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NUCLEIC ACID ISOTHERMAL AMPLIFICATION TECHNOLOGIES— A REVIEW

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□ *Nucleic acid amplification technologies are used in the field of molecular biology and recombinant DNA technologies. These techniques are used as leading methods in detecting and analyzing a small quantity of nucleic acids. The polymerase chain reaction (PCR) is the most widely used method for DNA amplification for detection and identification of infectious diseases, genetic disorders and other research purposes. However, it requires a thermocycling machine to separate two DNA strands and then amplify the required fragment. Novel developments in molecular biology of DNA synthesis in vivo demonstrate the possibility of amplifying DNA in isothermal conditions without the need of a thermocycling apparatus. DNA polymerase replicates DNA with the aid of various accessory proteins. Recent identification of these proteins has enabled development of new in vitro isothermal DNA amplification methods, mimicking these in vivo mechanisms. There are several types of isothermal nucleic acid amplification methods such as transcription mediated amplification, nucleic acid sequence-based amplification, signal mediated amplification of RNA technology, strand displacement amplification, rolling circle amplification, loop-mediated isothermal amplification of DNA, isothermal multiple displacement amplification, helicase-dependent amplification, single primer isothermal amplification, and circular helicase-dependent amplification. In this article, we review these isothermal nucleic acid amplification technologies and their applications in molecular biological studies.*

Keywords PCR; TMA; NASBA; SMART; SDA; RCA; LAMP; IMDA; HDA; SPIA; cHDA.

INTRODUCTION

Innovations in biotechnology that combine molecular biology, micro-fabrication and bioinformatics are moving nucleic acid technologies from futuristic possibilities to common laboratory techniques and modes for disease diagnoses. In this way, amplification of nucleic acids is widely used in research, forensics, medicine, and agriculture.[1] One of the most widely used amplification methods is the polymerase chain reaction (PCR), which is a target amplification method.[2] A PCR reaction typically utilizes two oligonucleotide primers, which are hybridized to the 5′ and 3′ borders of the target sequence, and a DNA polymerase, which can extend the annealed primers by adding on deoxyribonucleoside-triphosphates (dNTPs) to generate double-stranded products (Figure 1). By raising and lowering the temperature of the reaction mixture, the two strands of the DNA product are separated and can serve as templates for the next round of annealing and extension, and the process is repeated.[3] Although PCR has been widely used by researchers, it requires thermocycling to separate the two DNA strands and this characteristic has been limited its application in the field. On the other hand, several isothermal amplification techniques have been developed in the two past decades without using thermocycler machine. These non-PCR based methods have been developed according to some new findings in molecular biology of DNA/RNA synthesis and some accessory proteins and their mimicking in vitro for nucleic acid amplification. We describe here the best known isothermal amplification methods (such as transcription mediated amplification (TMA) or self-sustained sequence replication (3SR), nucleic acid sequence-based amplification (NASBA), signal mediated amplification of RNA technology (SMART), strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated isothermal amplification of DNA (LAMP), isothermal multiple displacement amplification (IMDA), helicase-dependent amplification (HDA), single primer isothermal amplification (SPIA), and circular helicase-dependent amplification (cHDA)) and their applications in molecular diagnosis.

1. TRANSCRIPTION MEDIATED AMPLIFICATION (TMA)/NUCLEIC ACID SEQUENCE-BASED AMPLIFICATION (NASBA)

One of the isothermal amplification technologies is transcription mediated amplification (TMA) that is very similar to nucleic acid sequence-based amplification (NASBA).[4] These techniques utilize the function of an RNA polymerase to make RNA from a promoter engineered in the primer region, and a reverse transcriptase, to produce DNA from
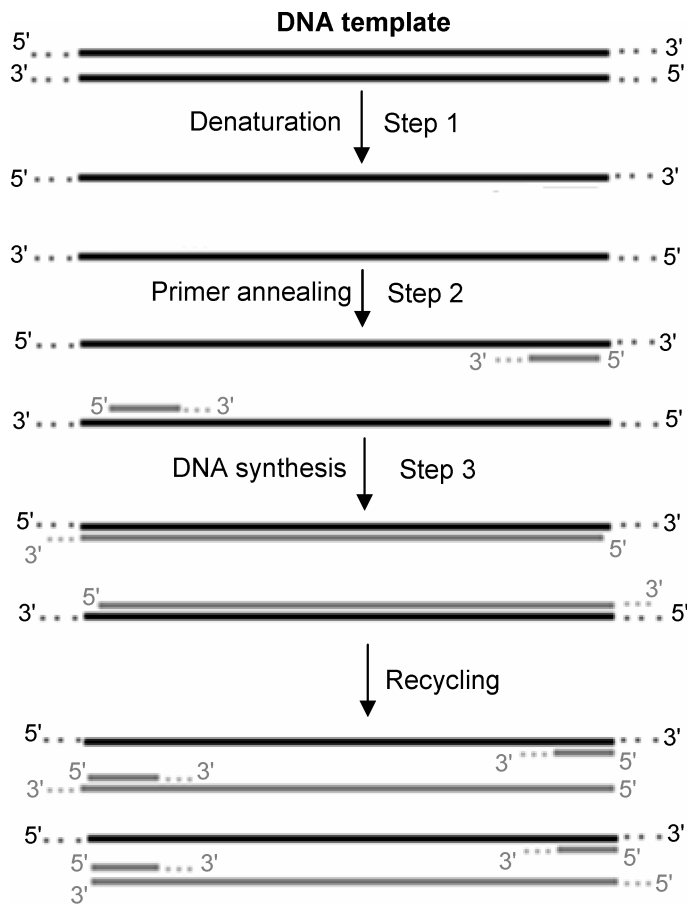
DNA template

FIGURE 1 Polymerase chain reaction (PCR). This process includes 30–40 cycles the following steps: step1, initial denaturation of DNA template by heat; step 2, primer annealing to denatured targeted DNA at specific temperature; step 3, DNA synthesis by thermostable DNA polymerase. (If single stranded RNA is the desired target for PCR, it has to be converted to double stranded DNA firstly by reverse transcriptase.)

the RNA templates (Figure 2). This RNA amplification technology has been further improved by introducing a third enzymatic activity, Rnase H, to remove the RNA from cDNA without the heat-denatured step. Thus, the thermocycling step has been eliminated, generating an isothermal amplification method named self-sustained sequence replication (3SR).[5] The end products of NASBA can be detected using gel electrophoresis, fluorescence probes (real-tmie NASBA), and colorimetric assay (NASBA-ELISA).[6−8] Food and Drug Administration office of United States of America (FDA) has approved the technique in NucliSence formulation (NASBA-ECL) for molecular detection of some microorganisms such as HCV and HIV-1.[9,10]
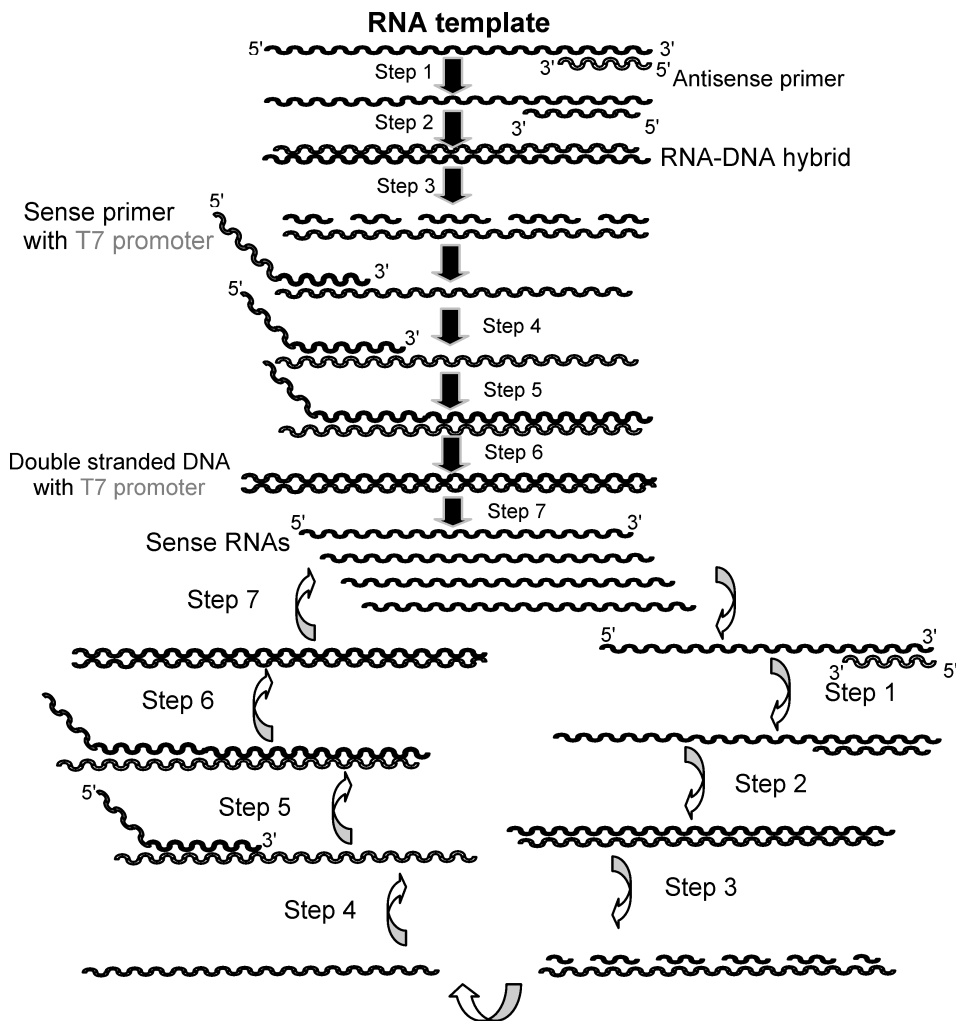
FIGURE 2 NASBA process. Step 1, the RNA template is targeted selectively with the antisense primer. Step 2, the primer anneals to the specific sequence. Step 3, the complement DNA is extended by reverse transcriptase (RNA dependent DNA polymerase activity) against early RNA. Step 4, the RNA-DNA hybrid is treated with RNase H and the early RNA is degenerated. Step 5, the sense primer-containing T7 promoter is annealed to the specific sequence of the newly synthesized single-stranded DNA. Step 6, the complement DNA strand is extended by reverse transcriptase (DNA dependent DNA polymerase activity). Step 7, the double stranded DNA with T7 promoter, which acts as a self-sustained template of the process, is formed. Then using this DNA as template, T7 RNA polymerase synthesizes the sense target RNAs. Each of synthesized RNAs can be participated in these steps again and more RNA amplicons are accumulated.

2. SIGNAL MEDIATED AMPLIFICATION OF RNA TECHNOLOGY (SMART)

This technology is based on the formation of a three-way junction (3WJ) structure. The method relies on signal amplification and does not
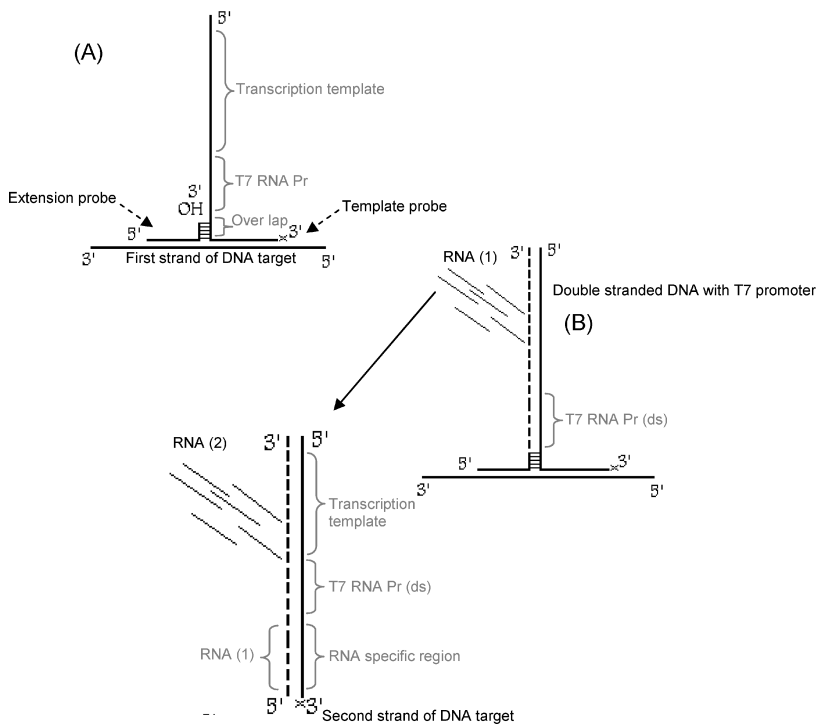
FIGURE 3 SMART process. (A) Formation of a 3WJ. Extension and template probes anneal to the target, and only then to each other. The short extension probe has a free 3′-OH to allow extension. The template probe includes a single-stranded T7 promoter (Pr) and sequence to allow the capture and detection of the RNA signal. The 3′ end of the template probe is blocked (×) by phosphorylation to prevent extension. (B) Extension and transcription generate an RNA signal. Bst DNA polymerase extension of the extension probe generates a double-stranded (ds), hence functional, T7 RNA polymerase promoter (Pr), and allowing transcription of multiple copies of an RNA signal by T7 RNA polymerase. If required, early RNA signals anneal to a second template, leading to further extension and transcription by the DNA and RNA polymerases to generate increased amounts of a second RNA signals.

require thermal cycling or involve the copying of target sequences. The assay generates a signal that is highly target dependent and is appropriate for the detection of DNA or RNA targets.[11] The SMART consists of two single-stranded oligonucleotide probes in corresponding to extension and template: each probe includes one region that can hybridize to the target at adjacent positions and another, much shorter, region that hybridizes to the other probe. The two probes are annealed to each other in the presence of the specific target, so forming a 3WJ (Figure 3A). Following 3WJ formation, Bst DNA polymerase extends the short (extension) probe by copying the opposing template probe to produce a double stranded T7 RNA polymerase promoter sequence (Figure 3B). The formed promoter allows T7 RNA polymerase to generate multiple copies of an RNA amplicons and therefore being produced only when a specific target is present to allow

3WJ formation. Each RNA amplicons may itself be amplified by binding to a second template oligonucleotide (probe for amplification) and is extended by DNA polymerase to generate a double-stranded promoter, leading to transcription which increases the RNA amplicons can be detected by an enzyme linked oligosorbent assay (ELOSA) or in real time format.[12,13] This process is in fact a signal amplification method that the target sequence is not itself amplified.[14]

3. STRAND DISPLACEMENT AMPLIFICATION (SDA)

A third isothermal amplification technology is known as strand displacement amplification (SDA).[15] SDA combines the ability of a restriction endonuclease to nick the unmodified strand of its target DNA and the action of an exonuclease-deficient DNA polymerase to extend the 3′ end at the nick and displace the downstream DNA strand. The displaced strand serves as a template for an antisense reaction and vice versa, resulting in exponential amplification of the target DNA (Figure 4). In the originally-designed SDA, a target DNA sample is heat denatured. Four primers (B1, B2, S1, and S2), present in excess, and bind the target strands at positions flanking the sequence to be amplified. Primers S1 and S2 have HincII recognition sequences (5′ GTTGAC 3′) located 5′ to the target complementary sequences. The four primers are simultaneously extended by exo- klenow using dGTP, dCTP, TTP, and dATP(αS). Extension of B1 displaces the S1 primer extension product, S1-ext. Likewise, extension of B2 displaces S2-ext. B2 and S2 bind to displaced S1-ext. B, and S1 bind to displaced S2-ext. Extension and displacement reactions on templates S1-ext and S2-ext produce two fragments with a hemiphosphorothioate HincII at each end and two longer fragments with a hemiphosphorothioate HincII site at just one end. HincII nicking and exo-kle now extension/displacement reactions initiate at these four fragments, automatically entering the SDA reaction cycle. These reaction steps continuously cycle during the course of amplification. Present in excess are two SDA primers (S1 and S2). The 3′-end of S1 binds to the 3′-end of the displaced target strand T1, forming a duplex with 5′-overhangs. Likewise, S2 binds T2. The 5′-overhangs of S1 and S2 contain the HincII recognition sequence (5′ GTTGAC 3′). Exo- klenow extends the 3′-ends of the duplexes using dGTP, dCTP, TTP, and dATP(αS), which produces hemiphosphorothioate recognition sites on S1:T1 and S2:T2. HincII nicks the unmodified primer strands of the hemiphosphorothioate recognition sites, leaving intact the modified complementary strands. Exo- klenow extends the 3′-end at the nick on S1:T1 and displaces the downstream strand that is equivalent to T2. Likewise, extension at the nick on S2:T2 results in displacement of T1. Nicking
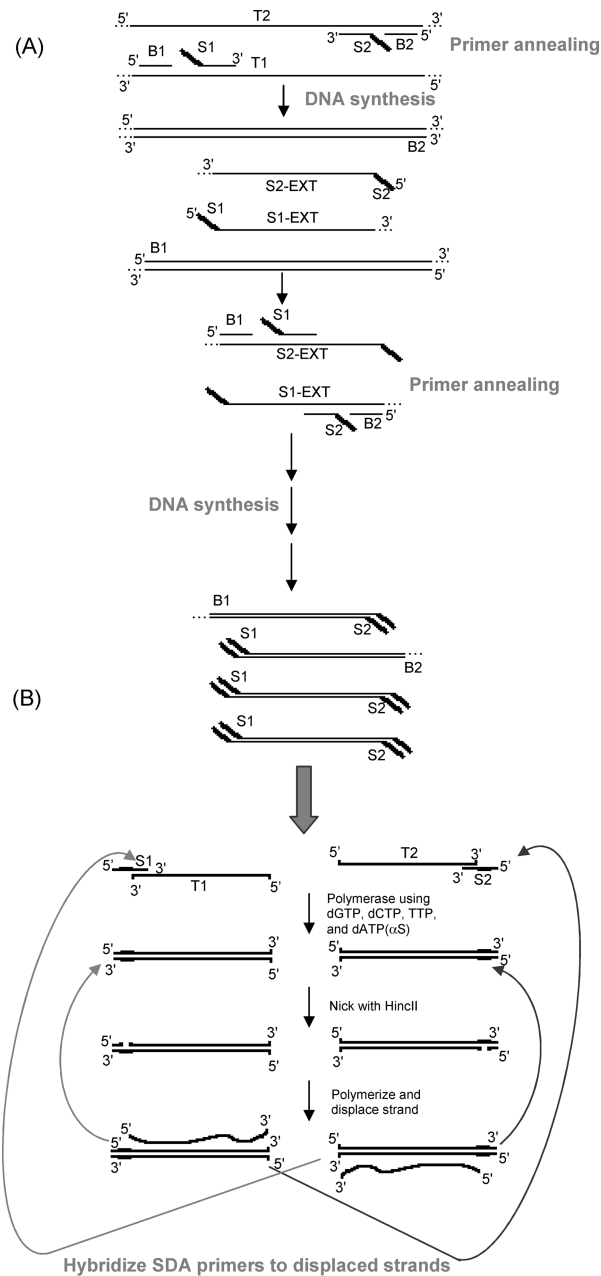
FIGURE 4 Target generation scheme for SDA. (A) This figure depicts the initial steps in an SDA reaction which transform the original target sequence into the amplification cycle. The DNA was first cleaved by a restriction enzyme in order to generate an amplifiable target fragment with defined 5′ and 3′-ends but the requirement of a restriction enzyme cleavage site limited the choice of target DNA sequences. Sense and antisense DNA strands are differentiated by thin and thick lines. HincII recognition sequences are depicted by (━━■■━━). (B) The SDA reaction cycle. This technique has been circumvented by the utilization of bumper primers, which flank the region to be amplified.
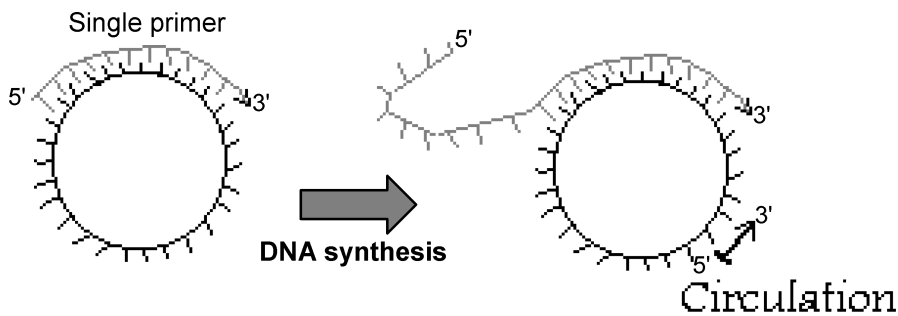
FIGURE 5 Schematic of RCA process. The RCA reaction carrying on a free DNA minicircle with use of a single primer. The reaction is initiated by the hybridization of a linear DNA single strand to a specific DNA minicircle. The amplification products generally exhibit a wide, essentially continuous distribution over length and are normally seen in gel-electrophoresis images as a broad of smear of stranded nature of high-molecular weight DNAs.

and polymerization/displacement steps cycle continuously on S1:T1 and S2:T2 because extension at a nick regenerates a nickable HincII recognition site. Target amplification is exponential because strands displaced from S1:T1 serve as target for S2 while strands displaced from S2:T2 serve as target for S1.[16] SDA technology has been used mainly for clinical diagnosis of infectious diseases such as chlamydia and gonorrhea.[17–20] Also, this technique can be used for isothermal amplification of RNA templates in RT-SDA format by adding reverse transcriptase to the original process.[21,22]

4. ROLLING CIRCLE AMPLIFICATION (RCA)

The rolling circle amplification (RCA) generates multiple copies of a sequence for the use in vitro DNA amplification adapted from in vivo rolling circle DNA replication.[23,24] In its original formulation, the RCA reaction involves numerous rounds of isothermal enzymatic synthesis in which Φ29 DNA polymerase extends a circle-hybridized primer by continuously progressing around the circular DNA probe of several dozen nucleotides to replicate its sequence over and over again (Figure 5).[25,26] The single stranded nature of amplicons in case of linear RCA may be beneficial for subsequent manipulations with these DNAs towards their detection.[24] This reaction is widely used for diagnostic purposes in direct or indirect detection of different DNA/RNA, protein, and other biomarkers via a set of various bimolecular recognition events. A similar reaction was described for RNA polymerases as well, but the RNA-generated process does not require any hybridization-dependent priming.[27] Therefore, the latter is only used to produce functional RNA sequences, such as RNA ladders and self-processing ribozymes.
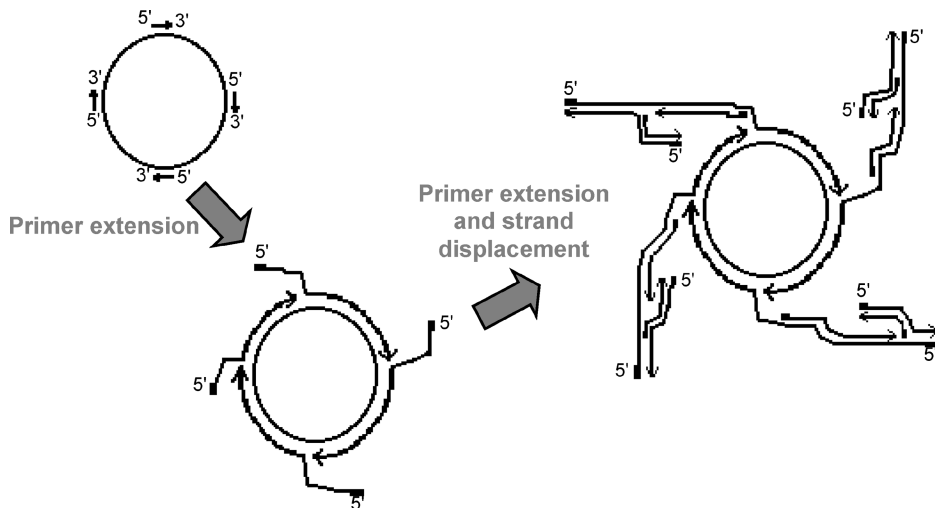
FIGURE 6 Scheme for multiply-primed rolling circle amplification. Oligonucleotide primers complementary to the amplification target circle are hybridized to the circle. The 3′-ends of the DNA strands are indicated by arrowheads to show the polarity of polymerization. Thickened lines indicate the location of the original primer sequences within the product strands. The addition of DNA polymerase and deoxynucleoside triphosphates (dNTPs) to the primed circle results in the extension of each primer, and displacement of each newly synthesized strand results from elongation of the primer behind it. Secondary priming events can subsequently occur on the displaced product strands of the initial rolling circle amplification step.

Recently, RCA has been further developed in a technique, named multiply-primed rolling circle amplification (multiply-primed RCA) that uses the unique properties of Φ29 DNA polymerase and random primers to achieve a 10,000-fold amplification (Figure 6). The process allows amplification of circular DNA directly from cells or plaques, generating, or cloning.[28] RCA-based approaches have recently been attracting attention of diagnostics-oriented biotech companies and research centers for gene tests and immunoassays, SNP scoring and sequencing template preparation, single-cell analysis systems, and gene expression studies.[26]

5. LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP)

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification technology that amplifies DNA under isothermal condition.[29] The LAMP method requires a set of four (to six) specific designed primers and a DNA polymerase with strand displacement activity. The amplification products are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops (Figure 7). The LAMP method is also a highly efficient amplification method that
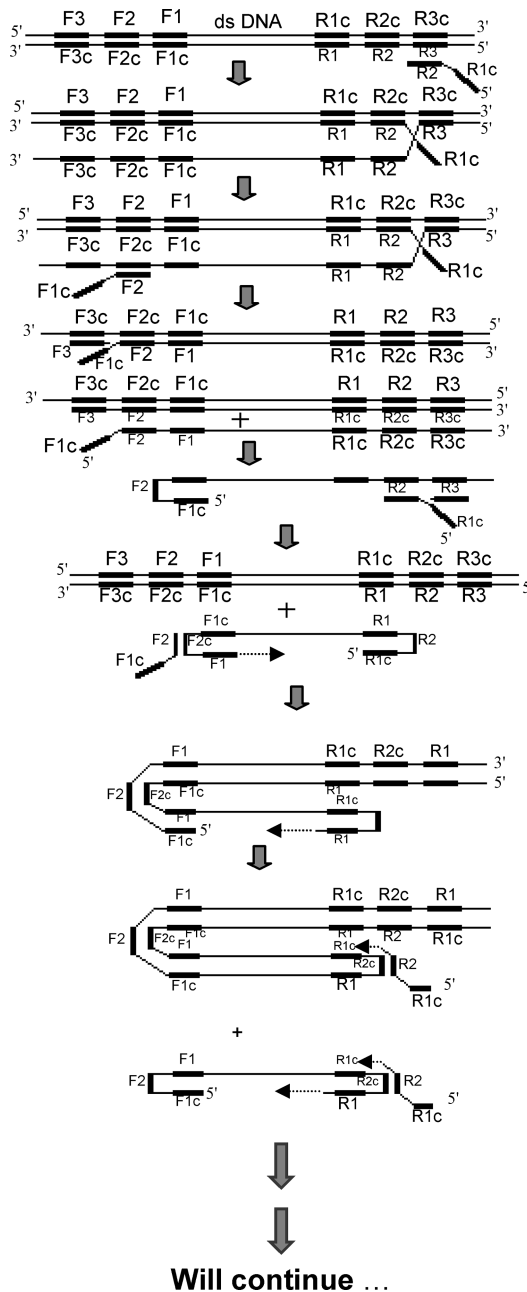
FIGURE 7 Schematic representation of the LAMP mechanism. Steps in the original LAMP reaction. In the first step, in which starting material is produced; the dumbbell-like DNA form is generated. Then, in cycling amplification step, DNAs of this form are generated continuously. The elongation reactions are started from the sub-products of the cycling amplification step, generating various sizes of the products. F2c and B2c are hybridized by the inner primers.

allows the synthesis of large amounts of DNA in a short time. As a result, pyrophosphate ions are produced in large amounts and form white precipitates of magnesium pyrophosphate. Judging the presence or absence of this white precipitate allows easy distinction of whether nucleic acid was amplified by the LAMP method.[30] However the other formats such as gel electrophoresis, real-time turbidimetry, and fluorescence probes have been used for detection of LAMP products.[31,32] This technology has widely been used for molecular detection of several microorganisms by researchers and it can be a suitable choice for design and development of rapid molecular tests in the field.[33,34]

6. ISOTHERMAL MULTIPLE DISPLACEMENT AMPLIFICATION (IMDA)

The technology is based on strand displacement replication of the nucleic acid sequences by multiple primers.[35] In one preferred form of the method, referred to as multiple strand displacement amplification, two sets of primers are used, a right set and a left set (Figure 8). The primers in the right set are complementary to one strand of the nucleic acid molecule to be amplified and the primers in the left set are complementary to the opposite strand. The 5′ ends of primers in both sets are distal to the nucleic acid sequence of interest when the primers have hybridized to the nucleic acid sequence molecule to be amplified. Amplification proceeds by replication initiated at each primer and continuing through the nucleic acid sequence of interest. A key feature of this method is the displacement of intervening primers during replication by the polymerase. In another preferred form of the method, referred to as whole genome strand displacement amplification, a random set of primers is used to randomly prime a sample of genomic nucleic acid.[36,37] Amplification proceeds by replication with a highly processive polymerase initiated at each primer and continuing until spontaneous termination. In this way, multiple overlapping copies of the entire genome to be synthesized in a short time.[38–40]

7. HELICASE-DEPENDENT AMPLIFICATION (HDA)

Helicase-dependent amplification (HDA) is based on the unwinding activity of a DNA helicase.[41] This process uses a helicase, rather than heat, to separate the two strands of a DNA duplex generating single-stranded templates for the purpose of in vitro amplification of a target nucleic acid.[42] Sequence-specific primers hybridize to the templates and are then extended by DNA polymerases to amplify the target sequence. This process repeats itself so that exponential amplification can be achieved at a single
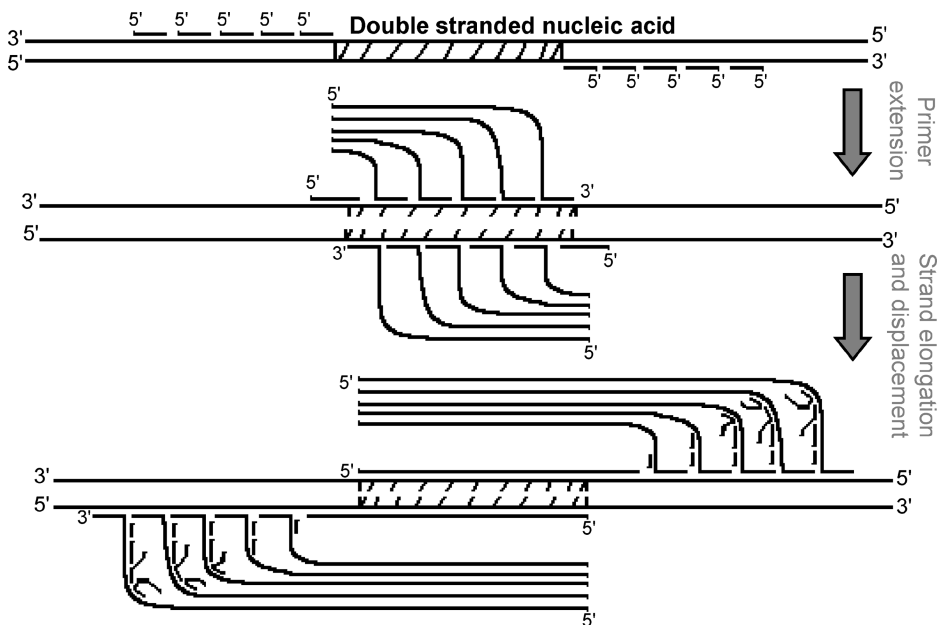
FIGURE 8 Schematic representation of IMDA mechanism. This is a diagram of an example of isothermal multiple strand displacement amplification. Diagramed at the top is a double stranded nucleic acid molecule which contains a nucleic acid of interest (hatched area). Hybridized to the nucleic acid molecules are right and left sets of primers. Diagramed at the middle are the multiple strands of replicated nucleic acid being elongated from each primer. The polymerase at the end of each elongating strand displaces the elongating strand of the primer ahead of it. Diagramed at the bottom are the multiple strands of replicated nucleic acid further elongated. Also shown are the next sets of primers, which hybridize to their complementary sites on the newly replicated strands. The newly replicated strands are made available for hybridization to the primers through displacement by the polymerase elongating the following strand.

temperature (Figure 9). This process allows multiple cycles of replication to be performed at a single incubation temperature, completely eliminating the need for thermo cycling equipment.[3] The HDA amplicons can be detected using gel electrophoresis, real-time format, and enzyme-linked immunosorbent assay (ELISA).[41–45]

8. SINGLE PRIMER ISOTHERMAL AMPLIFICATION (SPIA)

This amplification technology uses a single chimeric primer for amplification of DNA (SPIA) and RNA (Ribo-SPIA).[46] SPIA employs a single, target-specific chimeric primer composed of deoxyribonucleotides at the 3′ end and ribonucleotides at its 5′ end, RNase H, and a DNA polymerase with a strong strand displacement activity. Amplification is initiated by hybridizing the chimeric primer to a complementary sequence in the target DNA molecule. DNA polymerase initiates primer extension of the hybridized primer and extends along the target DNA strand. Following
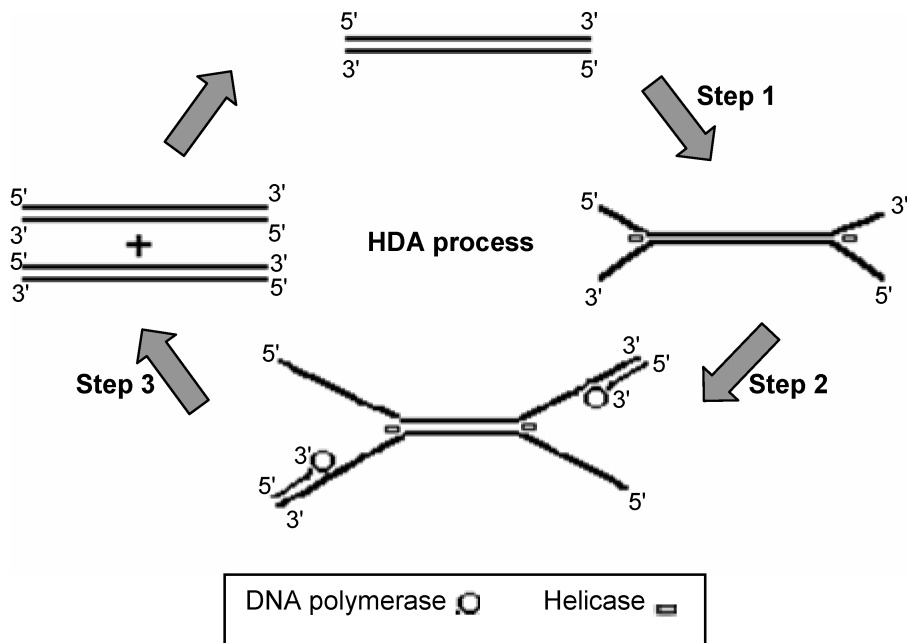
FIGURE 9 The figure shows HDA process. Step 1, the helicase unwinds DNA duplexes. Step 2, the primers anneal to the ssDNA. Step 3, DNA polymerase extends the primers; one duplex is amplified to two duplexes. The dsDNAs are separated by helicase and this chain reaction repeats itself.

initiation of the primer extension step, the 5′ RNA portion of the extended primer (RNA-DNA hybrid) is cleaved by RNase H, thus freeing part of the primer-binding site on the target DNA strand form binding of a new chimeric primer. The newly bound primer competes with the previous primer extension product for binding to the complementary DNA target sequence and is stabilized by binding of DNA polymerase and displaces the 5′ end of the previous extension product. As replication is again initiated by primer extension, RNase H cleavage of the 5′ RNA portion of the newly extended primer again frees part of the primer binding site for subsequent primer binding and replication cycle is repeated. SPIA amplification can be used for global genomic DNA amplification and for the amplification of specific genomic sequences and synthetic oligonucleotide DNA targets. Ribo-SPIA is similarly suitable for global and target-specific RNA amplification (Figure 10).[47–49] Ribo-SPIA technology provides an elegant method for linear, isothermal amplification of the mRNA species in a total RNA population. Replication is initiated and repeated up to 10,000 times off of each original transcript. Therefore, this process can be used for amplification of large populations of nucleic acid species, which are limited in biological samples, as are commonly encountered in clinical researches.[46]
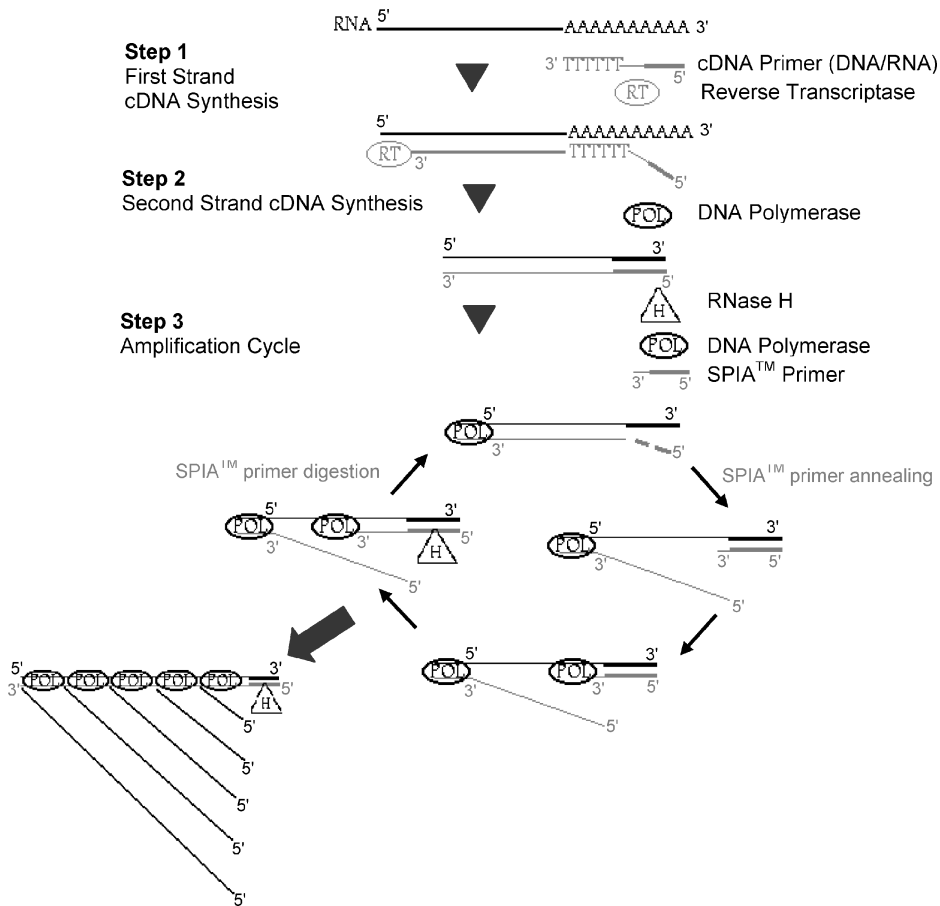
FIGURE 10 Schematic representation of the 3′-initiated Ribo-SPIA process. Step 1, first strand cDNA is produced using a unique DNA/RNA chimeric primer and reverse transcriptase. Step 2, the RNA template is partially degraded in a heating step which also serves to denature the reverse transcriptase. DNA polymerase is added to the reaction mixture to carry out second strand cDNA synthesis along the first strand cDNA product from the first step. The DNA polymerase elongates the product along the RNA portion of the chimeric primers, by its RNA-dependent DNA polymerase activity, thus forming a double-stranded cDNA with a unique RNA/DNA heterodouplex at one end. This unique product serves as a substrate for the subsequent SPIA DNA amplification step. Step 3, amplification is initiated by adding RNase H, DNA polymerase, and a second chimeric DNA/RNA heterodouplex at the end of the double stranded cDNA. This exposes 3′ single strand at one end of the second strand cDNA whose unique sequence is complementary to the SPIA primer. The SPIA primer binds to this site, and the primer is extended by a strand displacing DNA polymerase. As soon as this extension begins, RNase H can again digest the RNA portion of the primer at the 5′ end of the new strand, thus revealing another priming site for the RNA portion of a new primer molecule to bind. This continuous and isothermal cycle of degradation the RNA/DNA heterodouplexes, annealing of new SPIA primer, cDNA extension and strand displacement continues in a linear fashion, producing microgram quantities of anti-sense cDNA.

9. CIRCULAR HELICASE-DEPENDENT AMPLIFICATION (cHDA)

The cHDA is used for amplifying nucleic acids from a circular DNA template. This system combines a DNA polymerase and a helicase preparation
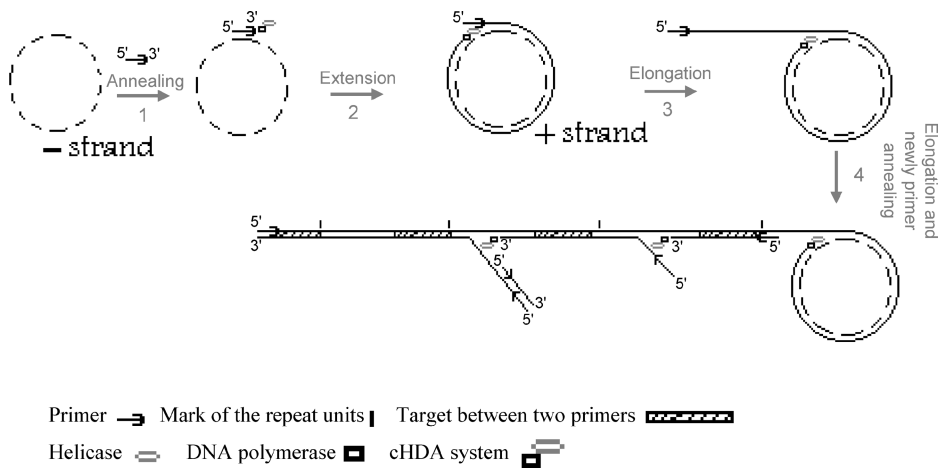
FIGURE 11 cHDA mechanism. This figure shows an antisense primer annealing to the template. Primer extension produces a concatemer of the template. Multiple sense primers anneal to the concatemer and are extended by the DNA polymerase. As the polymerization reaches the downstream primer extension product, the helicase/DNA polymerase complex displaces the non-template strand. Multiple rounds of displacement and polymerization produce a specific target DNA defined by two primers and multimers of the DNA template.

to amplify a target sequence as well as the entire circular DNA template containing the target sequence.[50] The technique is based on the T7 replication machinery, which includes the processive T7 helicase, an exonuclease-deficient T7 DNA polymerase (T7 sequenase) and the T7 Gp2.5 single-stranded DNA binding (SSB) protein. After the duplex DNA template is unwound by T7 helicase, specific primers anneal to the separated DNA strands and T7 sequenase extends the $3'$ end of each primer by a rolling circle mechanism to amplify not only a region defined by the primers but also continuous concatemers of the template (Figure 11). The process can be carried out at one temperature (25°C) for the entire process. Amplification can be performed using purified plasmid DNA or crude cell lysate can amplify inserts as large as 10 kilo base pairs.[50]

CONCLUSION

In this study, we described the best known isothermal technologies for DNA or RNA amplification that offer several advantages over PCR in that they eliminate the need for an expensive and cost-intensive thermocycler. However, these isothermal amplification technologies have advantages or weaknesses that limit their use in some aspects of molecular biology (Table 1) like PCR. For example, SDA uses four primers to generate initial amplicons and modified deoxynucleotides to provide strand-specific nicking.[15,16] However, it is inefficient at amplifying long target sequences.

TABLE 1 Properties of various isothermal amplification methods and PCR

Property	PCR	NASBA	SMART	SDA	RCA	LAMP	HDA	SPIA
DNA amplification	+	+	+	+	+	+	+	+
RNA amplification	+	+	+	+	+	+	+	+
Temperature (s)°C	94, 55–60, 72	37–42	41	37	37	60–65	Room*, 37, 60–65	45, 50
Number of enzyme (s)	1	2–3	2–3	2	1	1	2	3
Primer design	Simple	Simple	Complex	Complex	Simple	Complex	Simple	Simple
Multiplex amplification	+	+	–	–	+	–	+	–
Product detection method	Gel electrophoresis, ELISA, Real-time	Gel electrophoresis, ELISA, Real-time, ECL	ELOSA, Real-time	Gel electrophoresis, Real-time	Gel electrophoresis, Real-time	Gel electrophoresis, turbidity, real-time	Gel electrophoresis, ELISA, real-time	Bioanalyzer
Tolerance to biological components	–	–	–	–	–	+	+	–
Need to template denaturation	+	+	+	+	–	–	–	+
Denaturing agent (s)	Heat	RNase H	RNase H	Restriction enzymes, Bumper primers	Strand-displacement property of Φ29 DNA polymerase	Betaine	Helicase	RNase H

*Room: 22–24° C.
ELISA: Enzyme-linked immunosorbent assay.
ELOSA: Enzyme-linked oligosorbent assay.
ECL: Electrochemiluminescence.

LAMP requires to four to six specific primers that their designs are complicated for new user.[51] Also, its final product is a complex mixture of stem-loop cauliflower-like DNA structures of various sizes. Nagamine et al. have devised extra steps to obtain uniform single-stranded DNA from LAMP products. This is preferable for various hybridization techniques. The advanced method uses the thermo stable TspRI restriction enzyme to digest amplification product,[52] and an additional primer hybridized to the 9-nt 3′ overhang at the TspRI cleavage site to displace single-stranded DNA by primer extension.[53]

TMA needs three different enzymatic steps (transcription/cDNA synthesis/RNA degradation) to accomplish an isothermal RNA amplification,[4] and its starting material is limited to single stranded nucleic acid/RNA.

Due to its robustness and simplicity, RCA holds a distinct position in DNA diagnostics among other single-temperature amplification techniques. As compared with RCA, all other isothermal methods of signal, probe, or target DNA amplification, such as transcription-based system, strand-displacement approach or loop-mediated techniques are rather complicated and in most cases they require prior assay optimization.[26,54] Although, SDA and RCA are described as isothermal amplification systems, both methods require an initial heat denaturation step.

On the other hand, some of these methods such as HDA have a simple reaction scheme, in which a target sequence can be amplified by two flanking primers, similar to PCR.[41,42] One of the most important advantages of the isothermal amplification techniques is related to their tolerances to some inhibitory materials that affect the PCR efficiency. Recently, Kaneko et al. evaluated the tolerance of LAMP to a culture medium and some biological substances.[55] According to this study, the sensitivity of LAMP was less affected by the various components of the clinical samples than was PCR; therefore, DNA purification can be omitted. Another example is about HDA; a pathogen genomic DNA can even be detected in a human blood sample.[41] This demonstrates that HDA can be performed on crude samples and has the potential to be used as a diagnostic tool.

Another important advantage for the isothermal amplification techniques is no need to initial heat denaturation at a high temperature followed by amplification at a lower temperature. This property has been reported about some isothermal amplification methods. For example, because there is no necessity for heat denaturation of the template DNAs, LAMP can be used more easily and rapidly in molecular medicine.[56] As DNA helicase can melt double-stranded target DNA at the beginning of the reaction, the entire HDA reaction can be performed at one temperature.[41]

In conclusion, these technologies differ in their requirements for sample volume, specimen preparation, and methods of amplification and detection. There is no doubt that these capabilities should help isothermal

amplifications to successfully compete with its widely used non-isothermal predecessor (PCR) for the number of diagnostic applications. The simplicity and isothermal nature of these methods offer great potentials for the development of hand-held DNA diagnostic devices that could be used to detect pathogens at point-of-care or in the field.

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