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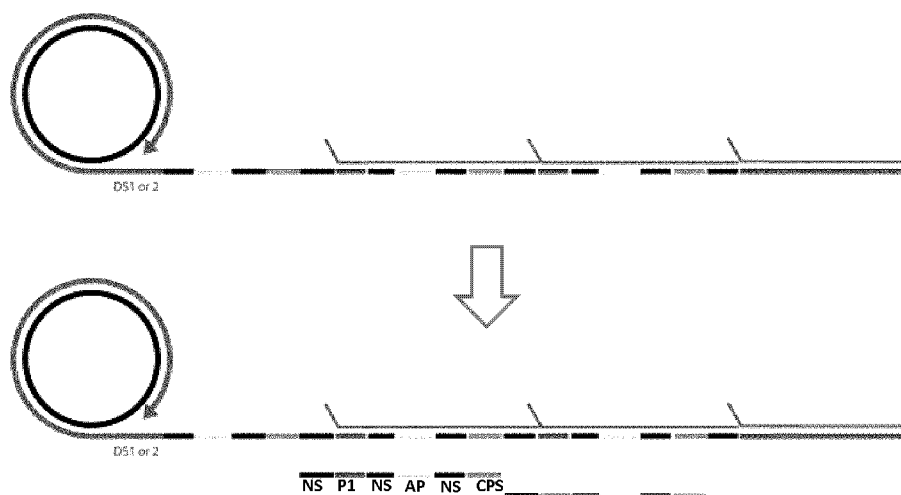
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(54) Title: METHOD, KITS AND COMPOSITIONS FOR AMPLIFYING NUCLEIC ACID SEQUENCES USING NICK-ASE-MEDIATED SINGLE STRANDED ROLLING CIRCLE ASSISTED STRAND DISPLACEMENT AMPLIFICATION

Figure 6



(57) Abstract: The present invention relates to an improved method for amplifying nucleic acid sequences using isothermal strand displacement amplification coupled with a nicking enzyme assisted rolling circle amplification and a temperature stable polymerase enzyme. Compositions for amplifying a target polynucleotide region of a nucleic acid molecule include four target-specific primers; a thermostable polymerase and a single- stranded polynucleotide circle.

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**Method, Kits and Compositions for Amplifying Nucleic Acid Sequences Using
Nickase-mediated Single Stranded Rolling Circle Assisted Strand Displacement
Amplification**

This application claims priority to United States provisional patent application 62/569,202 filed October 6, 2017.

FIELD OF THE INVENTION

[0001] The invention relates to isothermal amplification and detection of DNA or RNA sequences at a constant temperature.

BACKGROUND OF THE INVENTION

[0002] Nucleic acid amplification tests (NAATs) have become the cornerstone for microbiology laboratories, providing a same day diagnosis for a wide range of infections. Although polymerase chain reaction (PCR) has served laboratories well since its inception, PCR tests have significant disadvantages as they are labor intensive and relatively slow. Point-of-care (POC) tests that are being designed to provide rapid and actionable results for healthcare providers at the time and place when patients first encounter the health care system require more rapid NAATs.

[0003] Traditional diagnostic testing for bacterial and viral infections involve virus isolation in cell culture, ELISA, serology, and direct fluorescent antigen (DFA) staining of nasopharyngeal (NP) specimens and shell vial culture (SVC) using a panel of monoclonal antibodies. In the early 1990s, specific monoclonal antibodies were developed and respiratory viruses could be detected within 3 hours through DFA staining of viral antigens or within 1-2 days using SVC for slowly growing viruses. This was far superior to the 8-10 days required for cell culture. Rapid EIA tests developed in the 1980s and 1990s for point-of-care testing for bacteria and viruses lack sensitivity; the clinical sensitivities of these tests ranged from 20 to 90%, varying widely with the patient population being tested. These rapid EIA tests are therefore not recommended for use in critical care settings due to their low sensitivities.

[0004] NAATs, especially real time PCR, multiplex PCR, and more recently isothermal amplification methods, have replaced the conventional methods for detecting bacteria and viruses largely because these molecular tests detect 30 to 50% more positives. The movement towards isothermal amplification tests allows for the development of POC diagnostic tests, which should improve the detection and diagnosis of infections in clinical settings such as emergency rooms and walk in clinics as well as non-clinical settings such as the home or in the field.

[0005] Various amplification techniques have been developed that require multiple steps and more than a single temperature. Transcription-Mediated Amplification (TMA) employs a reverse transcriptase with RNase activity, an RNA polymerase, and primers with a promoter sequence at the 5' end. The reverse transcriptase synthesizes cDNA from the primer, degrades the RNA target, and synthesizes the second strand after the reverse primer binds. RNA polymerase then binds to the promoter region of the dsDNA and transcribes new RNA transcripts which can serve as templates for further reverse transcription. The reaction is rapid and can produce 10E9 copies in 20-30 minutes. This system is not as robust as other DNA amplification techniques. This amplification technique is very similar to Self-Sustained Sequence Replication (3SR) and Nucleic Acid Sequence Based Amplification (NASBA), but varies in the enzymes employed. Single Primer Isothermal Amplification (SPIA) also involves multiple polymerases and RNaseH. First, a reverse transcriptase extends a chimeric primer along an RNA target. RNaseH degrades the RNA target and allows a DNA polymerase to synthesize the second strand of cDNA. RNaseH then degrades a portion of the chimeric primer to release a portion of the cDNA and open a binding site for the next chimeric primer to bind and the amplification process proceeds through the cycle again. The linear amplification system can amplify very low levels of RNA target in roughly 3.5 hrs. The Q-Beta replicase method is a probe amplification method. A probe region complementary or substantially complementary to the target of choice is inserted into MDV-1 RNA, a naturally occurring template for Q-Beta replicase. Q-Beta replicates the MDV-1 plasmid so that the synthesized product is itself a template for Q-Beta replicase, resulting in exponential amplification as long as there is excess replicase to template. Because the Q-Beta replication process is so sensitive and can amplify whether the

target is present or not, multiple wash steps are required to purge the sample of non-specifically bound replication plasmids. The exponential amplification takes approximately 30 minutes; however, the total time including all wash steps is approximately 4 hours.

[0006] Factors that adversely affect the outcome of amplification methods are numerous and include inhibitors of polymerase activity and other components found in clinical specimens that reduce amplification efficiency, reduce amplification efficiencies due to secondary structure of primers or template, and template-independent amplification resulting from primer-dimer formation that decreases amplification efficiency and specificity leading to false positives. The negative effects are amplified at room temperature following the setup of reaction mixtures before they are moved to the amplification temperature presenting specificity problems for labs batching a large number of specimens. This can occur when a large number of reactions are prepared for a single run resulting in holding of reactions at room temperature. This is a common occurrence in large laboratories that process a high specimen volumes and where batch processing is required for high throughput of results. High throughput is therefore often negatively impacted by set up at room temperature and key requirements for molecular diagnostic testing including consistency, reproducibility and accuracy can be negatively impacted.

[0007] To further accelerate DNA detection assays, signal amplification approaches are becoming more common. This involves an early specific-sequence detection step followed by an exponential cascade of DNA production that is no longer reliant on the initial target being present. Examples of signal amplification include Nucleic Acid Sequence Based Amplification (NASBA), Transcription Mediated Amplification (TMA) and SMART.

[0008] These and other amplification methods are discussed in, for example, Van Ness. J, et al. PNAS 2003 100 (8): 4504-4509; Tan, E., et al. Anal. Chem. 2005, 77:7984-7992; Lizard, P., et al. Nature Biotech 1998, 6:1197-1202.

SUMMARY

[0009] In one embodiment, there is provided a method of amplifying a target polynucleotide region of a nucleic acid molecule, comprising: contacting the nucleic acid molecule with four target-specific primers; a thermostable polymerase; and a single-stranded polynucleotide circle; under conditions that promote strand displacement amplification and nickase enzyme-mediated rolling circle amplification of the target polynucleotide region.

[0010] In one embodiment, the single-stranded polynucleotide circle contains a polynucleotide region that is complementary to the target polynucleotide region.

[0011] In one embodiment, the four target-specific primers comprise: (a) a first primer, wherein the first primer is a polynucleotide primer having a target recognition region at the 3' end that is complementary to a first strand of the target polynucleotide region and a 5' tag that comprises a single stranded nicking enzyme site; (b) a second primer, having a target recognition region at a 3' end that is complementary to a second strand of the target polynucleotide region and a 5' tag that comprises a single stranded nicking enzyme site; (c) a third primer, wherein the third primer is a polynucleotide primer complementary to a target region immediately upstream of the target recognition region of the first primer; (d) a fourth primer, wherein the fourth primer is a polynucleotide primer complementary to a target region immediately upstream of the target recognition region of the second primer.

[0012] In one embodiment, a second single-stranded circular polynucleotide is used.

[0013] In one embodiment, a padlock probe is generated during amplification of single-stranded circular polynucleotides.

[0014] In one embodiment, the single-stranded circular polynucleotides contain specific nicking enzyme sites that are generated by filling in the single-stranded circular product and creating a double-stranded intermediate.

[0015] In one embodiment, the nicking reaction releases polynucleotides that can activate more single-stranded circular polynucleotides.

[0016] In one embodiment, DNA complementary to the single-stranded polynucleotide circle contains one or more activating primers flanked by DNA nickase sites, and wherein the method further comprises contacting the nucleic acid molecule with an accelerating primer, which hybridizes to the DNA complementary to the single-stranded polynucleotide circle generating a double-stranded intermediate that allows for DNA nicking and release of the one or more activating primers.

[0017] In one embodiment, the method includes combining a single stranded binding protein (SSB) with the thermostable polymerase, the four primers and the nucleic acid molecule in a reaction buffer at a first temperature; and immediately or after a lag time at a temperature above 4°C but below 70°C, performing an isothermal strand displacement amplification reaction at a second temperature, wherein the increase is determined with respect to the same mixture without the SBB.

[0018] Also provided is a composition for amplifying a target polynucleotide region of a nucleic acid molecule comprising: four target-specific primers; a thermostable polymerase; a single-stranded polynucleotide circle; and a buffer.

[0019] In one embodiment, the four target-specific primers comprise: (a) a first primer, wherein the first primer is a polynucleotide primer having a target recognition region at the 3' end that is complementary to a first strand of the target polynucleotide region and a 5' tag that comprises a single stranded nicking enzyme site; (b) a second primer, having a target recognition region at a 3' end that is complementary to a second strand of the target polynucleotide region and a 5' tag that comprises a single stranded nicking enzyme site; (c) a third primer, wherein the third primer is a polynucleotide primer complementary to a target region immediately upstream of the target recognition region of the first primer; and (d) a fourth primer, wherein the fourth primer is a polynucleotide primer complementary to a target region immediately upstream of the target recognition region of the second primer.

[0020] In one embodiment, the composition includes two different polynucleotide circles.

[0021] In one embodiment, the buffer has a pH in the range of pH 6-pH 9, and includes a stabilization agent selected from the group consisting of BSA, glycerol, a detergent and mixtures thereof.

[0022] In one embodiment, the buffer includes a monovalent salt having a concentration in the range of 0-500 mM and/or a divalent metal cation having a concentration of 0.5 mM-10 mM.

[0023] In one embodiment, the buffer has a pH in the range of pH 6-pH 9, and includes a monovalent salt having a concentration in the range of 0-500 mM, and a divalent metal cation having a concentration of 0.5 mM-10 mM and optionally a stabilizing agent selected from the group consisting of bovine serum albumin (BSA), glycerol, a detergent and mixtures thereof.

[0024] In one embodiment, the thermostable polymerase has strand displacement activity and is active at temperatures greater than 50°C.

[0025] In one embodiment, the buffer includes a single stranded binding protein (SSB) in the range of 0.5 ug to 2 ug per reaction.

[0026] In one embodiment, DNA complementary to the single-stranded polynucleotide circle contains one or more activating primers flanked by DNA nickase sites, and the composition further includes an accelerating primer, which hybridizes to the DNA complementary to the single-stranded polynucleotide circle generating a double-stranded intermediate that allows for DNA nicking and release of the one or more activating primers.

[0027] Also provided is a kit for amplifying a target polynucleotide region of a nucleic acid molecule comprising, in one or more containers, four target-specific primers, a thermostable polymerase, a single-stranded polynucleotide circle, and a buffer. In various embodiments, the four target-specific primers, the thermostable polymerase, and the buffer are as described above.

[0028] In one embodiment, the kit includes two different polynucleotide circles.

[0029] In one embodiment, DNA complementary to the single-stranded polynucleotide circle contains one or more activating primers flanked by DNA nickase sites, and the kit further includes an accelerating primer, which hybridizes to the DNA complementary to the single-stranded polynucleotide circle generating a double-stranded intermediate that allows for DNA nicking and release of the one or more activating primers.

BRIEF DESCRIPTION OF THE FIGURES

[0030] Figure 1 shows the generation of displaced strand (DS) following polymerase activation at natural nick sites, extension of primer 1 (P1), bumping off of extended strands by bumper primer 1 (B1).

[0031] Figure 2 shows the generation of displaced strands DS1 and DS2 following binding and extension of primer 2 (P2) and single strand nicking at two nickase sites.

[0032] Figure 3 shows the binding of DS1 and DS2 to single stranded DNA circles (C1 or C2).

[0033] Figure 4 shows rolling circle amplification of single stranded circles (C1 or C2) following binding of DS1 and DS2 generating complimentary strands containing nickase sites (NS), activator primers, complimentary probe binding sites and P1/P2 sites.

[0034] Figure 5 shows the generation of double strand DNA product from compliments of the single strand circles by extension of P1 and P2 and activation of the nickase sites.

[0035] Figure 6 shows the generation of single stranded DNA sequences containing probe binding sites (cPS) and activating primer (AP) sequences after nicking of the dsDNA generated from the circles.

[0036] Figure 7 shows the regeneration of DS1 or DS2 following circle activation and nickase activity. These regenerated DS1 and DS2 can cycle back into the iSDA amplification, ensuring that the iSDA reaction continues.

[0037] Figure 8 shows the priming of additional circles by released activating primers and subsequent rolling circle amplification generating more circles for signal amplification.

[0038] Figure 9 shows generation of signal following binding of probes to released complementary probe binding sequences.

[0039] Figure 10 shows iSDA amplification of decreasing copy numbers of a generic target. Amplification was performed at 55°C using the Genie II instrument, and fluorescence was measured using a Pleaides probe sequence targeting the amplified DNA.

[0040] Figure 11 shows amplification of single stranded circles (SSCs) using activator primer. Activation was measured using a Genie II instrument based on SYBR green detection of amplified DNA.

[0041] Figure 12 shows that the addition of an accelerator primer improves circle amplification compared with activating primer alone. Amplification was measured using a Genie II instrument based on SYBR green detection of amplified DNA.

[0042] Figure 13 shows increased circle activation and amplification in the presence of a nickase enzyme resulting in decreased time to positivity.

[0043] Figure 14 shows that the combination of iSDA and single stranded circle amplification (RCASDA) increases the signal and decreases time to positivity compared with iSDA alone.

[0044] Figure 15 shows that RCASDA detects target DNA more quickly and with a higher signal than iSDA alone without circle amplification.

[0045] Figure 16 shows that RCASDA has a lower detection limit than iSDA alone. Amplification was measured using a Genie II instrument and SYBR green detection of amplified DNA.

DESCRIPTION OF THE EMBODIMENTS

[0046] As used herein, in reference to two nucleic acid sequences “complementary” refers to the ability of the nucleic acid sequences to form sufficient hydrogen bonding therebetween to stabilize a double-stranded nucleic acid sequence formed by hybridization of the two nucleic acid sequences.

[0047] Isothermal amplification techniques have been developed to circumvent the need for temperature cycling. In this context “isothermal amplification” refers to nucleic acid amplification that following denaturation of the target can be performed without requiring changes to the reaction temperature, such that the amplification may be performed without use of a thermocycler.

[0048] Strand displacement amplification (SDA) uses two sets of primers, a strand displacing polymerase, and a restriction endonuclease. The bumper primers serve to displace the initially extended primers to create a single-strand for the next primer to bind. A restriction site is present in the 5' region of the primer. Thiol-modified nucleotides are incorporated into the synthesized products to inhibit cleavage of the synthesized strand. This modification creates a nick site on the primer side of the strand, which the polymerase can extend. This approach requires an initial heat denaturation step for double-stranded targets. The reaction is then run at a temperature below the melting temperature of the double-stranded target region. Products 60 to 100 bases in length are usually amplified in 30-45 minutes using this method.

[0049] SDA involves restriction endonuclease nicking of a recognition site in an unmodified strand, followed by strand-displacing polymerase extension of the nick at the 3' end, which displaces the downstream strand. The displaced strand can then act as a target for an antisense reaction, ultimately leading to exponential amplification of DNA.

[0050] Rolling circle replication was first characterized as the mechanism through which viral circular genomes are replicated. It can be applied as both an exponential DNA amplification tool and a rapid signal amplification tool. In this approach, a small

circular piece of DNA is primed by the target, after which a strand displacement polymerase enzyme continues around the circular DNA, displacing the complementary strand. Ultimately, the synthesized DNA remains attached to the circle as more DNA is generated, generating 10^9 or more copies of the circle within 90 minutes.

[0051] Isothermal amplification of a nucleic acid sequence requires specificity in the early stages of amplification combined with exponential DNA amplification for maximal sensitivity of DNA detection. In the inventive method provided herein, nucleic acid sequences are amplified by isothermal strand displacement amplification (iSDA) coupled with a nickase enzyme-mediated rolling circle amplification, where the product of the rolling circle amplification feeds back into the iSDA to improve the lower limit of detection and shorten the time-to-positivity. Using a combination of four primers, single stranded DNA circles (SSCs) for signal amplification, and a thermostable polymerase (for example Bst 3.0 or WarmStart RTx from New England Biolabs, Ipswich, MA, USA or LavaLamp polymerase from Lucigen, Middleton, WI, USA) specific nucleic acid sequences of viral, bacterial, fungal pathogens, or eukaryotic DNA (see e.g. Table 1) can be amplified and generate a specific product for detection using a variety of DNA binding dyes or DNA-specific probes.

TABLE 1

Examples of oligonucleotides used for RCASDA (primers)

Target	Primer sequence (5'-3')
--------	-------------------------

<i>Generic DNA</i>	P1 ATGACGATATGTGGATGCGAGTCGGACACCCTAAGGACACACTTTAACAATAGGC
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(SEQ ID NO: 01)

P2

ATGACGATATGTGGATGCGAGTCGGACATTCCCATGCTCCATTGGCACAT

(SEQ ID NO: 02)

B1 AATGAACTACCAAACGTTTCT (SEQ ID NO: 03)

B2 TGTTTACAGAGAATTGCGATAC (SEQ ID NO: 04)

*S. aureus*P1 GCATAATACTACCAGTCTCCTCAGCAAGCTACGCATTTTCATTAG
(SEQ ID NO: 05)

Methicillin resistant

P2 TAGAATAGTCGCATACTTCCTCAGCCATAACATCTCCTCGAACT
(SEQ ID NO: 06)

B1 AGGTAATGGTGCAGTAGGT (SEQ ID NO: 07)

B2 CCAGCTTTCACACGAAC (SEQ ID NO: 08)

Streptococcus pyogenes

P1

GCGTCCTTCCTAACTCATCTAATTTTGGTACTAGTCAGATTACTCC

(SEQ ID NO: 09)

P2 CTGCTAGAGGTACATTGACTTATGCCGGGGTTTTGATTTTACCG
(SEQ ID NO: 10)

B1 TTCAATGACAGTCCCAACT (SEQ ID NO: 11)

B2 GGTTTCCAGTCCATCCTG (SEQ ID NO: 12)

*Chlamydia trachomatis*P1 ACAGCCATGCAGCACCTGTGCATGTATATGACCGCGGCAG
(SEQ ID NO: 13)P2 GTTGGGTAAAGTCCCGCAACGAGGCAGTCTCGTTAGAGTTCC
(SEQ ID NO: 14)

B1 AGGACCTTACCTGGGTTTGA (SEQ ID NO: 15)

B2 TCGCCTTCCTCCTGGTTA (SEQ ID NO: 16)

Neisseria gonorrhoeae P1 CCAAAATTCCTCCACTGCTGCCTGGGTCTGAGAGGATGATCCG

(SEQ ID NO: 17)

P2 AAGCCTGATCCAGCCATGCCACAGCCTTTTCTTCCCTGAC

(SEQ ID NO: 18)

B1 GGTAAGGCCACCAAGG (SEQ ID NO: 19)

B2 TCAGGTACCGTCATCGGC (SEQ ID NO: 20)

Human B-actin

P1 GAGCCACACGCAGCTCATTGTACACGGCATCGTCACCAAC

(SEQ ID NO: 21)

P2 CTGAACCCCAAGGCCAACCGGCTGGGGTGTGAAGGTC

(SEQ ID NO: 22)

B1 CCCTGAAGTACCCCATCGA (SEQ ID NO: 23)

B2 ACAGCCTGGATAGCAACGT (SEQ ID NO: 24)

C. difficile Toxin P1 TCGGGTAGCTGAATTAAATAGTGAACCATAGAGTCTGACAATAACTTC

(SEQ ID NO: 25)

P2 AGCACCATACTTACAAGTAGGTTTTTAAGCTCCTGGACCACTT

(SEQ ID NO: 26)

B1 AACCAACACCTTAACCCA (SEQ ID NO: 27)

B2 GAAATCATAGTAAGCTGATGCA (SEQ ID NO: 28)

[0052] This isothermal amplification mixture consists of four primers (P1, P2, B1, B2), 1 or 2 single-stranded DNA circles, amplification buffer, and a strand displacement DNA polymerase.

[0053] Two of the target-specific primers (P1 and P2) bind to a region between 25 – 35 bp in the target genomic DNA. These two primers contain a 5' DNA tag that includes a single stranded nicking enzyme site, which is only active in a double-stranded form. These primers will extend in the 5' to 3' direction, generating a complement of the target genomic DNA with a single-stranded DNA nickase site sequence on the 5' end. Figures 1 and 2 show the generation of displaced strands (DS) with primer 1 and 2 (P1 and P2) and bumper primers 1 and 2 (B1 and B2). Genomic DNA is double-stranded, but single-stranded nicks occur naturally across the genome (100). Strand displacement polymerase can recognize these nicks and begin unwinding the double stranded DNA, providing a single-stranded intermediate. P1 contains a region complementary to the target genomic DNA (102) as well as a 5' tag containing a single-stranded nickase site that is only activated in its double-stranded form, and binds to the single stranded intermediate. P1 is then extended, generating a double stranded product (104). B1 then binds slightly upstream of P1 (106), and extends down the genomic DNA template. This process displaces the product generated by P1, resulting in the displaced strand (108).

[0054] Two additional primers (B1 and B2) bind slightly upstream on the genomic target of primers P1 and P2, acting as “bumper primers”. These primers will extend in the 5' to 3' direction, releasing the P1 primer product from the genomic target. These primers are required only for initiation of the amplification reaction but add specificity to the reaction since amplification cannot be initiated without their binding to target DNA. Figure 2 shows the generation of displaced strand (DS) 1 and DS2 following single-stranded DNA nicking. The released displaced strand contains a complementary region for primer 2 (P2) (110). P2 also contains a 5' overhang with a single-stranded DNA nickase site that is activated in the double stranded form. Upon binding of P2, both strands fill in, generating a double stranded product with nickase sites on both ends (112). These sites are then recognized by specific nickase enzymes, creating a single-stranded nick (114) that can be recognized by a strand-displacement polymerase

generating DS1 and DS2 (116) which are generated continuously through the action of nicking and extension by the strand displacement polymerase. Extension of the nicked strand continues to the end of P2, which contains the single-stranded DNA nickase site. Upon formation of a double stranded nick site, nicking can occur and the same reaction extends the nick site in the 5' to 3' direction, regenerating the nick site originally created by P1.

[0055] Methods for generating primers P1 and P2 and B1 and B2 are within the purview of persons of skill in the art. The preferred size of P1 and P2 primers is 30-40 nt and B1 and B2 primers is 18-22 nt depending on their CG content, shorter primers for lower GC content.

[0056] These reactions result in two single stranded pieces of DNA (DS1 and DS2) being continually generated and released, generating a specific signal. DS1 and DS2 can only be generated in the presence of target DNA. Figure 3 shows the binding of DS1 and DS2 to single stranded circles C1 or C2. Two circles are required in order to target both halves of the iSDA amplification reaction (targeted by DS1 and D2). Released DS1 and DS2 are generated in the presence of specific target in a linear fashion. Upon release of single stranded DS1 and DS2, they target and prime single-stranded circular DNA, which contains a complementary region to the 3' end of DS1 or DS2.

[0057] These reactions generate DS1 and DS2 in a linear fashion. Once generated, these strands have free 3' ends that can be used to prime an additional amplification reaction. Single stranded circular probes are present in the reaction mixture, with an activation site that includes the complement of the 3' end of DS1 or DS2 (two circles targeting either DS1 or DS1; C1 and C2, respectively). Upon activation by DS1 or DS2, the circles generate large amounts of single-stranded DNA that is complementary to the circle and amplifies the detection signal. Figure 4 shows rolling circle amplification of C1 and C2 following binding of DS1 and DS2. After priming of the circle by DS1 or DS2, extension occurs around the circle (118), ultimately displacing the double stranded DNA as more product is generated. This results in a concatemer of DNA that is complementary to the circle being generated and extended (120). Within this

complementary region, there is an activating primer (AP, Figure 4) sequence that can be released and activate more circles, a complementary probe sequence (CPS) that can be used as a measure of DNA production, nickase sites (NS) that are activated in their double-stranded state that flank both the AP and complementary probe sequence, and a P1 or P2 primer binding site (PBS) that allows the single-stranded piece of DNA to be extended and form a double-stranded product.

[0058] To achieve exponential signal amplification, the complementary circle DNA contains additional activating primers flanked by DNA nickase sites that can be recognized by a single-stranded DNA nickase enzyme, such as BstNBI. An accelerating primer recognizes the complementary circle DNA and creates a double-stranded intermediate, allowing for DNA nicking and subsequent release of the activating primers. These activating primers can activate secondary circles, which then undergo the same process. The region of the circle that is activated by DS1 or DS2 contains a modification within the nicking enzyme site to prevent nicking of the circle upon formation of the double-stranded product, suitably a modified phosphorothioate nucleotide. (The phosphorothioate (PS) bond substitutes a sulfur atom for a non-bridging oxygen in the phosphate backbone of an oligonucleotide within the nicking enzyme site to prevent nicking of the circle upon formation of the double-stranded product (DNA oligonucleotides containing a phosphorothioated nucleotide can be sourced e.g. from Integrated DNA Technologies, Skokie, IL, USA).) Figure 5 shows the generation of double stranded rolling circle amplification product using primer P2 (122). As the extended single-stranded piece of DNA is generated from the circle, P1 or P2 can bind the primer binding sites (Figure 5, PBS) and create a double-stranded product (124). This allows for activation of the nickase sites embedded within the DNA sequence. Figure 6 shows the generation of probe binding sequence (CPS) and AP after nickase treatment. Upon formation of the double stranded product, nickase enzyme can bind and cleave the single-stranded DNA, creating single-stranded nicks that can be recognized by strand displacement polymerases. Subsequently, the strand displacement polymerases can bind and “kick off” both the AP and CPS.

[0059] In addition to activating additional circles, the original DS1 and DS2 that are used to activate one of the two circles are re-generated by circles themselves, ensuring that iSDA will continue to generate more DS1 and DS2 and further accelerate the reaction and increase the signal. Figure 7 shows the regeneration of DS1 or DS2 after activation of the circle and generation of the nickase sites. These regenerated DS1 and DS2 can cycle back into the iSDA amplification, ensuring that the iSDA reaction continues. Figure 8 shows the priming of additional C1 and C2 by released activating primers (AP) (126). Upon release of AP from the complementary circle product, they can target unique sites on other, unactivated circles (128). This results in an exponential increase in DNA as more circles are activated, which then release more APs which activate more circles.

[0060] By increasing the rate of the reaction, single stranded circles (SSC) also significantly decrease background DNA amplification, improving the specificity of the reaction. Figure 9 shows signal generation using a probe that binds to a complementary probe binding sequence (CPS). Upon release of the CPS from the complementary circle sequence (130), the CPS can be targeted by a variety of detection methods including molecular beacons, Pleiades probes, DNA/BNA probes, DNA binding dyes, etc. (132). Figure 10 shows iSDA amplification of a generic target. Detection could be detected from 10^9 to 10^3 copies of a generic target. Amplification was performed at 55°C using the Genie II instrument, and fluorescence was measured using a Pleiades probe sequence targeting the amplified DNA. Figure 11 shows amplification of SSCs using activator primer. Circle alone, circle + non-specific activator, or circle + accelerator primer did not activate the circles. Activation was measured using a Genie II instrument using SYBR green detection of amplified DNA. Figure 12 shows that the addition of an accelerator primer improves circle activation. The addition of an accelerator primer with the activator primer significantly increases the fluorescence level and decreases the time to positivity of circle amplification. Figure 13 shows circle activation in the presence of a nickase enzyme. Addition of the nicking enzyme to the circle + activator mixture (Circle + Nick) increases the signal intensity and decreases the time-to-positivity by releasing AP, which subsequently activate secondary circles. The addition of nickase enzyme increased the absolute fluorescent levels and decreased the time-to-positivity

[0061] Methods of generating SSCs suitable for use in methods of the present invention are within the purview of persons of skill in the art. A single stranded DNA Ligase (suitably CircLigase™ ssDNA Ligase from Epicenter, Madison, WI) is used to circularize the oligonucleotide to make SSCs. A circle sequence construct used in methods described in the examples is shown in SEQ ID NO: 29 and in the table below:

SEQ ID NO: 29

5'atgacgatatgtggatgCGagtcggacagaggcaccattcccatgctcttttgactcagaggcaccat tcccatgctccattgtttgactcctaattcatcaacaatgcttttgactcagaggcaccattcccatgctccat gttttgactcctaattcatcaacaatgcttttgactcacaatgc3'
--

[0062] Figure 14 shows the combination of iSDA and single stranded circles (SSCs), known as rolling circle assisted strand displacement amplification or RCASDA. By applying RCASDA, an improved time-to-positivity and improved sensitivity was shown. Figure 14 shows that RCASDA using 10^8 circles detected 10^6 copies of generic target DNA with a higher fluorescent signal that iSDA without circles. In addition to an increased signal the time to positivity for RCASDA decreased. Figure 15 shows that RCASDA detected 10^3 copies of target DNA with both an increased fluorescent signal and shorter time to positivity. The peak fluorescence of RCASDA reached 15,000 RFU at 30 minutes, while iSDA alone only reached 2,000 RFU at 30 minutes. The time-to-positivity as detected on the Genie II instrument was 21:30 minutes for RCASDA, while iSDA alone at this same time was below the threshold of positivity. Figure 16 shows the detection of 10^2 copies by iSDA and RCASDA. The RCASDA positive control was 10^4 copies and showed a fluorescence level of 15,000 within 30 minutes while the negative control DNA showed no detectable fluorescence signal. Using RCASDA 10^2 copies gave a fluorescence signal of 1,500 within 30 minutes passing the positivity threshold while for iSDA, there was no fluorescence detected by 30 minutes. This evidences that RCASDA has a higher sensitivity than iSDA alone.

[0063] This approach can be used to target numerous organisms by modification of only P1, P2, B1, and B2, which provide assay specificity. Accordingly, the methods described herein provide a universal signal amplification approach that can be directed at any organism or target of interest without modification or optimization.

[0064] The product of RCASDA is large amounts of complementary circle DNA that can be detected using numerous approaches. The simplest approach is a visual detection using either a DNA binding dye (such as PicoGreen or GelRed) or a pH-based color changing dye. Using a dye allows for visual detection by comparing the color in a well containing target DNA to a well containing no DNA or control DNA. This detection approach can be enhanced by using a colorimetric detector capable of RGB analysis, for quantification of the color change. In addition, to color changes, the DNA concentration can be monitored using methylene blue with cyclic voltammetry, which is a sensitive approach to measure changes in DNA concentrations. To improve specificity, probe-based systems can be applied to monitor the production of specific circle DNA. For example, molecular beacons, TaqMan probes, Scorpions Probes, and Pleaides probes can be targeted to the DNA generated by the rolling circle amplification. This will allow for detection of specific amplification but avoiding detection of non-specific DNA amplification.

[0065]

REFERENCES

Walker, G. T. et al. (1992) Strand displacement amplification – an isothermal in vitro DNA amplification technique. *Nucleic Acids Res* 20:1691-1696.

Walker, G. T. et al. (1992). Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. *Proc Natl Acad Sci USA* 89:392-396.

Little, M.C. et al. (1999). Strand displacement amplification and homogeneous real-time detection incorporated in a second generation DNA probe system, BDProbe TecET. *Clin. Chemistry* 45:6 777-784.

Fire A, Xu S-Q. Rolling replication of short DNA circles. *Proc. Natl. Acad. Sci. USA*, 92 (1995) 4641– 4645.

Liu D, Daubendiek SL, Zillman MA, Ryan K, Kool ET. Rolling circle DNA synthesis: small circular oligonucleotides as efficient templates for DNA polymerases. *J Am Chem Soc*. 1996;118(7):1587–94.

Lizardi P, Huang X, Zhu Z, Bray-Ward Z, Thomas D, et al. Mutation detection and single molecule counting using isothermal rolling-circle amplification. *Nature genetics*. 19 (1998) 225-232.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K et al. Loop-mediated isothermal amplification of DNA. *Nucleic Acid Res*. 28 (2000) e63.

Wahed A, Patel P, Heidenreich D, Hufert F, Weidmann M. Reverse transcription recombinase polymerase amplification assay for the detection of middle East respiratory syndrome coronavirus. *PLoS Current*. 5 (2013).

Vincent M, Xu Y, Kong, H. Helicase-dependent isothermal DNA amplification. *EMBO reports*. 5 (2004) 795–800.

Hall M, Wharam S, Weston A, Cardy D, Wilson W Use of signal mediated amplification of RNA technology (SMART) to detect marine cyanophage DNA. *BioTechniques*. 32 (2002) 604-611.

EXAMPLES

Example 1

Detection of DNA using iSDA

[0066] Target genomic DNA is denatured at 95°C, generating a single stranded DNA intermediate that can be targeted by primer 1(P1), primer 2 (P2), bumper 1 (B1), and

bumper 2 (B2) (Figure 1). These primers are targeted specifically to genomic DNA from the pathogen of interest. Pathogens that can be targeted include but are not limited to RSV, Influenza, *E. coli*, *Streptococcus pneumoniae*, *Chlamydia pneumoniae*, and *Chlamydia trachomatis*. Primers P1 and P2 contain 5' tags, including a single-stranded DNA nickase site, and recognize specific target DNA sequences. B1 and B2 bind slightly upstream of P1 and P2, functioning as bumper primers to displace the P1 and P2 DNA product from the target DNA. Upon extension and displacement of P1 and P2, BstNBI recognizes the double-stranded nickase site and creates a single-stranded DNA nick. This nick is recognized by a strand-displacing DNA polymerase (such as Bst 2.0 or Bst 3.0), which extends starting at the nick and displaces any DNA downstream. After extension and release of DNA that is complementary to the genomic target, a second primer (either P1 or P2) binds to the released DNA and creates a double stranded intermediate containing a nickase site. After recognition and nicking by BstNBI, the strand-displacing polymerase binds and continuously releases a single-stranded piece of DNA, displaced strand 1 (DS1) or DS2. This creates a cyclical reaction in which released DS1 is targeted by Primer 2, generating another reaction in which DS2 is generated. Alternatively, DS2 is targeted by Primer 1, creating a reaction in which DS1 is generated. In this way, increasing amounts of specific DS1 and DS2 DNA are obtained. In addition, DS1 and DS2 contain a probe-binding site, which could include a Pleaides probe, a molecular beacon, a PNA probe, or a TaqMan probe. In this way, the specific production of DS1 and DS2 are monitored, which can only be generated upon activation of this circular cascade by specific target DNA. As shown in Figure 10, iSDA alone could detect 10^3 copies of target DNA within 20 minutes, reaching a peak fluorescence level of 5,000, using the Pleaides probe to monitor the production of DS1 and DS2. For this experiment, the reaction mixture was first made, consisting of:

Table 2

dH ₂ O	To 40 µl
10 x Amp Buffer II	1x
10 mM dNTP	0.65 mM
BstNBI Primer #1HPLC (02-21-2017) 100	0.5 µM

μM^*	
Generic Primer #2 08-28-2017 100 μM^*	4 μM

[0067] The enzyme mixture was made separately, consisting of:

Table 3

Pleiades Probe 10 μM	0.2 μM
Cpn F3 Orientation 1 Bumper 10 μM	0.25 μM
Cpn B3 (09-01-2017)10 μM	0.25 μM
BstNBI 500U	8U
Bst 3.0 400U	6U

[0068] Template DNA was added to the reaction mixture, which was denatured at 94°C for 2 minutes and incubated at 58°C for 5 minutes. The enzyme mixture was then added and amplified for 30 minutes at 58°C.

Example 2

Signal amplification using single stranded circles (SSCs)

[0069] Rolling circle amplification (RCA) is a well-established amplification technique that requires several pre-amplification steps, including ligation and single-stranded nicking. Using single-stranded circles (SSCs), the requirement for any pre-amplification preparation steps can be avoided. The SSCs can amplify DNA signals by recognizing either target or amplified DNA, after which the circles enter an activated state. Circle activation occurs by priming of the circle by the target DNA (Figure 3), after which a strand-displacing polymerase extends the primer around the circle, generating complementary circle DNA (Figure 4). To achieve exponential signal amplification, the complementary circle DNA contains additional activating primers flanked by DNA nickase sites that can be recognized by a single-stranded DNA nickase enzyme, such as BstNBI. An accelerating primer (Figure 5) recognizes the complementary circle DNA

and creates a double-stranded intermediate, allowing for DNA nicking and subsequent release of the activating primers. These activating primers can activate secondary circles, which then undergo the same process (Figure 6 to 8). For signal detection, it is possible to monitor total DNA concentrations using non-specific DNA binding dyes such as SybrGreen, PicoGreen, or Evan's Green, as well as probe-based systems with as the Pleiades probe, a TaqMan probe, molecular beacons, or PNA probes. The probe region is also present in the complementary circle DNA, which increases the specificity of the reaction (Figure 9). The basic reagents required for circle amplification include:

Table 4

Reagent	Concentration
Isothermal Master Mix	1X
Circle 1	0.5 μ M
Circle 2	0.5 μ M
Activating primer	0.2 μ M
Accelerating primer	0.2 μ M
Probe/DNA binding dye	0.2 μ M
Nickase (BstNbl)	8 Units

[0070] After the reagent mixture is prepared, Bst 2.0 or Bst 3.0 strand displacement DNA polymerase is added and allowed to incubate at 58°C for 40 minutes (Figure 11). The signal intensity peaked within 15 minutes, reaching an absolute fluorescence level of 55,000. The circle alone or circles with non-specific primer did not show any background. In addition, addition of the accelerator primer (compared to circles with the activator primer alone) showed improved time-to-positivity and increased absolute fluorescence (reaching 80,000 absolute fluorescence units) (Figure 12). In addition, including the nickase to release additional activating primers significantly improved both the time-to-positivity and the absolute fluorescence level (Figure 13), confirming that circle DNA product is being nicked and releasing additional activating primers, creating an exponential activation cascade.

Example 3

Enhanced target detection by RCASDA

[0071] To ensure that DS1 and DS2 are continuously generated and are not consumed by circle activation, the circles release DS1 and DS2 (Figure 7). As shown in Figure 7, nickase sites are generated after the circle is extended and double-stranded DNA is generated after binding and filling in of the activator primer. Once these nickase sites are cleaved, DS1 or DS2 (from C2 and C1, respectively) is nicked and released. This DS1 and DS2 can then either activate additional circles or can be recycled into the iSDA reaction.

[0072] DNA amplification requires both sensitivity and specificity, as well as a rapid time-to-positivity. The iSDA method described in the preceding paragraph has inherent specificity by requiring the binding of four target-specific primers before amplification and detection can occur. These four primers provide target-specificity and reduce assay background. To improve the sensitivity and time-to-positivity of the assay, an SSC signal amplification approach can be combined with the iSDA approach, providing a sensitive, specific, and rapid assay. The reaction mixture is as follows:

Table 5

Reagent	Concentration
Isothermal Master Mix	1X
Circle 1	0.5 μ M
Circle 2	0.5 μ M
Primer 1	0.5 μ M
Primer 2	4.0 μ M
Bumper 1	0.25 μ M
Bumper 2	0.25 μ M
Probe/DNA binding dye	0.2 μ M
Nickase (BstNbl)	8 Units
Bst 3.0 DNA polymerase	6 units

[0073] As described in Example 1, target genomic DNA is denatured at 95°C, generating a single stranded DNA intermediate that can be targeted by primer 1 (P1), primer 2 (P2), bumper 1 (B1), and bumper 2 (B2) (Figure 1). These primers are targeted specifically to genomic DNA from the pathogen of interest. Primers P1 and P2 contain 5' tags, including a nickase site. Upon extension and displacement of P1 and P2, BstNBI recognizes the double-stranded nickase site and creates a single-stranded DNA nick. This nick is recognized by a strand-displacing DNA polymerase (such as Bst 2.0 or Bst 3.0), which extends starting at the nick and displaces any DNA downstream. After extension and release of DNA that is complementary to the genomic target, a second primer (either P1 or P2) binds to the released DNA and creates a double stranded intermediate containing a nickase site. After recognition and nicking by BstNBI, the strand-displacing polymerase binds and continuously releases a single-stranded piece of DNA, called either DS1 or DS2. DS1 and DS2 contain sequences that are complementary to the circle at the 5' end, which act as circle primers (Figure 4). This step activates the circle, as described in Example 2, after which a strand-displacing polymerase extends the primer around the circle, generating complementary circle DNA (Figure 4). To achieve exponential signal amplification, the complementary circle DNA contains additional activating primers flanked by DNA nickase sites that can be recognized by a single-stranded DNA nickase enzyme, such as BstNBI. An accelerating primer (Figure 5) recognizes the complementary circle DNA and creates a double-stranded intermediate, allowing for DNA nicking and subsequent release of the activating primers. These activating primers can activate secondary circles, which then undergo the same process (Figure 6 to 8).

[0074] As shown in Figure 14, iSDA alone detects 10^6 copies of target DNA within 6 minutes compared to background DNA levels, reaching a peak fluorescence of 25,000 absolute fluorescence units. In combination with SSCs, RCASDA detects 10^6 copies of target DNA within 4 minutes compared to background, reaching an fluorescence level of 35,000 absolute fluorescent units. Thus, combination of iSDA and SSCs improves both the time-to-positivity and signal-to-noise ratio.

Example 4

RCASDA shows improved sensitivity compared to iSDA alone

[0075] Using iSDA, the limit of detection was 10^3 copies of target DNA, which may not be sufficient for clinical use. RCASDA was used to amplify the signal and improve the sensitivity level of iSDA alone. As shown in Figure 15, RCASDA has a stronger signal-to-noise ratio than iSDA alone when detecting 10^3 copies of target DNA. However, as shown in Figure 16, 10^2 copies of target DNA was not be detected using iSDA alone, whereas detection of 10^2 copies was possible within 30 minutes using RCASDA. The following enzyme mixture was applied:

Table 6

Reagent	Concentration
Isothermal Master Mix	1X
Circle 1	0.5 μ M
Circle 2	0.5 μ M
Primer 1	0.5 μ M
Primer 2	4.0 μ M
Bumper 1	0.25 μ M
Bumper 2	0.25 μ M
Probe/DNA binding dye	0.2 μ M
Nickase (BstNbl)	8 Units
Bst 3.0 DNA polymerase	6 units

[0076] After a heat denaturation step at 94°C for 2 minutes, the mixture was incubated at 58°C for 30 minutes to allow for DNA amplification and detection.

[0077] In this example, Pleiades probes were used to detect DNA detection.

WHAT IS CLAIMED:

1. A method of amplifying a target polynucleotide region of a nucleic acid molecule, comprising:
 - contacting the nucleic acid molecule with
 - four target-specific primers;
 - a thermostable polymerase; and
 - a single-stranded polynucleotide circle;under conditions that promote strand displacement amplification and nickase enzyme-mediated rolling circle amplification of the target polynucleotide region.
2. The method according to claim 1, wherein the single-stranded polynucleotide circle contains a polynucleotide region that is complementary to the target polynucleotide region.
3. The method according to claim 1, wherein the four target-specific primers comprise:
 - (a) a first primer, wherein the first primer is a polynucleotide primer having a target recognition region at the 3' end that is complementary to a first strand of the target polynucleotide region and a 5' tag that comprises a single stranded nicking enzyme site;
 - (b) a second primer, having a target recognition region at a 3' end that is complementary to a second strand of the target polynucleotide region and a 5' tag that comprises a single stranded nicking enzyme site;
 - (c) a third primer, wherein the third primer is a polynucleotide primer complementary to a target region immediately upstream of the target recognition region of the first primer;
 - (d) a fourth primer, wherein the fourth primer is a polynucleotide primer complementary to a target region immediately upstream of the target recognition region of the second primer.
4. The method according to any one of claims 1 to 3, wherein a second single-stranded circular polynucleotide is used.

5. The method according to any one of claims 1 to 4 wherein a padlock probe is generated during amplification of single-stranded circular polynucleotides.
6. The method according to any one of claims 1 to 4, wherein the single-stranded circular polynucleotides contain specific nicking enzyme sites that are generated by filling in the single-stranded circular product and creating a double-stranded intermediate.
7. The method according to claim 6, wherein the nicking reaction releases polynucleotides that can activate more single-stranded circular polynucleotides.
8. The method according to any one of claims 1 to 7, wherein DNA complementary to the single-stranded polynucleotide circle contains one or more activating primers flanked by DNA nickase sites, and wherein the method further comprises contacting the nucleic acid molecule with an accelerating primer, which hybridizes to the DNA complementary to the single-stranded polynucleotide circle generating a double-stranded intermediate that allows for DNA nicking and release of the one or more activating primers.
9. The method according to any one of claims 1 to 8, comprising:
 - (a) combining a single stranded binding protein (SSB) with the thermostable polymerase, the four primers and the nucleic acid molecule in a reaction buffer at a first temperature; and
 - (b) immediately or after a lag time at a temperature above 4°C but below 70°C, performing an isothermal strand displacement amplification reaction at a second temperature, wherein the increase is determined with respect to the same mixture without the SSB.
10. A composition for amplifying a target polynucleotide region of a nucleic acid molecule comprising: four target-specific primers; a thermostable polymerase; a single-stranded polynucleotide circle; and a buffer.

11. The composition according to claim 10, wherein the four target-specific primers comprise:

(a) a first primer, wherein the first primer is a polynucleotide primer having a target recognition region at the 3' end that is complementary to a first strand of the target polynucleotide region and a 5' tag that comprises a single stranded nicking enzyme site;

(b) a second primer, having a target recognition region at a 3' end that is complementary to a second strand of the target polynucleotide region and a 5' tag that comprises a single stranded nicking enzyme site;

(c) a third primer, wherein the third primer is a polynucleotide primer complementary to a target region immediately upstream of the target recognition region of the first primer; and

(d) a fourth primer, wherein the fourth primer is a polynucleotide primer complementary to a target region immediately upstream of the target recognition region of the second primer.

12. The composition according to claim 10 or 11, comprising two different polynucleotide circles.

13. The composition according to any one of claims 10 to 12, wherein the buffer has a pH in the range of pH 6-pH 9, and comprises a stabilization agent selected from the group consisting of BSA, glycerol, a detergent and mixtures thereof.

14. The composition according to any one of claims 10 to 12, wherein the buffer comprises a monovalent salt having a concentration in the range of 0-500 mM and/or a divalent metal cation having a concentration of 0.5 mM-10 mM.

15. The composition according to any one of claims 10 to 12, wherein the buffer has a pH in the range of pH 6-pH 9, and comprises a monovalent salt having a concentration in the range of 0-500 mM, and a divalent metal cation having a

concentration of 0.5 mM-10 mM and optionally a stabilizing agent selected from the group consisting of BSA, glycerol, a detergent and mixtures thereof.

16. The composition according to any one of claims 10 to 15, wherein the thermostable polymerase has strand displacement activity and is active at temperatures greater than 50°C.

17. The composition according to any one of claims 10 to 16, wherein the buffer comprises a single stranded binding protein (SSB) in the range of 0.5 ug to 2 ug per reaction.

18. The composition according to any one of claims 10 to 17, wherein DNA complementary to the single-stranded polynucleotide circle contains one or more activating primers flanked by DNA nickase sites, and wherein the composition further includes an accelerating primer, which hybridizes to the DNA complementary to the single-stranded polynucleotide circle generating a double-stranded intermediate that allows for DNA nicking and release of the one or more activating primers.

19. A kit for amplifying a target polynucleotide region of a nucleic acid molecule comprising, in one or more containers, four target-specific primers, a thermostable polymerase, a single-stranded polynucleotide circle, and a buffer.

20. The kit according to claim 19, wherein the four target-specific primers comprise:

(a) a first primer, wherein the first primer is a polynucleotide primer having a target recognition region at the 3' end that is complementary to a first strand of the target polynucleotide region and a 5' tag that comprises a single stranded nicking enzyme site;

(b) a second primer, having a target recognition region at a 3' end that is complementary to a second strand of the target polynucleotide region and a 5' tag that comprises a single stranded nicking enzyme site;

(c) a third primer, wherein the third primer is a polynucleotide primer complementary to a target region immediately upstream of the target recognition region of the first primer;

(d) a fourth primer, wherein the fourth primer is a polynucleotide primer complementary to a target region immediately upstream of the target recognition region of the second primer.

21. The kit according to claim 19 or 20, comprising two different polynucleotide circles.
22. A kit according to any one of claims 19 to 21, wherein the buffer has a pH in the range of pH 6-pH 9, and comprises a stabilization agent selected from the group consisting of BSA, glycerol, a detergent and mixtures thereof.
23. A kit according to any one of claims 19 to 21, wherein the buffer comprises a monovalent salt having a concentration in the range of 0-500 mM and/or a divalent metal cation having a concentration of 0.5 mM-10 mM.
24. A kit according to any one of claims 19 to 21, wherein the buffer has a pH in the range of pH 6-pH 9, and comprises a monovalent salt having a concentration in the range of 0-500 mM, and a divalent metal cation having a concentration of 0.5 mM-10 mM and optionally a stabilizing agent selected from the group consisting of BSA, glycerol, a detergent and mixtures thereof.
25. A kit according to any one of claims 19 to 24, wherein the thermostable polymerase has strand displacement activity and is active at temperatures greater than 50°C.
26. A kit according to any one of claims 19 to 25, wherein the buffer comprises a single stranded binding protein (SSB) in the range of 0.5 ug to 2 ug per reaction.
27. The kit according to any one of claims 19 to 26, wherein DNA complementary to the single-stranded polynucleotide circle contains one or more activating primers flanked by DNA nickase sites, and wherein the kit further includes an accelerating primer, which hybridizes to the DNA complementary to the single-stranded polynucleotide circle generating a double-stranded intermediate that allows for DNA nicking and release of the one or more activating primers.

Figure 1

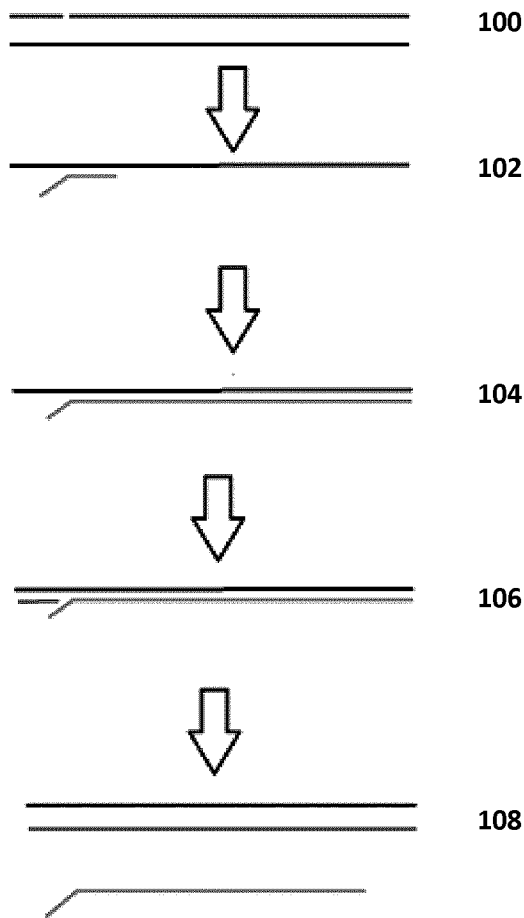


Figure 2

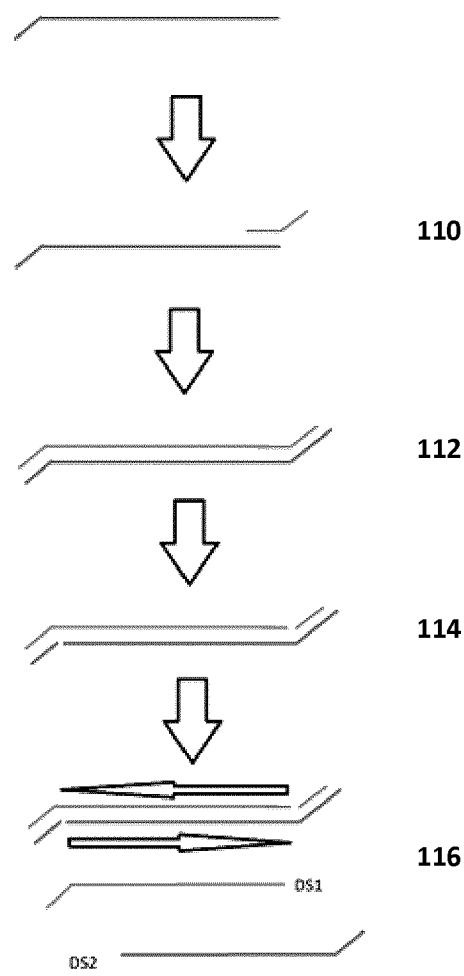


Figure 3

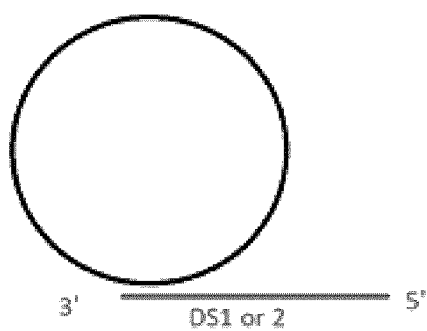


Figure 4

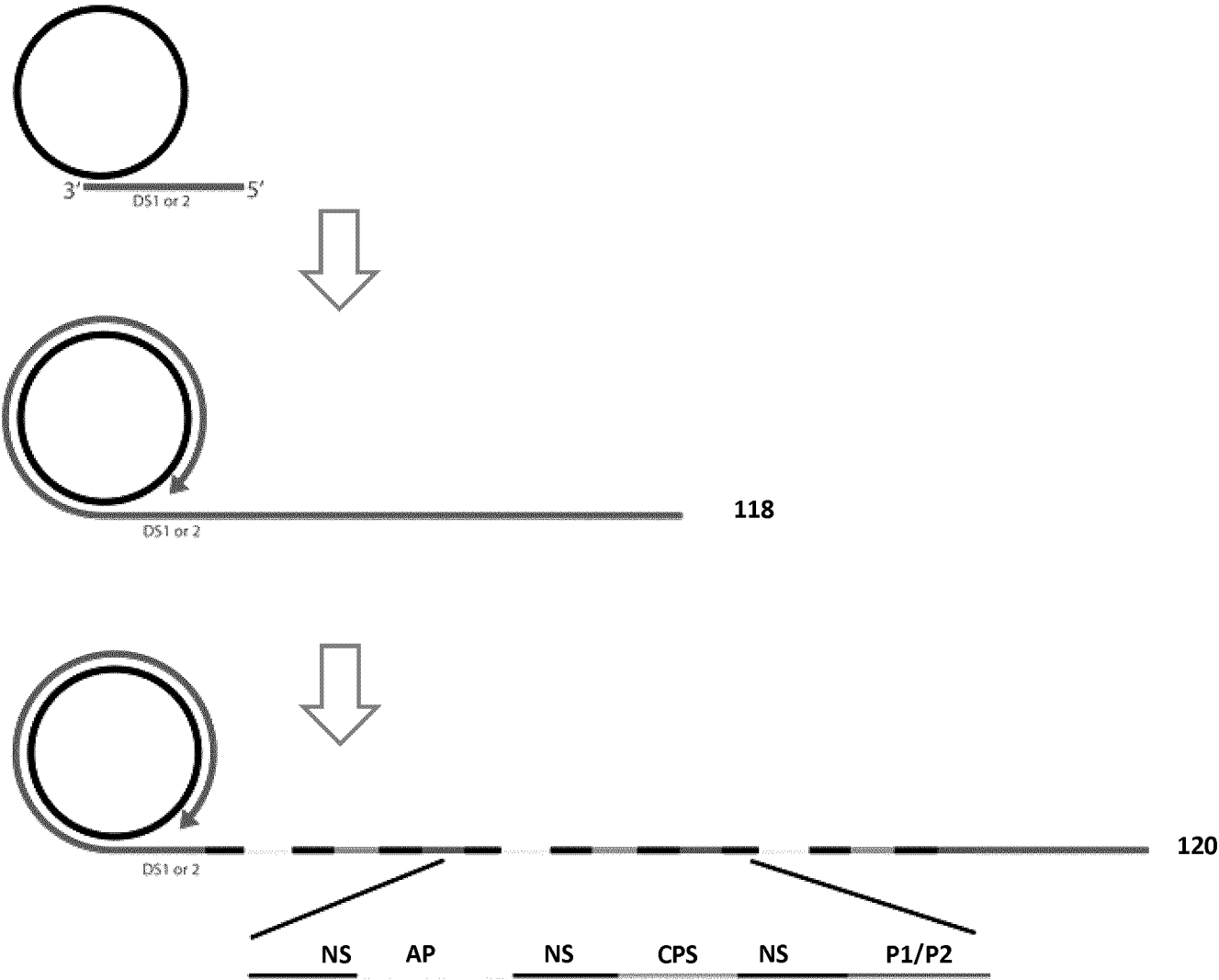


Figure 5

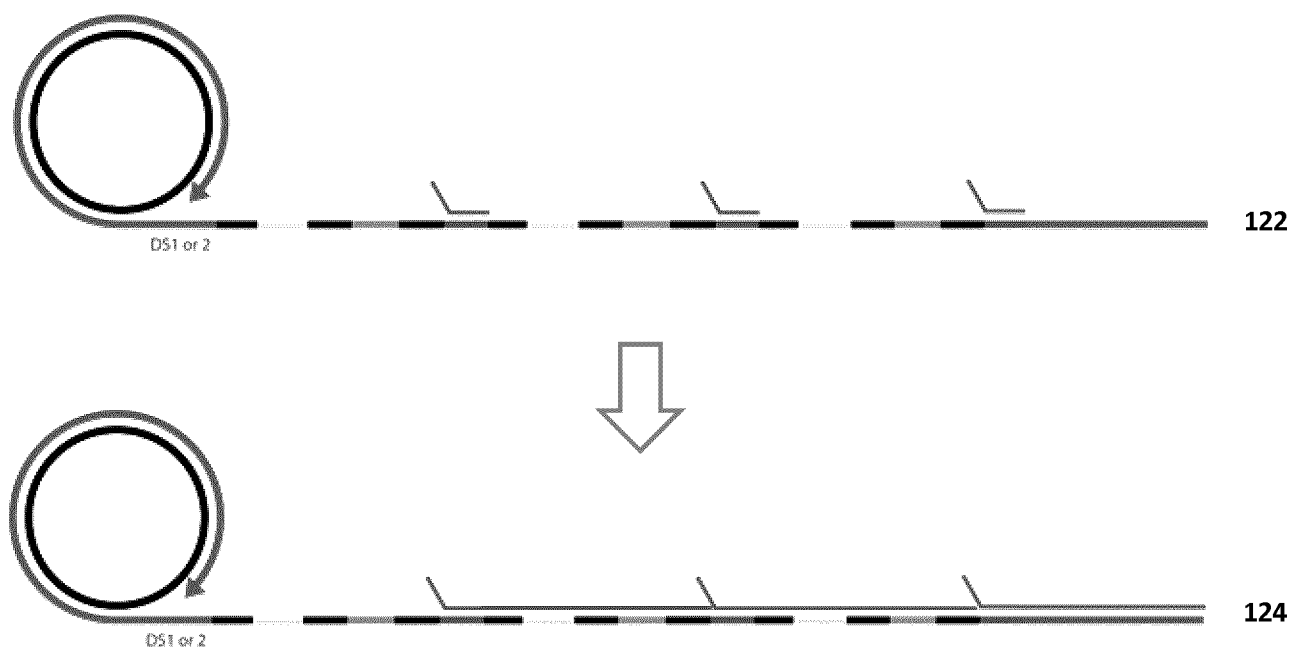


Figure 6

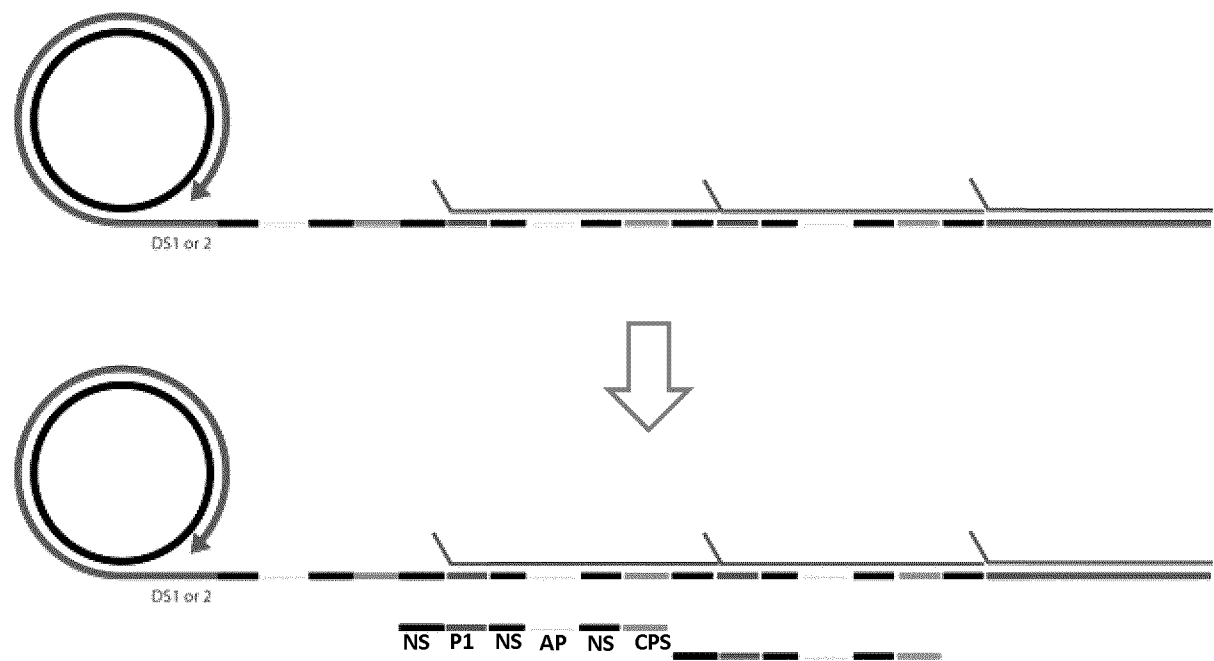


Figure 7

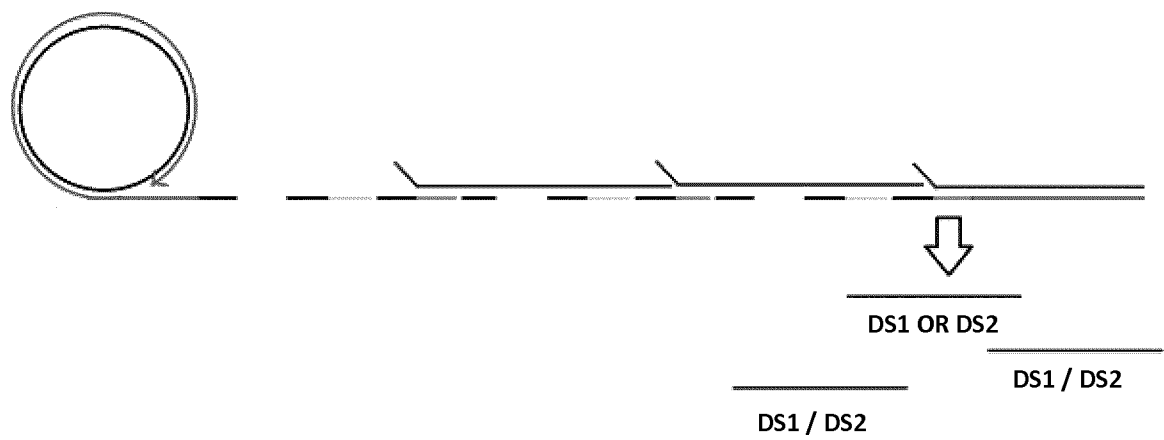


Figure 8

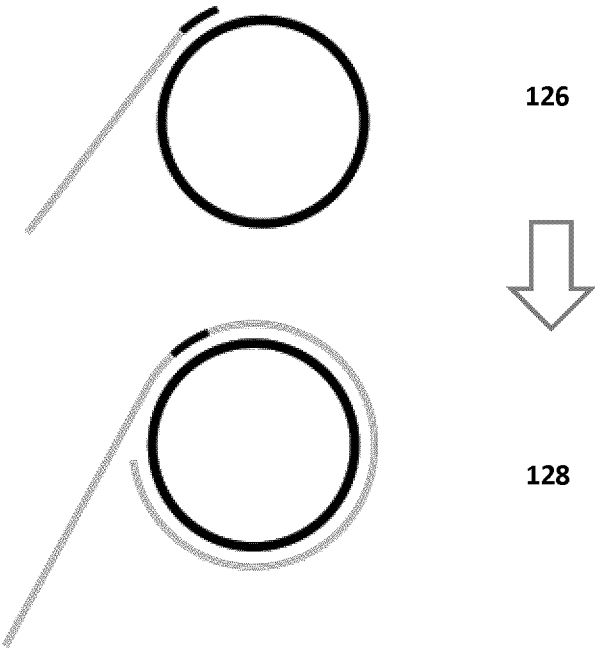


Figure 9



Figure 10

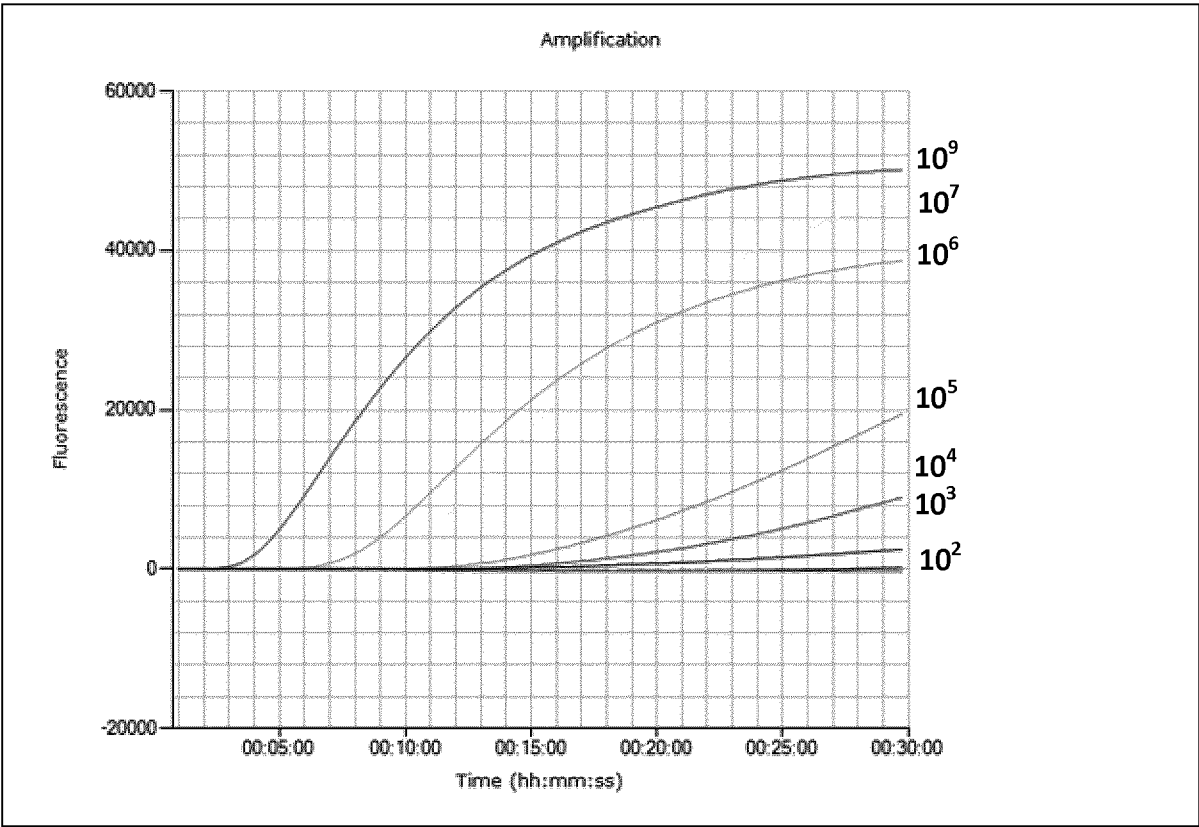


Figure 11

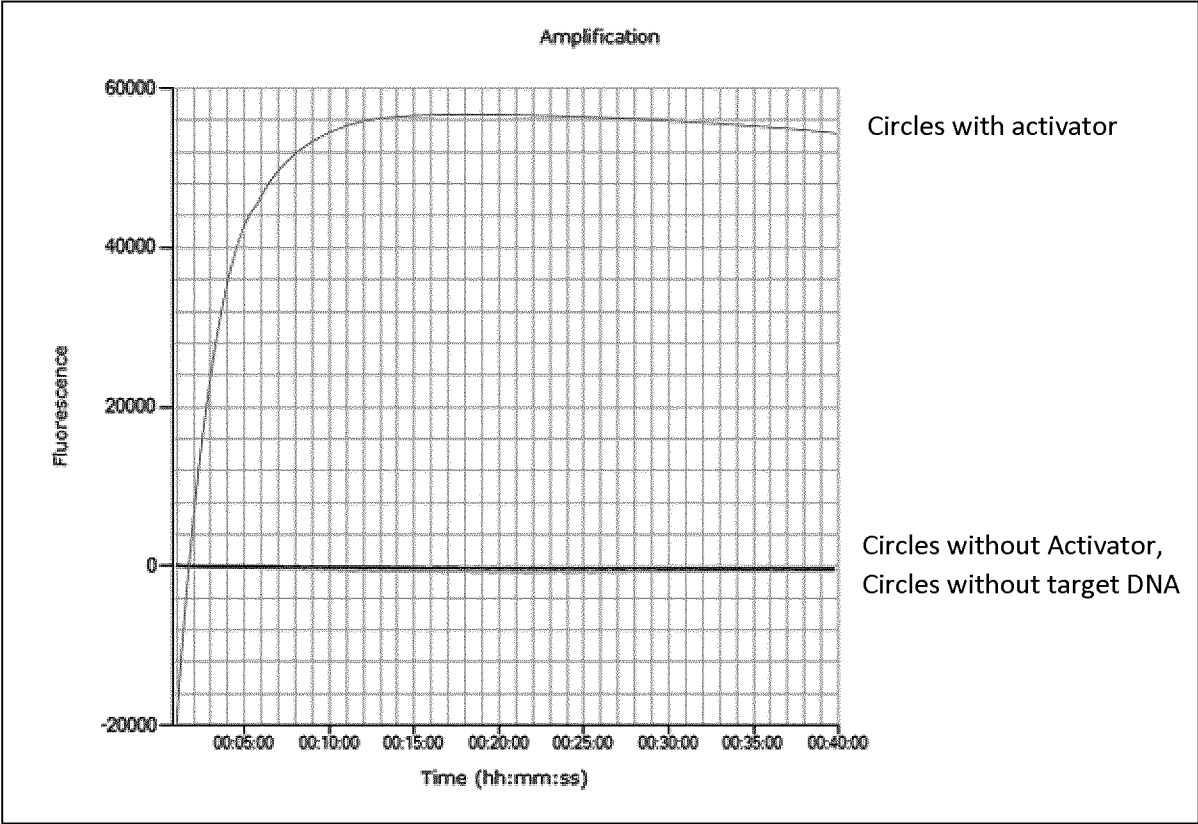


Figure 12

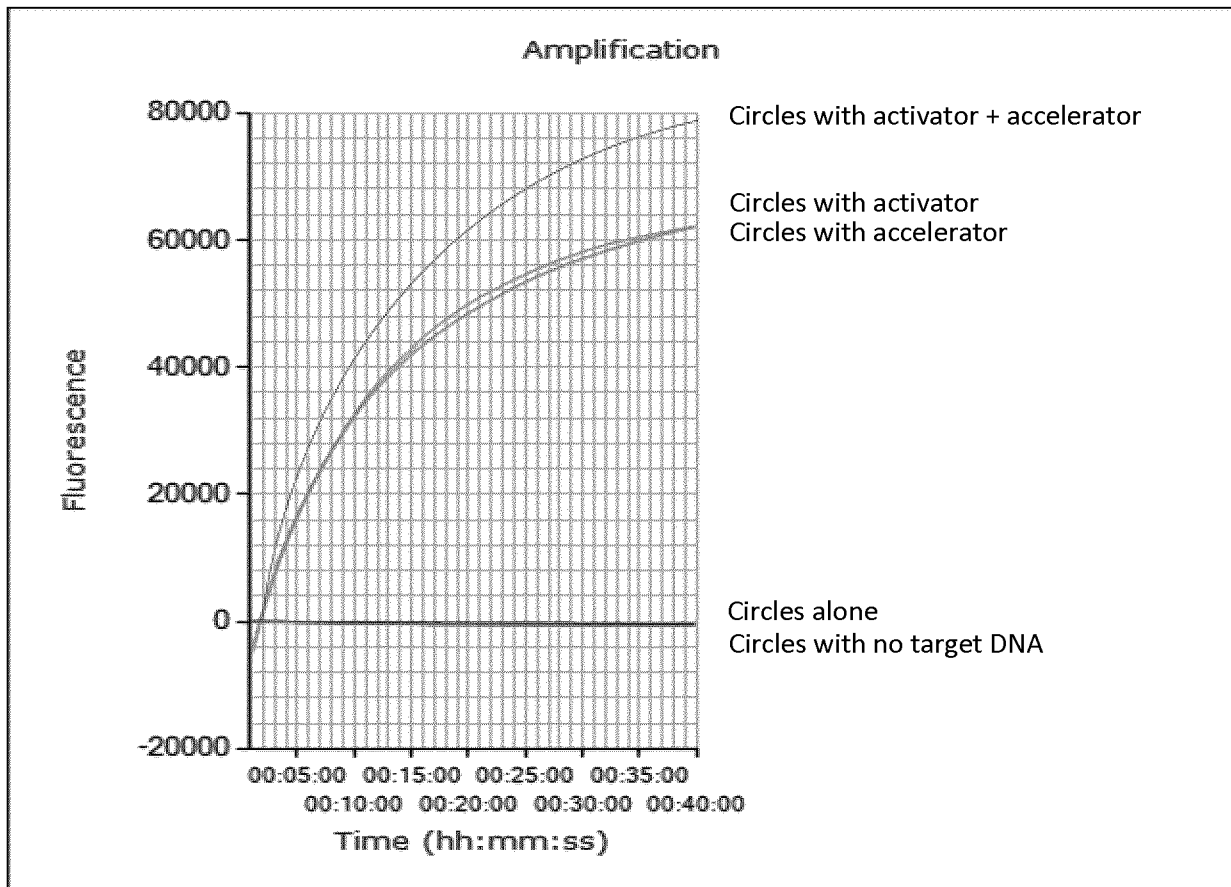


Figure 13

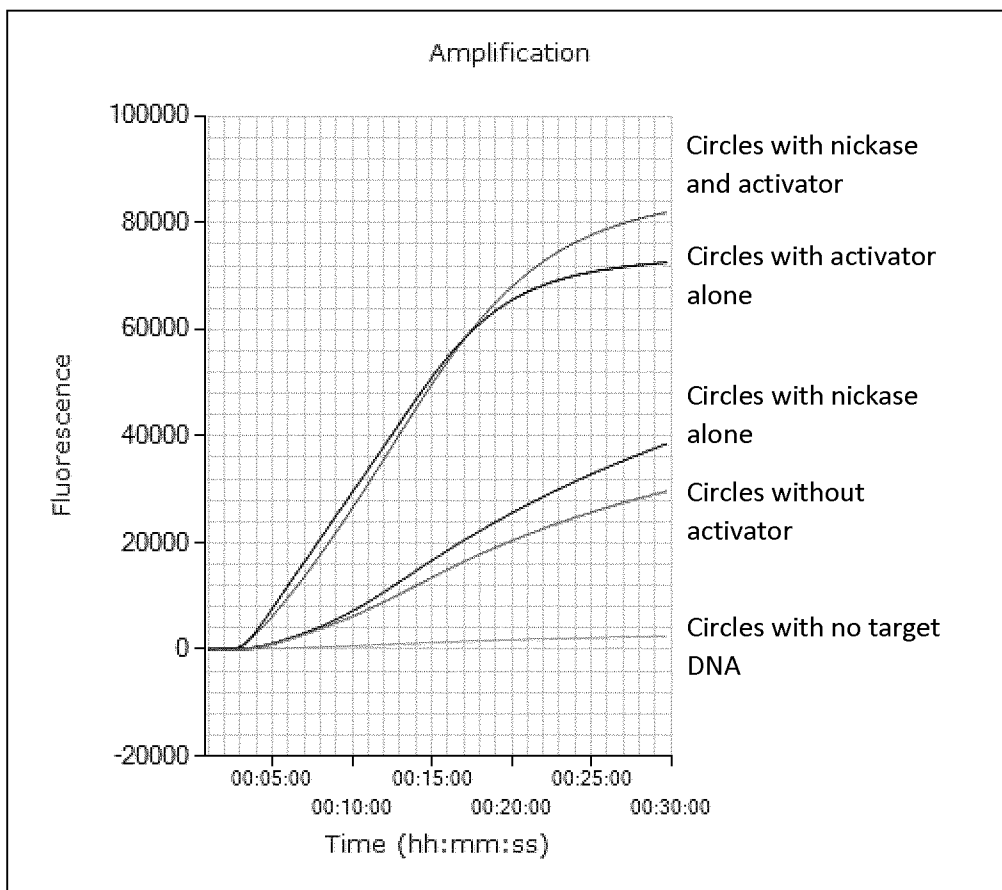


Figure 14

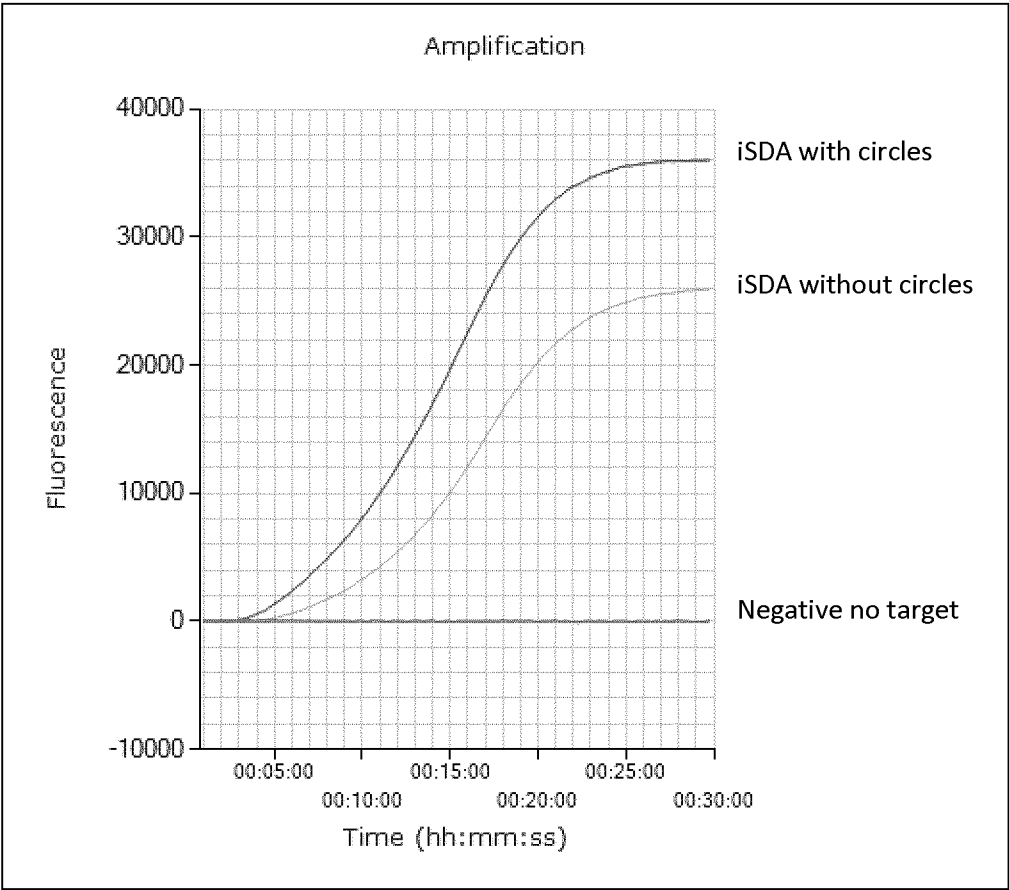


Figure 15

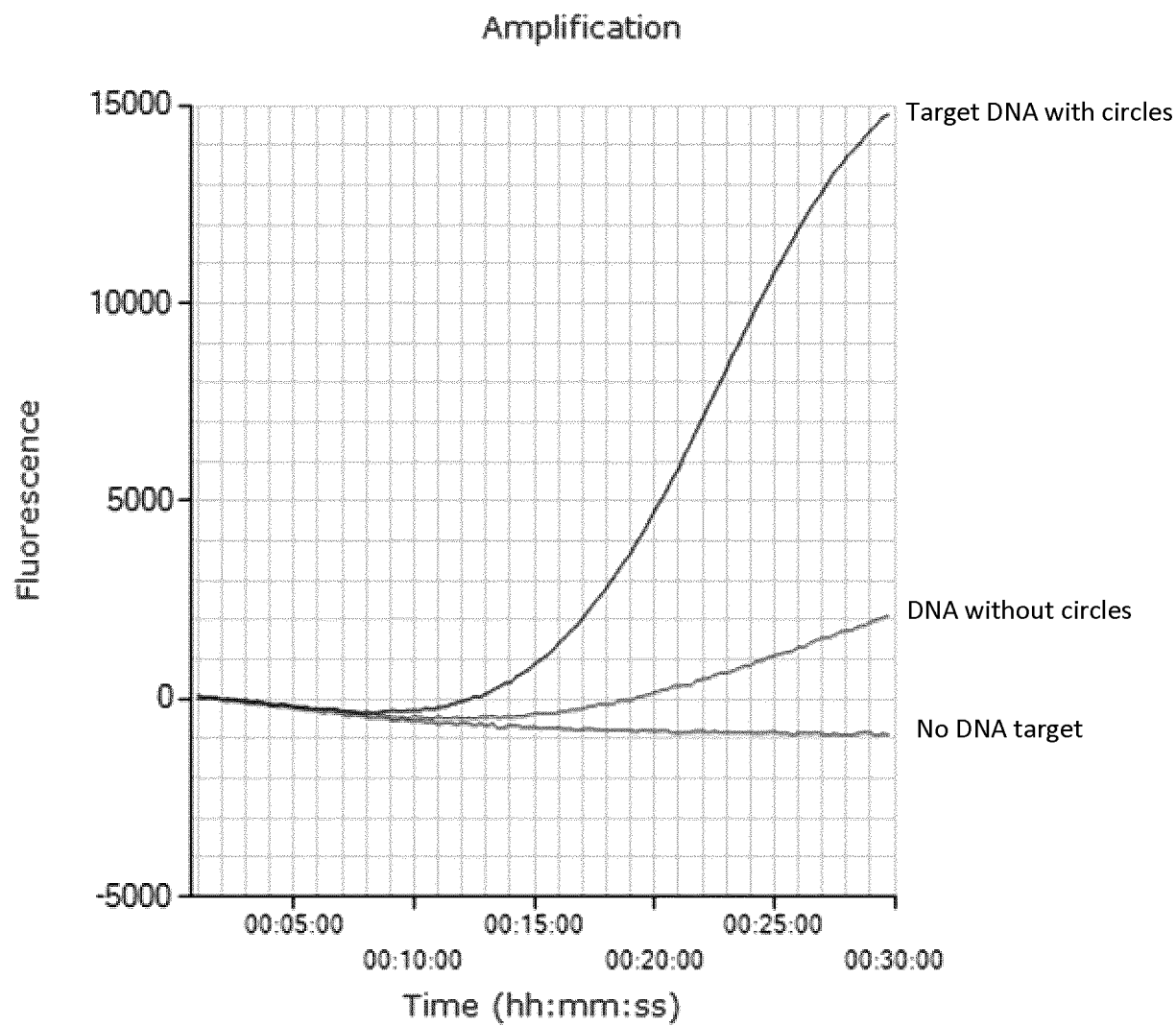
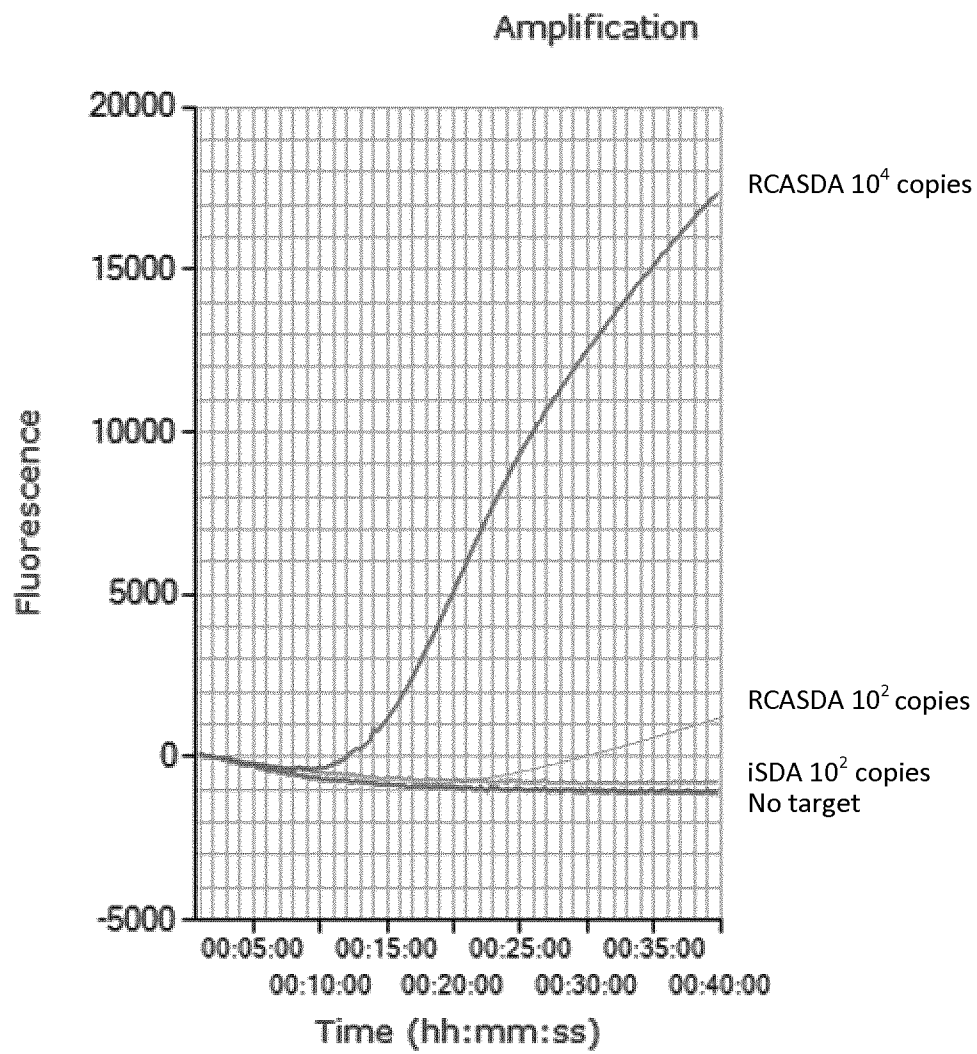


Figure 16



INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2018/051272

A. CLASSIFICATION OF SUBJECT MATTER
 IPC: **C12Q 1/6844** (2018.01), **C12P 19/34** (2006.01), **C12Q 1/6848** (2018.01)

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)
 keywords used across the whole IPC

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
 Databases: Google, Medline, Scopus, Canadian Patent Data Base
 Keywords: strand-displacement amplification (SDA), rolling circle amplification (RCA), combination SDA and RCA, 4 primers

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LI, Y. et al., " <i>Sensitive SERS detection of DNA and lysozyme based on polymerase assisted cross strand-displacement amplification</i> ". Chem Commun, 2012, Vol. 48, pp. 10892–10894, ISSN 1359-7345 [online] [retrieved on 2 January 2019 (02-01-2019)]. Retrieved from the Internet: < https://pubs.rsc.org/en/Content/ArticleLanding/2012/CC/C2CC35688K#!divAbstract >	1, 2, 10, 13, 16, and 19
X	ZHANG, D. Y. et al., " <i>Amplification of target-specific, ligation-dependent circular probe</i> ". Gene, 1998, Vol. 211, pp. 277–285, ISSN 1879-0038 [online] [retrieved on 2 January 2019 (02-01-2019)]. Retrieved from the Internet: < https://www.sciencedirect.com/science/article/pii/S0378111998001139 >	1, 2, 10, 13, 16, and 19

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* "A" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier application or patent but published on or after the international filing date 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) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" "X" "Y" "&"	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 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 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
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Date of the actual completion of the international search

Date of mailing of the international search report
 04 January 2019 (04-01-2019)

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Authorized officer

Damiano Conte (819) 639-7784

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA2018/051272

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZHOU, Y. et al., " <i>Fluorometric determination of microRNA based on strand displacement amplification and rolling circle amplification</i> ". <i>Microchim Acta</i> , 30 August 2017 (30-08-2017), Vol. 184(11), pp. 4359–4365, ISSN 1436-5073 [online] [retrieved on 2 January 2019 (02-01-2019)]. Retrieved from the Internet: < https://doi.org/10.1007/s00604-017-2450-6 >	1-27