的第二拷贝分隔开。CCAT还是第二引物106的尾区的5’-3’顺序的序列。类似地，在图1K的多联体120中，双链核酸模板的第二链的第一拷贝通过5’-3’顺序的序列ATGG与双链核酸模板的第二链的第二拷贝分隔开。ATGG还是第一引物105的尾区的5’-3’顺序的序列。在多联体内，第一多联体链的CCAT与第二多联体链的ATGG退火。

[0145] 在一些实施方式中，使得形成交换结构118或交换结构的链的延伸产物的条件可包括足以支持基于聚合酶的核酸合成的任何条件，包括本文其他地方讨论的任何条件。该条件可与如用于生成第一引物的第一拷贝的延伸产物111、第二引物的延伸产物112或第一引物的第二拷贝的延伸产物111的条件相同，或者它们可以是不同的条件。

[0146] 在一些实施方式中，本文提供的多联体可根据图1I-1K中的总体概述的过程进一步增加长度。例如，可处理如图1K所示的多联体分子120中的两个，使得它们形成与图1J中所示的结构相似的交换结构(除了具有更长的链)，随后生成包含核酸模板102的四个拷贝的更大的多联体分子。在另一实例中，二级核酸115和多联体120可形成交换结构，随后生成含有核酸模板102的三个拷贝的更大的多联体分子。在一些实施方式中，在本文提供的方法中，可同时生成多个不同长度的多个不同的多联体。

[0147] 在实施方式中，可参考图8对本文提供的方法和组合物进行描述。多核苷酸模板800(图8A)可以是用于扩增的靶标。多核苷酸模板800是单一核酸链。该多核苷酸模板链可作为游离的单链分子存在，或者它可作为双链分子的一部分存在，在该双链分子中该多核苷酸模板链与其互补链退火。多核苷酸模板800可以是DNA或RNA。

[0148] 多核苷酸模板800可含有至少第一部分801(也用“(i)”表示)和第二部分802(也用“(ii)”表示)。第一部分801和第二部分802可各自含有任何数目的核苷酸，诸如各自少于1000、500、400、300、200、100、90、80、70、60、50、40、30、20、15、10、9、8、7、6、5、4或3个核苷酸或各自至少1000、500、400、300、200、100、90、80、70、60、50、40、30、20、15、10、9、8、7、6、5、4或3个核苷酸。通常，第一部分801和第二部分802可以各自含有10至30个核苷酸，尽管第一部分801和第二部分802也可以是如本文其他地方所述的其他长度。第一部分801和第二部分802可含有相同或不同数目的核苷酸。在一些实施方式中，除第一部分801和第二部分802之外，多核苷酸模板800还可含有其他部分。在其他实施方式中，第一部分801和第二部分802可构成多核苷酸模板800的全部。多核苷酸模板800可具有本文其他地方所述的核酸模板的长度。多核苷酸模板800可以是一级核酸的一部分。多核苷酸模板可存在于从受试者获得的样品中。

[0149] 多核苷酸模板800可在含有第一引物810和第二引物820的反应混合物中进行温育(图8B)。通常，该反应混合物含有每个引物的多个拷贝，例如，每个引物的至少4、5、6、10、15、20、50、100、1000、10,000、100,000或1百万个拷贝或更多个拷贝。第一引物810可具有第

一区域811(也用“(i)”表示)和第二区域812(也用“(ii)”表示)。第一引物的第一区域811可具有5’端,并且第一引物的第二区域812可具有3’端。第一引物的第一区域可具有与第一引物的第二区域的核苷酸序列不同的核苷酸序列。第二引物820可具有第一区域821(也用“(i)”表示)和第二区域822(也用“(ii)”表示)。第二引物的第一区域821可具有5’端,并且第二引物的第二区域822可具有3’端。第二引物的第一区域可具有与第二引物的第二区域的核苷酸序列不同的核苷酸序列。

[0150] 第一引物的第二区域812可具有与多核苷酸模板的第一部分801的核苷酸序列互补的核苷酸序列。在图8中,多核苷酸模板的第一部分801的核苷酸序列由字母A表示,并且第一引物的第二区域812的核苷酸序列由字母A’表示,其中A和A’彼此互补。第二引物的第二区域822可具有与配偶体核苷酸序列830互补的核苷酸序列,其中配偶体核苷酸序列830与多核苷酸模板的第二部分802的核苷酸序列互补。在图8中,多核苷酸模板的第二部分802的核苷酸序列由字母B表示,配偶体核苷酸序列830的核苷酸序列由字母B’表示,并且第二引物的第二区域822的核苷酸序列由字母B表示,其中B和B’彼此互补。换言之,如图8所示,多核苷酸模板的第二部分802的核苷酸序列(B)可与第二引物的第二区域822的核苷酸序列(B)相同。通过采用本文提供的组合物和方法,可生成配偶体核苷酸序列830,例如,作为来自第一引物的3’端的延伸产物的一部分。在实施方式中,第一引物的第一区域811可与第二引物的第一区域821互补。在图8中,第一引物的第一区域的核苷酸序列由字母C表示,并且第二引物的第一区域的核苷酸序列由字母C’表示,其中C和C’彼此互补。

[0151] 在实施方式中,图8的第一引物和第二引物可分别具有本文其他地方所述的第一引物和第二引物的任何特征(例如,如图1中的)。而且,在实施方式中,图8的第一引物的第一区域可具有本文其他地方所述的第一引物的尾区的任何特征。在实施方式中,图8的第一引物的第二区域可具有本文其他地方所述的第一引物的模板结合区的任何特征。在实施方式中,图8的第二引物的第一区域可具有本文其他地方所述的第二引物的尾区的任何特征。在实施方式中,图8的第二引物的第二区域可具有本文其他地方所述的第二引物的模板结合区的任何特征。

[0152] 在实施方式中,含有多核苷酸模板800和第一引物810的至少两个拷贝以及第二引物820的至少两个拷贝的反应混合物可含有一个或多个可支持如本文其他地方所述的基于聚合酶的核酸合成的其他组分(例如,聚合酶、核苷酸、缓冲液、水等)。相似地,含有多核苷酸模板800和第一引物810的至少两个拷贝以及第二引物820的至少两个拷贝的反应混合物可在本文其他地方所述的用于支持核酸模板的扩增或多联体的生成的任何反应条件下进行温育。

[0153] 在实施方式中,在如本文所述在反应混合物中温育多核苷酸模板800和第一引物810的至少两个拷贝以及第二引物820的至少两个拷贝时,可形成一个或多个多联体链(图8C)。在实施方式中,形成的多联体链840可具有5’和3’端,并可具有含有5’至3’方向的通用结构C’-T-C’-T-X-C’的序列,其中C’是第二引物的第一区域的核苷酸序列,T是多核苷酸模板的序列或其类似序列,并且X是任何数目和序列的核苷酸。因此,如由多联体链的通用结构所示的,多联体链可含有多核苷酸模板的序列或其类似序列的至少两个拷贝,其中多核苷酸模板的序列的拷贝由至少第二引物的第一区域的序列分隔开。在实施方式中,T可以是多核苷酸模板的类似序列。如本文所用的,多核苷酸模板的“类似序列”指的是含有作为多

核苷酸模板的相似序列，而非含有多核苷酸模板中的核苷酸的一个或多个相似的核苷酸的序列。因此，例如，如果多核苷酸模板含有RNA序列，则其类似序列可以是相同序列的DNA形式(即，RNA序列中的尿嘧啶在类似的DNA序列中是胸腺嘧啶)，或反之亦然。继续该实例，如果多核苷酸模板含有5'UACCUG 3'的RNA序列，则类似的序列是5'TACCTG 3'的DNA序列。

[0154] 在实施方式中，在含有5'至3'方向的通用结构C'-T-C'-T-X-C'的序列中，X是零个核苷酸(即，X的任一侧上的T和C序列直接连接)。在其他实施方式中，X是少于10,000、5000、4000、3000、2000、1000、500、400、300、200、100、90、80、70、60、50、40、30、20、15、10、9、8、7、6、5、4或3个核苷酸。X中的核苷酸(如果有)可具有任何序列。在实施方式中，X含有具有5'至3'方向的通用结构[$(C'-T)_N$]的序列，其中C'是第二引物的第一区域的核苷酸序列，T是多核苷酸模板的序列或其类似序列，并且N是小于1000、500、200、100、50或10的任何整数，诸如1至500、1至200、1至100、1至50或1至10之间的整数。例如，如果当X含有5'至3'方向的通用结构[$(C'-T)_N$]的序列时“N”是3，则在结构C'-T-C'-T-X-C'的X位置上存在3个连续重复的(C'-T)，使得相应的结构可以写成：C'-T-C'-T-<u>C'-T-C'-T-C'-T</u>-C'，其中，带下划线的C'和T是式[$(C'-T)_3$]的C'-T的重复。因此，例如，根据本文提供的组合物和方法，可形成含有多核苷酸模板的数十或数百个拷贝的多联体链。在实施方式中，如图8所提供的多联体链可具有本文其他地方所述的多联体链的任何特征。而且，在一些实施方式中，在生成多联体链的过程中，在具有通用结构C'-T-C'-T-X-C'的序列中的C'或T的一个或多个位置处，可在C'与T之间插入少量的核苷酸(例如，15个或更少、10个或更少、5个或更少、或3个或更少)。而且，在一些实施方式中，在生成多联体链的过程中，在具有通用结构C'-T-C'-T-X-C'的序列中的C'或T的一个或多个位置处，少量的核苷酸(例如，15个或更少、10个或更少、5个或更少、或3个或更少)可从C'或T序列中缺失。因此，根据本文提供的方法生成的多联体链可包括具有如本文提供的通用结构的链，但该链可在多联体链的不同序列组分之间的一个或多个接合点(即，模板序列与引物序列/T和C'之间的接合)处添加或去除少量核苷酸。而且，在实施方式中，如本文提供的多联体链可在含有多联体(该多联体含有具有通用结构C'-T-C'-T-X-C'的序列)的分子的5'或3'端含有附加的核苷酸(例如，多达3、5、10、20、50、100、500、1000个或更多个附加的核苷酸)。在实施方式中，通用结构C'-T-C'-T-X-C'的C'可具有本文其他地方所述的第二引物的尾区的任何特征(例如，长度、序列)。在实施方式中，通用结构C'-T-C'-T-X-C'的T可具有本文其他地方所述的多核苷酸模板链的任何特征(例如，长度、序列)。

[0155] 在实施方式中，根据本文提供的方法生成的多联体链840可以是单链分子的一部分。在其他实施方式中，根据本文提供的方法生成的多联体链840可以是双链多联体的一部分，在该双链多联体中多联体链840与互补于多联体链850的链退火。虽然图8C描绘了含有具有5'至3'方向的通用结构C'-T-C'-T-X-C'(链840)或C-T'-C-T'-X'-C(链850)的序列的多联体链(其中X是0个核苷酸)，但根据图8的示意图生成的多联体链可具有上文或本文其他地方所述的多联体链的任何特征。而且，关于图8C，序列A和B是模板链的一部分，并且因此不在通用结构C'-T-C'-T-X-C'或C-T'-C-T'-X'-C中单独用符号表示。

[0156] 为了进一步说明在图8的实施方式中元件的关系，图8提供了区块(blocks)860、862、864、866和868。区块860(具有反斜线样填充)指示总多核苷酸模板序列(即元件800)或其互补序列的相对位置。区块862(具有垂直线填充)指示所述多核苷酸模板的第一部分的

序列(即元件801的序列的相对位置)或其互补序列的相对位置。因此,区块862还表示第一引物的第二区域(即元件812)的序列,它与元件801互补。区块864(具有垂直和水平线填充)指示所述多核苷酸模板的第二部分的序列(即元件802的序列的相对位置)或其互补序列的相对位置。因此,区块864还表示配偶体核苷酸序列(即元件830)的序列,它与元件802互补。区块864还指示第二引物的第二区域822的序列的相对位置,该序列可以是与所述多核苷酸模板的第二部分(元件802)的序列相同的序列,或相似的序列。换言之,在实施方式中,822的核苷酸序列可以与802的核苷酸序列相同或相似。例如,如图8A中所示,在实施方式中,在多核苷酸模板中具有不是该多核苷酸模板的第一部分或第二部分的一部分的核苷酸。例如,在一个实例中,所述多核苷酸模板的第一部分可含有15个核苷酸,所述多核苷酸模板的第二部分可含有15个核苷酸,并且所述多核苷酸模板的第一部分与第二部分之间为25个核苷酸。通常,所述多核苷酸模板的第一部分和所述多核苷酸模板的第二部分各自为10至30个核苷酸的长度,尽管也考虑其他数目。例如,所述多核苷酸模板的第一部分和第二部分中的每一个可以为至少5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50或100个核苷酸,不多于5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50或100个核苷酸,或至少5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45或50个核苷酸且不多于6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50或100个核苷酸(上限高于下限)。在实施方式中,在所述多核苷酸模板中第一部分与第二部分之间可以具有例如至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、100、200或500个核苷酸,不多于1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、100、200或500个核苷酸,或至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、100或200个核苷酸且不多于2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、100、200或500个核苷酸(上限高于下限)。区块866(具有波浪线填充)指示第一引物的第一区域的序列(即元件811/区域“C”的序列的相对位置)或其互补序列的相对位置。因此,区块866还表示第二引物的第一区域(元件821/区域“C”)的序列,它与元件811互补。区块868(具有菱形填充)指示多联体链(即元件840)的序列或其互补序列的相对位置。因此,区块868还表示与所述多联体链互补的链(元件850)的序列。如图8C中所示,在多联体链或其互补体(868)中存在所述多核苷酸模板序列或其互补体(860)的两个拷贝,这两个拷贝被第一引物的第一区域的核苷酸序列或其互补体(866)分隔开。此外,在多联体链或其互补体(868)中,第一引物的第一区域的核苷酸序列或其互补体(866)在两端侧接所述多核苷酸模板序列或其互补体(860)。而且,在多联体链或其互补体(868)中,具有第一引物的第二区域的序列或其互补体(862)的两个拷贝和第二引物的第二区域的序列或其互补体(864)的两个拷贝,它们均作为所述多核苷酸模板序列或其互补体的两个拷贝的一部分而以两个拷贝存在。区块860、862、864、866和868不应被解释为规定图8的元件之间的任何所需的特定大小关系;相反,在本文提供的某些实施方式中,区块860、862、864、866和868仅用于帮助说明图8的元件相对于彼此的一般位置。

[0157] 根据本文提供的方法和组合物生成的多联体可以是任何长度的核苷酸。在一些实施方式中,本文生成的多联体分子可以是至少30、40、50、60、70、80、90、100、150、200、250、300、400、500、600、700、800、900、1000、1500、2000、3000、4000、5000、6000、7000、8000、

9000、10,000、15,000、20,000或25,000个核苷酸的长度。在一些实施方式中,本文生成的多联体分子可以是不多于30、40、50、60、70、80、90、100、150、200、250、300、400、500、600、700、800、900、1000、1500、2000、3000、4000、5000、6000、7000、8000、9000、10,000、15,000、20,000或25,000个核苷酸的长度。在一些实施方式中,本文生成的多联体分子可具有选自最小值为30、40、50、60、70、80、90、100、150、200、250、300、400、500、600、700、800、900、1000、1500、2000、3000、4000、5000、6000、7000、8000、9000、10,000、15,000或20,000个核苷酸的长度且最大值为40、50、60、70、80、90、100、150、200、250、300、400、500、600、700、800、900、1000、1500、2000、3000、4000、5000、6000、7000、8000、9000、10,000、15,000、20,000或25,000个核苷酸的长度的范围的长度。在一些实施方式中,根据本文提供的方法或组合物生成的至少一些多联体具有上文所述的特征。在一些实施方式中,根据本文提供的方法或组合物生成的至少10%、20%、30%、40%、50%、60%、70%、80%、90%或95%的多联体具有上文所述的特征。

[0158] 根据本文提供的方法和组合物生成的多联体可含有核酸模板或特定核酸的任何数目的拷贝。在一些实施方式中,本文生成的多联体分子可含有核酸模板或特定核酸的至少2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、60、70、80、90或100个拷贝。在一些实施方式中,本文生成的多联体分子可含有核酸模板或特定核酸的不多于2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、60、70、80、90或100个拷贝。在一些实施方式中,本文生成的多联体分子可具有核酸模板或特定核酸的选自最小值为2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、60、70、80或90个拷贝且最大值为3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、60、70、80、90或100个拷贝的范围的多个拷贝。在一些实施方式中,根据本文提供的方法或组合物生成的至少一些多联体具有上文所述的特征。在一些实施方式中,根据本文提供的方法或组合物生成的至少10%、20%、30%、40%、50%、60%、70%、80%、90%或95%的多联体具有上文所述的特征。

[0159] 本文提供的方法的过程可以采用多种不同方式进行监测。在实施方式中,可测定反应的核酸扩增产物(例如,产物的量或其生成速率)。在其他实施方式中,可测定反应的聚合酶沿核酸模板的活性(例如,聚合酶沿模板链的移动)。因此,在一些实施方式中,由于由方法产生的产物的积累(可能在该方法的步骤期间或完成后)或由于在方法的步骤期间发生的可检测事件,使得可观察到本文提供的方法的事件。

[0160] 可测定扩增的核酸的存在,例如,通过检测反应产物(扩增的核酸或反应副产物)或通过检测与反应进展相关的探针。

[0161] 在一些实施方式中,可通过用染料将产物染色来鉴定反应产物。在一些实施方式中,染料在与核酸结合时比未与核酸结合时可具有更大的荧光。在实施方式中,染料可插入双链核酸或它可与核酸的外部区域结合。可与本文提供的方法和组合物一起使用的核酸染料包括,例如,花青染料、PicoGreen ®、OliGreen ®、RiboGreen ®、SYBR®染料、SYBR®Gold、SYBR ®Green I、SYBR ®Green II、溴化乙锭、二氢乙锭、BlueView™、TOTO ®染料、TO-PRO ®染料、POPO ® 染料、YOYO ®染料、BOBO ®染料、JOJO ®染料、LOLO ®染料、SYTOX ®染料、SYTO ®染料、碘化丙啶、碘化己啶(hexidium iodide)、亚甲蓝、DAPI、吖啶橙、奎纳克林(quinacrine)、吖啶二聚体(acridine

dimer)、9-氨基-6-氯-2-甲氧基吖啶、双苯酰亚胺染料、Hoechst染料、7-氨基放线菌素D、放线菌素D、羟芪巴胨、派洛宁Y(pyronin Y)、Diamond™染料、GelRed™、GelGreen™和LDS 751。

[0162] 在一些实施方式中，可通过分析扩增反应的浊度来鉴定反应产物。例如，在实施方式中，增加的浊度可能与反应产物和反应副产物(例如，与镁复合的焦磷酸)的形成相关。

[0163] 在一些实施方式中，可通过分离根据本文方法通过凝胶电泳进行的反应并随后用核酸染料将凝胶染色来鉴定反应产物。该染料可以是本文公开的或本领域以其他方式已知的任何核酸染料。

[0164] 在一些实施方式中，本领域已知的用于检测核酸或用于生成核酸的任何方法或组合物可与本文提供的方法和组合物一起使用。

[0165] 在一些实施方式中，含有与核酸模板链(或具有相似或相同的序列的链)的一部分互补的核苷酸序列和含有荧光报道分子(荧光团)和猝灭剂中的一种或两种的核酸探针包含在本文提供的反应中。

[0166] 在实例中，核酸探针可含有在其5'或3'末端的荧光报道分子，以及在另一末端的猝灭剂。该探针可进一步具有含有按顺序的至少第一、第二和第三区域的核苷酸序列，在该核苷酸序列中第一和第三区域彼此互补，并且第二区域的至少一部分与核酸模板链(探针“检测序列”)的一部分互补。在一些实施方式中，第二区域的长度可大于第一或第三区域的长度。在一些实施方式中，第二区域的长度可为10至40个核苷酸，并且第一和第三区域的长度可为4至10个核苷酸。该探针可具有至少两种不同的构型：(A)其中探针不与其检测序列退火且第一和第三区域彼此退火的构型；该构型可以是“茎-环”结构，在该结构中第一和第三区域形成茎而第二区域形成环，以及(B)其中探针与其检测序列退火的构型；在该构型中，第二区域或其一部分与其检测序列退火，并且第一和第三区域彼此不退火。在探针的构型(A)中，荧光报道分子和猝灭剂(它们位于探针的相对末端/第一和第三区域的外端)可彼此紧密靠近(均在通过第一和第三区域退火形成的茎结构的末端处)，以使得荧光报道分子被猝灭。在探针的构型(B)中，荧光报道分子和猝灭剂可不彼此紧密靠近，以使得荧光报道分子不被猝灭。探针可用于监测所选反应产物的积累，例如，在探针可形成茎环结构或与其检测序列退火的反应条件下。在一些实施方式中，如果存在检测序列，则探针可与检测序列退火，并且探针可响应于荧光团的激发光谱的波长的光而发荧光。相反，如果不存在检测序列，则探针可形成茎环结构，并且探针不响应于荧光团的激发光谱的波长的光而发荧光。

[0167] 在另一实例中，核酸探针可在其5'或3'末端含有荧光报道分子，并且它可与含有猝灭剂的核酸引物退火。含有猝灭剂的核酸引物可在引物的位置上含有猝灭剂，使得当核酸探针与引物退火时，荧光报道分子被猝灭。探针可用于监测所选反应产物的积累，例如，在探针可与引物退火或与其检测序列(在反应产物中)退火的反应条件下。在一些实施方式中，如果存在检测序列，则探针可与检测序列退火，并且探针可响应于荧光团的激发光谱的波长的光而发荧光(由此指示反应产物的存在)。相反，如果不存在检测序列，则探针可保持与引物配对，并且探针不响应于荧光团的激发光谱的波长的光而发荧光。

[0168] 在含有荧光报道分子和猝灭剂对的探针中，可选择荧光报道分子和猝灭剂，以使得猝灭剂可有效地猝灭报道分子。在一些实施方式中，荧光报道分子与猝灭剂配对，其中荧光报道分子的发射最大值类似于猝灭剂的吸收最大值。可用作荧光报道分子的荧光团包括，例如，CAL Fluor Gold、CAL Fluor Orange、Quasar 570、CAL Fluor Red 590、CAL

Fluor Red 610、CAL Fluor Red 610、CAL Fluor Red 635、Quasar 670(Biosearch Technologies)、VIC、NED(Life Technologies)、Cy3、Cy5、Cy5.5(GE Healthcare Life Sciences)、Oyster 556、Oyster 645(Integrated DNA Technologies)、LC red 610、LC red 610、LC red 640、LC red 670、LC red 705(Roche Applies Science)、德克萨斯红(Texas red)、FAM、TET、HEX、JOE、TMR和ROX。可使用的猝灭剂包括，例如，DDQ-I、DDQ-II(Eurogentec)、Eclipse(Epoch Biosciences)、Iowa Black FQ、Iowa Black RQ(Integrated DNA Technologies)、BHQ-1、BHQ-2、BHQ-3(Biosearch Technologies)、QSY-7、QSY-21(分子探针)和Dabcyl。

[0169] 在一些实施方式中，本文提供的组合物或方法使用的探针可以是肽核酸(PNA)探针。肽核酸是合成的DNA类似物，在其中，例如，磷酸二酯骨架被N-(2-氨乙基)甘氨酸的重复单元代替，并且嘌呤和嘧啶碱基经由甲基羰基连接体附接至所述合成DNA类似物上。任选地，在本文提供的反应中可以提供PNA探针，它进一步含有能与PNA-DNA双链体结合并且当与PNA-DNA双链体结合时改变颜色的染料。这样的染料的一个实例是3,3'-二乙基噻二碳菁碘化物[$DiSc_2(5)$]。$DiSc_2(5)$在与PNA-DNA双链体结合时从蓝色变为紫色，并且通过图像分析，例如在分光光度计中，或通过人类肉眼，可以观察到这一颜色变化。例如，在Wilhelmsson等人,Nucleic Acids Research,2002,Vol.30,No.2e3中描述了使用PNA探针和$DiSc_2(5)$检测PNA-DNA双链体，该文献通过引用并入于此用于所有目的。在使用PNA探针的实施方式中，通常将PNA探针设计为与有待于在相关扩增反应中扩增的序列互补。因此，如果序列成功扩增，则PNA探针将与含有扩增的序列的DNA链退火，并且可以检测到此退火(例如，通过观察$DiSc_2(5)$与PNA-DNA双链体结合时的颜色变化)。因此，本文提供的实施方式使用PNA探针可以帮助准确地确定序列得到成功扩增的反应，并且帮助将这些反应与具有核酸的非特异性扩增的反应区分开。为了随本文提供的实施方式使用PNA探针，任选地，PNA探针可以在反应开始时包含在本文提供的反应混合物中。在其他实例中，可以在反应开始之后或反应结束之后的某一时间，向反应混合物中添加PNA探针。可以在添加PNA探针的同时或在不同时间向反应混合物中添加染料。例如，在实施方式中，可以在反应开始时向反应混合物中添加染料，而可以在反应开始之后的某一时间点添加PNA。在实施方式中，本文提供的试剂盒可进一步含有与有待通过该试剂盒的试剂扩增的核苷酸序列互补的PNA探针和能与PNA-DNA双链体结合的染料。

[0170] 在一些实施方式中，根据本文提供的方法进行的反应可在含有光源和光学传感器的装置中进行监测。在一些情况下，可将反应定位于来自光源的光路上，并且可测量由样品吸收(例如，在混浊的反应的情况下)、由样品散射(例如，在混浊的反应的情况下)或由样品发射(例如，在含有荧光分子的反应的情况下)的光。在一些实施方式中，本文提供的方法可在如在2013年2月18日提交的美国专利申请系列号13/769,779中公开的设备或其中的模块中进行或监测，该专利申请通过引用全文并入本文。

[0171] 通过使用本文提供的方法，感兴趣的核酸模板的特异性扩增产物可在扩增反应开始后例如30秒、1分钟、3分钟、5分钟、10分钟、15分钟、20分钟、30分钟、45分钟、60分钟、90分钟、120分钟、180分钟或240分钟内进行鉴定。在其他实例中，当生成模板的少至10、50、100、500、1000、5000、10,000、50,000、100,000、500,000或1,000,000个拷贝时，通过使用本文提供的方法可鉴定对于感兴趣的核酸模板为阳性的扩增反应。在其他实例中，通过使用本文

提供的方法，可鉴定样品中感兴趣的核酸模板的存在，该样品在该方法开始时含有感兴趣的模板的少至1、2、3、4、5、10、15、20、25、30、40、50、100、200、500、1000、5000或10,000个拷贝。

[0172] 在实施方式中，本文提供的方法可用于测定样品中的感兴趣的靶核酸。在某些实施方式中，可通过包括测定反应中核酸扩增的转变时间的方法来确定样品中感兴趣的靶核酸的存在或量。转变时间/转变点是扩增反应被确定为对核酸模板为阳性的时间或点。转变时间/转变点可以通过一个或多个指标来鉴定，例如，反应开始后在反应中已经生成所选量的核酸的时间，当反应的扩增速率由基线期变为指数期时的时间，或者当反应的扩增速率由指数期变为平台期(plateau phase)时的时间等。在实施方式中，转变时间/转变点可以基于反应的荧光或吸光度的变化或基于反应的荧光或吸光度达到所选值来鉴定。在某些实施方式中，样品中感兴趣的靶核酸的存在或量可通过包括比较反应的核酸扩增的转变时间来

[0173] 确定，该反应相对于以下反应中的一者或两者具有未知量的感兴趣的靶核酸：i)已知缺乏感兴趣的靶核酸的反应(即，阴性对照)；或ii)已知含有感兴趣的靶核酸的反应(即，阳性对照)。在实施方式中，可测量含有感兴趣的靶核酸的反应和不含有靶核酸的反应两者的所选的转变时间。在实施方式中，样品中感兴趣的靶核酸的存在可基于这样的方法来确定，该方法包括评价包含可含有或可不含有感兴趣的靶核酸的样品的反应的转变点与具有感兴趣状态(例如，已知其含有或不含有感兴趣的靶核酸)的已知靶核酸的一个或多个反应的转变时间之间的时间差异。例如，如果根据本文提供的方法的反应的转变时间比已知不含有感兴趣的靶核酸的相应反应早至少3、5、10、15、20、30、40、50、60、90、120或180分钟，则样品可被鉴定为含有感兴趣的靶核酸。在另一实例中，如果根据本文提供的方法的反应的转变时间比已知不含有感兴趣的靶核酸的相应反应晚不多于3、5、10、15、20、30、40、50、60、90、120或180分钟，则样品可被鉴定为含有感兴趣的靶核酸。

[0174] 本文提供的方法可进行任意时间长度。通常，该方法将进行足以监测例如核酸复制速率、聚合酶活性的出现或扩增产物的积累的时间长度。在一些实施方式中，本文提供的方法可进行总计少于10秒、30秒、1分钟、5分钟、10分钟、20分钟、30分钟、45分钟、1小时、2小时、3小时、4小时、6小时、8小时、12小时、16小时或24小时，到该时间时测量核酸复制速率、聚合酶活性的出现或扩增产物的积累。

[0175] 本文提供的方法可以以各种方式终止。在一个实施方式中，方法的步骤可在浓度降低时或该方法的一个或多个步骤中包含的一种或多种试剂(例如，dNTP)完全消耗时结束。在另一个实施方式中，方法的步骤可在该方法的一个或多个步骤中包含的一种或多种酶(例如，聚合酶)失活时结束。酶可通过各种方式失活。例如，由于影响酶结构的随机事件，导致酶可随时间逐渐失去酶活性，或者酶可暴露于加速酶活性失活的条件(例如，高热量、极端pH等)。

[0176] 在一些实施方式中，一级核酸可以是单链或双链的。单链一级核酸在本文也可被称为“一级多核苷酸”。一级核酸可以是线形或环形的。一级核酸可以包含核酸模板。在一些实施方式中，整个一级核酸可以是核酸模板。在其他实施方式中，一级核酸可以含有不是核酸模板的一部分的一个或多个核苷酸(例如，一级核酸可具有比包含在一级核酸内的核酸模板更长的长度)。在一些实施方式中，一级核酸可含有核酸模板的两个或更多个拷贝。一

级核酸可含有DNA、RNA或其混合物。双链线形一级核酸可具有平端或粘端(“粘端”指的是具有突出链的末端,该突出链具有一个或多个未配对的核苷酸)。

[0177] 一级核酸可以是任何长度的核苷酸。例如,一级核酸可以是至少2、3、4、5、6、7、8、9、10、12、14、16、18、20、25、30、35、40、45、50、60、70、80、90、100、150、200、250、300、350、400、500、750、1000或1500个核苷酸的长度。在另一个实例中,一级核酸可以是2至100,000、5至100,000、10至100,000、15至100,000、20至100,000、25至100,000、30至100,000、50至100,000、70至100,000、100至100,000、200至100,000、2至10,000、5至10,000、10至10,000、15至10,000、20至10,000、25至10,000、30至10,000、50至10,000、70至10,000、100至10,000、200至10,000、2至5,000、5至5,000、10至5,000、15至5,000、20至5,000、25至5,000、30至5,000、50至5,000、70至5,000、100至5,000、200至5,000、2至3,000、5至3,000、10至3,000、15至3,000、20至3,000、25至3,000、30至3,000、50至3,000、70至3,000、100至3,000、200至3,000、2至1,000、5至1,000、10至1,000、15至1,000、20至1,000、25至1,000、30至1,000、50至1,000、70至1,000、100至1,000、200至1,000、2至500、5至500、10至500、15至500、20至500、25至500、30至500、50至500、70至500、100至500或200至500个核苷酸碱基的长度。

[0178] 在一些实施方式中,核酸模板可以是单链或双链的。核酸模板的单链在本文可被称为“多核苷酸模板”。如本文提及的“多核苷酸模板”并不排除与其互补序列结合。换言之,“多核苷酸模板”可以是例如整个单链核酸模板,或者它可以是双链核酸模板的一条链。核酸模板可以包含在一级核酸分子中。在一些实施方式中,核酸模板可构成整个一级核酸分子。在其他实施方式中,核酸模板可包含在含有不是核酸模板的一部分的一个或多个核苷酸的一级核酸中(例如,核酸模板可具有比含有核酸模板的一级核酸更短的长度)。

[0179] 核酸模板可以是任何长度的核苷酸。例如,核酸模板可以是至少2、3、4、5、6、7、8、9、10、12、14、16、18、20、25、30、35、40、45、50、60、70、80、90、100、150、200、250、300、350、400、500、750、1000或1500个核苷酸的长度。在另一个实例中,核酸模板可以是2至100,000、5至100,000、10至100,000、15至100,000、20至100,000、25至100,000、30至100,000、50至100,000、70至100,000、100至100,000、200至100,000、2至10,000、5至10,000、10至10,000、15至10,000、20至10,000、25至10,000、30至10,000、50至10,000、70至10,000、100至10,000、200至10,000、2至5,000、5至5,000、10至5,000、15至5,000、20至5,000、25至5,000、30至5,000、50至5,000、70至5,000、100至5,000、200至5,000、2至3,000、5至3,000、10至3,000、15至3,000、20至3,000、25至3,000、30至3,000、50至3,000、70至3,000、100至3,000、200至3,000、2至1,000、5至1,000、10至1,000、15至1,000、20至1,000、25至1,000、30至1,000、50至1,000、70至1,000、100至1,000、200至1,000、2至500、5至500、10至500、15至500、20至500、25至500、30至500、50至500、70至500、100至500或200至500个核苷酸碱基的长度。

[0180] 如本文所用的“引物”可以指这样一种多核苷酸,该多核苷酸i)能够与原始核酸链杂交,并且ii)充当合成新的核酸链的起始点,其中新的核酸链是引物的延伸产物并与原始链互补。引物可在其3’末端具有游离的-OH基团,其可充当延伸产物合成的起点。

[0181] 引物可含有标准核苷酸[例如,标准DNA脱氧核糖核苷酸(脱氧腺苷单磷酸、脱氧鸟苷单磷酸、胸苷单磷酸、脱氧胞苷单磷酸)或标准RNA核糖核苷酸(腺苷单磷酸、鸟苷单磷酸、尿苷单磷酸、胞苷单磷酸)]、替代性核苷酸(例如,肌苷)、修饰的核苷酸、核苷酸类似物或其组合。例如,寡核苷酸引物可包括肽核酸、吗啉代寡核苷酸(例如,磷二酰胺吗啉代寡核苷

酸)、锁定核酸[参见,例如,Kaur,H,等人,Biochemistry 45(23),7347-55(2006)]、乙二醇核酸或苏糖核酸。引物可具有骨架,包括,例如,磷酸二酯键、硫代磷酸酯键(用硫替代非桥接O)或肽键(作为肽核酸的一部分)。替代性核苷酸、修饰的核苷酸以及核苷酸类似物可在本文中通称为“非标准核苷酸”。

[0182] 引物中非标准核苷酸的存在可影响该引物的各种特性。在一些实施方式中,在引物中包含非标准核苷酸可增加或降低引物对其互补序列的热力学稳定性。例如,具有增加的热力学稳定性的引物可含有锁定核酸。具有降低的热力学稳定性的引物可含有,例如,肌苷(由Auer等人,Nucl.Acids Res.24;5021-5025(1996)描述的)或带负电荷的化学基团,诸如羧酸。

[0183] 本文提供的第一引物或第二引物可以是任何长度。第一引物和第二引物可含有相同数目的核苷酸或不同数目的核苷酸。在一些实施方式中,第一或第二引物可以为至少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、35、40、45、50、60、70、80、90、100、150、200、250、300、350、400、500、750、1000或1500个核苷酸的长度。在一些实施方式中,第一或第二引物可以为不多于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、35、40、45、50、60、70、80、90、100、150、200、250、300、350、400、500、750、1000或1500个核苷酸的长度。在一些实施方式中,第一或第二引物可具有选自最小值为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、35、40、45、50、60、70、80、90、100、150、200、250、300、350、400、500、750或1000个核苷酸的长度并且最大值为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、35、40、45、50、60、70、80、90、100、150、200、250、300、350、400、500、750、1000或1500个核苷酸的长度的范围的长度。

[0184] 本文提供的第一引物或第二引物的尾区可以是任何长度。通常,针对于同一模板的第一引物和第二引物的尾区含有相同数目的核苷酸。在一些实施方式中,第一或第二引物的尾区可以为至少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、35、40、45、50、60、70、80、90、100、150、200、250、300、350、400、500、750、1000或1500个核苷酸的长度。在一些实施方式中,第一或第二引物的尾区可以为不多于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、35、40、45、50、60、70、80、90、100、150、200、250、300、350、400、500、750、1000或1500个核苷酸的长度。在一些实施方式中,第一或第二引物的尾区可具有选自最小值为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、35、40、45、50、60、70、80、90、100、150、200、250、300、350、400、500、750或1000个核苷酸的长度并且最大值为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、35、40、45、50、60、70、80、90、100、150、200、250、300、350、400、500、750、1000或1500个核苷酸的长度的范围的长度。

[0185] 本文提供的第一引物或第二引物的模板结合区可以是任何长度。针对于同一模板的第一引物和第二引物的模板结合区可含有相同数目的核苷酸或不同数目的核苷酸。在一些实施方式中,第一或第二引物的模板结合区可以为至少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、35、40、45、50、60、70、80、90、

100、150、200、250、300、350、400、500、750、1000或1500个核苷酸的长度。在一些实施方式中，第一或第二引物的模板结合区可以为不多于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、35、40、45、50、60、70、80、90、100、150、200、250、300、350、400、500、750、1000或1500个核苷酸的长度。在一些实施方式中，第一或第二引物的模板结合区可具有选自最小值为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、35、40、45、50、60、70、80、90、100、150、200、250、300、350、400、500、750或1000个核苷酸的长度且最大值为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、35、40、45、50、60、70、80、90、100、150、200、250、300、350、400、500、750、1000或1500个核苷酸的长度的范围的长度。

[0186] 在一些实施方式中，引物可以是任何长度，并含有在用于包括该引物的方法或其步骤的温度下允许该引物与其互补序列足够稳定且特异性地退火的任何核苷酸序列。引物所需的确切长度可能依赖于多种因素，包括反应的温度、引物的化学组成以及包括引物的反应。在一些实施方式中，引物的模板结合区可以是任何长度，并含有在用于包括该引物的方法或其步骤的温度下允许该引物的模板结合区与其互补序列足够稳定且特异性地退火的任何核苷酸序列。引物的模板结合区所需的确切长度可能依赖于多种因素，包括反应的温度、引物的模板结合区的化学组成以及包括引物的反应。与缺乏非标准核苷酸的相应引物的长度相比，在引物中包含一个或多个非标准核苷酸可改变在本文提供的方法中使用的引物的所需长度。例如，如果采用本文提供的方法，期望有具有某一解链温度(“Tm”)的引物，在一些实施方式中，相比于如果引物仅含有标准核苷酸，如果引物含有至少一些非标准核苷酸，具有所选Tm的引物可以具有更短的长度。通常，核苷酸序列的“解链温度”指的是50％的具有核苷酸序列的核酸与其互补序列碱基配对(即在双链分子中)，而50％的具有核苷酸序列的核酸是单链形式时的温度。

[0187] 在一些实施方式中，本文提供的第一引物或第二引物中的一个或两者可被设计为在引物的尾区中含有与靶核酸序列内的内部基序(即核苷酸序列)互补的核苷酸序列。例如，再次参考图1，第一引物105的尾区可含有与双链核酸模板的第二链104中的内部基序互补的核苷酸序列，第一引物的第一拷贝的延伸产物111或第一引物的第二拷贝的延伸产物113。类似地，仍然参考图1，第二引物106的尾区可含有与双链核酸模板的第一链103中的内部基序互补的核苷酸序列或第二引物的延伸产物112。在至少一些情况下，如本文所述，在引物的尾区中具有与靶核酸序列内的内部基序互补的核苷酸序列可以提高反应的速度或特异性。

[0188] 不受理论束缚，根据图9中概括的机理，可以认为在引物的尾区中具有与靶核酸序列内的内部基序互补的核苷酸序列可以提高反应的速度或特异性。为简洁起见，图9和这一机理仅就第一引物的第一拷贝来描述，但根据具体情况，同样可应用于第一引物的第二拷贝、第二引物等。另外，图9和这一机理仅就第一引物的第一拷贝的延伸产物111来描述，该延伸产物与第二引物的延伸产物112退火，但其同样可应用于含有本文所述的引物的延伸产物的其他分子。现在转向图9，第一引物的尾区的至少一部分可含有与在第一引物的第一拷贝的延伸产物111中的内部基序902(反斜线样填充)互补的核苷酸序列901(棋盘状填充)，并且该第一引物可被整合到第一引物的第一拷贝的延伸产物(如本文其他地方所述)

111中。第一引物的第一拷贝的延伸产物111可与第二引物的延伸产物112退火。在含有第一引物的第一拷贝的延伸产物111和第二引物的延伸产物112的双链体核酸的“呼吸(breathing)”过程中，引物的尾区901可向存在于第一引物的第一拷贝的延伸产物中的内部基序902移动并可与之暂时退火。在与第一引物的第一拷贝的延伸产物中的内部基序902暂时退火之后，引物的尾区901可退回到与第二引物的延伸产物112退火的原始位置，并且任选地，它可以在这些结构之间反复地来回移动(由图9中的箭头表示)。在尾区901的核苷酸序列与内部基序902退火的时间过程中，第二引物的延伸产物112的3’末端处的一个或多个核苷酸可能未退火，并且可用于结合第一引物的新拷贝或另一双链体核酸(例如，二级核酸)中的互补序列。在第二引物的延伸产物112的3’末端处的一个或多个核苷酸结合第一引物的新拷贝或另一双链体核酸(例如，二级核酸)中的互补序列的事件中，这些事件中的任意一个均可以如本文其他地方所述提高反应的速率，例如，通过生成第一引物的新延伸产物(在其生成过程中，它代替原始的第一引物延伸产物)。而且，即使在第一引物中不具有与内部基序902互补的核苷酸序列901，在本文提供的方法中在双链体核酸中的互补序列之间仍然可以存在“呼吸”。然而，当在第一引物中具有与内部基序902互补的核苷酸序列901时，双链体核酸可以“呼吸”的时间长度可以增加，并且根据本文提供的方法这可能转而提高反应的速率。

[0189] 在实施方式中，内部基序可以含有至少2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、75或100个核苷酸。在实施方式中，内部基序可以含有不多于2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、75或100个核苷酸。在实施方式中，内部基序可以含有至少2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50或75个且不多于3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、75或100个核苷酸(其中上限值高于下限值)。通常，在引物中与内部基序互补的核苷酸序列含有与该内部基序中相同数目的核苷酸。而且，在引物中与内部基序互补的核苷酸序列可与该内部基序完全互补，或者在引物与内部基序中的核苷酸序列之间可以存在一个或多个错配的核苷酸。内部基序的起点可与引物的3’末端核苷酸隔开任意距离，该3’末端核苷酸延伸以形成含有该内部基序的引物延伸产物，但是该内部基序的起点通常可与该引物的3’末端核苷酸隔开例如至少2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、75或100个核苷酸。在实施方式中，内部基序的起点可与引物的3’末端核苷酸隔开不多于2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、75或100个核苷酸，该3’末端核苷酸延伸以形成含有该内部基序的引物延伸产物。在实施方式中，内部基序的起点可与引物的3’末端核苷酸隔开至少2、3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50或75个且不多于3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19、20、25、30、35、40、45、50、75或100个核苷酸(其中上限数目大于下限数目)，该3’末端核苷酸延伸以形成含有该内部基序的引物延伸产物。

[0190] 如本文所用的，在单一靶核酸链/多核苷酸模板的情况下，“内部基序”是该靶核酸链的一部分，它与用于扩增本文提供的靶核酸的引物对的一个引物的尾区互补，并且具有与该引物对的另一引物的尾区相同或相似的核苷酸序列。因此，例如，如果靶核酸中的内部基序具有5’至3’方向的核苷酸序列GGGATGGA，则用来根据本文提供的方法扩增靶核酸的引

物对的一个引物的尾区/第一区域将具有5'至3'方向的核苷酸序列TCCATCCC(或类似序列),并且该引物对的另一引物的尾区/第一区域将具有5'至3'方向的核苷酸序列GGGATGGA(或类似序列)。在双链靶核酸的情况下,术语"内部基序"是指两条链的任意一条的相关区域,如以上对于单链所描述的。在本文描述的引物延伸产物的情况下,"内部基序"是指与引物延伸产物的一部分互补的延伸产物的一部分,所述引物延伸产物的一部分对应于延伸以形成特定引物延伸产物的引物的尾区。而且,如果提到与特定链中的"内部基序"互补的不同链中的核苷酸序列,该互补序列可在本文中被称为"内部基序互补体"。值得注意的是,在存在两条互补核酸链的情况下(例如,在含有两个互补引物延伸产物的双链分子中),每条链将具有不同的"内部基序"(所述不同链的"内部基序"将彼此互补),因此在适当的情况下,本文提供的"内部基序"的描述可根据相关链或引物来限定。例如,可以描述双链核酸,其中该核酸的第一链以5'-3'顺序具有部分C'-B-C-A-C',并且该核酸的第二链以5'-3'顺序具有部分C-A'-C'-B'-C,其中第一链的部分C'与第二链的部分C退火,第一链的部分A与第二链的部分A'退火,以此类推。如果第一链的部分C'对应于本文提供的引物的尾区,并且部分C'与部分C互补,那么第一链的部分C可被称为第一链的"内部基序",并且第二链的部分C'可被称为相对于第一链的"内部基序互补体"。同时,如果第二链的部分C也对应于本文提供的引物的尾区,并且它与部分C'互补,那么第二链的部分C'可被称为第二链的"内部基序",并且第一链的部分C可被称为相对于第二链的"内部基序互补体"。

[0191] 在实施方式中,本文提供的方法和组合物可以结合图10进行描述。可按照与图8大体相同的方式描述图10的特征,除了图10的模板还包括内部基序之外。

[0192] 多核苷酸模板1000(图10A)可以是扩增的靶标。多核苷酸模板1000是单核酸链。多核苷酸模板链可作为游离的单链分子而存在,或者它可作为双链分子的一部分而存在,其中该多核苷酸模板链与其互补链退火。多核苷酸模板1000可以是DNA或RNA。

[0193] 多核苷酸模板1000可以含有至少第一部分1001(也用"(i)"和字母"A"表示)和第二部分1002(也用"(ii)"和字母"B"表示)。第一部分1001和第二部分1002可各自含有任意数目的核苷酸,例如对于图8中的模板的第一部分和第二部分所描述的。第一部分1001和第二部分1002可含有相同或不同数目的核苷酸。此外,多核苷酸模板1000可含有第三部分1003(也用"(iii)"和字母"C"表示),它还可被称为"内部基序"。内部基序通常含有6至10个核苷酸,尽管任选地可含有其他数目的核苷酸,诸如至少4、5、6、7、8、9、10、11或12个核苷酸,不多于6、7、8、9、10、11、12、13、14、15、16、17、18、19或20个核苷酸,或至少4、5、6、7、8、9、10、11或12个核苷酸且不多于6、7、8、9、10、11、12、13、14、15、16、17、18、19或20个核苷酸(上限值大于下限值)。内部基序1003通常与第一部分1001和第二部分1002在任一侧隔开2至25个核苷酸,尽管可以隔开其他数目的核苷酸,诸如至少1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、45或50个核苷酸,不多于1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、45、50或60个核苷酸,或1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、45或50个核苷酸至2、3、4、5、6、7、8、9、10、11、12、13、14、15、20、25、30、35、40、45、50或60个核苷酸(上限值大于下限值)。在实施方式中,第一部分1001与内部基序1003之间相比于第二部分1002与内部基序1003之间可具有相同数目的核苷酸(例如,第一部分与内部基序之间可具有6个核苷酸,并且第二部分与内部基序之间可具有6个核苷酸)。在其他实施方式中,第一部分1001与内部基序1003之间可具有不同于第二部分1002与内部基序

1003之间的核苷酸数目(例如,第一部分与内部基序之间可具有6个核苷酸,而第二部分与内部基序之间可具有8个核苷酸)。在实施方式中,内部基序与第一部分1001或第二部分1002之间没有核苷酸(例如在实施方式中,内部基序可以直接挨着第一部分1001或第二部分1002中的一个或两者)。

[0194] 多核苷酸模板1000可以在含有第一引物1010和第二引物1020的反应混合物中温育(图10B)。通常,该反应混合物含有每个引物的多个拷贝,例如,每个引物的至少4、5、6、10、15、20、50、100、1000、10,000、100,000个或1百万个或更多拷贝。第一引物1010可以具有第一区域1011(也用“(i)”表示)和第二区域1012(也用“(ii)”表示)。第一引物的第一区域1011可具有5'端,而第一引物的第二区域1012可具有3'端。第一引物的第一区域可具有与第一引物的第二区域的核苷酸序列不同的核苷酸序列。第二引物1020可以具有第一区域1021(也用“(i)”表示)和第二区域1022(也用“(ii)”表示)。第二引物的第一区域1021可具有5'端,而第二引物的第二区域1022可具有3'端。第二引物的第一区域可具有与第二引物的第二区域的核苷酸序列不同的核苷酸序列。

[0195] 第一引物的第二区域1012可具有与多核苷酸模板的第一部分1001的核苷酸序列互补的核苷酸序列。在图10中,多核苷酸模板的第一部分1001的核苷酸序列由字母A表示,而第一引物的第二区域1012的核苷酸序列由字母A'表示,其中A与A'彼此互补。第二引物的第二区域1022可具有与配偶体核苷酸序列1030互补的核苷酸序列,其中配偶体核苷酸序列1030与多核苷酸模板的第二部分1002的核苷酸序列互补。在图10中,多核苷酸模板的第二部分1002的核苷酸序列由字母B表示,配偶体核苷酸序列1030的核苷酸序列由字母B'表示,并且第二引物的第二区域1022的核苷酸序列由字母B表示,其中B与B'彼此互补。换言之,如图10所示,多核苷酸模板的第二部分1002(B)的核苷酸序列可与第二引物的第二区域1022(B)的核苷酸序列相同。采用本文提供的组合物和方法,可以生成配偶体核苷酸序列1030,例如,作为从第一引物的3'端延伸的延伸产物的一部分。在实施方式中,第一引物的第一区域1011可与第二引物的第一区域1021互补。在图10中,第一引物的第一区域的核苷酸序列由字母C表示,而第二引物的第一区域的核苷酸序列由字母C'表示,其中C与C'彼此互补。

[0196] 此外,第二引物的第一区域1021可与多核苷酸模板中的内部基序1003互补。在图10中,多核苷酸模板的内部基序1003的核苷酸序列由字母C表示,其中C与C'彼此互补。因此,如图10所示,在实施方式中,第一引物的第一区域1011可具有与多核苷酸模板的内部基序1003相同的核苷酸序列,而第二引物的第一区域1021具有与内部基序1003互补的序列。此外,第二引物的第一区域1021与第一引物的第一区域1011和内部基序都互补,而第一引物的第一区域1011与第二引物的第一区域1021和内部基序互补体互补。

[0197] 在实施方式中,图10的第一引物和第二引物可分别具有在本文其他地方(例如,如图1或8中)所述的第一引物和第二引物的任何特征。而且,在实施方式中,图10的第一引物的第一区域可具有在本文其他地方所述的第一引物的尾区的任何特征。在实施方式中,图10的第一引物的第二区域可具有在本文其他地方所述的第一引物的模板结合区的任何特征。在实施方式中,图10的第二引物的第一区域可具有在本文其他地方所述的第二引物的尾区的任何特征。在实施方式中,图10的第二引物的第二区域可具有在本文其他地方所述的第二引物的模板结合区的任何特征。

[0198] 在实施方式中,含有多核苷酸模板1000和第一引物1010的至少两个拷贝以及第二

引物1020的至少两个拷贝的反应混合物可含有如本文其他地方所述可支持基于聚合酶的核酸合成的一个或多个其他组分(例如聚合酶、核苷酸、缓冲液、水等)。类似地,含有多核苷酸模板1000和第一引物1010的至少两个拷贝以及第二引物1020的至少两个拷贝的反应混合物可在本文其他地方所述用于支持核酸模板的扩增或多联体的生成的任何反应条件下温育。

[0199] 在实施方式中,在多核苷酸模板1000和第一引物1010的至少两个拷贝以及第二引物1020的至少两个拷贝在反应混合物中如本文所述进行温育时,可形成一条或多条多联体链(图10C)。在实施方式中,形成的多联体链1040可具有5'端和3'端,并且可具有含有5'至3'方向的通用结构C'-T-C'-T-X-C'的序列,其中C'是第二引物的第一区域的核苷酸序列,T是多核苷酸模板的序列或其类似序列,并且X是任意数目和序列的核苷酸。如图10C进一步所示,在链1040的多核苷酸模板的序列内,在5'至3'方向上,存在该模板的以下部分的序列:B(多核苷酸模板的第二部分),C(内部基序),和A(多核苷酸模板的第一部分)。因此,如图10C所示,在本文提供的实施方式中,内部基序/内部基序互补体的序列在多联体链中的多个位置处存在。特别地,在链1040中,内部基序互补体(C')的序列出现在多联体链中所述多核苷酸模板的序列的两个拷贝之间和该链的末端处,并且内部基序(C)的序列在多核苷酸模板的序列内的同一条链中存在。因此,如多联体链1040的通用结构所示,多联体链可含有多核苷酸模板的序列或其类似序列的至少两个拷贝,其中多核苷酸模板的序列的拷贝由至少第二引物的第一区域的序列分隔开。在实施方式中,在以上提供的通用结构C'-T-C'-T-X-C'中的T可以是多核苷酸模板的类似序列。

[0200] 在实施方式中,根据本文提供的方法生成的多联体链1040可以是单链分子的一部分。在其他实施方式中,根据本文提供的方法生成的多联体链1040可以是双链多联体的一部分,其中多联体链1040与互补于多联体链的链1050退火。尽管图10C描绘了含有具有5'至3'方向的通用结构C'-T-C'-T-X-C'(链1040)或C-T'-C-T'-X'-C(链1050)的序列的多联体链,其中X为0个核苷酸,但根据图10的示意图生成的多联体链可具有以上或本文其他地方描述的多联体链的任意特征。而且,如图10C所示,序列A和B和C是模板链的一部分,因此在通用结构C'-T-C'-T-X-C'或C-T'-C-T'-X'-C中没有单独以符号表示。

[0201] 为了进一步说明在图10的实施方式中元件的关系,图10提供了区块1060、1062、1064、1066和1068。区块1060(具有反斜线样填充)指示总多核苷酸模板序列(即元件1000)或其互补序列的相对位置。区块1062(具有垂直线填充)指示所述多核苷酸模板的第一部分的序列(即元件1001的序列的相对位置)或其互补序列的相对位置。因此,区块1062还表示第一引物的第二区域(即元件1012)的序列,它与元件1001互补。区块1064(具有垂直和水平线填充)指示所述多核苷酸模板的第二部分的序列(即元件1002的序列的相对位置)或其互补序列的相对位置。因此,区块1064还表示配偶体核苷酸序列(即元件1030)的序列,它与元件1002互补。区块1064还指示第二引物的第二区域1022的序列的相对位置,该序列可以是与所述多核苷酸模板的第二部分(元件1002)的序列相同的序列,或相似的序列。换言之,在实施方式中,1022的核苷酸序列可以与1002的核苷酸序列相同或相似。例如,如图10A中所示,在实施方式中,在多核苷酸模板中具有不是该多核苷酸模板的第一部分、第二部分或第三部分(内部基序)的一部分的核苷酸。区块1066(具有波浪线填充)指示第一引物的第一区域的序列(即元件1011/区域“C”的序列的相对位置)或其互补序列的相对位置。因此,区块

1066还表示第二引物的第一区域(即元件1021/区域“C”)的序列,它与元件1011互补。此外,区块1066还表示内部基序的序列或其互补序列。值得注意的是,内部基序的序列或其互补序列既在模板序列中又在第一和第二引物的尾区中。如本文其他地方所述,内部基序可以直接邻近所述多核苷酸模板的第一部分或第二部分中的一个或两者,或者在内部基序与第一部分或第二部分之间可以具有额外的核苷酸。区块1068(具有菱形填充)指示多联体链(即元件1040)的序列或其互补序列的相对位置。因此,区块1068还表示与多联体链互补的链(元件1050)的序列。如图10C中所示,在多联体链或其互补体(1068)中,存在所述多核苷酸模板序列或其互补体(1060)的两个拷贝,这两个拷贝被第一引物的第一区域的核苷酸序列或其互补体(1066)分隔开。这种将所述多核苷酸模板的拷贝分隔开的序列也是内部基序或其互补体(1066)的序列。此外,在多联体链或其互补体(1068)中,第一引物的第一区域的核苷酸序列或其互补体(1066)在两端侧接所述多核苷酸模板序列或其互补体(1060)。而且,在多联体链或其互补体(1068)中,具有第一引物的第二区域的序列或其互补体(1062)的两个拷贝和第二引物的第二区域的序列或其互补体(1064)的两个拷贝,它们均作为所述多核苷酸模板序列或其互补体的两个拷贝的一部分而以两个拷贝存在。区块1060、1062、1064、1066和1068不应被解释为规定图10的元件之间的任何所需的特定大小关系;相反,在本文提供的某些实施方式中,区块1060、1062、1064、1066和1068仅用于帮助说明图10的元件相对于彼此的一般位置。

[0202] 本文提供的引物可通过任何合适的方法进行制备。例如,引物可化学合成。在另一实例中,天然存在的核酸可进行分离、裂解(例如,采用限制酶)和/或修饰以生成或变为本文所述的引物的一部分。

[0203] 在一些实施方式中,标记物可附接至引物。标记物包括,例如,结合配体(例如,地高辛或生物素)、酶、荧光分子/荧光团、发光的分子、猝灭剂分子或放射性同位素。在其他实施方式中,可将寡核苷酸的碱基替代为荧光类似物,诸如2-氨基嘌呤(参见,例如,Proc.Acad.Sci.USA,91,6644—6648(1994),该文献通过引用全文并入本文)。

[0204] 在一些实施方式中,使得i)第一引物的第一拷贝的模板结合区与核酸模板链退火,ii)第二引物的模板结合区与第一引物的第一拷贝的延伸产物退火,iii)第一引物的第二拷贝的模板结合区与第二引物的延伸产物退火,或iv)二级核酸的第一拷贝的第一引物的第二拷贝的延伸产物的3’末端区与二级核酸的第二拷贝的第二引物的延伸产物的3’末端区退火,以产生包含这些链的交换结构的条件,可各自包括(即,i)、ii)、iii)或iv)中的任何一个可包括)在使得双链核酸分子的链以一定程度“呼吸(breathe)”(即经历连接碱基对的氢键的局部断裂的短暂时期)的温度下温育核酸,该程度足以促进引物或不同的核酸链进入双链分子的链之间,并足以使引物或不同的核酸链与打开的双链核酸分子的一条链退火。在一些实施方式中,方法或其步骤可在至少10、15、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、50、51、52、53、54、55、56、57、58、59、60、61、62、63、64、65、66、67、68、69、70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、90或95℃的温度下执行或温育。在一些实施方式中,方法或其步骤可在不高于10、15、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、50、51、52、53、54、55、56、57、58、59、60、61、62、63、64、65、66、67、68、69、70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、90或95℃的温度

下执行或温育。在一些实施方式中，方法或其步骤可在10、15、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、50、51、52、53、54、55、56、57、58、59、60、61、62、63、64、65、66、67、68、69、70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85或90℃至15、20、21、22、23、24、25、26、27、28、29、30、31、32、33、34、35、36、37、38、39、40、41、42、43、44、45、46、47、48、49、50、51、52、53、54、55、56、57、58、59、60、61、62、63、64、65、66、67、68、69、70、71、72、73、74、75、76、77、78、79、80、81、82、83、84、85、90或95℃之间的温度下执行或温育。

[0205] 在一些实施方式中，对于本文提供的方法或其步骤，在低于相关潜在配对的核苷酸链或其区域(例如，第一引物的模板结合区与核酸模板的第一链配对，第二引物的模板结合区与第一引物的第一拷贝的延伸产物配对，第一引物与第二引物的延伸产物配对，第二引物的延伸产物的3'末端区与其互补序列配对，第一引物的第一拷贝的延伸产物的3'末端区与其互补序列配对，等等)的解链温度(Tm)的温度下执行步骤或方法。在一些实施方式中，对于本文提供的方法或其步骤，在高于相关潜在配对的核苷酸链或其区域(例如，第一引物的模板结合区与核酸模板的第一链配对，第二引物的模板结合区与第一引物的第一拷贝的延伸产物配对，第一引物与第二引物的延伸产物配对，第二引物的延伸产物的3'末端区与其互补序列配对，第一引物的第一拷贝的延伸产物的3'末端区与其互补序列配对，等等)的Tm的温度下执行步骤或方法。在一些实施方式中，对于本文提供的方法或其步骤，在处于相关潜在配对的核苷酸链或其区域(例如，第一引物的模板结合区与核酸模板的第一链配对，第二引物的模板结合区与第一引物的第一拷贝的延伸产物配对，第一引物与第二引物的延伸产物配对，第二引物的延伸产物的3'末端区与其互补序列配对，第一引物的第一拷贝的延伸产物的3'末端区与其互补序列配对，等等)的Tm或在其+/-1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、20或25℃或+/-1、2、3、4、5、6、7、8、9、10、11、12、13、14、15、20或25℃以内的温度下执行步骤或方法。

[0206] 在一些实施方式中，核酸聚合酶包含在本文提供的方法或组合物中。聚合酶可生成引物的延伸产物。引物及其延伸产物可与模板核酸链互补。通常，核酸聚合酶将在引物的3'端处引发引物的延伸产物的合成。在一些实施方式中，DNA聚合酶包含在本文提供的方法或组合物中。如本文所用的，“DNA聚合酶”指的是对DNA模板具有主要的或专一的聚合酶活性的核酸聚合酶。在一些实施方式中，逆转录酶包含在本文提供的方法或组合物中。如本文所用的，“逆转录酶”指的是可从RNA模板合成DNA链的核酸聚合酶。在一些实施方式中，RNA聚合酶可包含在本文提供的方法或组合物中。如本文所用的，“RNA聚合酶”指的是可从DNA或RNA模板合成RNA链的核酸聚合酶。

[0207] 在一些实施方式中，本文提供的聚合酶可具有链置换活性。具有链置换活性的聚合酶包括，例如，exo-Bca DNA聚合酶、phi29DNA聚合酶、大肠杆菌DNA聚合酶I的Klenow片段、$Vent_R$ DNA聚合酶、Deep $Vent_R$ DNA聚合酶、9°N_m DNA聚合酶、Bst 2.0DNA聚合酶以及Bst DNA聚合酶的大片段。也可以使用具有链置换活性的其他聚合酶。

[0208] 聚合酶的修饰形式也可以与本文提供的方法和组合物一起使用，条件是修饰的聚合酶具有序列依赖性核酸合成活性。聚合酶的修饰形式(“修饰的聚合酶”)可以具有，例如，100个或更少的、70个或更少的、50个或更少的、40个或更少的、30个或更少的、20个或更少的、10个或更少的、5个或更少的、4个或更少的、3个或更少的、2个或更少的或1个与聚合酶

的亲代形式的序列不同的氨基酸。在一些实施方式中，修饰的聚合酶可含有比亲代聚合酶多或少不多于1000、700、500、400、300、200、100、50、40、30、20、10或5个氨基酸。在一些实施方式中，修饰的聚合酶可含有亲代聚合酶的片段。在一些实施方式中，修饰的聚合酶可包含具有来源于聚合酶的一部分和来源于非聚合酶蛋白质的一部分的嵌合多肽。在一些实施方式中，修饰的聚合酶可具有，例如，与亲代聚合酶相比提高的催化活性、提高的稳定性或提高的热稳定性。

[0209] 在一些实施方式中，本文提供的聚合酶是热稳定的。热稳定的聚合酶在高达25、30、35、40、45、50、55、60、65、70、75、80、85、90或95℃的温度下可具有例如至少5、10、15、20、30、40、50、60、90、120或180分钟的半衰期。在一些实施方式中，修饰的聚合酶可以是热稳定的。

[0210] 在一些实施方式中，本文提供的方法和其步骤包括足以支持基于聚合酶的核酸合成的条件或在该条件下进行。针对基于聚合酶的核酸合成的示例性条件是本领域已知的并提供于例如Green和Sambrook(同上)中。用于基于聚合酶的核酸合成反应的非限制性组分可包括以下的一种或多种：聚合酶(例如，以每50微升反应体积0.01至10个单位的酶，或者其中的任何范围，包括例如，每50微升反应体积0.01-1、0.1-10、0.1-5、0.5-10、0.5-5、0.5-2、1-10或1-5个单位的酶的浓度，其中1单位的酶在75℃下将使15nmol的dNTP在30分钟内掺入聚合产物中)；模板(以每个反应至少例如1、10、100、1,000、10,000或100,000个拷贝的浓度)；引物(以例如0.01至10微摩尔，或者其中的任何范围，包括例如0.01-1、0.1-10、0.1-5、0.5-5或0.5-2微摩尔的浓度)；dNTP(例如dATP、dTTP、dGTP和dCTP，以例如每个dATP、dTTP、dGTP和dCTP 50至500微摩尔，或者其中的任何范围，包括例如每个dATP、dTTP、dGTP和dCTP 50-350、100-500、100-300、200-500或300-400微摩尔的浓度)；盐(例如KCl或乙酸钾，以例如1至200毫摩尔，或者其中的任何范围，包括例如1-100、1-50、1-20、1-10、10-20、10-50或10-200毫摩尔的浓度)；缓冲液(例如Tris-HCl或Tris-乙酸盐(Tris-acetate)，pH 7.8-8.5，以例如1至100毫摩尔，或者其中的任何范围，包括例如1-50、1-20、1-10、1-5、10-100、20-100或50-100毫摩尔的浓度)；以及镁离子(以例如0.1至10毫摩尔，或者其中的任何范围，包括例如，0.1-5、0.1-1、0.5-10、0.5-5或0.5-2.5毫摩尔的浓度)。用于基于聚合酶的核酸合成反应的另外的非限制性组分可提高反应速度、提高反应的保真度或提高反应中的酶或DNA的稳定性，并且可以包括以下的一种或多种：明胶(以例如0.0001％至0.1％w/v的浓度)、BSA(以例如0.01至1微克每微升的浓度)、蔗糖(以例如0.01摩尔至0.8摩尔的浓度)、海藻糖(以例如0.01摩尔至0.8摩尔的浓度)、DMSO(以例如0.01％至10％v/v的浓度)、甜菜碱(以例如0.1至10摩尔的浓度)、甲酰胺(以例如0.1％至10％v/v的浓度)、甘油(以例如0.1％至20％v/v的浓度)、聚乙二醇(以例如0.1％至20％v/v的浓度)、非离子型去污剂[例如NP-40(以例如0.01％至1％v/v的浓度)]、Tween-20(以例如0.01％至1％v/v的浓度)或Triton X-100(以例如0.01％至1％v/v的浓度)]、铵离子[例如硫酸铵(以例如1至100毫摩尔的浓度)]和EDTA(以例如0.001至0.1毫摩尔的浓度)。其他的试剂也可存在于本文提供的基于聚合酶的核酸合成反应中。例如，可以使用足以合成RNA反应产物或含有非标准核苷酸的反应产物的试剂。足以支持基于聚合酶的核酸合成的条件可包括多个温度和pH值。例如，基于聚合酶的核酸合成反应的pH可为例如pH 6.0至pH 10.0，诸如pH 6.5、7、7.5、7.8、7.9、8、8.1、8.2、8.5、9或9.5。基于聚合酶的核酸合成反应的温度可以是恒定的或变化的。恒定的温度

可以在例如10℃至95℃之间，诸如20、25、30、35、37、40、42、45、50、55、60、65、70、75、80或85℃。变化的温度可以是例如10℃至95℃之间的两个或更多个不同的温度，诸如选自20、25、30、35、37、40、42、45、50、55、60、65、70、75、80或85℃的两个或更多个温度。

[0211] 本文提供的方法可在多个温度下进行。在一些实施方式中，方法的所有步骤在相同的温度下进行。因此，诸如在PCR中的温度循环在本文公开的方法中是不必要的。在一些实施方式中，本文提供的方法可在两个或更多个不同的温度下进行。在一些实施方式中，含有用于本文提供的方法的试剂的反应混合物在两个或更多个不同的温度下进行温育。在一些实例中，可选择不同的温度来优化速率、准确度或本文提供的方法的不同步骤的其他特征。例如，可选择温度来提高聚合酶的酶活性。在一些实例中，可选择不同的温度来提高引物与模板的结合特异性或提高模板对引物的可及性(例如较高的温度可促进双链体模板核酸的分离)。在一些实施方式中，本文提供的方法的所有步骤在不高于80、70、60、50、40、30、20或10℃的温度下进行。在一些实施方式中，本文提供的方法在20-60、30-70、40-80、20-40、30-50、40-60、50-70、60-80、30-40、35-45、40-50、45-55、50-60、55-65℃的温度下进行。在某些实施方式中，含有靶核酸的样品可在本文提供的方法开始之前加热至大于40、50、60、70、80、90或95℃的温度。在某些实施方式中，本文提供的反应混合物可在本文提供的方法开始之前或之后一次加热至大于40、50、60、70、75、80、85、90或95℃的升高的温度。将反应混合物加热至升高的温度之后，反应混合物可维持在如本文其他地方提供的较低的温度下(例如，在40-70℃的温度下)用于进行该方法的其余部分。在实施方式中，如果在本文提供的方法开始之前将反应混合物或样品加热至升高的温度，则可在反应混合物或样品已经加热至升高的温度并且反应混合物或样品已经回到如本文提供的较低温度之后，将核酸聚合酶添加至反应混合物或样品中。本文公开的方法可在有或没有热循环仪的情况下进行。

[0212] 作为一种考虑，可选择用于本文提供的方法或其步骤的温度以适合在该方法的步骤中使用的酶。在一些实施方式中，对于使用聚合酶的方法，选择反应的温度，使得该温度不会显著损害聚合酶的活性(例如，可选择反应的温度使得聚合酶具有至少24、12、6、4、3、2、1、0.75、0.5、0.25或0.1小时的半衰期)。或者，可在损害方法中使用的酶的活性的温度下执行方法(例如，可选择反应的温度使得反应中的酶具有不多于24、12、6、4、3、2、1、0.75、0.5、0.25或0.1小时的半衰期)。在一些实施方式中，如果在损害一种或多种酶的活性的温度或其他条件(例如pH)下执行方法，则可在该方法开始后以一个或多个间隔向反应中加入另外的酶以补充受损的酶的活性。

[0213] 在一些实施方式中，本文提供的方法的一个或多个步骤在相同的反应容器(例如，管、尖端、器皿等)中发生。在一些实施方式中，方法的所有步骤均在相同的反应容器中发生。

[0214] 用于本文提供的方法的试剂可在反应开始时一起提供，或者它们可以按顺序加入，其中在一个、两个或更多个步骤之后向反应中添加新的试剂。在一些情况下，可在反应过程期间向反应容器中添加新的试剂(例如，酶、引物)，以增加可作用于底物或代替已变得失活的试剂(例如酶)的功能的试剂的量。可在本文提供的方法的反应开始后的一个或多个选定的时间间隔(例如，在反应开始后1、3、5、7、10、15、20、30、45或60分钟)向反应中添加新的试剂。

[0215] 在一些实施方式中，本文提供的方法的一个或多个步骤可同时发生。例如，在生成

第一引物的第一拷贝的延伸产物的单一拷贝后，可由该第一引物的第一拷贝的延伸产物的单一拷贝按顺序生成第二引物的延伸产物的多个拷贝。由于第二引物的延伸产物的拷贝由第一引物的第一拷贝的延伸产物的单一拷贝按顺序生成，因此第二引物的延伸产物的单独的拷贝可以例如，充当用于形成第一引物的第二拷贝的延伸产物的模板，是二级核酸的组分，是交换结构的组分，或者可以是用于形成第二引物/第一多联体链的延伸产物的延伸产物的起始点。在另一实例中，二级核酸的两个拷贝可在由核酸模板的第一链生成第一引物的第一拷贝的延伸产物的同时形成交换结构。在另一实例中，在由核酸模板的第一链生成第一引物的第一拷贝的延伸产物的一个拷贝的同时，由第一引物的第一拷贝的延伸产物的不同拷贝生成第二引物的延伸产物。本文提供的其他步骤也可同时发生。此外，在本文提供的包括双链核酸模板的方法和组合物中，核酸模板的任一条链可被认为是“第一链”，并且本文提供的引物对的任一引物可被认为是“第一引物”。因此，在一些实例中，双链核酸的两条链可以根据本文提供的方法同时用作核酸的“第一链”，且引物对的相对引物充当不同的“第一链”的“第一引物”。而且，根据本文提供的方法生成的各种结构可任选地进入本文提供的方法的不同途径。例如，在实施方式中，第一二级核酸可与第二二级核酸配对以形成如本文其他地方所述的交换结构。在其他实施方式中，第一二级核酸可被第一引物侵入，并且第二引物的延伸产物可充当用于生成第一引物的新的延伸产物的模板。为生成第一引物的新的延伸产物，置换作为二级核酸的一部分的第一引物的延伸产物。在另一实例中，在实施方式中，第一多联体可与另一多联体配对以形成交换结构。在其他实施方式中，第一多联体可被第一引物侵入，并且第一多联体链可充当用于生成新的第二多联体链的模板。为生成新的第一多联体链，置换作为多联体的一部分的第一多联体链。其他类似事件也可在本文提供的方法的过程中发生。

[0216] 本文提供的反应和组合物可含有第一引物、第二引物、一级核酸、二级核酸、第一引物的第一拷贝的延伸产物、第一引物的第二拷贝的延伸产物、第二引物的延伸产物、交换结构、多联体等的多个拷贝。因此，本文提供的方法可包括这样的过程，其中本文提供的多个步骤同时发生，并且这样的步骤可与相关分子的多个拷贝一起发生。

[0217] 在一些实施方式中，在本文提供的方法或组合物中提供了两组或更多组第一和第二引物，其中每一组均含有第一引物和第二引物，并且其中不同的引物组与不同的核酸模板互补。组中的第一和第二引物两者的模板结合区可与同一双链核酸模板的不同链互补。通常，给定组中的第一和第二引物的尾区具有不同于将在同一方法或组合物中使用的其他引物组的第一和第二引物的尾区的非互补序列，使得来自不同引物组的引物尾部不是互补的。这可能是理想的以便防止形成含有来源于不同核酸模板的链的杂合交换结构。或者，在两个或更多个不同的引物组中的第一和第二引物的尾区可具有互补序列，以便形成杂合交换结构和含有来源于不同核酸模板的链的多联体。在本文提供的方法或组合物中包含两个或更多个引物组可支持在同一反应容器中同时扩增多个不同的核酸模板。这例如对于扩增样品中感兴趣的多个模板或对于测定样品中多个不同模板的存在可能是有用的。在一些实施方式中，在本文提供的方法中提供了1、2、3、4、5、6、7、8、9、10、15、20、25、30、40、50、60、70、100、200、500组或更多组第一和第二引物，以便扩增1、2、3、4、5、6、7、8、9、10、15、20、25、30、40、50、60、70、100、200、500个或更多个不同的核酸模板或测定该核酸模板的存在。

[0218] 在一些实施方式中，在本文提供的方法中，可快速扩增核酸模板。例如，在一些实

施方式中，在该方法开始后0.1、0.5、1、3、5、10、15、20、30、40、50、60、90、120或180分钟内核酸模板可扩增至少500倍。在另一实例中，在一些实施方式中，在该方法开始后0.1、0.5、1、3、5、10、15、20、30、40、50、60、90、120或180分钟内核酸模板可扩增至少10,000倍。在另一实例中，在一些实施方式中，在该方法开始后0.1分钟、0.5分钟、1分钟、3分钟、5分钟、10分钟、15分钟、20分钟、30分钟、40分钟、50分钟、60分钟、90分钟、2小时、3小时、4小时、6小时、8小时、12小时、16小时或24小时内，核酸模板相对于该方法开始时反应混合物中存在的核酸模板的原始量可扩增至少5、10、25、50、100、250、500、1,000、5,000、10,000、50,000、100,000、500,000或1,000,000倍。在一些实施方式中，当方法开始时，用于该方法的第一步骤的所有试剂均在含有用于该方法的反应混合物的容器中。在一些实施方式中，当方法开始时，用于该方法的所有步骤的所有试剂均在含有用于该方法的反应混合物的容器中。

[0219] 在一些实施方式中，在本文提供的方法中，核酸模板可以以比线性速率更高的速率进行扩增。在一些实施方式中，在本文提供的方法中，核酸模板可以以指数方式进行扩增。在一些实施方式中，在本文提供的方法中，在该方法开始后每1、2、3、5、10、15、20、25、30、45、60、90、120、180或240分钟，核酸模板的数目可至少加倍。在一些实施方式中，相对于该方法开始时反应中存在的核酸模板的原始量，核酸模板可扩增至少5、10、25、50、100、250、500、1,000、5,000、10,000、50,000、100,000、500,000、1,000,000或10,000,000倍。

[0220] 根据本文提供的方法生成的多联体中核酸模板的多个拷贝的存在可有助于根据本文提供的方法快速扩增核酸模板。特别地，由于核酸模板的链的多个拷贝可存在于单一多联体链中，因此将单一聚合酶负载至单一多联体链上可导致随着聚合酶沿着该多联体链移动而生成核酸模板的链的多个拷贝。在一些情况下，核酸聚合酶遇到并负载至核酸链上所需的时间可显著影响扩增反应的总体速度。例如，如果聚合酶在复制反应期间遇到的每个核酸链仅含有核酸模板的链的单一拷贝，则在生成该模板的链的每个拷贝后聚合酶可能需要遇到并负载至新的模板链上。相比之下，通过采用多联体，在聚合酶遇到并负载至多联体链上后，它可合成模板的链的多个拷贝而无需使多联体链留下或遇到并负载至另一条链上。

[0221] 在一些实施方式中，本文提供了用于由单链或双链RNA模板生成双链DNA多联体的方法和组合物。除了逆转录酶(例如，AMV逆转录酶、M-MLV逆转录酶、Superscript II™逆转录酶、Superscript III™逆转录酶或ThermoScript™逆转录酶)也包含在本文提供的方法中外，可如本文所述执行该方法。第一引物的第一拷贝可与RNA模板退火，并且逆转录酶可生成第一引物的第一拷贝的延伸产物，该延伸产物通过逆转录的过程形成为DNA链。用于RNA的逆转录的方法和条件是本领域已知的并公开在例如RNA:A Laboratory Manual，D.Rio等人，Cold Spring Harbor Laboratory Press(2011)中，该文献通过引用全文并入本文。在通过逆转录酶(由RNA链)生成第一引物的第一拷贝的延伸产物(为DNA链)后，用于生成多联体的其余步骤可与本文其他地方所述的相同。

[0222] 在一些实施方式中，本文提供的方法和组合物可包括一个或多个“暴沸(bumping)引物”。暴沸引物可与本文提供的方法和组合物一起使用，以通过例如提高从核酸模板的第一链置换核酸模板的第二链的速率，提高从第一引物延伸产物置换核酸模板的第一链的速率或者提高从第二引物延伸产物置换第一引物延伸产物的速率来例如提高生成反应产物的速率或特异性。如本文所用的“暴沸引物”指的是与在核酸模板的第一链或第二链上的序

列互补的引物，该序列在与第一引物或第二引物的模板结合区结合的相同链上的序列的下游。因此，当暴沸引物与同一核酸链(第一引物或第二引物与该核酸链退火)退火时，暴沸引物的3'末端朝向第一引物或第二引物的5'末端(即，第一引物或第二引物的5'末端核苷酸和尾区)。当与核酸聚合酶一起温育并在支持生成引物延伸产物的条件下时，暴沸引物的延伸产物可通过该聚合酶由暴沸引物的3'末端形成。由于暴沸引物的3'末端朝向第一引物或第二引物的5'末端，因此随着暴沸引物的延伸产物的长度增加，它可最终遇到作为延伸产物的第一引物或第二引物的5'末端。聚合酶可随后从链以及第一引物或第二引物的任何延伸产物中置换第一引物或第二引物。因此，暴沸引物可加快本文提供的反应。

[0223] 在一些实施方式中，本文提供了含有一种或多种酶、引物或本文提供的其他试剂的容器。容器可包含能够支持或含有液体或固体材料的任何结构，并可包括管、器皿、尖端等。在一些实施方式中，容器壁可允许光穿过壁透射。容器可以是光学透明的。容器可含有，例如，分离的核酸聚合酶、分离的DNA聚合酶、分离的逆转录酶、第一引物、第二引物、核酸染料或核酸探针中的任意一种或多种，如本文其他地方所述。可提供容器的任何内容物的任何数目的拷贝(例如，第一拷贝、第二拷贝、第三拷贝等)。容器的内容物可以是流体连通的。在一些实施方式中，容器可进一步含有核酸模板。在一些实施方式中，容器可进一步含有核苷酸、缓冲液、盐、水或本文提供的用于核酸扩增的其他试剂。在一些实施方式中，容器可含有两个或更多个引物组，其中每个引物组包含第一引物和第二引物，并且不同的引物组与不同的核酸模板互补。

[0224] 可将对于本文提供的方法有用的两种或更多种试剂包装并作为试剂盒提供。例如，试剂盒可包含以下的任意两种或更多种：核酸模板、第一引物、第二引物、核酸聚合酶、DNA聚合酶、逆转录酶、缓冲液、核酸染料、核酸探针或dNTP，如本文其他地方所述。在试剂盒内，两种或更多种试剂可包装于分开的容器或同一容器中。在一些实施方式中，试剂盒可进一步含有核苷酸、缓冲液、盐、水或本文提供的用于核酸扩增的其他试剂。

[0225] 在实施方式中，如本文提供的第一引物和第二引物可作为引物组一起提供。引物组可作为单独的试剂盒或组合物提供，或者该引物组可与用于执行本文提供的方法的一种或多种其他试剂一起提供于试剂盒中。

[0226] 在一些实施方式中，核酸连接酶可包含在本文提供的方法或组合物中。连接酶催化核苷酸之间(通常在一个核苷酸的5'磷酸与另一个核苷酸的3'羟基基团之间)磷酸二酯键的形成。相比于不包含连接酶的反应，本文提供的反应通过在反应中包含连接酶可以以更高的速率扩增靶核酸。连接酶可以例如增加存在于本文提供的反应中的多联体的大小或数目。

[0227] 核酸连接酶包括大肠杆菌DNA连接酶、Taq DNA连接酶、T3DNA连接酶、T4DNA连接酶、T7DNA连接酶、Ampligase™、T4RNA连接酶1和T4RNA连接酶2。

[0228] 为催化连接反应，某些连接酶需要ATP(例如T4DNA连接酶)或NAD+(大肠杆菌DNA连接酶)。在一些实施方式中，连接酶可连接具有平端的核酸。在一些实施方式中，连接酶可连接具有粘端的核酸。在一些实施方式中，连接酶可连接具有平端和粘端两者的核酸。

[0229] 连接酶的修饰形式也可与本文提供的方法和组合物一起使用，条件是修饰的连接酶具有催化核苷酸之间磷酸二酯键的形成的能力。连接酶的修饰形式(“修饰的连接酶”)可以具有例如，100个或更少的、70个或更少的、50个或更少的、40个或更少的、30个或更少的、

20个或更少的、10个或更少的、5个或更少的、4个或更少的、3个或更少的、2个或更少的、或1个与天然存在的亲代连接酶形式的序列不同的氨基酸。在一些实施方式中，修饰的连接酶可含有比亲代连接酶多或少不多于1000、700、500、400、300、200、100、50、40、30、20、10或5个的氨基酸。在一些实施方式中，修饰的连接酶可含有亲代连接酶的片段。在一些实施方式中，修饰的连接酶可包含具有来源于连接酶的一部分和来源于非连接酶蛋白质的一部分的嵌合多肽。在一些实施方式中，修饰的连接酶可具有，例如，与亲代连接酶相比提高的催化活性、提高的稳定性或提高的热稳定性。

[0230] 在一些实施方式中，本文提供的连接酶是热稳定的。热稳定的连接酶在高达25、30、35、40、45、50、55、60、65、70、75、80、85、90或95℃的温度下可具有例如至少5、10、15、20、30、40、50、60、90、120或180分钟的半衰期。在一些实施方式中，修饰的连接酶可以是热稳定的。

[0231] 在一些实施方式中，包含在本文提供的方法和组合物中的连接酶可以是在本文中被称为“p50-Tth”的修饰的连接酶，其具有氨基酸序列：MGHHHHHHHHHHSSGHIEGRAS*ADGPYLQILEQPKQRGFRFRYVCEG*

PSHGGLPGASSEKNKKSYPQVKICNYVGPAKVIVQLVTNGKNIHLHAHSL

VGKHCEDGICTVTAGPKDMVVGFANLGILHVTKKKVFETLEARMTEACI

RGYNPGLLVHPDLAYLQAEGGGDRQLGDREKELIRQAALQQTKEMDLS

VVRLMFTAFLPDSTGSFTRRLEPVVSDAIYDSKAPNASNLKIVRMDRTAGC

VTGGEEIYLLCDKVQKDDIQIRFYEEEENGGVWEGFGDFSPTDVHRQFA

IVFKTPKYKDINITKPASVFVQLRRKSDLETSEPKPFLYYPEIKDKEEVQRK

*RQK*GSSGTSGGGSGGGMTLEEARKRVNELRDLIRYHNYRYYVLADPEISDAEYDRLLRELKELEER

FPELKSPDSPTLQVGARPLEATFRPVRHPTRMYSLDNAFNLDELKAFEERIERALGRKGPFAYTVEHKVDGLSVNLY

YEEGVLVYGATRGDGEVGEEVTQNLLTIPTIPRRLKGVPERLEVRGEVYMPIEAFLRLNEELEERGERIFKNPRNAA

AGSLRQKDPRITAKRGLRATFYALGLGLEEVEREGVATQFALLHWLKEKGFPVEHGYARAVGAEGVEAVYQDWLKKR

RALPFEADGVVVKLDELALWRELGYTARAPRFAIAYKFPAEEKETRLLDVVFQVGRTGRVTPVGILEPVFLEGSEVS

RVTLHNESYIEELDIRIGDWVLVHKAGGVIPEVLRVLKERRTGEERPIRWPETCPECGHRLLKEGKVHRCPNPLCPA

KRFEAIRHFASRKAMDIQGLGEKLIERLLEKGLVKDVADLYRLRKEDLVGLERMGEKSAQNLLRQIEESKKRGLERL

LYALGLPGVGEVLARNLAARFGNMDRLLEASLEELLEVEEVGELTARAILETLKDPAFRDLVRRLKEAGVEMEAKEK

GGEALKGLTFVITGELSRPREEVKALLRRLGAKVTDSVSRKTSYLVVGENPGSKLEKARALGVPTLTEEELYRLLEA

RTGKKAEELV(SEQ ID NO:79)。连接酶p50-Tth在至少60℃的温度下具有热稳定的平端连接活性。连接酶p50-Tth是嵌合蛋白，其包括含His10的前导序列、来自人类NF-kappa-B蛋白登录号NP_003989氨基酸40-366的p50序列(以斜体字表示)、柔性富甘氨酸序列以及来自嗜热栖热菌(Thermus Thermophilus)HB8，登录YP_144363的Tth DNA连接酶序列(用下划线表示)。在一些实施方式中，p50-Tth连接酶的修饰形式可与本文提供的方法和组合物一起使用(例如具有100个或更少的、70个或更少的、50个或更少的、40个或更少的、30个或更少的、20个或更少的、10个或更少的、5个或更少的、4个或更少的、3个或更少的、2个或更少的、或1个与p50-Tth连接酶不同的氨基酸)。在实施方式中，与本文提供的组合物或方法一起使用

的连接酶可以是描述于2013年3月15日提交的美国临时专利申请号61/802,124或2014年3月15日提交的PCT申请号PCT/US14/30003中的连接酶，这两个专利申请均通过引用全文并入本文用于所有目的。

[0232] 本文提供的用于扩增核酸模板/生成含有该模板的至少两个拷贝的多联体的各种方法和组合物可实现先前已通过用于等温的依赖热循环仪的核酸扩增的其他方法和组合物实现的许多功能。根据本文提供的方法用于扩增的核酸模板在本文也可称为“靶核酸”等。本文提供的方法和组合物可用于，例如感兴趣的核酸的分离和克隆、基因表达分析、核酸的诊断鉴定、新型核酸的合成、核酸探针合成和标记、受试者的法医学鉴定、受试者的等位基因鉴定、遗传筛查、核酸测序以及相关的应用。靶核酸分子可以是任何类型，包括单链或双链DNA或RNA(例如，mRNA)。靶核酸可以是任何类型或功能(例如，编码蛋白质的序列、调节序列、内含子等)。靶核酸可以是整个基因或其一部分。

[0233] 在一些实施方式中，本文提供的方法或组合物可用于检测样品中靶核酸的量(包括靶标的存在或不存在)，测量在所选时间段内由样品形成的靶标的扩增产物的量，或确定由样品生成模板的一定数目的拷贝所必需的时间量。可与本文提供的方法和组合物一起使用的样品在本文其他地方进行了描述，并且可包括，例如受试者的体液、分泌物或组织。在实施方式中，可在测定中使用样品之前处理样品以根据本文提供的方法扩增样品中的靶核酸。样品的处理可包括如本文其他地方所述的任何处理步骤，并可包括例如声处理或化学裂解步骤。

[0234] 在一些实施方式中，可执行本文提供的方法以在同一反应容器中同时测定至少2、3、4、5、6、7、8、9、10、12、15、20、25、30、35、40、45、50、75、100种或更多种不同的靶核酸。通常，对于每种感兴趣的靶核酸，提供了第一引物和第二引物，每一个均与核酸靶标的链或其互补序列互补。可通过例如使用对不同靶核酸中的检测序列具有序列特异性的核酸探针和不同的荧光团，来监测在同一容器中不同靶核酸的扩增。

[0235] 在一些实施方式中，本文提供的方法或组合物可用于检测靶核酸中感兴趣的特定核苷酸的存在或不存在(例如，在突变或SNP的情况下)。例如，可选择与靶核酸中包括感兴趣的核苷酸或与之相邻的区域选择性结合的第一或第二引物。该引物可设计为使得其选择性地：i)当该区域含有感兴趣的核苷酸时与该区域结合，或ii)当该区域含有感兴趣的核苷酸时不与该区域结合。可用所选引物来执行如本文所述的方法，并且扩增反应的结果可提供关于靶核酸中感兴趣的核苷酸存在或不存在的信息。例如，如果第一引物的模板结合区被设计为具有与包含感兴趣的特定核苷酸(例如突变)的靶核酸中的序列互补的核苷酸序列，则用所选引物从样品中成功扩增靶核酸可表明该样品含有具有感兴趣的特定核苷酸的靶核酸。在一些实施方式中，用于分析靶核酸中感兴趣的核苷酸的引物可在该引物的3’末端含有关键核苷酸(即对应于靶核酸中感兴趣的核苷酸的同一位置的核苷酸)。在这样的情况下，引物的3’末端核苷酸的退火可能依赖于靶核酸中感兴趣的核苷酸的存在。如果引物的3’末端核苷酸不与靶核酸中的核苷酸退火(例如由于核苷酸之间的错配)，则该错配可显著影响核酸聚合酶由引物合成延伸产物。因此，在一些实施方式中，具有对应于感兴趣的核苷酸的3’末端核苷酸的引物可用于确定靶核酸中特定核苷酸的存在或不存在。在这样的实施方式中，在一些情况下可选择在引物的3’末端的关键核苷酸与靶核酸中感兴趣的核苷酸互补，并且在一些其他情况下可选择在引物的3’末端的关键核苷酸与靶核酸中感兴趣的核

苷酸不互补。感兴趣的核苷酸可代表例如靶核酸的野生型形式、突变体形式或多态性。

[0236] 在其他实施方式中，可通过选择引物，使得感兴趣的核苷酸存在于不与第一或第二引物的模板结合区互补的区域中的靶核酸中，来检测靶核酸中感兴趣的特定核苷酸(例如突变或SNP)。例如，感兴趣的核苷酸可以大约在靶核酸序列的中间。在实施方式中，感兴趣的核苷酸可以在本文其他地方所述的“内部基序”中。在实施方式中，当感兴趣的核苷酸在内部基序中时，可以将引物对制备成在引物的尾区中含有与内部基序或其互补体互补，并且可用于确定在靶序列中的内部基序中存在或不存在感兴趣的核苷酸的核苷酸序列。如本文其他地方所解释的，引物的尾区中的核苷酸序列与该引物的延伸产物中的内部基序暂时退火可提高本文提供的反应的速率。在一些情况下，引物的尾区中的核苷酸序列对该引物的延伸产物中的内部基序的亲和力越大，反应可越快发生。而且，通常，在引物的尾区中的核苷酸序列中可与该引物的延伸产物中的内部基序中的核苷酸结合的核苷酸数目越大，该延伸产物的尾区中的核苷酸序列对于延伸产物中的内部基序的亲和力越大。因此，在实施方式中，采用本文提供的组合物和方法，可以通过使用引物来确定在靶序列中存在或不存在感兴趣的核苷酸，所述引物在引物的尾区中具有能与靶序列中的内部基序或其互补体结合的核苷酸序列，并且在尾区内具有或不具有与内部基序或其互补体中感兴趣的特定核苷酸特异性结合的核苷酸。通常，当引物的尾区中的核苷酸序列含有与该引物的延伸产物中的核苷酸(对应于靶标中感兴趣的核苷酸)互补的核苷酸时，将比当引物的尾区中的相关核苷酸不与该引物的延伸产物中的核苷酸(对应于靶标中感兴趣的核苷酸)互补时更快地发生反应。例如，野生型形式的靶核酸链的内部基序可具有核苷酸序列：5’TATTGCAT 3’。然而，在群体中的许多个体中，该序列中的“G”可频繁突变为“A”。为了确定特定靶核酸是否在序列中含有“G”或其他核苷酸，可以制备在其尾区中含有具有序列5’ATGCAATA 3’的核苷酸序列的引物。如果使用该引物(和合适的第二引物)根据本文提供的方法扩增靶核酸，则当“G”存在于内部基序中时，相对于当在该内部基序中“G”的位置处存在不同的核苷酸时，扩增反应可具有特定的反应速率。这是因为如果“G”存在于内部基序中，则引物的尾区中的核苷酸序列将与内部基序完全互补，但是如果在G的正常位置处存在非“G”的核苷酸，则将不会完全互补。通常，在这一实例中，如果“G”存在于内部基序中，与不存在“G”相比，引物的尾区将与内部基序更频繁地退火，并且这可以转而导致存在“G”时比不存在“G”时更快的反应速率。采用本文提供的系统和方法，可以制备引物，以确定存在或不存在感兴趣的核苷酸(例如，可以制备引物，使得当存在突变形式的核苷酸时比存在野生型形式的核苷酸时更快地发生反应，或反之亦然)。而且，在实施方式中，可以制备引物，以便确定在靶标中的内部基序中在感兴趣的位置处的未知核苷酸的身份。例如，可以制备四个不同的引物对，每一个根据情况含有对应于靶核酸中感兴趣的核苷酸的A、T、G或C。在实施方式中，产生最快反应结果的引物对可能指示哪个核苷酸存在于靶核酸中的内部基序中感兴趣的位置处。例如，靶核酸可具有通常具有5’至3’方向的序列GTAACGAG的内部基序。然而，靶核酸的不同变体可在内部基序的第5位置(即“C”的位置)处具有不同的核苷酸。继续该实例，如果提供含有靶核酸的样品，其中不知道在内部基序的第5位置处是何种核苷酸，则可将该样品分成至少4个部分，并且这四个部分可用于第一反应混合物、第二反应混合物、第三反应混合物和第四反应混合物。向第一反应混合物中提供第一引物对，其中引物分别具有包含5’至3’方向的序列CTCGTTAC和GTAACGAG的尾区。如果“C”存在于内部基序的第5位置处，则这些尾区将

与该内部基序或其互补体完全互补。因此，如果靶标在这一反应混合物中扩增最快，则表明“C”存在于该靶标中的内部基序中感兴趣的核苷酸的位置处。向第二反应混合物中提供第二引物对，其中引物分别具有包含5’至3’方向的序列CTCATTAC和GTAATGAG的尾区。如果“T”存在于内部基序的第5位置处，则这些尾区将与该内部基序或其互补体完全互补。因此，如果靶标在这一反应混合物中扩增最快，则表明“T”存在于该靶标中的内部基序中感兴趣的核苷酸的位置处。向第三反应混合物中提供第三引物对，其中引物分别具有包含5’至3’方向的序列CTCCTTAC和GTAAGGAG的尾区。如果“G”存在于内部基序的第5位置处，则这些尾区将与该内部基序或其互补体完全互补。因此，如果靶标在这一反应混合物中扩增最快，则表明“G”存在于该靶标中的内部基序中感兴趣的核苷酸的位置处。向第四反应混合物中提供第四引物对，其中引物分别具有包含5’至3’方向的序列CTCTTTAC和GTAAAGAG的尾区。如果“A”存在于内部基序的第5位置处，则这些尾区将与该内部基序或其互补体完全互补。因此，如果靶标在这一反应混合物中扩增最快，则表明“A”存在于该靶标中的内部基序中感兴趣的核苷酸的位置处。在实施方式中，如果例如不希望筛选所有可能的核苷酸变体(例如，如果在感兴趣的位置通常发现仅2个不同的核苷酸)，则以上概述的方法或组合物可以任选地仅用2或3种反应混合物来准备，这些反应混合物具有对应于在感兴趣的位置处仅有2或3个核苷酸选项的引物对。

[0237] 在实施方式中，根据本文提供的方法，含有靶核酸的样品可被分成至少第一和第二部分，其中第一部分在具有第一引物对的第一反应混合物中温育，并且第二部分在具有第二引物对的第二反应混合物中温育。在实施方式中，第一引物对和第二引物对彼此仅在引物各自的尾区/第一区域中相差一个核苷酸(例如，第一对的第一引物的第一区域的序列与第二对的第一引物的第一区域的序列仅相差一个核苷酸)。在引物的尾区中不同的单一核苷酸可对应于靶核酸中感兴趣的核苷酸的位置。通过比较在第一反应混合物中与在第二反应混合物中靶核酸的扩增速率或量，可以确定在靶核酸中的感兴趣的核苷酸的身份。在实施方式中，以上概述的方法可采用样品的第一、第二、第三和第四部分以及第一引物对、第二引物对、第三引物对和第四引物对进行，其中如上所述，第一引物对、第二引物对、第三引物对和第四引物对彼此仅在引物各自的尾区/第一区域中相差一个核苷酸。

[0238] 在实施方式中，可提供至少第一靶核酸和第二靶核酸，其中第一靶核酸和第二靶核酸如本文其他地方所述在内部基序中感兴趣的位置处相差一个核苷酸。在实施方式中，根据本文提供的方法，第一靶核酸可在含有本文所述的引物对的第一反应混合物中温育，并且第二靶核酸可在含有相同引物对的第二反应混合物中温育。通过比较在第一反应混合物中的第一靶核酸与在第二反应混合物中的第二靶核酸的扩增速率或量，或与绝对值比较，根据本文其他地方所述的原理，可以确定在第一靶核酸和第二靶核酸的一个或两者中感兴趣的位置处的核苷酸的身份。

[0239] 此外，在实施方式中，本文所述用于检测感兴趣的单一核苷酸的组合物和方法也可以用来检测多个核苷酸突变或多态性。

[0240] 本文提供的方法和组合物可用于从可含有核酸的任何样品中扩增核酸。样品的实例可包括各种流体样品。在一些情况下，样品可以是来自受试者的体液样品。样品可包含一个或多个流体组分。在一些情况下，可提供固体或半固体样品。样品可包括从受试者采集的组织。样品可包括受试者的体液、分泌物或组织。样品可以是生物样品。该生物样品可以是

体液、分泌物或组织样品。生物样品的实例可以包括但不限于，血液、血清、唾液、尿液、胃液和消化液、泪液、粪便、精液、阴道液、来源于肿瘤组织的间质液、眼部液体、汗液、粘液、耳垢、油、腺体分泌物、呼气、脊髓液、毛发、指甲、皮肤细胞、血浆、鼻拭子或鼻咽洗液、脊髓液、脑脊髓液、组织、咽喉拭子、活检物、胎盘液、羊水、脐带血、重点流体(emphatic fluid)、腔液、痰、脓、微生物群、胎粪、母乳或其他排泄物。样品可以由人或动物提供。样品可以来自植物、微生物(例如病毒、细菌)或其他生物物质。

[0241] 在一些实施方式中，本文提供的方法和组合物可在服务点位置(例如受试者的家中或工作地点、食品杂货店、药店、诊所或学校等)执行或使用。本文提供的方法和组合物可允许快速扩增来自受试者的样品中的核酸，以便帮助诊断或治疗受试者。例如，此处提供的方法和组合物可用于检测来自受试者的样品中来自病原体如病毒(例如流感病毒)或细菌(例如链球菌属(streptococcus))的核酸的存在。

[0242] 在实施方式中，本文提供的方法和组合物可用来检测病原体或病原体的特征(例如病原体内的基因或基因变异)。可采用本文提供的方法或组合物检测的病原体或其特征可包括，例如，鲍氏不动杆菌(Acinetobacter baumannii)、blaROB抗性基因、blaTEM抗性基因、Bordetalla holmesii、洋葱伯克霍尔德氏菌(Burkholderia cepacia)、白假丝酵母(Candida albicans)、艰难梭菌(Clostridium difficile)、索氏梭菌(Clostridium sordellii)、产气肠杆菌(Enterobacter aerogenes)、阴沟肠杆菌(Enterobacter cloacae)、粪肠球菌(Enterococcus faecaelis)、屎肠球菌(Enterococcus faecium)、大肠杆菌(Escherichia coli)、肺炎克雷伯氏菌(Klebsiella pneumoniae)、KPC抗性基因、侵肺军团菌(Legionella pneumophila)、mecA抗性基因、mecC抗性基因、卡他莫拉菌(Moraxella catarrhalis)、脓肿分枝杆菌(Mycobacterium abscessus)、铜绿假单胞菌(Pseudomonas aeruginosa)、粘质沙雷氏菌(Serratia marcescens)、金黄色葡萄球菌(Staphylococcus aureus)、金黄色葡萄球菌(万古霉素抗性)VRSA、无乳链球菌(Strep B)(Streptococcus agalactiae(Strep B))、肺炎链球菌(Streptococcus pneumonia)、肺炎链球菌(青霉素敏感性)、酿脓链球菌(Streptococcus pyogenes)、vanA抗性基因、vanB抗性基因、腺病毒B(Adenovirus B)、腺病毒C(Adenovirus C)、腺病毒E、博卡病毒1+3(Bocavirus 1+3)、博卡病毒2+4、副百日咳博德特氏菌(Bordetella parapertussis)、百日咳博德特氏菌(Bordetella pertussis)、肺炎衣原体(Chlamydia pneumonia)、索氏梭菌、冠状病毒229E(Coronavirus 229E)、冠状病毒HKU1、冠状病毒MERS、冠状病毒NL63、冠状病毒OC43、人偏肺病毒A(Human metapneumovirus A)、人偏肺病毒B、甲型流感-MP(Influenza A-MP)、乙型流感-MP(Influenza B-MP)、流感H1N1(2009)、流感H1N1(季节性)、流感H3N2、流感H5N1、流感H7N9(HA基因)、流感H7N9(NA基因)、麻疹病毒(measles virus)、腮腺炎病毒(mumps virus)、结核分枝杆菌(Mycobacterium tuberculosis)、肺炎支原体(Mycoplasma pneumonia)、副流感1(Parainfluenza 1)、副流感2、副流感3、副流感4a、副流感4b、人呼吸道合胞病毒A(RSV A)(Respiratory syncytial virus A(RSV A))、人呼吸道合胞病毒B(RSV B)、鼻病毒A(Rhinovirus A)、鼻病毒B、鼻病毒C、风疹病毒(rubella virus)、酿脓链球菌、白假丝酵母、砂眼衣原体(Chlamydia trachomatis)、人乳头瘤病毒(human papilloma virus)、HPV 16、HPV 18、HSV-1/2、生殖道支原体(mycoplasma genitalium)、淋病奈瑟氏球菌(Neisseria gonorrhoeae)、苍白密螺旋体(梅毒)(Treponema pallidum

(syphilis))、阴道毛滴虫(Trichomonas vaginalis)、水痘-带状疱疹病毒(Varicella-zoster virus)、EB病毒(Epstein-Barr Virus)、甲型肝炎(Hepatitis A)、乙型肝炎(Hepatitis B)、丙型肝炎(Hepatitis C)、丁型肝炎(Hepatitis D)、HIV、HIV-1组M、HIV-1组O1、HIV-1组O3、HIV-2组A、HIV-2组B、人型支原体(Mycoplasma hominis)、奇异变形菌(Proteus mirabilis)、彭氏变形菌(Proteus penneri)、普通变形菌(Proteus vulgaris)、腐生葡萄球菌(S.saprophyticus)、解脲支原体(Ureaplasma urealyticum)、炭疽芽孢杆菌(Bacillus anthracis)、鼠疫耶尔森氏菌(Yersina pestis)、登革病毒(Dengue virus)、疟原虫(Plasmodium)、克氏锥虫(锥虫病)(Tryponosoma cruzi(chagas))、西尼罗病毒1(West Nile Virus 1)、西尼罗病毒2、诺如病毒谱系1(Norovirus lineage 1)、诺如病毒谱系2、埃博拉病毒(Ebola virus)。在实施方式中,可使用本文提供的方法针对来自受试者的单一样品测定1、2、3、5、10、20、30、50个或更多个这样的病原体或其特征。在实施方式中,在2014年9月5日提交的国际申请号PCT/US2014/54424或2014年5月20日提交的美国临时专利申请号62/001,050中描述的任何基于核酸扩增的测定或测定组,可使用本文所述的核酸扩增方法进行,这些申请通过引用以全文并入于此用于所有目的。类似地,在国际申请号PCT/US2014/54424或美国临时专利申请号62/001,050中描述的任何病原体或其特征可采用本文提供的核酸扩增方法进行测定。

[0243] 在实施方式中,流感组可以使用本文提供的用于从一个或多个流感菌株扩增核酸的方法来进行。例如,使用本文提供的方法可以从甲型流感、甲型流感H3N2、甲型流感H5N1、甲型流感H7N9-HA基因、甲型流感H7N9-NA基因或乙型流感中的一个或多个扩增一个或多个核酸。可以使用本文提供的方法进行的其他组包括,例如,呼吸组、性传播疾病组、血源性病原体组或医源性感染组。这些组中的任何一个可包括针对至少2、3、4、5、10、15或20个不同的病原体或基因的测定。

[0244] 在实施方式中,本文提供的方法和组合物可用于甲型流感病毒检测和亚型分型。甲型流感亚型由以下两种病毒基因的类型确定:血凝素(HA)和神经氨酸酶(NA)。目前有18种已知的HA基因(H1-H18)和11种已知的NA基因(N1-N11)。这些基因在甲型流感病毒中的不同分子上编码,因而甲型流感亚型可能携带不同HA和NA基因的任何组合。为了准确地对甲型流感病毒进行检测和分型,可能需要测试可能含有或已知含有甲型流感病毒的样品的至少两种至高达全部18种已知的HA基因和至少两种至高达全部11种已知的NA基因。在实施方式中,来自受试者的单一样品可被分成两个或更多个部分,并且这些部分可在本文提供的反应条件下与具有对于所述18种已知HA基因和11种已知NA基因中的每一种为特异性的模板结合区的引物对(即29个不同的引物对)一起温育。在实施方式中,来自受试者的单一样品可被分成至少29个不同的部分,其中第一部分在本文提供的反应条件下与具有对于H1为特异性的模板结合区的引物对一起温育,其中第二部分在本文提供的反应条件下与具有对于H2为特异性的模板结合区的引物对一起温育,其中第三部分在本文提供的反应条件下与具有对于H3为特异性的模板结合区的引物对一起温育,其中第四部分在本文提供的反应条件下与具有对于H4为特异性的模板结合区的引物对一起温育,其中第五部分在本文提供的反应条件下与具有对于H5为特异性的模板结合区的引物对一起温育,其中第六部分在本文提供的反应条件下与具有对于H6为特异性的模板结合区的引物对一起温育,其中第七部分在本文提供的反应条件下与具有对于H7为特异性的模板结合区的引物对一起温育,其中第

八部分在本文提供的反应条件下与具有对于H8为特异性的模板结合区的引物对一起温育，其中第九部分在本文提供的反应条件下与具有对于H9为特异性的模板结合区的引物对一起温育，其中第十部分在本文提供的反应条件下与具有对于H10为特异性的模板结合区的引物对一起温育，其中第十一部分在本文提供的反应条件下与具有对于H11为特异性的模板结合区的引物对一起温育，其中第十二部分在本文提供的反应条件下与具有对于H12为特异性的模板结合区的引物对一起温育，其中第十三部分在本文提供的反应条件下与具有对于H13为特异性的模板结合区的引物对一起温育，其中第十四部分在本文提供的反应条件下与具有对于H14为特异性的模板结合区的引物对一起温育，其中第十五部分在本文提供的反应条件下与具有对于H15为特异性的模板结合区的引物对一起温育，其中第十六部分在本文提供的反应条件下与具有对于H16为特异性的模板结合区的引物对一起温育，其中第十七部分在本文提供的反应条件下与具有对于H17为特异性的模板结合区的引物对一起温育，其中第十八部分在本文提供的反应条件下与具有对于H18为特异性的模板结合区的引物对一起温育，其中第十九部分在本文提供的反应条件下与具有对于N1为特异性的模板结合区的引物对一起温育，其中第二十部分在本文提供的反应条件下与具有对于N2为特异性的模板结合区的引物对一起温育，其中第二十一部分在本文提供的反应条件下与具有对于N3为特异性的模板结合区的引物对一起温育，其中第二十二部分在本文提供的反应条件下与具有对于N4为特异性的模板结合区的引物对一起温育，其中第二十三部分在本文提供的反应条件下与具有对于N5为特异性的模板结合区的引物对一起温育，其中第二十四部分在本文提供的反应条件下与具有对于N6为特异性的模板结合区的引物对一起温育，其中第二十五部分在本文提供的反应条件下与具有对于N7为特异性的模板结合区的引物对一起温育，其中第二十六部分在本文提供的反应条件下与具有对于N8为特异性的模板结合区的引物对一起温育，其中第二十七部分在本文提供的反应条件下与具有对于N9为特异性的模板结合区的引物对一起温育，其中第二十八部分在本文提供的反应条件下与具有对于N10为特异性的模板结合区的引物对一起温育，并且其中第二十九部分在本文提供的反应条件下与具有对于N11为特异性的模板结合区的引物对一起温育。在一些实施方式中，如本文其他地方所述，针对2、3、4、5个或更多个不同的HA或NA亚型的引物对可以与来自受试者的样品的单一部分一起温育(例如在常规容器中)，并且可以使用序列特异性探针(例如DNA探针或PNA探针)鉴定扩增反应产物。在实施方式中，可以进行一组(panel)，其中单一样品用于以针对至少2、3、4、5、6、7、8、9、10、11、12、13、14、15、20、25个或全部上述29个HA和NA亚型的引物对进行扩增反应。在实施方式中，组(panel)可包括用来扩增H1、H2、H3、H5、H7或N9中的一个或多个的反应。在实施方式中，本文所述的用于检测甲型流感或其亚型的组或方法可进一步包括用来检测例如甲型流感基质蛋白基因(它存在于全部甲型流感亚型中，从而可用于鉴定样品中的甲型流感)、人RNA酶P(以确保从人类受试者中充足地收集样品；对于非人类受试者，可以使用其他合适的物种特异性对照基因)或掺加核酸对照的试剂或反应，该掺加核酸对照可在样品收集或处理步骤的过程中的一个或多个时间点添加，以便确保充分的样品收集或处理。在实施方式中，可提供试剂盒来进行以上提供的任何方法或任何组。

[0245] 在实施方式中，与可能使用其他核酸扩增方法(例如PCR或LAMP)采用一对引物进行扩增相比，通过使用本文提供的方法，可以使用一对引物来扩增更多种类的靶核酸序列。

例如，在实施方式中，本文提供的引物的模板结合区可与模板充分退火，以根据本文提供的方法扩增该模板，即使当引物的模板结合区与模板之间具有1、2、3、4、5个或更多个核苷酸错配时。在实施方式中，当在本文提供的方法中使用的至少第一引物和任选的第二引物的模板结合区中至少6％、8％、10％、12％、14％、16％、18％、20％、22％或24％的核苷酸不与模板序列中的相应核苷酸互补时，可进行本文提供的方法。因此，本文提供的引物和方法的使用可有助于用同一对引物扩增多个类似但不同的序列。例如，本文提供的引物和方法可用于检测甲型流感的多个不同的菌株，其中所述菌株具有类似但不同的核苷酸序列。例如，可能有甲型流感的两个不同的菌株，其中这两个菌株均被确定为H1N1亚型，但所述菌株具有略微不同的H1或N1核苷酸序列。在实施方式中，使用本文提供的组合物和方法，第一菌株和第二菌株均可使用相同的引物对得到扩增。

[0246] 此外，可基于病原体的核酸中的高度保守区选择用于从病原体中扩增核酸的引物，以进一步帮助根据本文提供的方法采用相同引物对检测病原体的多个不同的菌株。这样的高度保守区可通过例如在病原体的多个菌株之间的感兴趣的基因的序列比对来鉴定。例如，Genbank ID号为AEK98400、ABG47862、AEO00689、ABF17954、AFG99237、AFG99027、ABO33069、ABE12623、AFG72790、AAA87553、AAZ38605、AFN19700、ABB04371、ABC39805的HA3序列来自H3N2甲型流感病毒的不同隔离群。这些HA3基因各自具有略微不同的核苷酸序列。尽管如此，通过细心选择引物，可根据本文提供的方法采用同一组引物扩增全部这些HA3基因。例如，根据本文提供的方法可选择以5'-3'顺序具有序列AACCTCACTACGTAAGTCTTGACGTAAACGGTTACCCACGTAGACTAGAGTAATAACTCGAAAAGGGT(SEQ ID NO:91)的HA3基因的一部分作为扩增靶标(该序列以DNA形式，而非RNA形式提供)。以5'至3'方向描述该序列，第一下划线序列是模板的第二部分，第二下划线序列是内部基序，第三下划线序列是模板的第一部分，如这些术语在例如图10中使用一样。在这个和其他这样的实例中，可以选择用于扩增的总靶序列以及充当模板的第一部分、模板的第二部分和内部基序的特定序列，使得如本文所述的引物可用于根据本文提供的方法成功地扩增范围广泛的相关序列。在实施方式中，可提供如本文所述的第一引物，其中该引物的模板结合区与以上模板的第一部分互补，并且可以提供如本文所述的第二引物，其中该引物的模板结合区与同该模板的第二部分互补的配偶体序列互补。任选地，所述引物可进一步具有与内部基序或其互补序列互补的尾区。这样的引物可在例如本文提供的组合物或方法中提供。

[0247] 采用本文提供的方法，可以在样品或反应混合物中在低至例如小于1000、500、100、50、10、5、2或1个拷贝/微升的浓度下明确鉴定病原体或基因。在实施方式中，本文提供的方法可从一种类型的病原体或基因特异性地扩增核酸，并且不从相关的病原体或基因扩增核酸。例如，本文提供的用于扩增甲型流感基质蛋白基因的试验可从甲型流感的多个不同菌株中扩增该基因，但不从乙型流感中扩增遗传物质。

[0248] 在实施方式中，本文提供的方法可在一种或多种潜在干扰性物质的存在下成功进行。潜在干扰性物质的实例包括BSA、葡萄糖、胆红素、亚硝酸盐、β-hCG、丙酮、低pH条件、高pH条件、醋氨酚、阿司匹林、黄体酮、炔雌醇、尿防腐剂、精液、人体润滑剂、避孕凝胶剂、杀精剂、女性粉剂、痔疮乳膏、咪康唑、人类基因组DNA、洗剂、通用转运培养基(病毒的)、艾米斯(amies)转运培养基(细菌的)、血液、粘蛋白、阿昔洛韦、冷疱治疗、尿、粪便、血红蛋白、甘油三酯、EDTA、肝素、胆固醇、γ-球蛋白、氨苄青霉素、烟碱、可替宁、喷鼻剂、滴鼻剂或它们的

任意组合。在实施方式中，本文提供的方法可在潜在干扰性物质的存在下进行，该物质的浓度可达至少与2014年5月20日提交的美国临时专利申请号62/001,050中提供的一样大，该申请通过引用并入于此用于所有目的。在实施方式中，本文提供的方法可在潜在干扰性物质的存在下进行，该物质的浓度可达比美国临时专利申请号62/001,050中提供的大至少10％、25％、50％或100％。

[0249] 本文公开的测定和方法可在用于处理样品的装置或系统上执行。本文公开的测定和方法可容易地并入用于处理样品的装置或用于处理样品的系统中并在其中使用，该装置或系统可以是自动化的测定装置或者可以是自动化的测定系统。这样的装置以及这样的系统可用于实施本文公开的方法。例如，装置可用于接收样品。装置可用于制备或用于处理样品。装置可用于对样品进行测定。装置可用于从样品获得数据。装置可用于传送从样品中获得的数据。装置可用于处理或测定样品之后处置样品。

[0250] 装置可以是系统的一部分，该系统的组件可以是样品处理装置。装置可以是样品处理装置。样品处理装置可配置为促进样品的采集、制备用于临床检验的样品或执行使用一种或多种试剂的方法，如本文所公开的。样品处理装置可配置为从样品获得数据。样品处理装置可配置为传送从样品中获得的数据。样品处理装置可配置为分析来自样品的数据。样品处理装置可配置为与另一装置或实验室或附属于实验室的个体通信，以分析从样品中获得的数据。

[0251] 样品处理装置可配置为放置在受试者体内或受试者上。样品处理装置可配置为直接或间接地接纳来自受试者的样品。样品可以是，例如，血液样品(例如，由手指针刺或由静脉穿刺获得的样品或动脉血样品)、尿液样品、活检样品、组织切片、粪便样品或其他生物样品；水样品、土壤样品、食物样品、空气样品；或其他样品。血液样品可以包括，例如，全血、血浆或血清。样品处理装置可通过装置的外壳接收来自受试者的样品。样品采集可发生在样品采集地点或其他地方。可在样品采集地点向装置提供样品。

[0252] 在一些实施方式中，样品处理装置可配置为接收或容纳筒匣。在一些实施方式中，样品处理装置可包含筒匣。该筒匣可从样品处理装置中移除。在一些实施方式中，可向样品处理装置的筒匣提供样品。或者，可向样品处理装置的另一部分提供样品。筒匣和/或装置可包含可配置为容纳样品的样品采集单元。

[0253] 筒匣可包含样品，并可包含用于在处理或测试样品中使用的试剂、用于在处理或测试样品中使用的一次性用品或其他材料。筒匣可包含本文公开的用于执行本文公开的方法的试剂。将筒匣放置在样品处理装置上或将筒匣插入样品处理装置之后，筒匣的一个或多个组件可与样品处理装置的其他组件流体连通。例如，如果样品在筒匣收集，则样品可转移至样品处理装置的其他部分。类似地，如果在筒匣上提供了一种或多种试剂，则该试剂可转移至样品处理装置的其他部分，或者样品处理装置的其他组件可得到该试剂。在一些实施方式中，筒匣的试剂或组件可保持在筒匣上。在一些实施方式中，不包含需要装管或需要维护(例如，人工或自动维护)的流体元件。

[0254] 样品或试剂可转移至装置，诸如样品处理装置。样品或试剂可在装置内转移。样品或试剂的这种转移可在不提供从筒匣到装置的连续流体途径的情况下完成。样品或试剂的这种转移可在不提供装置内的连续流体途径的情况下完成。在实施方式中，样品或试剂的这种转移可以通过样品处理系统(例如移液管)来完成；例如，样品、试剂或其等分试样可被

吸入顶端开放的转移组件，诸如移液管尖端，该转移组件可以可操作地连接到样品处理系统，该样品处理系统将该尖端以及包含在该尖端内的样品、试剂或其等分试样一起转移至样品处理装置之上或之内的位置。样品、试剂或其等分试样可放置在样品处理装置之上或之内的位置。样品和试剂或多种试剂可使用样品处理系统以相似的方式进行混合。筒匣的一个或多个组件可以以自动化方式转移至样品处理装置的其他部分，反之亦然。

[0255] 装置，诸如样品处理装置，可具有流体处理系统。流体处理系统可进行或可帮助进行运输、稀释、提取、等分、混合以及对于流体如样品的其他操作。在一些实施方式中，流体处理系统可包含在装置外壳内。流体处理系统可允许流体的采集、递送、处理和/或运输，干燥试剂的溶解，液体和/或干燥试剂与液体的混合，以及非流体组分、样品或材料的采集、递送、处理和/或运输。该流体可以是样品、试剂、稀释液、洗液、染料或可被装置使用的任何其他流体，并且可以包括但不限于均匀流体、不同的液体、乳液、悬浮液和其他流体。流体处理系统(包括但不限于移液管)还可用于在装置周围运输容器(其中含有或不含有流体)。流体处理系统可分配或抽吸流体。样品可包含漂浮在流体内的一个或多个微粒或固体物质。

[0256] 在实施方式中，流体处理系统可包含移液管、移液管尖端、注射器、毛细管或其他组件。流体处理系统可具有含内表面和外表面以及开放端的部分。流体处理系统可包括移液管(其可包含移液管主体和移液管的管嘴)并且可包括移液管尖端。移液管尖端可以从或不可以从移液管管嘴上拆除。在实施方式中，流体处理系统可使用与移液管尖端配对的移液管；移液管尖端可以是一次性的。尖端在与移液管配对时，可形成流体密封。移液管尖端可使用一次、两次或更多次。在实施方式中，流体处理系统可使用具有或不具有移液管尖端的移液管或相似装置来抽吸、分配、混合、运输或以其他方式处理流体。需要时，可从流体处理系统分配流体。在从例如移液管尖端的孔口中分配之前流体可包含在移液管尖端内。在实施方式或情况中，在使用期间，可分配全部流体；在其他实施方式或情况中，在使用期间，可分配尖端内流体的一部分。移液管可选择性地抽吸流体。移液管可抽吸所选量的流体。移液管可以能够致动搅拌机构以混合尖端或容器内的流体。移液管可合并尖端或容器，从而产生连续的流动回路以用于包括非液体形式的材料或试剂的混合。移液管尖端还可通过多个流体的同时或顺序计量递送来促进混合，诸如在两段式底物反应中。

[0257] 流体处理系统可包括一个或多个流体隔离的或液压独立的单元。例如，流体处理系统可包含一个、两个或更多个移液管尖端。移液管尖端可被配置为接纳并限制流体。尖端可以是彼此流体隔离或彼此液压独立的。包含在每个尖端内的流体可以与其他尖端内的流体以及与装置内的其他流体为流体隔离或液压独立的。流体隔离或液压独立的单元可以是相对于装置的其他部分和/或彼此可移动的。流体隔离或液压独立的单元可以是单独可移动的。流体处理系统可以包含一个或多个基部或支持体。基部或支持体可支持一个或多个移液管或移液管单元。基部或支持体可将流体处理系统的一个或多个移液管彼此连接。

[0258] 样品处理装置可配置为对从受试者获得的样品执行处理步骤或操作。样品处理可包括样品制备，包括，例如，样品稀释、将样品分为等分试样、提取、与试剂接触、过滤、分离、离心或其他的预备或处理操作或步骤。样品处理装置可配置为对样品执行一个或多个样品制备操作或步骤。任选地，样品可制备用于化学反应和/或物理处理步骤。样品制备操作或步骤可包括以下的一个或多个：离心、分离、过滤、稀释、富集、纯化、沉淀、温育、移液、运输、色谱分析、细胞裂解、细胞计数、粉碎、研磨、活化、超声处理、微柱处理、用磁珠处理、用纳米

颗粒处理或其他样品制备操作或步骤。例如，样品制备可包括将血液分离为血清和/或微粒部分或者将任何其他样品分离为各个组分的一个或多个步骤。样品制备可包括稀释和/或浓缩样品如血液样品或其他生物样品的一个或多个步骤。样品制备可包括向样品中加入抗凝剂或其他成分。样品制备还可包括样品的纯化。在实施方式中，所有的样品处理、制备或测定操作或步骤均由单一装置执行。在实施方式中，所有的样品处理、制备或测定操作或步骤均在单一装置的外壳内执行。在实施方式中，大多数的样品处理、制备或测定操作或步骤由单一装置执行，并且可在单一装置的外壳内执行。在实施方式中，许多的样品处理、制备或测定操作或步骤由单一装置执行，并且可在单一装置的外壳内执行。在实施方式中，样品处理、制备或测定操作或步骤可由多于一个装置执行。

[0259] 样品处理装置可配置为对样品运行一个或多个测定，并从该样品获得数据。样品处理装置可执行本文提供的方法以及附加的测定。测定可包括一种或多种物理或化学处理，并可包括运行一个或多个化学或物理反应。样品处理装置可配置为对体液的小样品执行一个、两个或更多个测定。可在具有一定体积的样品上进行一个或多个化学反应，如本文其他地方所述。例如，可在具有小于飞升(femtoliter)体积的丸剂中进行一个或多个化学反应。在实例中，样品采集单元被配置为接收相当于单滴或更少的血液或间质液体积的体液样品。在实施方式中，样品的体积可以是小体积，其中小体积可以是小于约1000μL、或小于约500μL、或小于约250μL、或小于约150μL、或小于约100μL、或小于约75μL、或小于约50μL、或小于约40μL、或小于约20μL、或小于约10μL、小于约5μL、小于约1μL、小于约0.5μL、小于约0.1μL的体积或其他的小体积。在实施方式中，对单一样品执行全部的样品测定操作或步骤。在实施方式中，所有的样品测定操作或步骤均由单一装置执行。在实施方式中，所有的样品测定操作或步骤均在单一装置的外壳内执行。在实施方式中，大多数的样品测定操作或步骤由单一装置执行，并且可在单一装置的外壳内执行。在实施方式中，许多的样品测定操作或步骤由单一装置执行，并且可在单一装置的外壳内执行。在实施方式中，样品处理、制备或测定操作或步骤可由多于一个装置执行。

[0260] 样品处理装置可配置为对样品进行多个测定。在一些实施方式中，样品处理装置可以配置为执行本文提供的方法以及一个、两个或更多个附加测定。在实施方式中，样品处理装置可配置为对单一样品进行多个测定。在实施方式中，样品处理装置可配置为对单一样品进行多个测定，其中该样品是小样品。例如，小样品可以具有为小于约1000μL、或小于约500μL、或小于约250μL、或小于约150μL、或小于约100μL、或小于约75μL、或小于约50μL、或小于约40μL、或小于约20μL、或小于约10μL、小于约5μL、小于约1μL、小于约0.5μL、小于约0.1μL的小体积或其他小体积的样品体积。样品处理装置可以能够对单一样品进行多重测定。多个测定可同时运行；可按顺序运行；或者一些测定可同时运行而其他测定按顺序运行。一个或多个对照测定和/或校准物(例如，包括具有用于测定/测试的校准物的对照的配置)也可并入装置中；对照测定和对校准物的测定可与对样品进行的测定同时进行，或可在对样品进行的测定之前或之后进行，或以其任何组合方式进行。在实施方式中，所有的样品测定操作或步骤均由单一装置执行。在实施方式中，全部多个样品测定操作或步骤均在单一装置的外壳内执行。在实施方式中，多个测定中的大多数的样品测定操作或步骤由单一装置执行，并且可在单一装置的外壳内执行。在实施方式中，多个测定中的许多的样品测定操作或步骤由单一装置执行，并且可在单一装置的外壳内执行。在实施方式中，样品处理、

制备或测定操作或步骤可由多于一个装置执行。

[0261] 在实施方式中，全部多个测定可在短的时间段内进行。在实施方式中，这样的短的时间段包括少于约三小时、或少于约两小时、或少于约一小时、或少于约40分钟、或少于约30分钟、或少于约25分钟、或少于约20分钟、或少于约15分钟、或少于约10分钟、或少于约5分钟、或少于约4分钟、或少于约3分钟、或少于约2分钟、或少于约1分钟或其他短的时间段。

[0262] 样品处理装置可配置为检测与样品相关的一种或多种信号。样品处理装置可配置为鉴定样品的一个或多个特性。例如，样品处理装置可配置为检测样品中一种分析物(例如靶核酸)或多种分析物或疾病状况的存在或浓度(例如，在体液、分泌物、组织或其他样品中或通过体液、分泌物、组织或其他样品)。或者，样品处理装置可配置为检测一种或多种可被分析以检测样品中一种或多种分析物(其可以指示疾病状况)或疾病状况的存在或浓度的信号。信号可在装置上或另一位置进行分析。运行临床检验可以包括或可以不包括对收集的数据的任何分析或比较。

[0263] 可在有或没有样品的情况下进行化学反应或其他处理步骤。可由装置制备或运行的步骤、测试或测定的实例可包括但不限于，免疫测定、核酸测定(例如本文所提供的方法)、基于受体的测定、细胞计数测定、比色测定、酶测定、电泳测定、电化学测定、光谱测定、色谱测定、显微测定、地形测定(topographic assay)、量热测定、浊度测定、凝集测定、放射性同位素测定、粘度测定、凝聚测定、凝固时间测定、蛋白质合成测定、组织学测定、培养测定、摩尔渗透压浓度测定和/或其他类型的测定，离心、分离、过滤、稀释、富集、纯化、沉淀、粉碎、温育、移液、运输、细胞裂解或其他样品制备操作或步骤或其组合。可由装置制备或运行的步骤、测试或测定可包括成像，包括显微镜检查、细胞计数以及制备或使用图像的其他技术。可由装置制备或运行的步骤、测试或测定可进一步包括对样品的组织学、形态学、运动学、动力学和/或状态的评估，该评估可包括对细胞的这种评估。

[0264] 装置可以能够在短的时间量内进行所有的机载步骤(例如，由单一装置执行的步骤或操作)。装置可以能够在短的时间量内对单一样品进行所有的机载步骤。例如，从受试者样品采集到传输数据和/或分析可能花费约3小时或更少、2小时或更少、1小时或更少、50分钟或更少、45分钟或更少、40分钟或更少、30分钟或更少、20分钟或更少、15分钟或更少、10分钟或更少、5分钟或更少、4分钟或更少、3分钟或更少、2分钟或更少、或1分钟或更少的时间。从将样品接纳在装置内到传输数据和/或关于这样的样品的装置分析的时间量可能依赖于对样品执行的步骤、测试或测定的类型或数目。从将样品接纳在装置内到传输数据和/或关于这样的样品的装置分析的时间量可能花费约3小时或更少、2小时或更少、1小时或更少、50分钟或更少、45分钟或更少、40分钟或更少、30分钟或更少、20分钟或更少、15分钟或更少、10分钟或更少、5分钟或更少、4分钟或更少、3分钟或更少、2分钟或更少或1分钟或更少的时间。

[0265] 装置可配置为准备样品以供处置或配置为在样品的处理或测定后处置样品，诸如生物样品。

[0266] 在实施方式中，样品处理装置可配置为传送从样品获得的数据。在实施方式中，样品处理装置可配置为通过网络进行通信。样品处理装置可包括可与网络接合的通信模块。样品处理装置可经由有线连接或无线地连接至网络。该网络可以是局域网(LAN)或广域网(WAN)诸如因特网。在一些实施方式中，该网络可以是个人局域网。该网络可包括云。样品处

理装置可连接至网络而无需中间装置，或者可能需要中间装置将样品处理装置连接至网络。样品处理装置可以通过网络与另一装置通信，该另一装置可以是任何类型的联网装置，包括但不限于个人计算机、服务器计算机或膝上型计算机；个人数字助理(PDA)，诸如Windows CE装置；电话，诸如蜂窝电话、智能电话(例如，iPhone、Android、Blackberry等)或位置感知的便携式电话(例如GPS)；漫游装置，诸如联网的漫游装置；无线装置，诸如无线电子邮件装置或能够与计算机网络无线通信的其他装置；或可以通过网络而可能通信并处理电子交易的任何其他类型的网络装置。这样的通信可包括向云计算基础设施或可被其他装置访问的任何其他类型的数据存储基础设施提供数据。

[0267] 样品处理装置可以向例如卫生保健专业人员、健康护理专业场所诸如实验室或其附属机构提供关于样品的数据。实验室、卫生保健专业人员或受试者中的一个或多个可具有能够接收或访问由样品处理装置提供的数据的网络装置。样品处理装置可配置为向数据库提供关于样品的数据。样品处理装置可配置为向电子病历系统、向实验室信息系统、向实验室自动化系统或其他系统或软件提供关于样品的数据。样品处理装置可以以报告的形式提供数据。

[0268] 实验室、装置或其他实体或软件可对关于样品的数据进行实时分析。软件系统可进行化学分析和/或病理分析，或者这些可分布在实验室、临床以及专业或专家人员的组合中。分析可包括样品的定性和/或定量评估。数据分析可包括随后的样品的定性和/或定量评估。任选地，报告可基于原始数据、预处理的数据或分析的数据生成。可制作这样的报告以便维持从样品获得的数据的机密性、关于从中获得样品的受试者的身份和其他信息、数据的分析以及其他机密信息。报告和/或数据可传输至卫生保健专业人员。可向数据库、电子病历系统、向实验室信息系统、向实验室自动化系统或其他系统或软件提供由样品处理装置获得的数据或对这样的数据的分析或报告。

[0269] 可使用或可与本文公开的方法、组合物或其他试剂一起使用的试剂、测定、方法、试剂盒、装置和系统的实例的描述和公开内容可发现于，例如，美国专利8,088,593；美国专利8,380,541；2013年2月18日提交的美国专利申请序列号13/769,798；2013年2月18日提交的美国专利申请序列号13/769,779；2011年9月26日提交的美国专利申请序列号13/244,947；2012年9月25日提交的国际申请号PCT/US2012/57155；2011年9月26日提交的美国申请序列号13/244,946；2011年9月26日提交的美国专利申请13/244,949；以及2011年9月26日提交的美国申请序列号61/673,245；2014年3月15日提交的国际申请号PCT/US14/30034；和2014年3月15日提交的美国专利申请号14/214,850，这些专利和专利申请的公开内容均通过引用全文并入于此。

[0270] 本申请要求2013年11月22日提交的美国临时专利申请号61/908,027；2014年5月20日提交的美国临时专利申请号62/001,050；2014年3月15日提交的美国非临时专利申请号14/214,850；以及2014年3月15日提交的国际专利申请号PCT/US14/30034的权益和优先权，其中每一个专利申请的公开内容通过引用全文并入于此用于所有目的。2013年3月15日提交的美国临时专利申请号61/800,606的公开内容也通过引用全文并入于此用于所有目的。

[0271] 实施例

[0272] 提供以下实施例仅仅是为了说明性目的，并非旨在以任何方式限制本发明。

[0273] 实施例1-用可变尾区长度的引物扩增模板核酸

[0274] 使用如本文提供的方法扩增靶核酸。准备反应以测定靶核酸T124A1的102核苷酸部分，其是A型流感病毒血凝素(HA3)基因(RNA分子)的464核苷酸部分。在SEQ ID NO:89中提供了T124A1的核苷酸序列。制备第一引物(“P1”)的12个变体和第二引物(“P2”)的12个变体。图2A中提供了全部引物变体的序列。所有的引物均含有15个核苷酸长度的模板结合区。第一引物的所有变体的模板结合区的序列为:CAAACCGTACCAACC(SEQ ID NO:80)(在5’-3’方向)。第二引物的所有变体的模板结合区的序列为:ATGCGGAATGTACC(SEQ ID NO:81)(在5’-3’方向)。不同的引物变体含有范围为8至22个核苷酸长度特别是8、9、10、11、12、13、14、15、16、18、20或22个核苷酸长度的尾区。第一引物的尾区具有碱基序列:CGCCGGATGGCTCTTGGGAAAC(SEQ ID NO:82)(在5’-3’方向)。碱基序列为22个核苷酸的长度;第一引物的较短尾区具有相同的序列，从尾区的5’端减去适当数目的核苷酸(例如具有18个核苷酸长度的尾区的引物具有含有与碱基序列减去碱基序列的前4个核苷酸(CGCC)相同的序列的尾区)。第二引物的尾区具有碱基序列:GTTTCCCAAGAGCCATCCGGCG(SEQ ID NO:83)(在5’-3’方向)。碱基序列为22个核苷酸的长度;第一引物的较短尾区具有相同的序列，从尾区的3’端减去适当数目的核苷酸(例如具有18个核苷酸长度的尾区的引物具有含有与碱基序列减去碱基序列的最后4个核苷酸(GGCG)相同的序列的尾区)。

[0275] 制备150微升的反应混合物，每一个含有:50mM乙酸钾，20mM Tris-乙酸盐(pH 7.9)，10mM乙酸镁，1mM DTT，30μg牛血清白蛋白(BSA)，0.8M甜菜碱，各为1.4mM的dATP、dTTP、dGTP和dCTP，2uMSYTO ®59(Life Technologies)，0.8单位/μl Bst DNA聚合酶(New England BioLabs)，0.016单位/μl AMV逆转录酶(New England Biolabs)，1单位/μl鼠核糖核酸酶抑制剂(New England Biolabs)，0.8μM的第一引物变体，0.8μM的第二引物变体以及每微升100,000个拷贝T124A1模板，并在59℃下在CFX 96Touch仪器(Bio-Rad)中温育100分钟。测定的转变点使用采用CFX Manager软件(Bio-Rad)的单一阈值方法来确定并示于图2B中。X轴提供了反应中使用的第一和第二引物的尾区的核苷酸长度，而Y轴提供了测定的转变时间(以分钟计)。对于每一个尾区核苷酸长度，示出了两个相邻的条:左边的条是含有模板的反应的转变时间，而右边的条是没有模板的反应[“无模板对照”(“NTC”)]的转变时间。对于超过90分钟的转变时间，不显示条。如图2B所示，在这些反应条件下，具有8-15个核苷酸并且尤其是8-11个核苷酸的尾区的引物支持最快的转变时间。无模板对照反应最后显示转变时间;这是由于随着时间的推移形成了背景非特异性产物。

[0276] 实施例2-用可变尾区长度和可变模板结合区长度的引物扩增模板核酸

[0277] 使用如本文提供的方法扩增靶核酸。准备反应以测定靶核酸T124A1，该靶核酸T124A1在上文实施例1中进行了描述。制备两个不同组的第一引物和第二引物对组。在第一组的引物对中，每一个引物均具有20个核苷酸长度的模板结合区(“20核苷酸模板结合区”组)。在第二组的引物对中，每一个引物均具有16个核苷酸长度的模板结合区(“16核苷酸模板结合区”组)。在各组中，制备具有8个不同尾区长度的第一和第二引物对组:7、8、9、10、11、12、14和16个核苷酸。图3提供了针对16核苷酸模板结合区组(图3A)和20核苷酸模板结合区组(图3B)的不同的引物对组的核苷酸序列。在图3A和3B中，“尾”序列指尾区，而“引物”区指引物的模板结合区。引物的序列以5’-3’方向显示。

[0278] 制备110微升的反应混合物，每一个含有:50mM乙酸钾，20mM Tris-乙酸盐(pH

7.9),10mM乙酸镁,1mM DTT,30μg牛血清白蛋白(BSA),0.8M甜菜碱,各为1.4mM的dATP、dTTP、dGTP和dCTP,2uMSYTO®59(Life Technologies),0.8单位/μl Bst DNA聚合酶(New England BioLabs),0.016单位/μl AMV逆转录酶(New England Biolabs),1单位/μl鼠核糖核酸酶抑制剂(New England Biolabs),0.8μM的第一引物变体,0.8μM的第二引物变体以及每微升100,000个拷贝T124A1模板,并在56℃下在CFX 96Touch仪器(Bio-Rad)中温育100分钟。测定的转变点示于图3C(20核苷酸模板结合区组)和3D(16核苷酸模板结合区组)中。在图3C和3D中,X轴提供了反应中使用的第一和第二引物的尾区的核苷酸长度,而Y轴提供了测定的转变时间(以分钟计)。对于每一个尾区核苷酸长度,示出了两个相邻的条:左边的条是含有模板的反应的转变时间,而右边的条是没有模板的反应的转变时间。如图3所示,在这些反应条件下,含有具有相对较短尾区的引物的反应显示了更快的转变时间以及含模板的反应与没有模板的反应的转变时间之间的更大的分离。例如,对于16bp和20bp引物,含有具有7、8或9个核苷酸的尾区长度的引物的反应显示了比含有具有12、14或16个核苷酸的尾区长度的引物的反应更快的转变时间。

[0279] 实施例3-在可变的温度下扩增模板核酸

[0280] 使用如本文提供的方法扩增靶核酸。准备反应以测定在上文实施例1中描述的靶核酸T124A1。第一引物“RLX0892”(序列:5'TTGGGAAACCAAACCGTACCAACC 3')(SEQ ID NO:20)和第二引物“RLX0893”(序列:5'GTTTCCCAAATGCGGAATGTACC 3')(SEQ ID NO:32)用于扩增T124A1。这两种引物均具有9核苷酸尾区,RLX0892具有15核苷酸模板结合区而RLX0893具有14核苷酸模板结合区。RLX0892的尾区为:5'TTGGGAAAC 3'(SEQ ID NO:84)而RLX0893的尾区为:5'GTTTCCCAA 3'(SEQ ID NO:85)。

[0281] 制备80微升的反应混合物,每一个含有:50mM乙酸钾,20mM Tris-乙酸盐(pH 7.9),10mM乙酸镁,1mM DTT,20μg牛血清白蛋白(BSA),0.8M甜菜碱,各为1.4mM的dATP、dTTP、dGTP和dCTP,2uMSYTO®59(Life Technologies),0.8单位/μl Bst DNA聚合酶(New England BioLabs),0.016单位/μl AMV逆转录酶(New England Biolabs),1单位/μl鼠核糖核酸酶抑制剂(New England Biolabs),0.8μM的第一引物RLX0892,0.8μM的第二引物RLX0893以及每微升10,000、1,000、100或0个拷贝T124A1模板,并一式三份在52、52.7、54、55.9、58.4、60.3、61.4或62℃下在CFX 96Touch仪器(Bio-Rad)中温育100分钟。测定的转变点示于图4中。X轴提供了反应的温育温度,而Y轴提供了测定的转变时间(以分钟计)。对于各个温度,示出了4个相邻的条,从左至右为:10,000个拷贝模板/微升、1000个拷贝模板/微升、100个拷贝模板/微升或无模板对照。如图4所示,在这些反应条件下,该试验在52-62℃的整个温度范围内在1000个拷贝模板/微升下,并且在一些温度下在至少低至100个拷贝模板/微升的模板浓度下有效地扩增了模板。

[0282] 实施例4-在人基因组DNA的存在下扩增模板核酸

[0283] 使用如本文提供的方法扩增靶核酸。在人DNA的存在下准备反应以测定靶核酸T124A1(上文所述的)。使用第一引物“RLX0892”(上文所述的)和第二引物“RLX0893”(上文所述的)来扩增T124A1。

[0284] 制备160微升的反应混合物,每一个含有:50mM乙酸钾,20mM Tris-乙酸盐(pH 7.9),10mM乙酸镁,1mM DTT,20μg牛血清白蛋白(BSA),0.8M甜菜碱,各为1.4mM的dATP、dTTP、dGTP和dCTP,0.4xSYTO®59(Life Technologies),0.8单位/μl Bst DNA聚合酶

(New England BioLabs),0.016单位/μl AMV逆转录酶(New England Biolabs),1单位/μl鼠核糖核酸酶抑制剂(New England Biolabs),0.8μM的第一引物RLX0892,0.8μM的第二引物RLX0893,和每微升1,000、100或0个拷贝T124A1模板,以及0、1、2.5、5或10纳克的人基因组DNA,并在56℃下在CFX 96Touch仪器(Bio-Rad)中温育100分钟。测定的转变点示于图5中。X轴提供了反应中人基因组DNA(“hDNA”)的量,而Y轴提供了测定的转变时间(以分钟计)。对于hDNA的每个量,示出了4个相邻的条,从左至右为:1000个拷贝模板/微升、100个拷贝模板/微升或无模板。如图5所示,在这些反应条件下,该试验在至少10ng hDNA的存在下在1000个拷贝模板/微升下有效地扩增了模板。而且,反应中加入hDNA仅引起了NTC转变时间相对较小的降低,即使在5或10ng hDNA的浓度下。

[0285] 实施例5-从病毒裂解物洗脱液扩增模板核酸

[0286] 使用如本文提供的方法从含有靶标的两个不同样品中扩增靶核酸。在以下两个样品中制备靶核酸T124A1:1)含有分离的T124A1RNA分子(如在实施例1中的)的样品,以及2)含有从甲型流感H3N2病毒样品中分离的核酸的样品。T124A1是HA3基因的一部分,并因此被预期存在于从甲型流感H3N2病毒中分离的核酸的样品中。根据制造商的说明用Chemagic病毒DNA/RNA试剂盒(PerkinElmer)制备甲型流感病毒裂解物。通过qPCR定量两个不同样品中的T124A1的浓度,并且稀释样品以将样品中T124A1的浓度归一化。使用第一引物“RLX0892”和第二引物“RLX0893”扩增T124A1。

[0287] 制备25微升的反应混合物,每一个含有:50mM乙酸钾,20mM Tris-乙酸盐(pH 7.9),10mM乙酸镁,1mM DTT,20μg牛血清白蛋白(BSA),0.8M甜菜碱,各为1.4mM的dATP、dTTP、dGTP和dCTP,2uMSYTO®59(Life Technologies),0.8单位/μl Bst DNA聚合酶(New England BioLabs),0.016单位/μl AMV逆转录酶(New England Biolabs),1单位/μl鼠核糖核酸酶抑制剂(New England Biolabs),0.8μM的第一引物RLX0892,0.8μM的第二引物RLX0893以及每微升10,000、1000或0个拷贝T124A1模板,并在56℃下在CFX 96Touch仪器(Bio-Rad)中温育100分钟。测定的转变点示于图6中。X轴提供了反应中的模板拷贝数/微升,而Y轴提供了测点的转变点(以分钟计)。对于模板拷贝数/微升的各个量,示出了2个相邻的条,从左至右为:分离的T124A1和甲型流感病毒裂解物。还示出了NTC的转变点。如图6中所示,在这些反应条件下,该试验有效地扩增了分离的T124A1模板以及甲型流感病毒裂解物中的T124A1模板。

[0288] 实施例6-模板核酸的扩增

[0289] 使用如本文提供的方法扩增靶核酸。准备反应以测定T129D1的54核苷酸部分,其为乙型流感病毒血凝素(HA)基因(RNA分子)的298核苷酸部分。在SEQ ID NO:90中提供了T129D1的核苷酸序列。第一引物“A8”(核苷酸序列:5’TCTTGAGAGAACCCACTAAC 3’)(SEQ ID NO:86)和第二引物“B8”(核苷酸序列:5’TCTCAAGAATTTGGTCTTCC 3’)(SEQ ID NO:87)用于扩增T129D1。在这两种引物中,前8个核苷酸(从5’末端)是尾区,而最后的12个核苷酸是模板结合区。这些引物一起靶向T129D1的54核苷酸部分。

[0290] 制备100微升的反应混合物,其含有:50mM乙酸钾,20mM Tris-乙酸盐(pH 7.9),10mM乙酸镁,1mM DTT,20μg牛血清白蛋白(BSA),0.8M甜菜碱,各为1.4mM的dATP、dTTP、dGTP和dCTP,0.4xSYTO®59(Life Technologies),0.8单位/μl Bst DNA聚合酶(New England BioLabs),0.016单位/μl AMV逆转录酶(New England Biolabs),1单位/μl鼠核糖

核酸酶抑制剂(New England Biolabs),0.8μM的第一引物A8,0.8μM的第二引物B8以及每微升100,000T129D1模板,并在58℃下在CFX 96Touch仪器(Bio-Rad)中温育100分钟。100分钟后,从反应中取出样品并将其连接入克隆载体。使用针对于克隆位点的载体特异性引物对含有反应产物的载体进行测序。多个示例性测序反应的结果的一部分示于图7中。测序结果表明形成了具有期望的结构的多联体。特别地,在这些实例中,在反应产物的单一链中,存在来自T129D1基因的靶向的54核苷酸序列的多个拷贝,这些拷贝由A8引物的尾区的序列(5'TCTTGAGA 3')(SEQ ID NO:88)分隔开。在图7中,仅显示了T129D1的第二次(从左至右)出现的一部分;序列在图中所示的核苷酸之外继续。

[0291] 实施例7-具有与内部基序互补的尾区的引物

[0292] 为了进一步描述本文提供的实施方式,其中引物尾区含有与靶核酸内的内部基序互补的序列,现将提供具有示例性核酸序列的实例。可提供54个核苷酸总长度的多核苷酸模板("实施例7模板")。实施例7模板对应于图10的元件1000。实施例7模板的序列如下:5'TCGCTTGAACTGAGGACTTAGCGCCTAGAAACTTGGCTCAGGTAACCAACTGGT 3'(SEQ ID NO:92)。以5'至3'方向描述实施例7模板,第一下划线序列是多核苷酸模板的第二部分(例如类似于图10中提供的元件1002),第二下划线序列是内部基序(例如对应于图10中提供的元件1003),第三下划线序列是多核苷酸模板的第一部分(例如对应于图10中提供的元件1001)。实施例7模板和本文提供的其他序列的特定核苷酸可就它们在5'至3'方向读取的各自序列中的位置而被提及。因此,例如,实施例7模板的5'端的"T"在第1位置,与5'端的"T"相邻的"C"在第2位置,以此类推。可提供24个核苷酸总长度的第一引物("实施例7第一引物")。实施例7第一引物对应于图10的元件1010。实施例7第一引物的序列如下:5'CTAGAAACACCAGTTGGTTACCTG 3'(SEQ ID NO:93)。下划线序列是实施例7第一引物的第一区域/尾区(对应于图10的元件1011),而非下划线序列是实施例7第一引物的第二区域/模板结合区(对应于图10的元件1012)。还应当注意以下事实,实施例7第一引物的第一区域具有与实施例7模板的内部基序相同的核苷酸序列。实施例7第一引物可如下与实施例7模板退火:当实施例7模板和实施例7第一引物被定向为使得实施例7模板的3'端和实施例7第一引物的5'端面向相同的方向时,实施例7第一引物的非下划线序列(以5'至3'方向读取,如ACCAG…)与实施例7模板的第一部分(以3'至5'方向读取,如TGGTC...)退火。更具体地,在实施例7第一引物的第9位置的"A"与在实施例7模板的第54位置的"T"配对,在实施例7第一引物的第10位置的"C"与在实施例7模板的第53位置的"G"配对,在实施例7第一引物的第11位置的"C"与在实施例7模板的第52位置的"G"配对,等等。当实施例7第一引物与实施例7模板退火时,如本文其他地方所述,具有链置换活性的聚合酶可延伸实施例7第一引物,以生成实施例7第一引物延伸产物。实施例7第一引物延伸产物将具有5'至3'方向的核苷酸序列:5'CTAGAAACACCAGTTGGTTACCTGAGCCAAGTTTCTAGGCGCTAAGTCCTCAGTTCAAGCGA 3'(SEQ ID NO:94)。以5'至3'方向描述实施例7第一引物延伸产物,第一非下划线序列是实施例7第一引物的第一区域的序列,第一下划线序列是与实施例7模板的第一部分互补的序列,第二下划线序列是实施例7第一引物延伸产物的内部基序的序列,第三下划线序列是与实施例7模板的第二部分互补的序列。在实施方式中,当实施例7第一引物延伸产物与实施例7模板退火时,在一些情况下,实施例7第一引物延伸产物的一部分(它是实施例7第一引物的第一区域的序列)可与实施例7第一引物延伸产物的内部基序暂时退火,使得在第1位置的"C"与在实施

例7第一引物延伸产物的第38位置的“G”退火，在第2位置的“T”与在第37位置的“A”退火，在第3位置的“A”与在第36位置的“T”退火，以此类推，直到在第8位置的“C”与在第31位置的“G”退火。当实施例7第一引物延伸产物的一部分(它是实施例7第一引物的第一区域的序列)与实施例7第一引物延伸产物的内部基序的一部分暂时退火时，这些部分之间的实施例7第一引物延伸产物的部分(包括，例如，与实施例7模板的第一部分互补的序列)可变为不与实施例7模板退火，从而有助于，例如，实施例7第一引物的另一拷贝与实施例7模板的结合，以及另外的实施例7第一引物延伸产物的后续生成。

[0293] 还可提供24个核苷酸总长度的第二引物(“实施例7第二引物”)。实施例7第二引物对应于图10的元件1020。实施例7第二引物的序列如下：5’GTTTCTAGTCGCTTGAACTGAGGA 3’(SEQ ID NO:95)。下划线序列是实施例7第二引物的第一区域(对应于图10的元件1021)，而非下划线序列是实施例7第二引物的第二区域(对应于图10的元件1022)。实施例7第二引物可如下与实施例7第一引物延伸产物退火：在实施例7第二引物的第9位置的“T”与在实施例7第一引物延伸产物的第62位置的“A”配对，在实施例7第二引物的第10位置的“C”与在实施例7第一引物延伸产物的第61位置的“G”配对，在实施例7第二引物的第11位置的“G”与在实施例7第一引物延伸产物的第60位置的“C”配对，以此类推。实施例7第二引物可由如本文其他地方所述具有链置换活性的聚合酶进行延伸，以产生实施例7第二引物延伸产物。实施例7第二引物延伸产物将具有5’至3’方向的核苷酸序列：5’GTTTCTAGTCGCTTGAACTGAGGACTTAGCGCCTAGAAACTTGGCTCAGGTAACCAACTGGTGTTTCTAG 3’(SEQ ID NO:96)。以5’至3’方向描述实施例7第二引物延伸产物，第一非下划线序列是实施例7第二引物的第一区域的序列，第一下划线序列是实施例7模板的第二部分的序列，第二下划线序列是实施例7第二引物延伸产物的内部基序的序列，第三下划线序列是实施例7模板的第一部分的序列，第四非下划线序列是实施例7第二引物的第一区域的序列和与实施例7第一引物的第一区域互补的序列的序列。在实施方式中，当实施例7第二引物延伸产物与实施例7第一引物延伸产物退火时，在一些情况下，实施例7第二引物延伸产物的一部分(它是实施例7第二引物的第一区域的序列)可与实施例7第二引物延伸产物的内部基序暂时退火，使得在第1位置的“G”与在实施例7第二引物延伸产物的第40位置的“C”退火，在第2位置的“T”与在第39位置的“A”退火，在第3位置的“T”与在第38位置的“A”退火，以此类推，直到在第8位置的“G”与在第33位置的“C”退火。当实施例7第二引物延伸产物的一部分(它是实施例7第二引物的第一区域的序列)与实施例7第二引物延伸产物的内部基序暂时退火时，这些部分之间的实施例7第二引物延伸产物的部分(包括，例如，实施例7模板的第二部分的序列)可变为不与实施例7第一引物延伸产物退火，从而有助于，例如，实施例7第二引物的另一拷贝与实施例7第一引物延伸的结合，以及另外的实施例7第二引物延伸产物的后续生成。

[0294] 在实施方式中，本文其他地方提供的方法可根据以上概述的一般机理进行。本文其他地方提供的关于引物、靶核酸、反应条件、反应产物等的任何描述和细节可应用于涉及以上在实施例7中描述的一般机理的反应。

[0295] 实施例8-SNP检测

[0296] 本文提供的方法用于鉴定在靶核酸中的SNP/突变。提供了靶核酸的两种版本，它们相差一个核苷酸。该核酸模板的版本1(“版本1模板”；还被称为“野生型”序列)的第一链具有5’至3’方向的序列：GCATTCATCACGTTTTTGCGAGTCCTTTCC*A*TCCCACCAACAGCAGGGATTCTGA

AGAGATGGGGACAGTT(SEQ ID NO:97)。以5'至3'方向描述版本1模板,第一下划线序列是版本1模板的第二部分,第二下划线序列是版本1模板的内部基序,第三下划线序列是版本1模板的第一部分。该核酸模板的版本2("版本2模板";还被称为"SNP"序列)的第一链具有5'至3'方向的序列:GCATTCATCACGTTTTTGCGAGTCCTTTCC***T***TCCCACCAACAGCAGGGATTCTGAAGAGATGGGGACAGTT(SEQ ID NO:98)。以5'至3'方向描述版本2模板,第一下划线序列是版本2模板的第二部分,第二下划线序列是版本2模板的内部基序,第三下划线序列是版本2模板的第一部分。版本1模板和版本2模板序列仅在各自模板的内部基序序列中相差一个核苷酸。具体地,版本1模板在序列的第31位置具有"A"(用粗体和斜体字体强调),而版本2模板在序列的第31位置具有"T"(用粗体和斜体字体强调)。还提供了第一引物和第二引物以扩增模板序列。第一引物("实施例8引物1")具有序列:5'TCCATCCCAACTGTCCCCATCT3'(SEQ ID NO:99)。下划线序列是实施例8引物1的第一区域/尾区,而非下划线序列是实施例8引物1的第二区域/模板结合区。第二引物("实施例8引物2")具有序列:5'GGGATGGAGCATTCATCACGTTT 3'(SEQ ID NO:100)。下划线序列是实施例8引物2的第一区域/尾区,而非下划线序列是实施例8引物2的第二区域/模板结合区。

[0297] 对于版本1模板或版本2模板进行如本文其他地方所述的单独的扩增反应,版本1模板和版本2模板的反应都使用实施例8引物1和实施例8引物2作为引物。对于每个模板进行相同的条件下的多个反应。

[0298] 在版本1模板反应中,当实施例8引物1与版本1模板退火且通过聚合酶生成实施例8引物1的延伸产物("版本1模板引物1延伸产物")时,版本1模板引物1延伸产物将具有如下序列:5'TCCATCCCAACTGTCCCCATCTCTTCAGAATCCCTGCTGTTGGTGGGA***T***GGAAAGGACTCGCAAAAACGTGATGAATGC(SEQ ID NO:101)。以5'至3'方向描述版本1模板引物1延伸产物,第一非下划线序列是实施例8引物1的第一区域/尾区的序列,第一下划线序列是与版本1模板的第一部分互补的序列,第二下划线序列是版本1模板引物1延伸产物的内部基序的序列(其中黑体、斜体"T"是感兴趣的核苷酸),第三下划线序列是与版本1模板的第二部分互补的序列。根据本文所述的方法,在实施方式中,对应于实施例8引物1序列的第一区域/尾区的序列的版本1模板引物1延伸产物的部分可与版本1模板引物1延伸产物的内部基序暂时退火,使得在第1位置的"T"与在第52位置的"A"退火,在第2位置的"C"与在第51位置的"G"退火,在第3位置的"C"与在第50位置的"G"退火,以此类推,直到在第8位置的"C"与在第45位置的"G"退火。值得注意的是,对应于实施例8引物1序列的第一区域/尾区的序列的版本1模板引物1延伸产物的部分与版本1模板引物1延伸产物的内部基序序列完全互补(即在8组配对的核苷酸中的任一组之间没有错配)。

[0299] 在版本2模板反应中,当实施例8引物1与版本2模板退火且通过聚合酶生成实施例8引物1的延伸产物("版本2模板引物1延伸产物")时,版本2模板引物1延伸产物将具有如下序列:5'TCCATCCCAACTGTCCCCATCTCTTCAGAATCCCTGCTGTTGGTGGGA***A***GGAAAGGACTCGCAAAAACGTGATGAATGC(SEQ ID NO:102)。以5'至3'方向描述版本2模板延伸产物,第一非下划线序列是实施例8引物1的第一区域/尾区的序列,第一下划线序列是与版本2模板的第一部分互补的序列,第二下划线序列是版本2模板引物1延伸产物的内部基序的序列(其中黑体、斜体"A"是感兴趣的核苷酸),第三下划线序列是与版本2模板的第二部分互补的序列。根据本文所述的方法,在实施方式中,对应于实施例8引物1序列的第一区域/尾区的序列的版本2模

板引物1延伸产物的部分可与版本2模板引物1延伸产物的内部基序暂时退火，使得在第1位置的“T”与在第52位置的“A”退火，在第2位置的“C”与在第51位置的“G”退火，在第3位置的“C”与在第50位置的“G”退火，以此类推，直到在第8位置的“C”与在第45位置的“G”退火。值得注意的是，对应于实施例8引物1序列的第一区域/尾区的序列的版本2模板引物1延伸产物的部分与版本2模板引物1延伸产物的内部基序序列不完全互补，因为在第4位置的“A”与在第49位置的“A”之间存在错配。因此，通过比较版本1模板引物1延伸产物与版本2模板引物1延伸产物，与版本2模板引物1延伸产物中的内部基序相比，对应于实施例8引物1的第一区域/尾区的各自延伸产物的部分将与版本1模板引物1延伸产物中的内部基序更大互补并且将更稳定地退火。具体地，版本1模板引物1延伸产物中的序列“TCCATCCC”与版本1模板引物1延伸产物中的内部基序完全互补(8个核苷酸中的8个可正确配对)，而版本2模板引物1延伸产物中的序列“TCCATCCC”与版本2模板引物1延伸产物中的内部基序不完全互补(8个核苷酸中仅有7个可正确配对)。通常，对应于引物尾区的延伸产物的部分与该延伸产物中的内部基序之间更大的互补性导致这两个部分之间更稳定的退火。当延伸产物被配置为使得这两个部分退火时，这可加快本文提供的反应的速率，例如，通过根据本文提供的方法，允许新的引物与经由延伸产物的区域的内部退火或经由提高的多联体形成速率而暴露的互补链中未退火的核苷酸的结合。

[0300] 对于版本1模板和版本2模板引物2延伸产物还存在如上所述的类似机理，其中延伸产物的尾区的部分对于版本1模板引物2延伸产物而言完全互补，但对于版本2模板引物2延伸产物而言不完全互补。

[0301] 图11显示采用这些模板和引物的扩增反应的结果，其中X轴表示时间，Y轴表示相对荧光单位(RFU)(指示扩增的DNA)。如图11所示，采用版本1模板(“野生型”序列)的扩增反应比采用版本2模板(“SNP”序列)的反应更快发生。因此，这些结果表明，本文提供的方法可用于例如区别具有单核苷酸变异的序列。

[0302] 实施例9-SNP检测

[0303] 本文提供的方法用于鉴定在人类靶核酸中的SNP/突变。BRCA1蛋白质的Q356R变异可能与增加的癌症风险有关。例如，当在野生型BRCA1蛋白质的356氨基酸位置处编码谷氨酰胺的密码子(核苷酸序列CAG)具有核苷酸序列CGG，使得该密码子编码精氨酸而非谷氨酰胺时，可发生Q356R变异。

[0304] 获得了BRCA1基因组序列的一部分的四种不同版本，该基因组序列的部分除了其他核苷酸之外还含有356氨基酸位置的以下密码子。第一版本(“野生型靶标”)在356氨基酸位置处含有编码谷氨酰胺的野生型密码子(序列CAG)，并且具有5’至3’方向的核苷酸序列：AAGATGTTCCTTGGATAACACTAAATAGCAGCATTC***A***GAAAGTTAATGAGTGGTTTTCCAGA(SEQ ID NO:103)。第二版本(“Q356R靶标”)在356氨基酸位置处含有编码精氨酸的密码子(序列CGG)，并且具有5’至3’方向的核苷酸序列：AAGATGTTCCTTGGATAACACTAAATAGCAGCATTC***G***GAAAGTTAATGAGTGGTTTTCCAGA(SEQ ID NO:104)。第三版本(“Q356P靶标”)在356氨基酸位置处含有编码脯氨酸的密码子(序列CCG)，并且具有5’至3’方向的核苷酸序列：AAGATGTTCCTTGGATAACACTAAATAGCAGCATTC***C***GAAAGTTAATGAGTGGTTTTCCAGA(SEQ ID NO:105)。第四版本(“Q356L靶标”)在356氨基酸位置处含有编码亮氨酸的密码子(序列CTG)，并且具有5’至3’方向的核苷酸序列：AAGATGTTCCTTGGATAACACTAAATAGCAGCATTC***T***

GAAAGTTAATGAGTGGTTTTCCAGA(SEQ ID NO:106)。在以上四个靶标的每一个中,在5'至3'方向上,第一下划线序列是靶标的第二部分,第二下划线序列是靶标的内部基序,第三下划线序列是靶标的第一部分。

[0305] 以上四个靶标序列仅在各自靶标的内部基序中相差一个核苷酸。具体地,野生型靶标在序列的第37位置具有"A"(用粗体和斜体字体强调),而Q356R靶标在序列的第37位置具有"G",Q356P靶标在序列的第37位置具有"C",而Q356L靶标在序列的第37位置具有"T"。还提供了第一引物和第二引物以扩增靶标序列。第一引物("实施例9引物1")具有序列:5'ATTCAGAATCTGGAAAACCACTC 3'(SEQ ID NO:107)。下划线序列是实施例9引物1的第一区域/尾区,而非下划线序列是实施例9引物1的第二区域/模板结合区。第二引物("实施例9引物2")具有序列:5'TTCTGAATAAGATGTTCCTTGGAT 3'(SEQ ID NO:108)。下划线序列是实施例9引物2的第一区域/尾区,而非下划线序列是实施例9引物2的第二区域/模板结合区。

[0306] 对于以上四个靶标中的每一个进行如本文所述的单独的扩增反应,全部四个靶标的反应均使用实施例9引物1和实施例9引物2作为引物。反应的条件如下:50mM乙酸钾,20mM Tris-乙酸盐,pH 7.9,10mM乙酸镁,1mM DTT,0.8M甜菜碱,2uMSYTO®59(Life Technologies),0.8单位/μl Bst DNA聚合酶(New England BioLabs),0.8μM实施例9引物1,0.8μM实施例9引物2,和10个拷贝的靶分子/μl。将反应在56℃下保持90分钟,并采用CFX Manager软件(Bio-Rad)使用单阈值方法确定每个反应的拐点。对于每个靶标进行相同的条件下的多个反应。

[0307] 所述扩增反应根据本文其他地方,例如,如实施例8中所述的机理而发生。例如,在每个各自的反应中,实施例9引物1和实施例9引物2延伸产物由以上每个靶标核酸形成,以形成例如野生型靶标实施例9引物1延伸产物,Q356R靶标实施例9引物1延伸产物,Q356P靶标实施例9引物1延伸产物和Q356L靶标实施例9引物1延伸产物,以及由实施例9引物2从每个靶标延伸的相应的延伸产物。

[0308] 值得注意的是,例如,对应于实施例9引物1的第一区域/尾区的野生型靶标实施例9引物1延伸产物的部分与野生型靶标实施例9引物1延伸产物中的内部基序完全互补,而对应于针对Q356R靶标、Q356P靶标和Q356L靶标引物1延伸产物中每一个的实施例9引物1的第一区域/尾区的延伸产物的部分与各自延伸产物中的内部基序不完全互补。具体地,如上所述,在涉及靶序列的第37位置的核苷酸的位置处具有单核苷酸错配。该错配可能影响本文提供的反应的速率。

[0309] 采用每一个不同靶标进行的测定的平均拐点时间如下:野生型靶标:24.5分钟;Q356R靶标:49.5分钟;Q356P靶标:55.7分钟,Q356L靶标:59.8分钟。因此,如所述测定的拐点时间所示,所述反应可容易地区分在内部基序中相差一个核苷酸的靶标。与其他靶标相比,采用野生型靶标进行的测定的显著更快的拐点时间可能是对应于实施例9引物1的第一区域/尾区的野生型靶标实施例9引物1延伸产物的部分与野生型靶标实施例9引物1延伸产物中的内部基序之间完全互补的结果,这不同于对应于实施例9引物1的第一区域/尾区的Q356R靶标、Q356P靶标和Q356L靶标实施例9引物1延伸产物的部分与各自的实施例9引物1延伸产物中的内部基序之间的不完全互补。类似的推论还适用于引物2延伸产物的延伸产物。

[0310] 实施例10-流感N9基因扩增

[0311] 使用本文提供的方法从含有甲型流感病毒亚型H7N9的样品中扩增神经氨酸酶基因(N9)的一部分。在5'至3'方向上,来自N9的靶序列为(DNA形式):AACTTTAAGGACGAACAAGAGAGAAGGTAAACCTACAACCACTACAAACAAAGTAATATTATCAACAAATAATAACAAACCGAACACAACCAACAAAGT(SEQ ID NO:109)。用于扩增所述模板的第一引物具有5'至3'方向的序列:CTTCCATTTGAAACAACCAACAC(SEQ ID NO:110)。描述第一引物,下划线区域是该引物的尾区/第一区域,非下划线区域是第二区域/模板结合区。用于扩增所述模板的第二引物具有5'至3'方向的序列:AATGGAAGTTGAAATTCCTGCTTG(SEQ ID NO:111)。描述第二引物,下划线区域是该引物的尾区/第一区域,非下划线区域是第二区域/模板结合区。如本文所述的多个反应使用第一引物和第二引物进行,其中所述反应含有模板序列或不含模板序列。含有所述模板的反应具有每一反应250,000个拷贝模板序列。所述反应的结果提供于图12中。X轴上的数字为分钟,Y轴值为相对荧光单位(RFU)。如图所示,在模板化反应中,RFU在约20分钟时快速升高,这表明模板序列的扩增。在无模板反应中约60分钟时及以后的RFU增加是非特异性背景产物的结果。

[0312] 除非另外指出,本文提供的核苷酸和氨基酸序列是人工序列。

[0313] 尽管本文中已经示出并描述了本发明的优选实施方式,但对于本领域技术人员显而易见的是,这些实施方式仅以示例的方式提供。前述的描述并非详尽的或将本发明限于所公开的精确的实施方式,并且根据以上教导在不脱离本发明的情况下其他的修改和变化是可能的。无论优选与否的任何特征均可与无论优选与否的任何其他特征相组合。还应当理解,虽然出于方便的目的,本文中已经使用有限数目的术语和短语描述了本文提供的发明,但本发明也可以使用本文未提供的同样准确描述本发明的其他术语和短语进行描述。所附权利要求书不应被解释为包括装置加功能的限定,除非这样的限定在给定的权利要求中使用短语"用于…的装置"而被明确阐述。应当理解,如此处的说明书和随后的权利要求书全文中所使用,"一个"、"一种"和"该"的含义包括复数指代对象,除非上下文另有明确规定。例如,提及"测定"可以指一个测定或多个测定。此外,如此处的说明书和随后的权利要求书全文中所使用,"之中"的含义包括"之中"和"之上",除非上下文另有明确规定。如在此处的说明书和随后的整个权利要求书中所用的,描述为含有第二个对象的"至少一部分"的第一对象可含有所有量的/完整的第二对象。如在此处的说明书和随后的整个权利要求书中所用的,术语"包含"、"包括"和"含有"以及相关的时态是包含在内且开放式的,并且不排除附加的、未叙述的要素或方法步骤。而且,在一些情况下,拓宽词语和短语诸如"一个或多个(一种或多种)"、"至少"、"但不限于"或其他类似的短语的存在不应被理解为在这类拓宽短语可能不存在的情况下意味着较窄的情况是有意或需要的。最后,如此处的说明书和随后的权利要求书全文中所使用,"或"的含义同时包括结合的和分离的,除非上下文另有明确规定。因此,除非上下文另外明确规定,术语"或"包括"和/或"。

[0314] 本文档包含受到版权保护的材料。版权所有者(本文的申请人)不反对对专利文件或专利公开内容中的任何一个的摹本复制,因为它们出现于美国专利商标局专利文件或记录中,但除此之外保留任何所有的版权。以下声明应当适用:Copyright 2013-14Theranos, Inc。

[0001]

序列表

SEQ ID NO: 1: AACGGTTGCTC

SEQ ID NO: 2: GAGCAACCGTT

SEQ ID NO: 3: ATGGGAGC

SEQ ID NO: 4: CCATAACG

SEQ ID NO: 5: ATGGGAGCAACCGTT

SEQ ID NO: 6: CCATAACGGTTGCTCCCAT

SEQ ID NO: 7: ATGGGAGCAACCGTTATGG

SEQ ID NO: 8: CCATAACGGTTGCTCCCATAACGGTTGCTCCCAT

SEQ ID NO: 9: ATGGGAGCAACCGTTATGGGAGCAACCGTTATGG

SEQ ID NO: 10:
CGCCGGATGGCTCTTGGGAAACCAAACCGTACCAACC

SEQ ID NO: 11:
CCGGATGGCTCTTGGGAAACCAAACCGTACCAACC

SEQ ID NO: 12: GGATGGCTCTTGGGAAACCAAACCGTACCAACC

SEQ ID NO: 13: ATGGCTCTTGGGAAACCAAACCGTACCAACC

SEQ ID NO: 14: TGGCTCTTGGGAAACCAAACCGTACCAACC

SEQ ID NO: 15: GGCTCTTGGGAAACCAAACCGTACCAACC

SEQ ID NO: 16: GCTCTTGGGAAACCAAACCGTACCAACC

SEQ ID NO: 17: CTCTTGGGAAACCAAACCGTACCAACC

SEQ ID NO: 18: TCTTGGGAAACCAAACCGTACCAACC

SEQ ID NO: 19: CTTGGGAAACCAAACCGTACCAACC

SEQ ID NO: 20: TTGGGAAACCAAACCGTACCAACC

SEQ ID NO: 21: TGGGAAACCAAACCGTACCAACC

SEQ ID NO: 22:
GTTTCCCAAGAGCCATCCGGCGATGCGGAATGTACC

SEQ ID NO: 23: GTTTCCCAAGAGCCATCCGGATGCGGAATGTACC

SEQ ID NO: 24: GTTTCCCAAGAGCCATCCATGCGGAATGTACC

SEQ ID NO: 25: GTTTCCCAAGAGCCATATGCGGAATGTACC

SEQ ID NO: 26: GTTTCCCAAGAGCCAATGCGGAATGTACC

SEQ ID NO: 27: GTTTCCCAAGAGCCATGCGGAATGTACC

SEQ ID NO: 28: GTTTCCCAAGAGCATGCGGAATGTACC

SEQ ID NO: 29: GTTTCCCAAGAGATGCGGAATGTACC

SEQ ID NO: 30: GTTTCCCAAGAATGCGGAATGTACC

SEQ ID NO: 31: GTTTCCCAAGATGCGGAATGTACC

SEQ ID NO: 32: GTTTCCCAAATGCGGAATGTACC

SEQ ID NO: 33: GTTTCCCAATGCGGAATGTACC

SEQ ID NO: 34: ATGGCTCTTGGGAAACTGAAACCGTACCAACC

SEQ ID NO: 35: GTTTCCCAAGAGCCATGGATGCGGAATGTACC

SEQ ID NO: 36: GGCTCTTGGGAAACTGAAACCGTACCAACC

SEQ ID NO: 37: GTTTCCCAAGAGCCGGATGCGGAATGTACC

SEQ ID NO: 38: CTCTTGGGAAACTGAAACCGTACCAACC

[0002] SEQ ID NO: 39: GTTTCCCAAGAGGGATGCGGAATGTACC

SEQ ID NO: 40: TCTTGGGAAACTGAAACCGTACCAACC

SEQ ID NO: 41: GTTTCCCAAGAGGATGCGGAATGTACC

SEQ ID NO: 42: CTTGGGAAACTGAAACCGTACCAACC

SEQ ID NO: 43: GTTTCCCAAGGGATGCGGAATGTACC

SEQ ID NO: 44: TTGGGAAACTGAAACCGTACCAACC

SEQ ID NO: 45: GTTTCCCAAGGATGCGGAATGTACC

SEQ ID NO: 46: TGGGAAACTGAAACCGTACCAACC

SEQ ID NO: 47: GTTTCCCAGGATGCGGAATGTACC

SEQ ID NO: 48: GGGAAACTGAAACCGTACCAACC

SEQ ID NO: 49: GTTTCCCGGATGCGGAATGTACC

SEQ ID NO: 50:

ATGGCTCTTGGGAAACTGCCTGAAACCGTACCAACC

SEQ ID NO: 51:

GTTTCCCAAGAGCCATACAGGGATGCGGAATGTACC

[0003]

SEQ ID NO: 52: GGCTCTTGGGAAACTGCCTGAAACCGTACCAACC

SEQ ID NO: 53: GTTTCCCAAGAGCCACAGGGATGCGGAATGTACC

SEQ ID NO: 54: CTCTTGGGAAACTGCCTGAAACCGTACCAACC

SEQ ID NO: 55: GTTTCCCAAGAGACAGGGATGCGGAATGTACC

SEQ ID NO: 56: TCTTGGGAAACTGCCTGAAACCGTACCAACC

SEQ ID NO: 57: GTTTCCCAAGAACAGGGATGCGGAATGTACC

SEQ ID NO: 58: CTTGGGAAACTGCCTGAAACCGTACCAACC

SEQ ID NO: 59: GTTTCCCAAGACAGGGATGCGGAATGTACC

SEQ ID NO: 60: TTGGGAAACTGCCTGAAACCGTACCAACC

SEQ ID NO: 61: GTTTCCCAAACAGGGATGCGGAATGTACC

SEQ ID NO: 62: TGGGAAACTGCCTGAAACCGTACCAACC

SEQ ID NO: 63: GTTTCCCAACAGGGATGCGGAATGTACC

SEQ ID NO: 64: GGGAAACTGCCTGAAACCGTACCAACC

SEQ ID NO: 65: GTTTCCCACAGGGATGCGGAATGTACC

SEQ ID NO: 66:
TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACA
GAAGGGGAAGACCAAATTCTTGAGA

SEQ ID NO: 67:
TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACA
GAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAA
GTACCATACATTT

SEQ ID NO: 68:
TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACA
GAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAA
GTACCATACATTT

SEQ ID NO: 69:
TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACA
GAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAA
GTACCATACATTT

[0004]

SEQ ID NO: 70:

TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACT
GAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAA
GTACCATACATTT

SEQ ID NO: 71:

TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACA
GAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAA
GTACCATACATTT

SEQ ID NO: 72:

TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACA
GAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAA
GTACCATACATTT

SEQ ID NO: 73:

TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACA
GAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAA
GTACCATACATTT

SEQ ID NO: 74:

TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACA
GAAGGGGAAGACCAAATTCTTGAGAGAACCCACTAACAGTAGAA
GTACCATACATTT

SEQ ID NO: 75:

TCTTGAGAGAACCCACTAACAGTAGAAGTACCATACATTTGTACA
GAAGGGGAAGACCAATTCTTGAGAGAACCCACTAACAGTAGAAG
TACCATACATTT

SEQ ID NO: 76:

GAACCCACTAACAGTAGAAGTACCATACATTTGTACAGAAGGGG
AAGACCAAATGAACCCACTAACAGTAGAAGTACCATACATTT

SEQ ID NO: 77:

TCTTGAGAGAACCCACTAACTCTTGAGAGAACCCACTAAC

[0005]

SEQ ID NO: 78: GGAAGACCAAATTCTTGAGA

SEQ ID NO: 79:

MGHHHHHHHHHHSSGHIEGRASADGPYLQILEQPKQRGFRFRYVCE
GPSHGGLPGASSEKNKKSYPQVKICNYVGPAKVIVQLVTNGKNIHLH
AHSLVGKHCEDGICTVTAGPKDMVVGFANLGILHVTKKKVFETLEA
RMTEACIRGYNPGLLVHPDLAYLQAEGGGDRQLGDREKELIRQAAL
QQTKEMDLSVVRLMFTAFLPDSTGSFTRRLEPVVSDAIYDSKAPNAS
NLKIVRMDRTAGCVTGGEEIYLLCDKVQKDDIQIRFYEEEENGGVW
EGFGDFSPTDVHRQFAIVFKTPKYKDINITKPASVFVQLRRKSDLETS
EPKPFLYYPEIKDKEEVQRKRQKGSSGTSGGGSGGGMTLEEARKRV
NELRDLIRYHNYRYYVLADPEISDAEYDRLLRELKELEERFPELKSPD
SPTLQVGARPLEATFRPVRHPTRMYSLDNAFNLDELKAFEERIERAL
GRKGPFAYTVEHKVDGLSVNLYYEEGVLVYGATRGDGEVGEEVTQ
NLLTIPTIPRRLKGVPERLEVRGEVYMPIEAFLRLNEELEERGERIFKN
PRNAAAGSLRQKDPRITAKRGLRATFYALGLGLEEVEREGVATQFAL
LHWLKEKGFPVEHGYARAVGAEGVEAVYQDWLKKRRALPFEADG
VVVKLDELALWRELGYTARAPRFAIAYKFPAEEKETRLLDVVFQVG
RTGRVTPVGILEPVFLEGSEVSRVTLHNESYIEELDIRIGDWVLVHKA
GGVIPEVLRVLKERRTGEERPIRWPETCPECGHRLLKEGKVHRCPNP
LCPAKRFEAIRHFASRKAMDIQGLGEKLIERLLEKGLVKDVADLYRL
RKEDLVGLERMGEKSAQNLLRQIEESKKRGLERLLYALGLPGVGEV
LARNLAARFGNMDRLLEASLEELLEVEEVGELTARAILETLKDPAFR
DLVRRLKEAGVEMEAKEKGGEALKGLTFVITGELSRPREEVKALLR
RLGAKVTDSVSRKTSYLVVGENPGSKLEKARALGVPTLTEEELYRLL
EARTGKKAEELV

SEQ ID NO: 80: CAAACCGTACCAACC

SEQ ID NO: 81: ATGCGGAATGTACC

SEQ ID NO: 82: CGCCGGATGGCTCTTGGGAAAC

SEQ ID NO: 83: GTTTCCCAAGAGCCATCCGGCG

[0006]

SEQ ID NO: 84: TTGGGAAAC

SEQ ID NO: 85: GTTTCCCAA

SEQ ID NO: 86: TCTTGAGAGAACCCACTAAC

SEQ ID NO: 87: TCTCAAGAATTTGGTCTTCC

SEQ ID NO: 88: TCTTGAGA

SEQ ID NO: 89:

UCAGCAUUUUCCCUCAGUUGCCUCCUUGUUUUUUCAAACAGUU
UGUUCAUUUCCGAGUCAGUCAGAUCAAUUGUAUGUUGGUUCUC
CAGAGCGACAAGAAGCUCCGCAUUGUAAGACCAGAGAUCUAUU
UUAGUGUCUUCAACGUAUUUCUCGAGGUCCUGAAUUCUCCCUU
CUACUUCUGAGAAUUCCUUUUCGAUUUGAUGGAAUUUCUCGUU
CGUCUUCUCGAUUACCCUAUUCAGUUUCCCAUUGAUUUGGUCG
AUGGCUGCUUGAGUGCUUUUAAGAUCUGCUGCUUGUCCUGUGC
CCUCGGAAUUUUGAUGCCUGAAACCGUACCAACCGUCUAUCAU
UCCCUCCCAACCAUUUUCUAUGAAACCUGCUAUUGCGCCGAAUA
UGCCUCUAGUUUGUUUCUCUGGUACAUUCCGCAUCCCUGUUGC
CAACUUCAGAGUGUUUUGCUUAACAUACUUGGG

SEQ ID NO: 90:

GUCCUGGUGUUCACCAUAAGGUGUUACUGUGUCUUGGGAGCAC
CGCGACCUUUCGCAACAAUGGCUUGGGCUGUCCCAAAGGACAA
CAACAAAAAUGCAACGAACCCACUAACAGUAGAAGUACCAUAC
AUUUGUACAGAAGGGGAAGACCAAAUCACUGUUUGGGGGUUCC
AUUCAGAUGACAAAACCCAAAUGAAGAACCUCUAUGGAGACUC
AAAUCCUCAAAAGUUCACCUCAUCUGCUAAUGGUUUUGCCGAC
UGAAGGGACACCCCCAGGGUCCUUGGGGGCAGUCAGGGAG

SEQ ID NO: 91:

AACCTCACTACGTAAGTCTTGACGTAAACGGTTACCCACGTAGAC
TAGAGTAATAACTCGAAAAGGGT

SEQ ID NO: 92:

[0007]

TCGCTTGAACTGAGGACTTAGCGCCTAGAAACTTGGCTCAGGTAA
CCAACTGGT

SEQ ID NO: 93: CTAGAAACACCAGTTGGTTACCTG

SEQ ID NO: 94:
CTAGAAACACCAGTTGGTTACCTGAGCCAAGTTTCTAGGCGCTAA
GTCCTCAGTTCAAGCGA

SEQ ID NO: 95: GTTTCTAGTCGCTTGAACTGAGGA

SEQ ID NO: 96:
GTTTCTAGTCGCTTGAACTGAGGACTTAGCGCCTAGAAACTTGGC
TCAGGTAACCAACTGGTGTTTCTAG

SEQ ID NO: 97:
GCATTCATCACGTTTTTGCGAGTCCTTTCCATCCCACCAACAGCAG
GGATTCTGAAGAGATGGGGACAGTT

SEQ ID NO: 98:
GCATTCATCACGTTTTTGCGAGTCCTTTCCTTCCCACCAACAGCAG
GGATTCTGAAGAGATGGGGACAGTT

SEQ ID NO: 99: TCCATCCCAACTGTCCCCATCT

SEQ ID NO: 100: GGGATGGAGCATTCATCACGTTT

SEQ ID NO: 101:
TCCATCCCAACTGTCCCCATCTCTTCAGAATCCCTGCTGTTGGTGG
GATGGAAAGGACTCGCAAAAACGTGATGAATGC

SEQ ID NO: 102:
TCCATCCCAACTGTCCCCATCTCTTCAGAATCCCTGCTGTTGGTGG
GAAGGAAAGGACTCGCAAAAACGTGATGAATGC

SEQ ID NO: 103:
AAGATGTTCCTTGGATAACACTAAATAGCAGCATTCAGAAAGTTA
ATGAGTGGTTTTCCAGA

SEQ ID NO: 104:
AAGATGTTCCTTGGATAACACTAAATAGCAGCATTCGGAAAGTTA

[0008]

ATGAGTGGTTTTCCAGA

SEQ ID NO: 105:

AAGATGTTCCTTGGATAACACTAAATAGCAGCATTCCGAAAGTTA

ATGAGTGGTTTTCCAGA

SEQ ID NO: 106:

AAGATGTTCCTTGGATAACACTAAATAGCAGCATTCTGAAAGTTA

ATGAGTGGTTTTCCAGA

SEQ ID NO: 107: ATTCAGAATCTGGAAAACCACTC

SEQ ID NO: 108: TTCTGAATAAGATGTTCCTTGGAT

SEQ ID NO: 109:

AACTTTAAGGACGAACAAGAGAGAAGGTAAACCTACAACCACTA

CAAACAAAGTAATATTATCAACAAATAATAACAAACCGAACACA

ACCAACAAAGT

SEQ ID NO: 110: CTTCCATTTGAAACAACCAACAC

SEQ ID NO: 111: AATGGAAGTTGAAATTCCTGCTTG

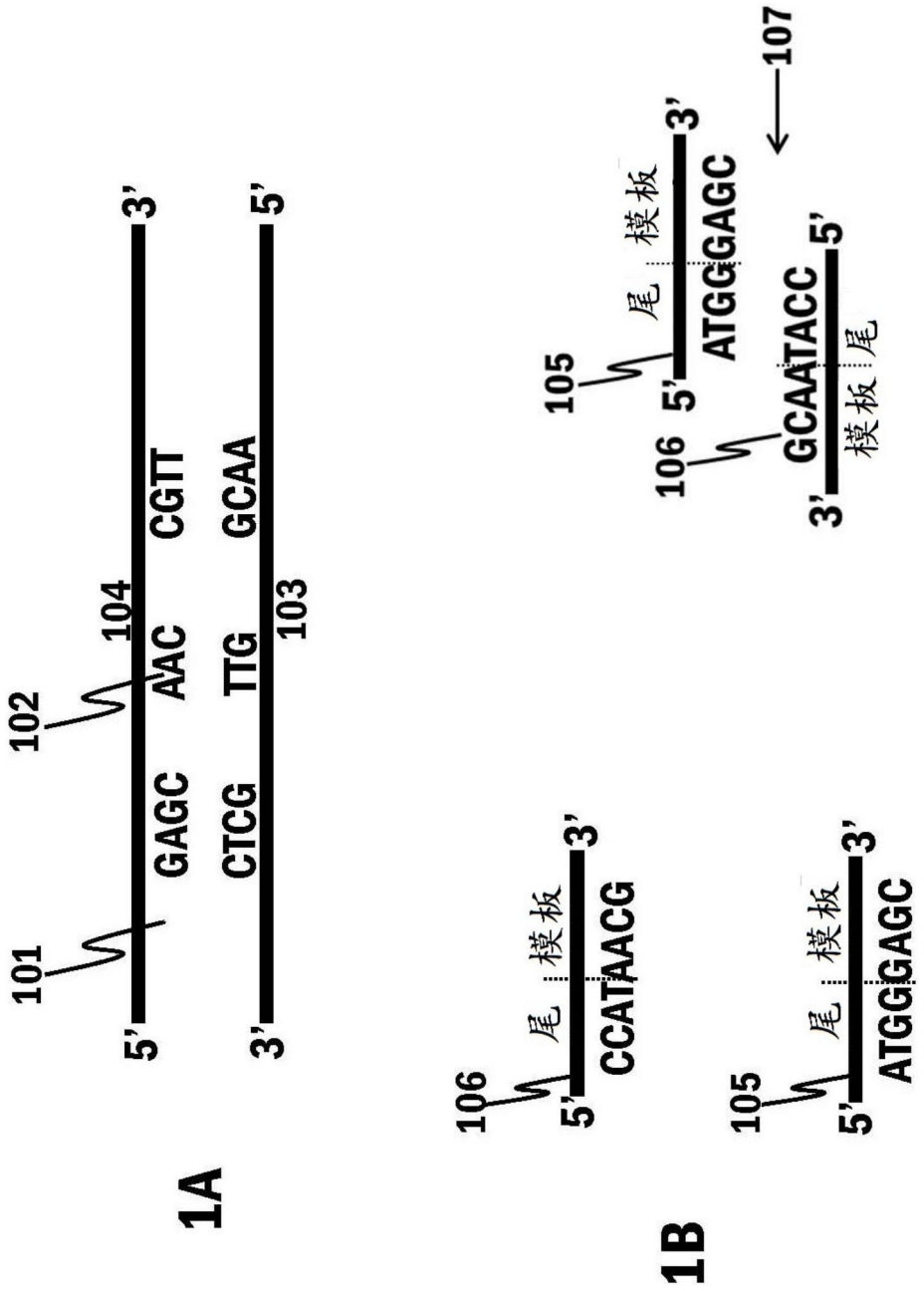

1A
101
102
104
103
5'
3'
GAGC
AAC
CGTT
CTCG
TTG
GCAA
1B
106
105
107
尾
模板
CCATAACG
ATGGGAGC
GCAATACC

图1A和图1B

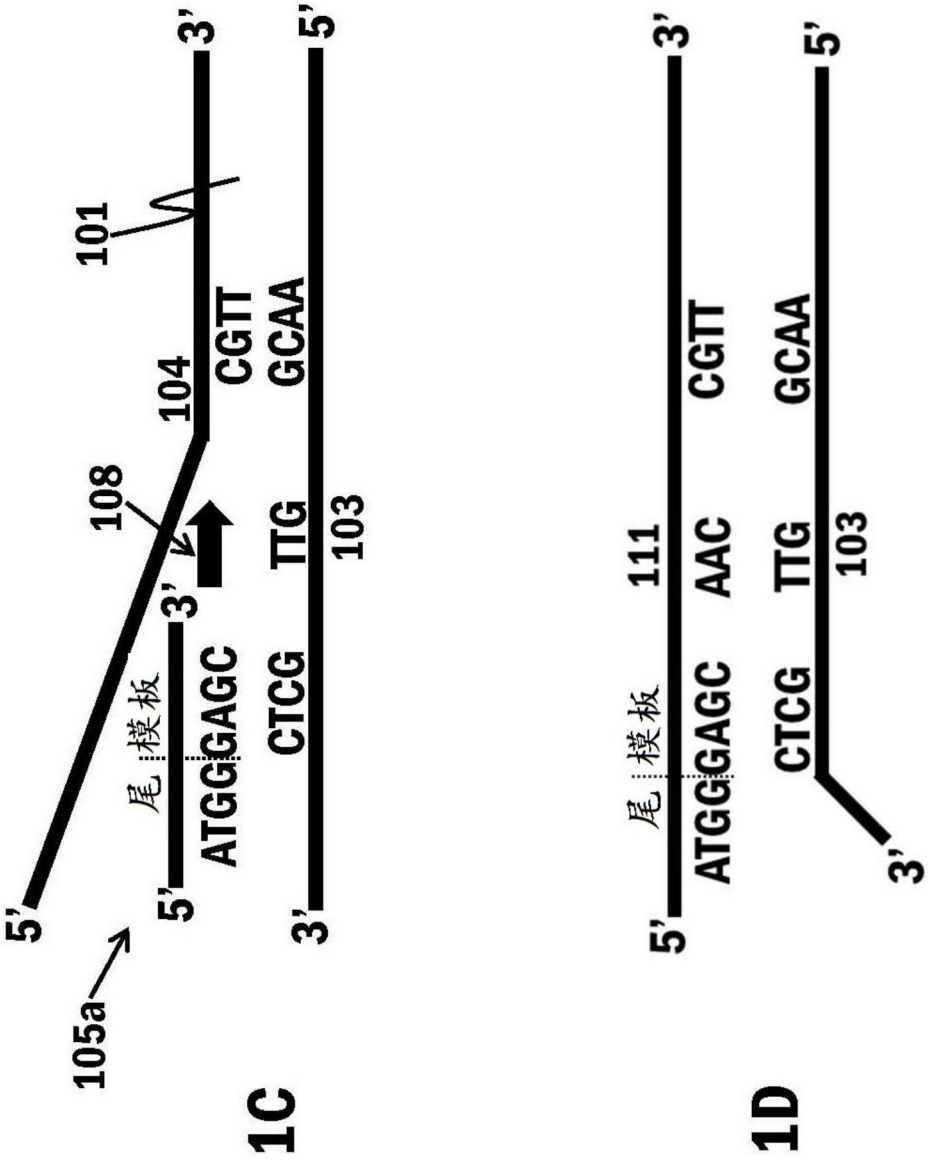

5'
105a
5'
尾
模板
ATGGGAGC
3'
108
104
101
3'
CTCG
TTG
CGTT
GCAA
5'
103
3'
1C
5'
尾
模板
ATGGGAGC
111
AAC
3'
CTCG
TTG
CGTT
GCAA
5'
103
3'
1D

图1C和图1D

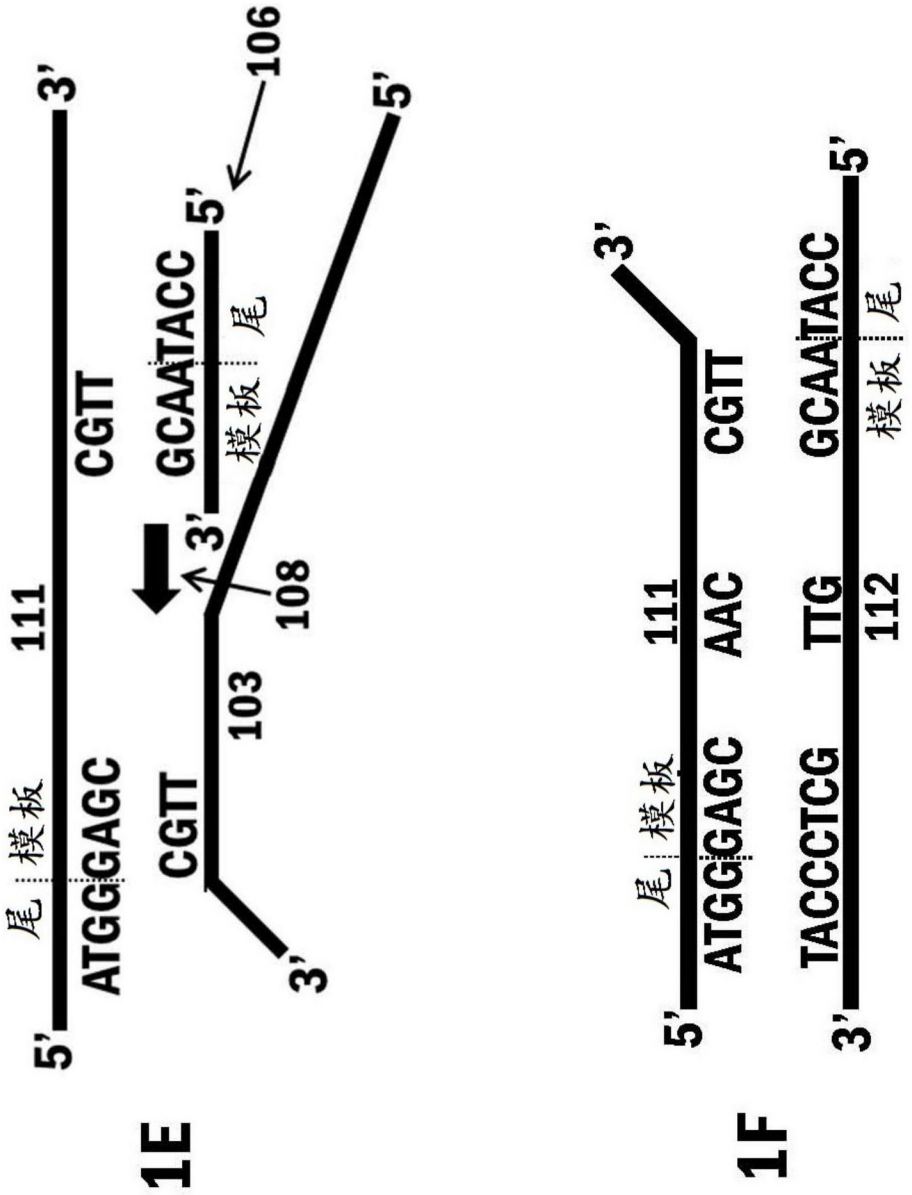

5'
尾
模板
ATGGGAGC
111
3'
CGTT
CGTT
3'
103
108
3'
5'
GCAATACC
尾
模板
106
5'
1E
5'
ATGGGAGC
尾
模板
111
AAC
CGTT
3'
3'
TACCCTCG
TTG
112
GCAATACC
尾
模板
5'
1F

图1E和图1F

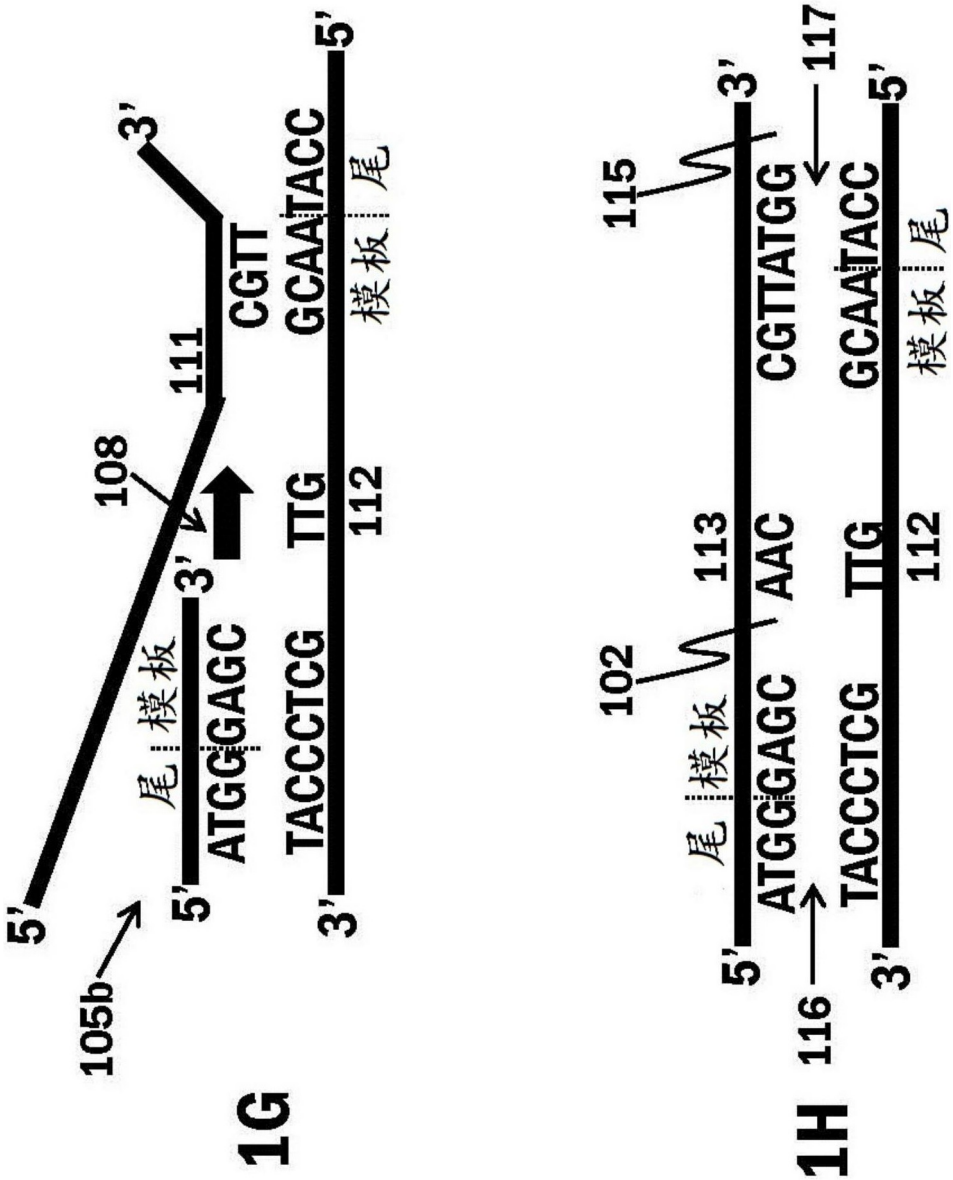

5'
105b
108
3'
111
5'
尾
模板
ATGGGAGC
3'
CGTT
TACCCTCG
TTG
GCAATACC
112
5'
模板
尾
3'
1G
5'
102
115
3'
117
尾
模板
ATGGGAGC
113
AAC
CGTTATGG
TACCCTCG
TTG
GCAATACC
112
5'
模板
尾
3'
116
1H

图1G和图1H

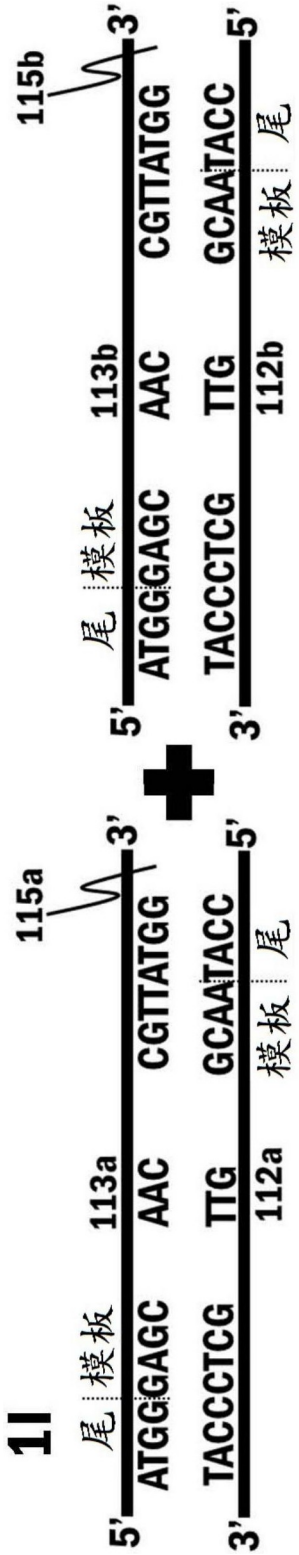
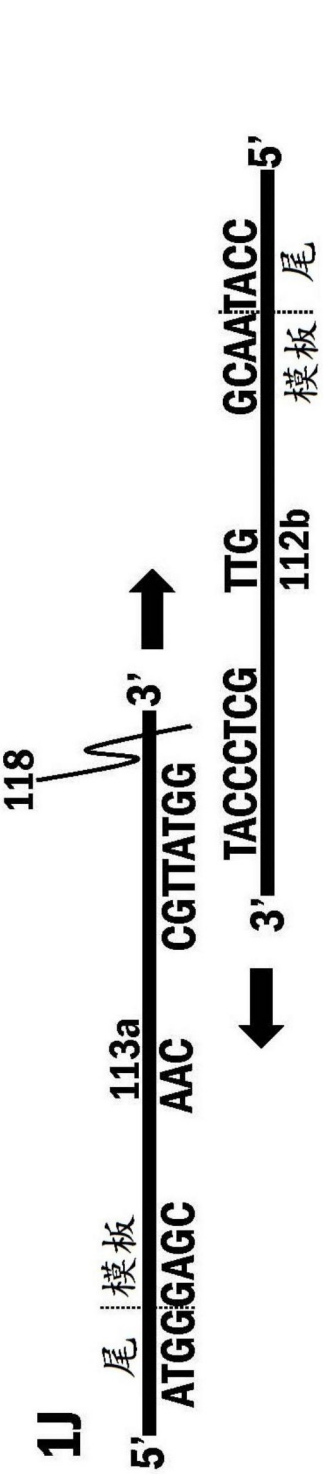

1I
115a
115b
113a
113b
112a
112b
尾
模板
ATGGGAGC
AAC
CGTTATGG
TACCCTCG
TTG
GCAATACC
5'
3'

图1I和图1J


1J
118
113a
112b
尾
模板
ATGGGAGC
AAC
CGTTATGG
TACCCTCG
TTG
GCAATACC
5'
3'

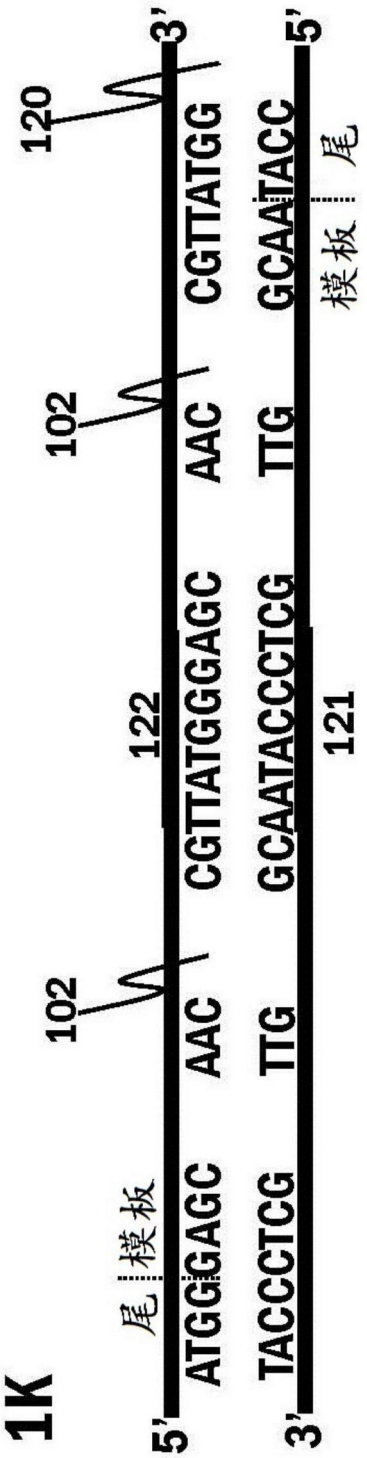

1K
120
102
122
121
尾
模板
ATGGGAGC
AAC
CGTTATGGGAGC
CGTTATGG
TACCCTCG
TTG
GCAATACCCTCG
GCAATACC
5'
3'

图1K

2A

T-引物 1(P1)	长度	尾长度	T*-引物 2(P2)	长度	尾长度
CGCCGGATGGCTCTTGGGAAACCAAACCGTACCAACC	37	22	GTTTCCCAAGAGCCATCCGGCGATGCGGAATGTACC	36	22
CCGGATGGCTCTTGGGAAACCAAACCGTACCAACC	35	20	GTTTCCCAAGAGCCATCCGGATGCGGAATGTACC	34	20
GGATGGCTCTTGGGAAACCAAACCGTACCAACC	33	18	GTTTCCCAAGAGCCATCCATGCGGAATGTACC	32	18
ATGGCTCTTGGGAAACCAAACCGTACCAACC	31	16	GTTTCCCAAGAGCCATATGCGGAATGTACC	30	16
TGGCTCTTGGGAAACCAAACCGTACCAACC	30	15	GTTTCCCAAGAGCCAATGCGGAATGTACC	29	15
GGCTCTTGGGAAACCAAACCGTACCAACC	29	14	GTTTCCCAAGAGCCATGCGGAATGTACC	28	14
GCTCTTGGGAAACCAAACCGTACCAACC	28	13	GTTTCCCAAGAGCATGCGGAATGTACC	27	13
CTCTTGGGAAACCAAACCGTACCAACC	27	12	GTTTCCCAAGAGATGCGGAATGTACC	26	12
TCTTGGGAAACCAAACCGTACCAACC	26	11	GTTTCCCAAGAATGCGGAATGTACC	25	11
CTTGGGAAACCAAACCGTACCAACC	25	10	GTTTCCCAAGATGCGGAATGTACC	24	10
TTGGGAAACCAAACCGTACCAACC	24	9	GTTTCCCAAATGCGGAATGTACC	23	9
TGGGAAACCAAACCGTACCAACC	23	8	GTTTCCCAATGCGGAATGTACC	22	8

图2A

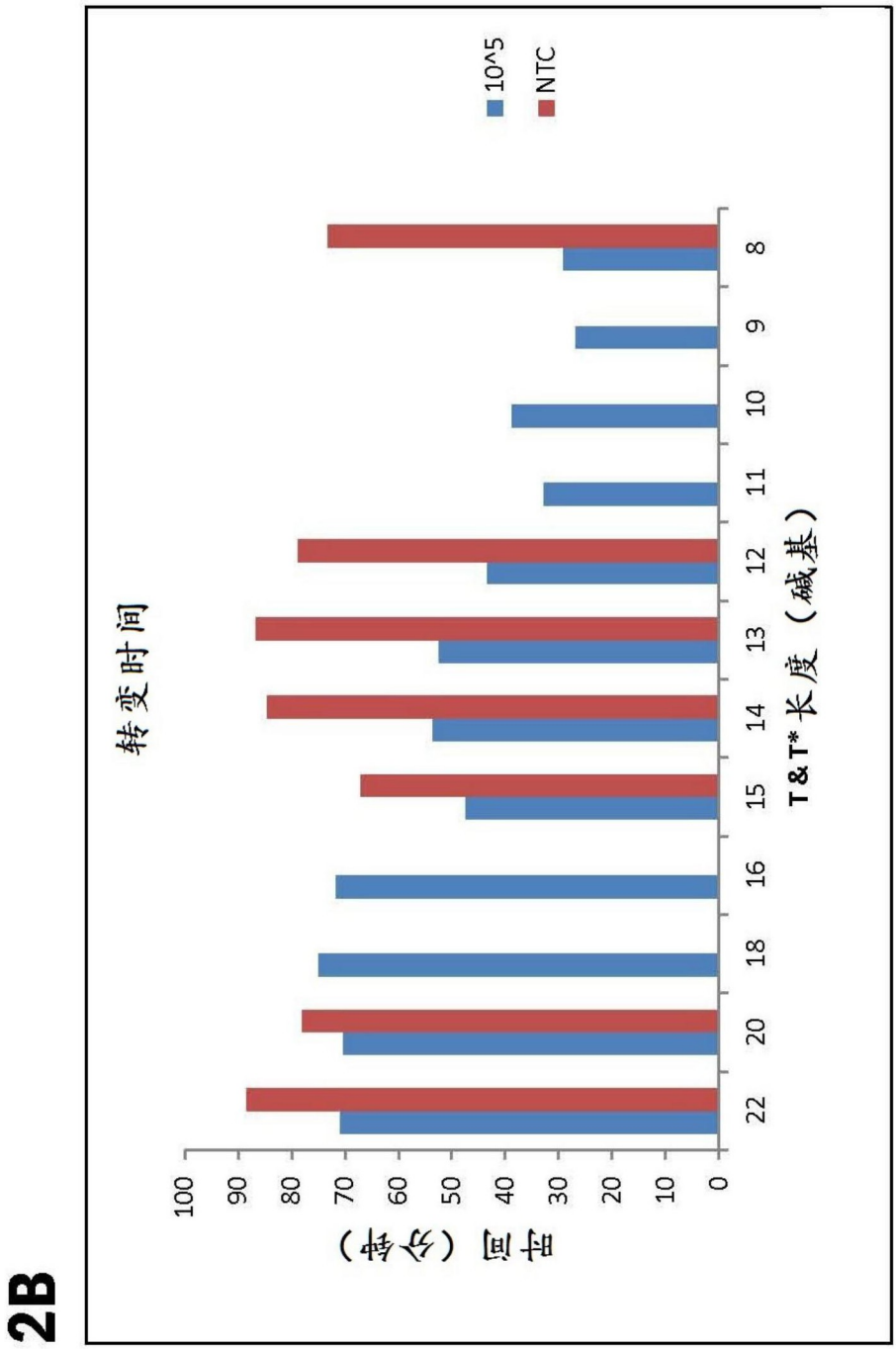

2B
转变时间
时间（分钟）
100
90
80
70
60
50
40
30
20
10
0
22
20
18
16
15
14
13
12
11
10
9
8
T & T* 长度（碱基）
10^5
NTC

图2B

3A

	尾		引物 (16nt)	
C5	ATGGCTCTTGGGAAAC		TGAAACCGTACCAACC	正向引物
D5	GTTTCCCAAGAGCCAT		GGATGCGGAATGTACC	反向引物
C6	GGCTCTTGGGAAAC		TGAAACCGTACCAACC	正向引物
D6	GTTTCCCAAGAGCC		GGATGCGGAATGTACC	反向引物
C7	CTCTTGGGAAAC		TGAAACCGTACCAACC	正向引物
D7	GTTTCCCAAGAG		GGATGCGGAATGTACC	反向引物
C8	TCTTGGGAAAC		TGAAACCGTACCAACC	正向引物
D8	GTTTCCCAAGA		GGATGCGGAATGTACC	反向引物
C9	CTTGGGAAAC		TGAAACCGTACCAACC	正向引物
D9	GTTTCCCAAG		GGATGCGGAATGTACC	反向引物
C10	TTGGGAAAC		TGAAACCGTACCAACC	正向引物
D10	GTTTCCCAA		GGATGCGGAATGTACC	反向引物
C11	TGGGAAAC		TGAAACCGTACCAACC	正向引物
D11	GTTTCCCA		GGATGCGGAATGTACC	反向引物
C12	GGGAAAC		TGAAACCGTACCAACC	正向引物
D12	GTTTCCC		GGATGCGGAATGTACC	反向引物

图3A

3B

	尾		引物 (20nt)	
A1	ATGGCTCTTGGGAAAC		TGCCTGAAACCGTACCAACC	正向引物
B1	GTTTCCCAAGAGCCAT		ACAGGGATGCGGAATGTACC	反向引物
A2	GGCTCTTGGGAAAC		TGCCTGAAACCGTACCAACC	正向引物
B2	GTTTCCCAAGAGCC		ACAGGGATGCGGAATGTACC	反向引物
A3	CTCTTGGGAAAC		TGCCTGAAACCGTACCAACC	正向引物
B3	GTTTCCCAAGAG		ACAGGGATGCGGAATGTACC	反向引物
A4	TCTTGGGAAAC		TGCCTGAAACCGTACCAACC	正向引物
B4	GTTTCCCAAGA		ACAGGGATGCGGAATGTACC	反向引物
A5	CTTGGGAAAC		TGCCTGAAACCGTACCAACC	正向引物
B5	GTTTCCCAAG		ACAGGGATGCGGAATGTACC	反向引物
A6	TTGGGAAAC		TGCCTGAAACCGTACCAACC	正向引物
B6	GTTTCCCAA		ACAGGGATGCGGAATGTACC	反向引物
A7	TGGGAAAC		TGCCTGAAACCGTACCAACC	正向引物
B7	GTTTCCCA		ACAGGGATGCGGAATGTACC	反向引物
A8	GGGAAAC		TGCCTGAAACCGTACCAACC	正向引物
B8	GTTTCCC		ACAGGGATGCGGAATGTACC	反向引物

图3B

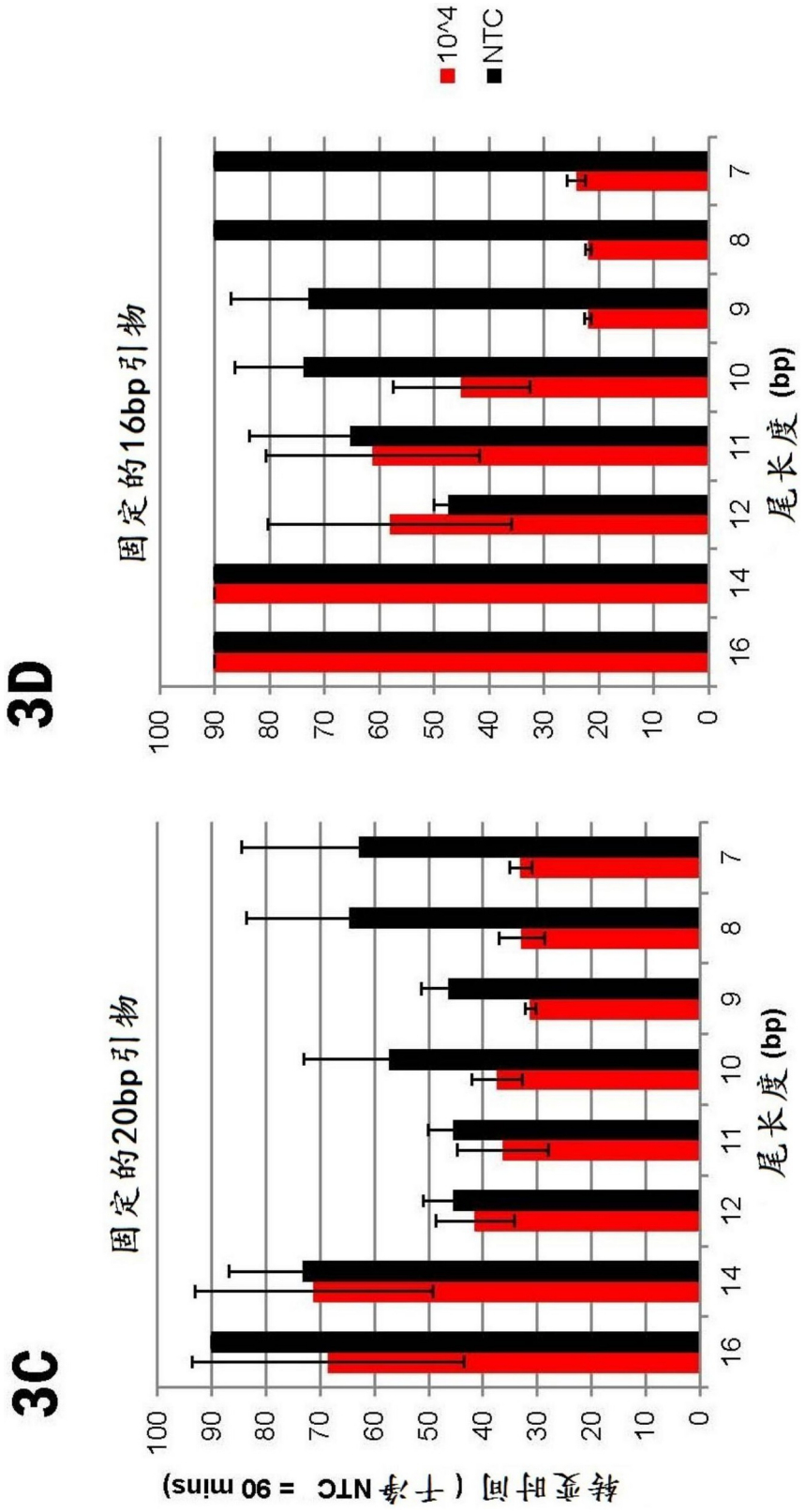

3C
固定的20bp引物
转变时间（千净NTC = 90 mins)
尾长度 (bp)
3D
固定的16bp引物
尾长度 (bp)
10^4
NTC

图3C和图3D

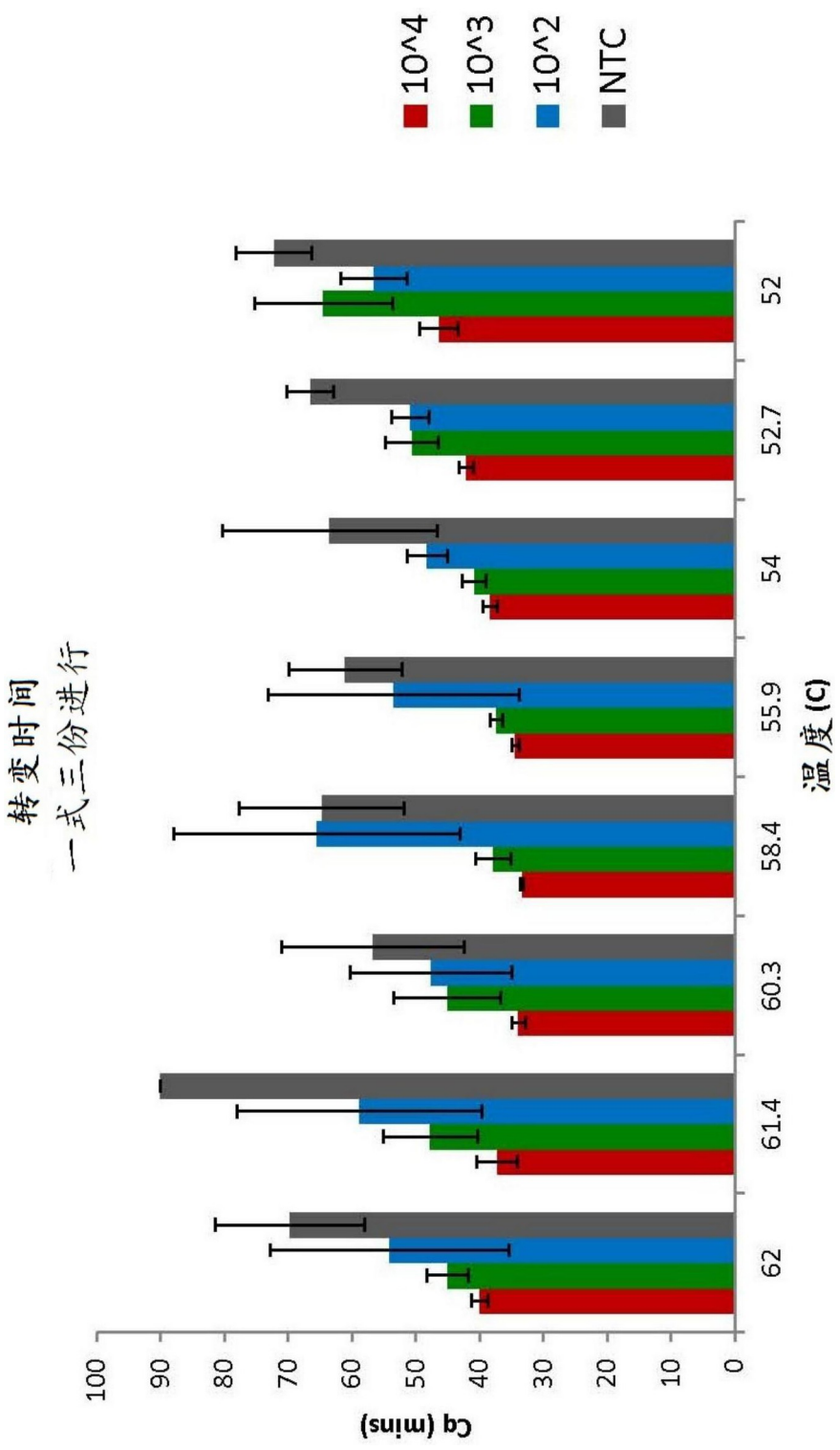

转变时间
一式三份进行
10^4
10^3
10^2
NTC
Cq (mins)
100
90
80
70
60
50
40
30
20
10
0
62
61.4
60.3
58.4
55.9
54
52.7
52
温度 (C)

图4

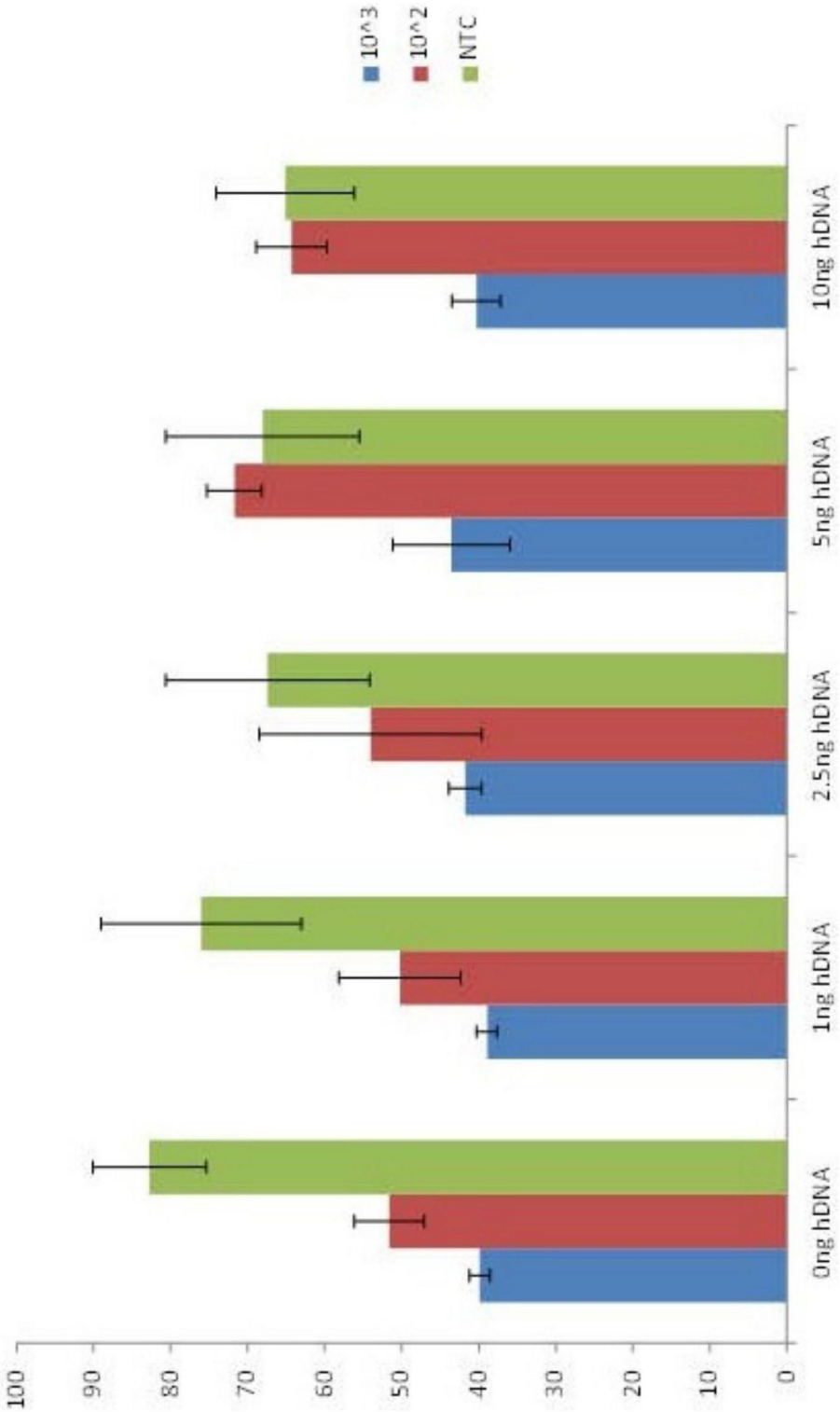

10^3
10^2
NTC
100
90
80
70
60
50
40
30
20
10
0
0ng hDNA
1ng hDNA
2.5ng hDNA
5ng hDNA
10ng hDNA

图5

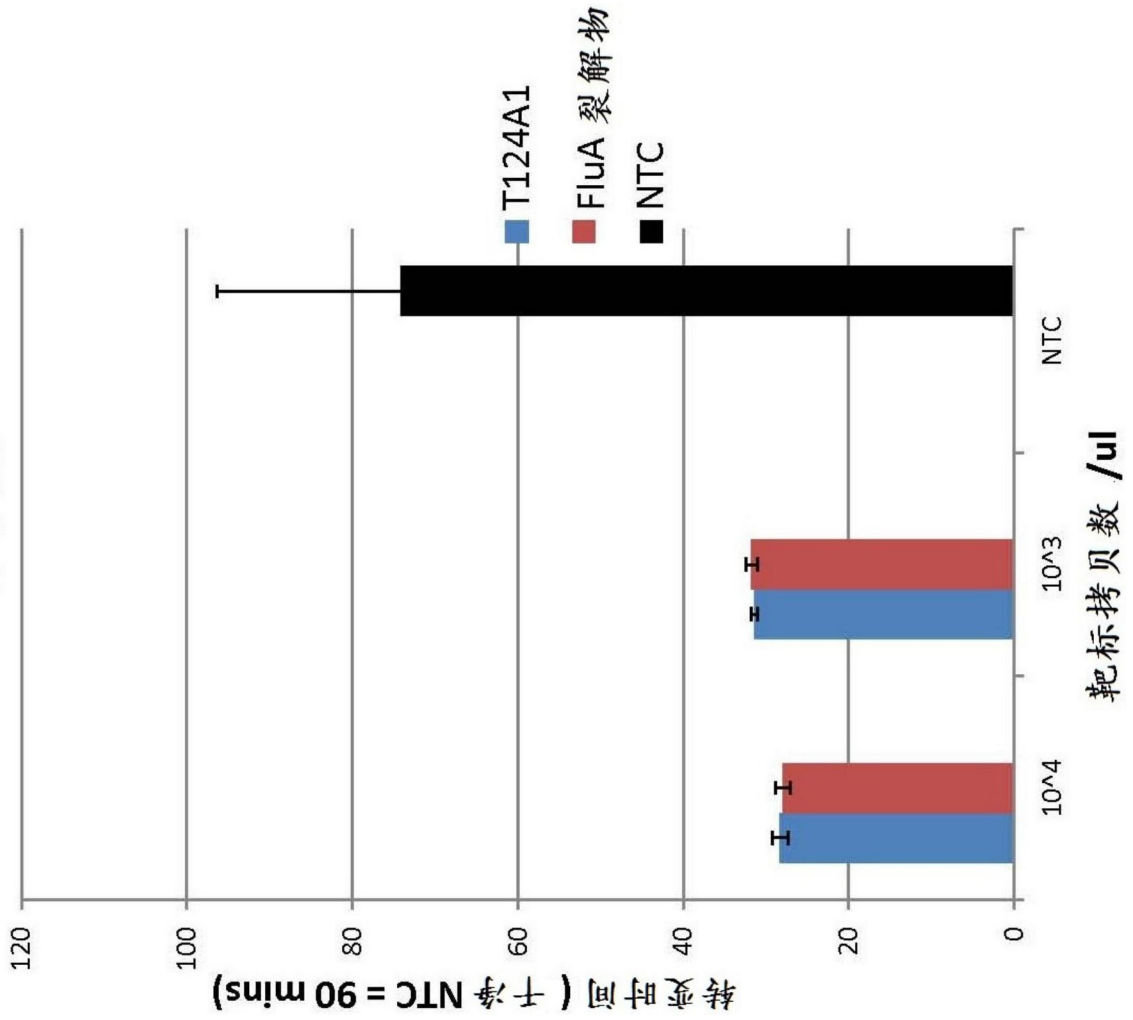

T124A1
FluA 裂解物
NTC
转变时间（千净 NTC = 90 mins）
120
100
80
60
40
20
0
10^4
10^3
NTC
靶标拷贝数 /ul

图6

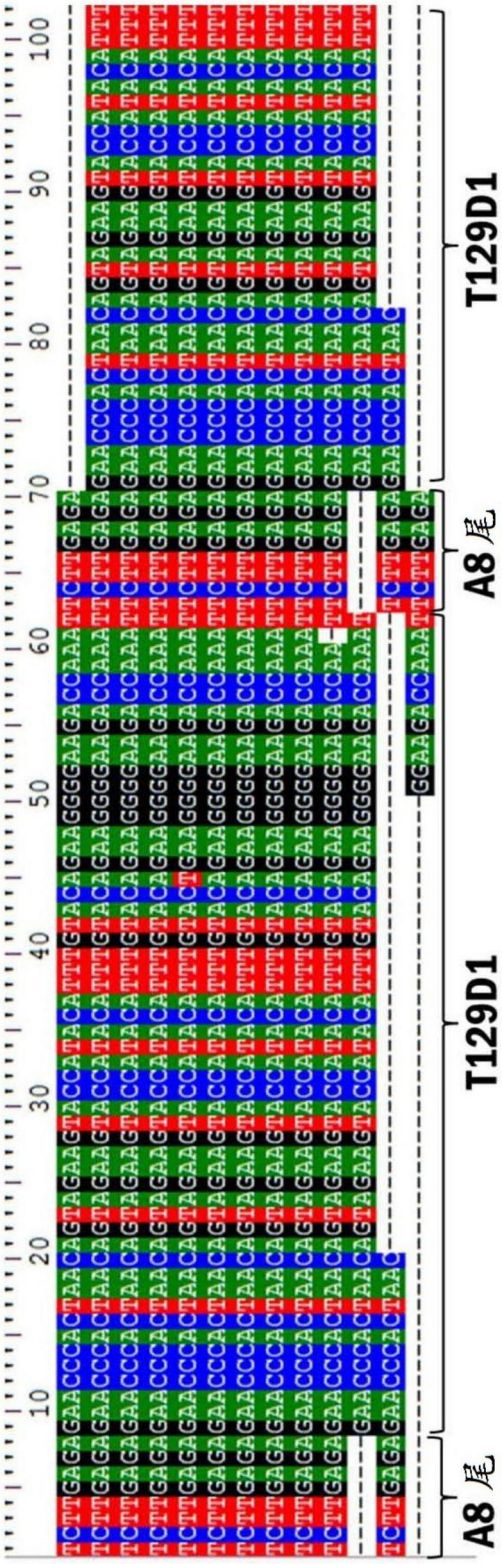


图7

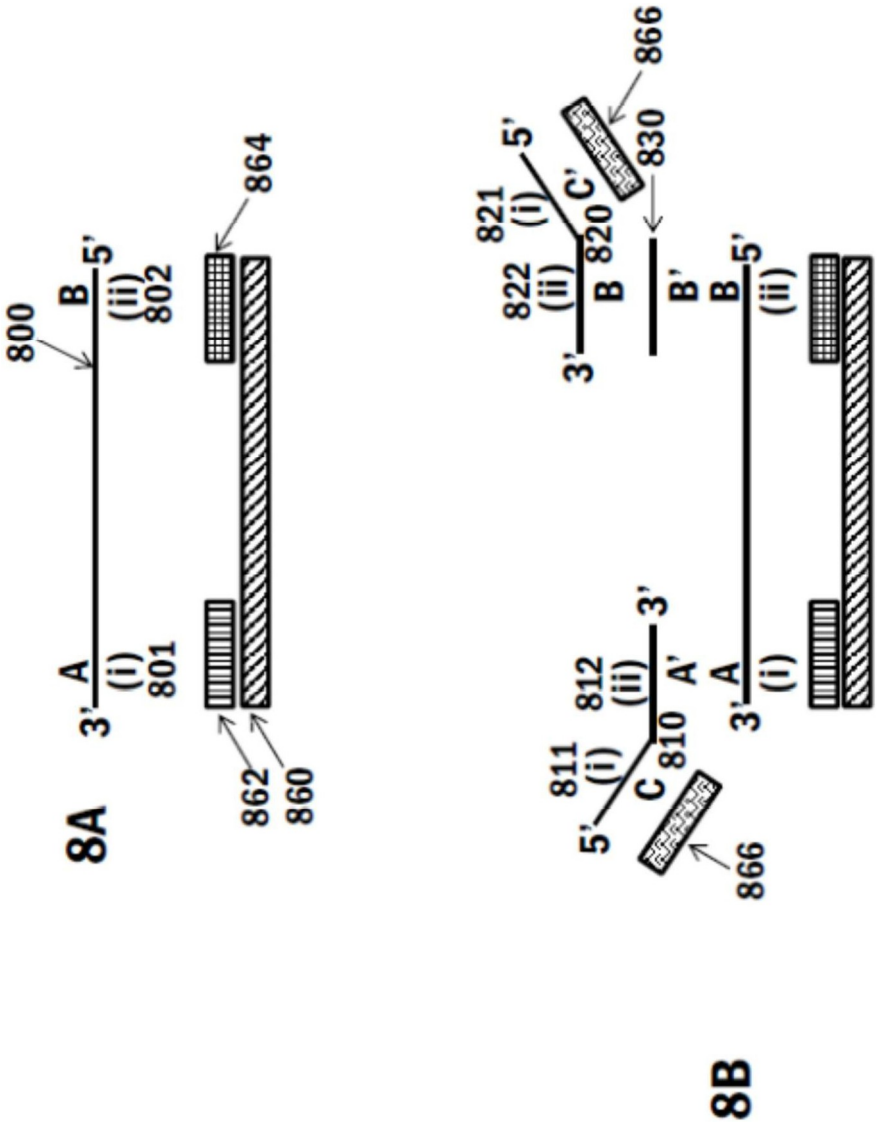

8A
800
3'
A
(i)
801
B
5'
(ii)
802
862
860
864
8B
811
(i)
5'
C
810
812
(ii)
3'
A'
3'
A
(i)
B'
B
5'
(ii)
822
(ii)
3'
B
820
821
(i)
5'
C'
866
830
866

图8A和图8B

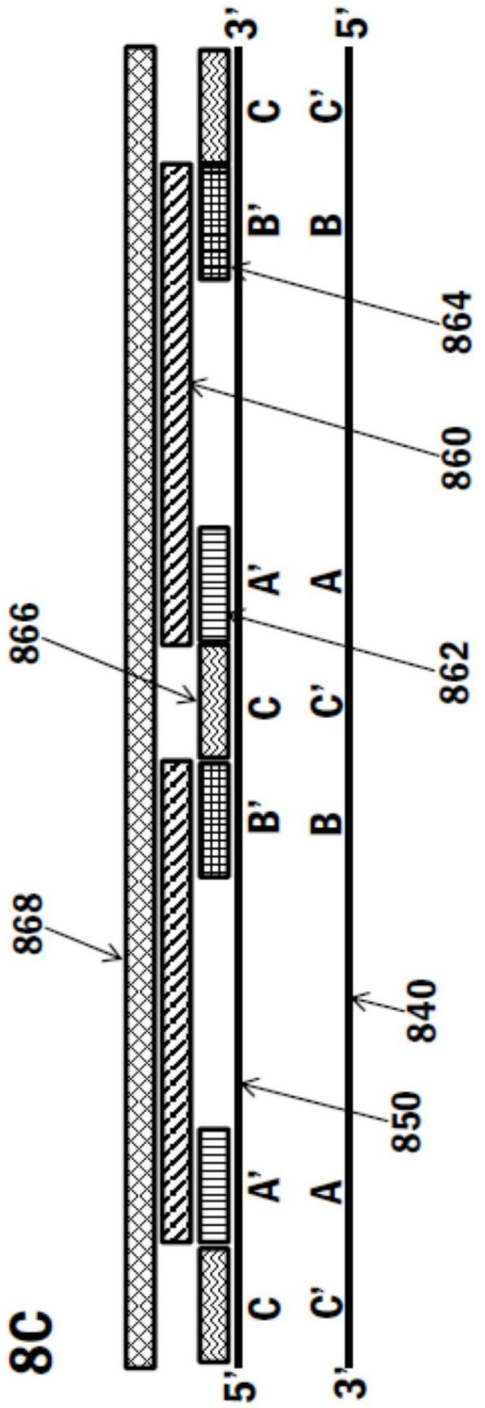

8C
5'
3'
C
C'
A'
A
B'
B
C
C'
B'
B
3'
5'
868
866
850
840
862
860
864

图8C

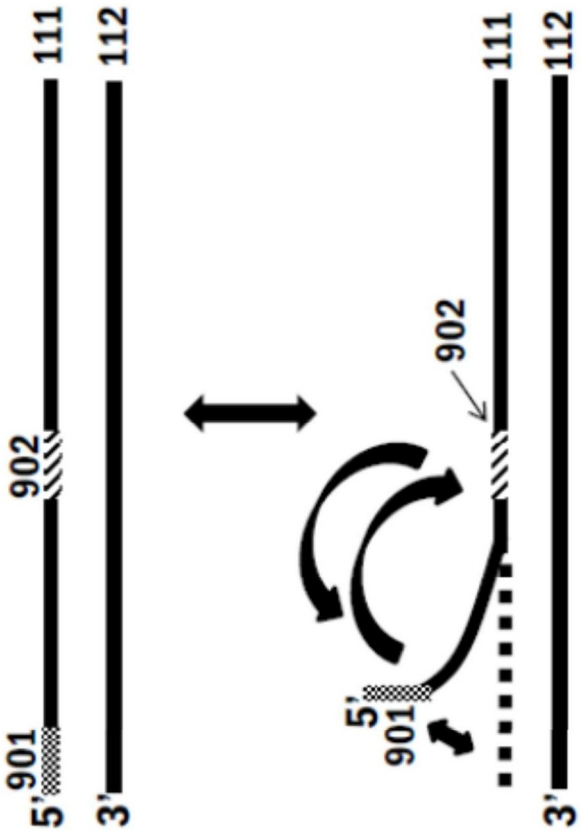

5'
901
902
3'
111
112
5'
901
902
3'
111
112

图9

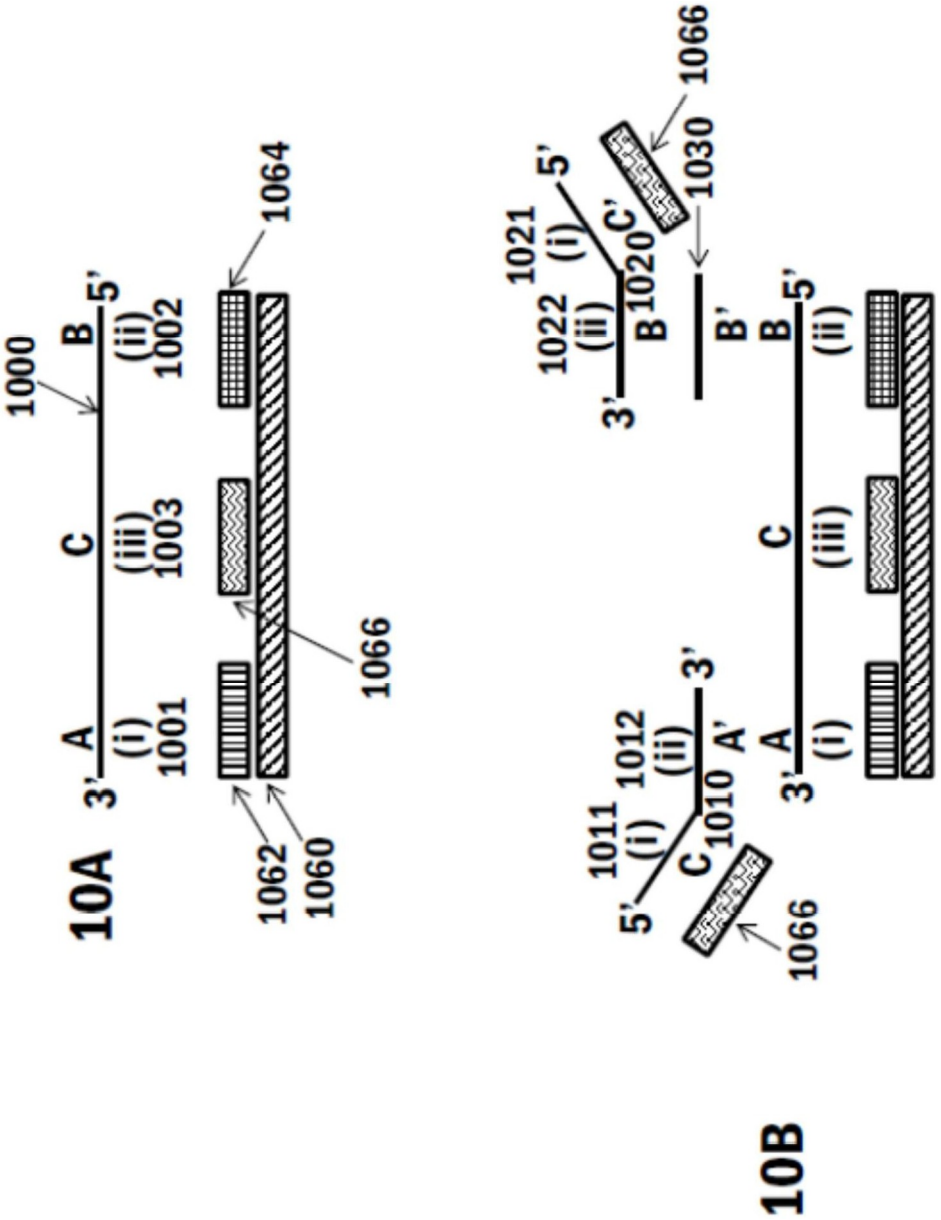

10A
1000
3'
A
(i)
1001
C
(iii)
1003
B
5'
(ii)
1002
1062
1060
1066
1064
10B
1011
(i)
5'
C
1010
1012
(ii)
3'
A'
1066
3'
A
(i)
C
(iii)
B
5'
(ii)
1021
(i)
5'
C'
1020
1022
(ii)
B
3'
B'
1030
1066

图10A和图10B

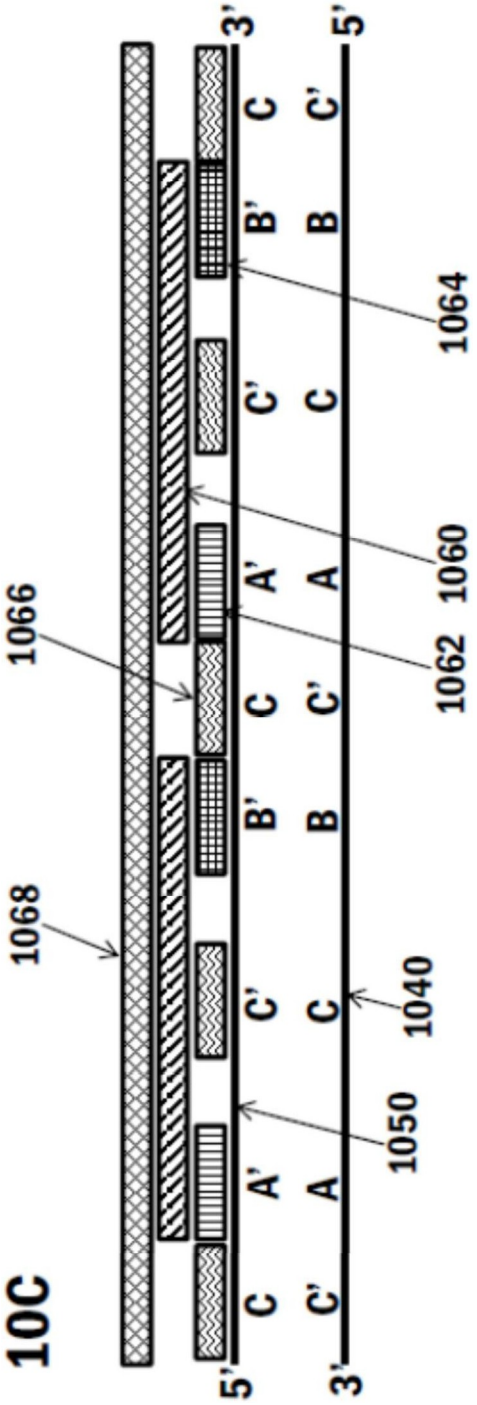

10C
1066
1068
1064
1060
1062
1040
1050
5'
3'
C
C'
A'
A
C'
C
B'
B
C'
C
A'
A
C'
C
B'
B
C'
C
3'
5'

图10C

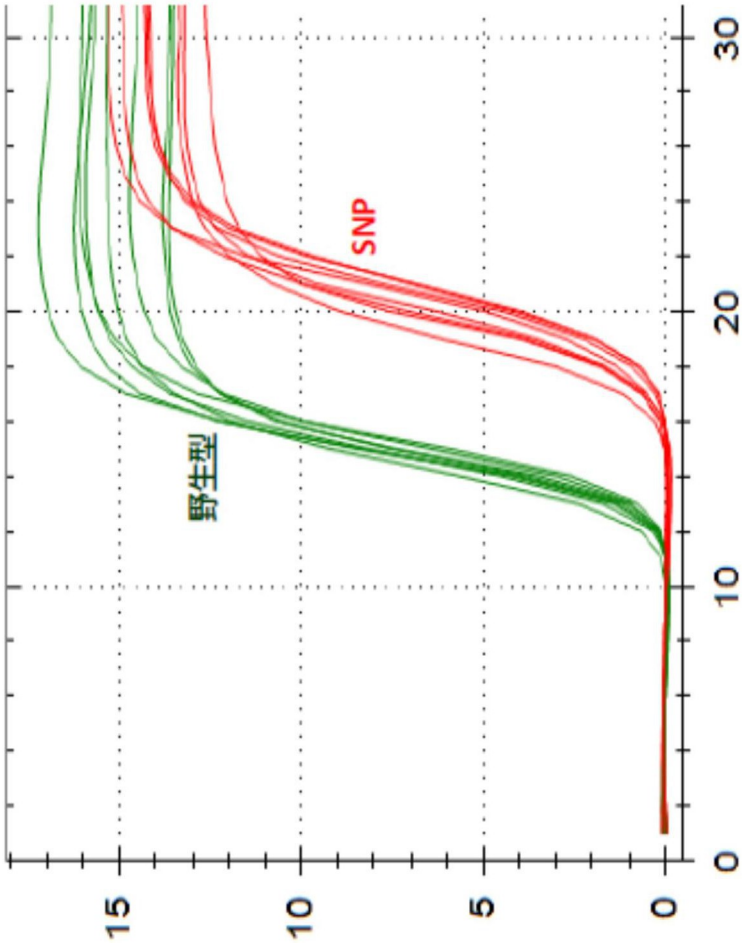


图11

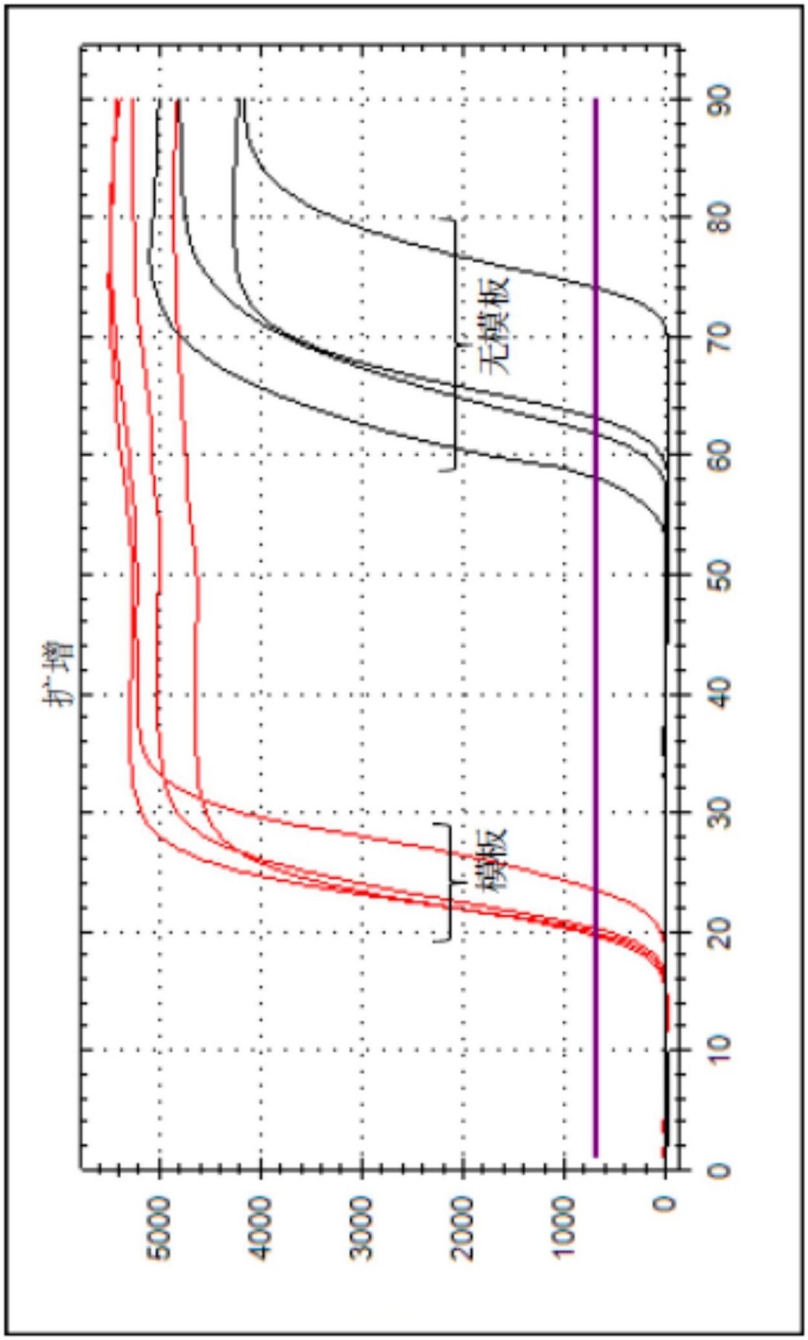


图12