A Review of the Current Isothermal Amplification Techniques: Applications, Advantages and Disadvantages

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ABSTRACT

Polymerase chain reaction is the most widely used method for DNA amplification for the detection and identification of infectious diseases, genetic disorders and for other research purposes. However, it requires a thermocycling machine to separate the 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 temperature without the need of a thermocycling apparatus. DNA polymerase replicates DNA with various accessory proteins. Therefore, with identification of these proteins, we are able to develop new *in vitro* isothermal DNA amplification methods by mimicking these *in vivo* mechanisms. There are several isothermal nucleic acid amplifications, such as transcription-mediated amplification or self-sustained sequence replication, 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 paper, we reviewed the nucleic acid amplification methods and their applications in molecular biology. We reviewed the best-known isothermal techniques for DNA/RNA amplification. The above information can be used for the application of valuable molecular diagnostic techniques for the detection of infectious agents in small-scale hospital laboratories in the field. With regards to the properties of isothermal DNA/RNA amplification techniques, the design and development of novel rapid molecular tests for application in field may be possible.

Key words: cHDA, HDA, IMDA, LAMP, NASBA, PCR, RCA, SDA, SMART, SPIA, TMA

INTRODUCTION

Innovations in biotechnology that combine molecular biology, microfabrication and bioinformatics are moving nucleic acid technologies from futuristic possibilities into common laboratory and clinical procedures. By this, amplification of nucleic acids can be widely used in research, forensics, medicine and agriculture.[1] One of the best-known amplification methods is the polymerase chain reaction (PCR), which is a target amplification method. 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.[2] Although PCR has been widely used by researchers, it requires thermocycling to separate the two DNA strands. This characteristic has limited its application in the field. On the other hand, several isothermal target amplification methods have been developed in the two past decades without using a thermocycler machine. These non-PCR-based techniques have been developed according to 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

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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 [HAD], single primer isothermal amplification [SPIA] and circular helicase-dependent amplification [cHDA]) and their applications in the field. These techniques differ in their requirements for sample volume, specimen preparation and methods of amplification and detection. There are strengths and weakness to each of the amplification systems and, therefore, there is probably no one assay best suited to all situations.[3]

TMA/NASBA

One of the isothermal amplification techniques is TMA, which is similar to NASBA.[3] 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 the RNA templates [Figure 2]. This RNA amplification technology has been further improved by introducing a third enzymatic activity, RnaseH, to remove the RNA from cDNA without the heat-denaturing step. Thus, the thermocycling step has been eliminated, generating an isothermal amplification method named 3SR.[4] The end products of NASBA can be detected using gel electrophoresis, fluorescence probes (real-time NASBA) and colorimetric assay (NASBA-enzyme-linked immunosorbent assay [ELISA]).[1,5,6] The United States Food and Drug Administration office (FDA) has approved the technique in NucliSence format (NASBA-ECL) for molecular detection of some microorganisms such as hepatitis C virus and human immunodeficiency virus-1.[7,8]

SMART

This technique is based on the formation of a three-way junction (3WJ) structure. The method relies on signal amplification and does not 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.[9] The SMART consists of two single-stranded oligonucleotide probes 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, 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 RNA. Amplicons are therefore produced only when a specific target is present to allow 3WJ formation. Each RNA amplicon 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 detected by an enzyme-linked oligosorbent assay (ELOSA) or in real-time format.[10,11] This process is in fact a signal amplification method, where the target sequence is not itself amplified.[12]

SDA

A second isothermal amplification system is known as SDA.[13] 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

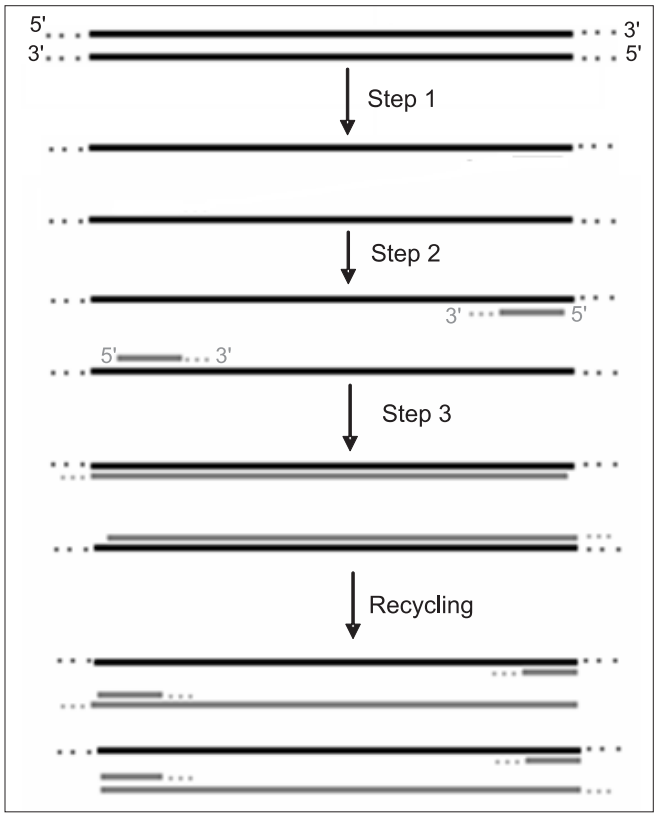

Figure 1: Polymerase chain reaction. This process includes 30–40 cycles of the following steps: Step 1, initial denaturation of DNA template by heat; Step 2, primer annealing to denatured targeted DNA at specific temperature; Step 3, DNA synthesis by thermostable DNA polymerase

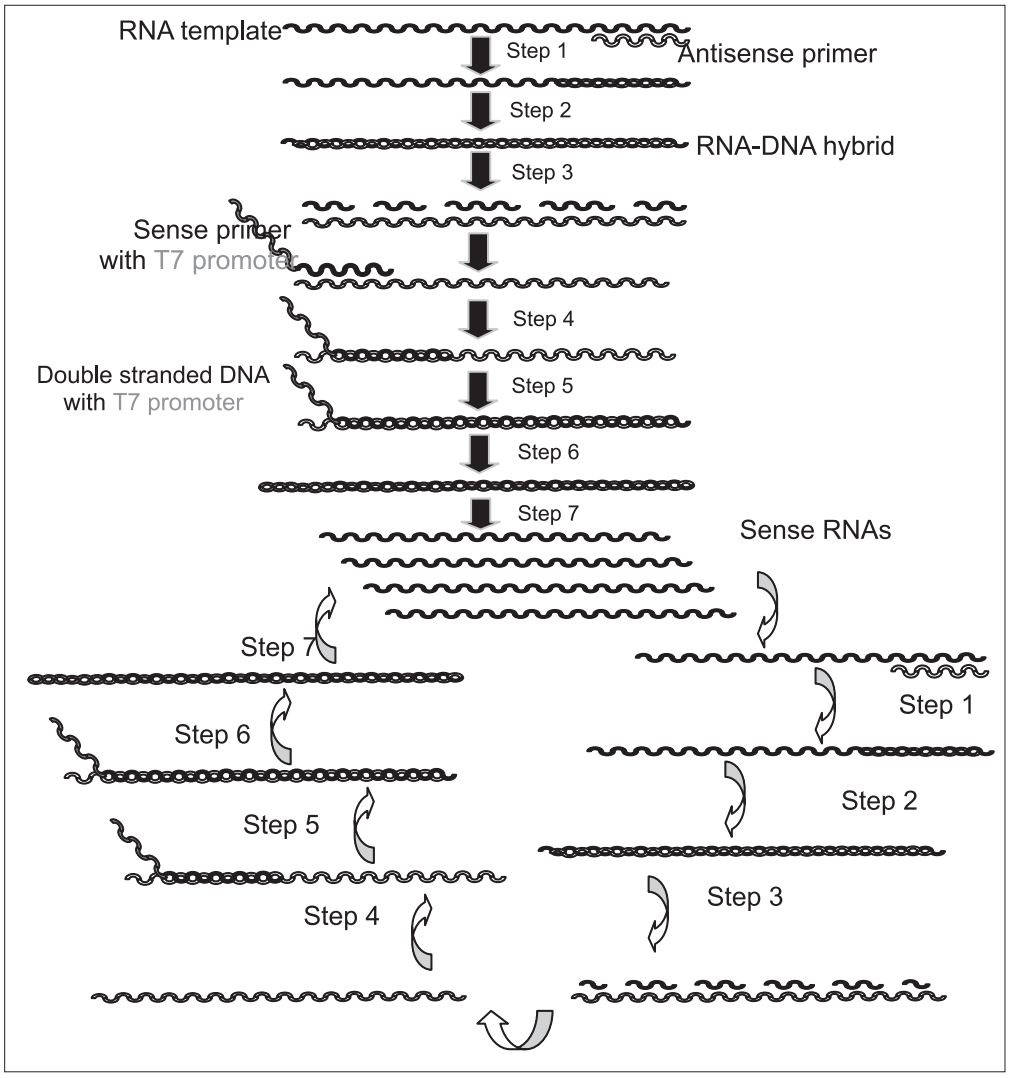

Figure 2: Nucleic acid sequence-based amplification process. Step 1: the RNA template is targeted selectively with the antisense primer. Step 2: the primer anneals to the specific sequence. Step 3: the complement DNA is extended by reverse transcriptase (RNA-dependent DNA polymerase activity) against early RNA. Step 4: the RNA–DNA hybrid is treated with RNaseH 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 the template, T7 RNA polymerase synthesizes the sense target RNAs. Each of the synthesized RNAs can be participated in these steps again and more RNA amplicons are accumulated

[Figure 4]. In the originally designed SDA, 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.[14] This technique has been circumvented by using bumper primers, which flank the region to be amplified. SDA technology has been used mainly for clinical diagnosis of infectious diseases such as chlamydia and gonorrhea.[15,16] One of the most attractive features of SDA is its operation at a single temperature, which circumvents the need for expensive instrumented thermal cycling. However, SDA is inefficient in amplifying long target sequences. Also, this technique can be used for isothermal amplification of RNA templates in the RT-SDA format by adding reverse transcriptase to the original process.[17]

RCA

The RCA generates multiple copies of a sequence for use *in vitro* and DNA amplification adapted from *in vivo* rolling circle DNA amplification.[18,19] In this reaction, a DNA polymerase extends a primer on a circular template, generating tandemly linked copies of the complementary sequence of the template using Φ29 DNA polymerase [Figure 5]. As a result, long DNA repeats will be formed that

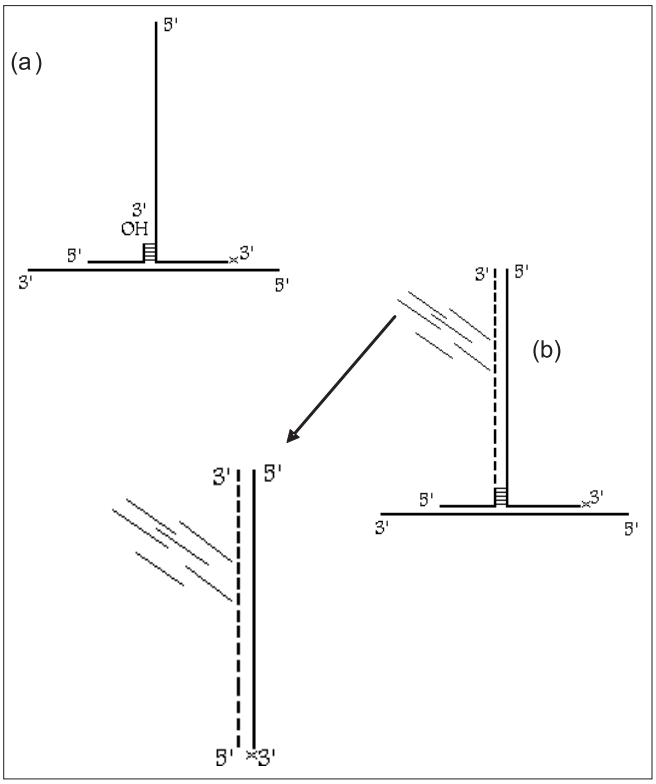

Figure 3: Signal-mediated amplification of RNA technology process. (a) Formation of a three-way junction. Extension and template probes anneal to the target, and only then to each other. The short extension probe has a free 3'-OH to allow extension. The template probe includes a single-stranded T7 promoter (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 allows transcription of multiple copies of an RNA signal by the 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 signal

can readily be detected. Importantly, RCA needs no special instrumentation to cycle the temperature, as is required with the widely used PCR-based DNA technologies.[20,21] RCA-based approaches have recently been attracting the attention of diagnostics-oriented biotech companies and research centers for gene tests and immunoassays, single nucleotide polymorphisms (SNP) scoring and sequencing template preparation, single-cell analysis systems and gene expression studies.[21]

LAMP

LAMP is a nucleic acid amplification technique that amplifies DNA under isothermal conditions.[22] The LAMP method requires a set of four (to six) specific designed primers and a DNA polymerase with strand displacement

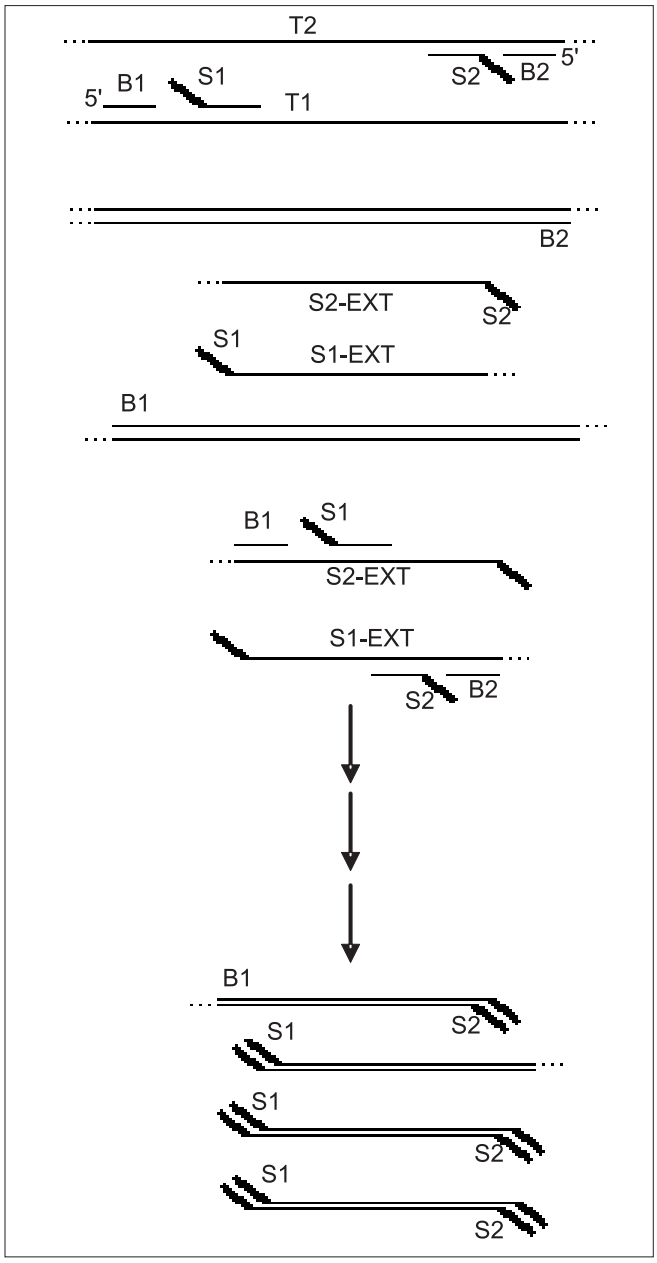

Figure 4: Target generation scheme for strand displacement amplification (SDA). This figure depicts the initial steps in an SDA reaction, which transform the original target sequence into the amplification cycle. A target DNA sample is heat denatured. Four primers (B1, B2, SI and S2), present in excess, bind the target strands at positions flanking the sequence to be amplified. Primers SI and S2 have HincII recognition sequences (GTTGAC) located 5' to the target complementary sequences. The four primers are simultaneously extended by exo-Klenow using dGTP, dCTP, TTP and dATPS. Extension of B1 displaces the SI primer extension product, S1-ext. Likewise, extension of B2 displaces S2-ext. B2 and S2 bind to the displaced SI-ext. B, and SI binds to the displaced S2-ext. Extension and displacement reactions on templates S-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-Klenow extension/displacement reactions initiate at these four fragments, automatically entering the SDA reaction cycle. Sense and antisense DNA strands are differentiated by thin and thick lines. HincId recognition sequences are also depicted

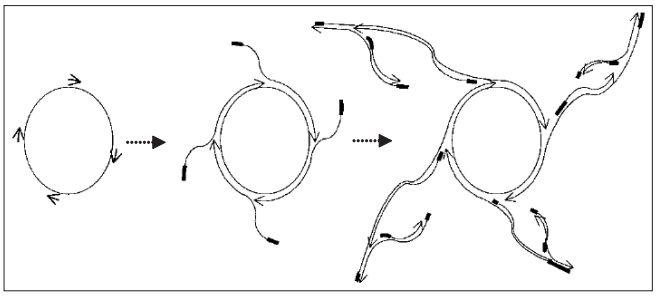

Figure 5: Scheme for multiply primed rolling circle amplification. Oligonucleotide primers complementary to the amplification target circle are hybridized to the circle. The 3` ends of the DNA strands are indicated by arrowheads to show the polarity of polymerization. Thickened lines indicate the location of the original primer sequences within the product strands. The addition of DNA polymerase and deoxynucleoside triphosphates 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

activity. The amplification products are stem–loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops [Figure 6]. The LAMP method is also a highly efficient amplification method that 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 or not.[23] However, the other formats such as gel electrophoresis, real-time turbidimetry and fluorescence probes have been used for the detection of LAMP products.[24,25] Despite its high specificity and effectiveness, this procedure, in its primary version, also has limitations.[26] LAMP requires a complicated design of multiple primers, which might cause trouble for beginners. Also, the 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.[27] This is preferable for various hybridization techniques. The advanced method uses the thermostable TspRI restriction enzyme to digest the amplification product,[27] and an additional primer hybridized to the 9-nt 3' overhang at the TspRI cleavage site to displace the single-stranded DNA by primer extension.[28] This technology has been widely used for the molecular detection of several microorganisms by researchers, and it can be a suitable choice for the design and development of rapid molecular tests in the field.

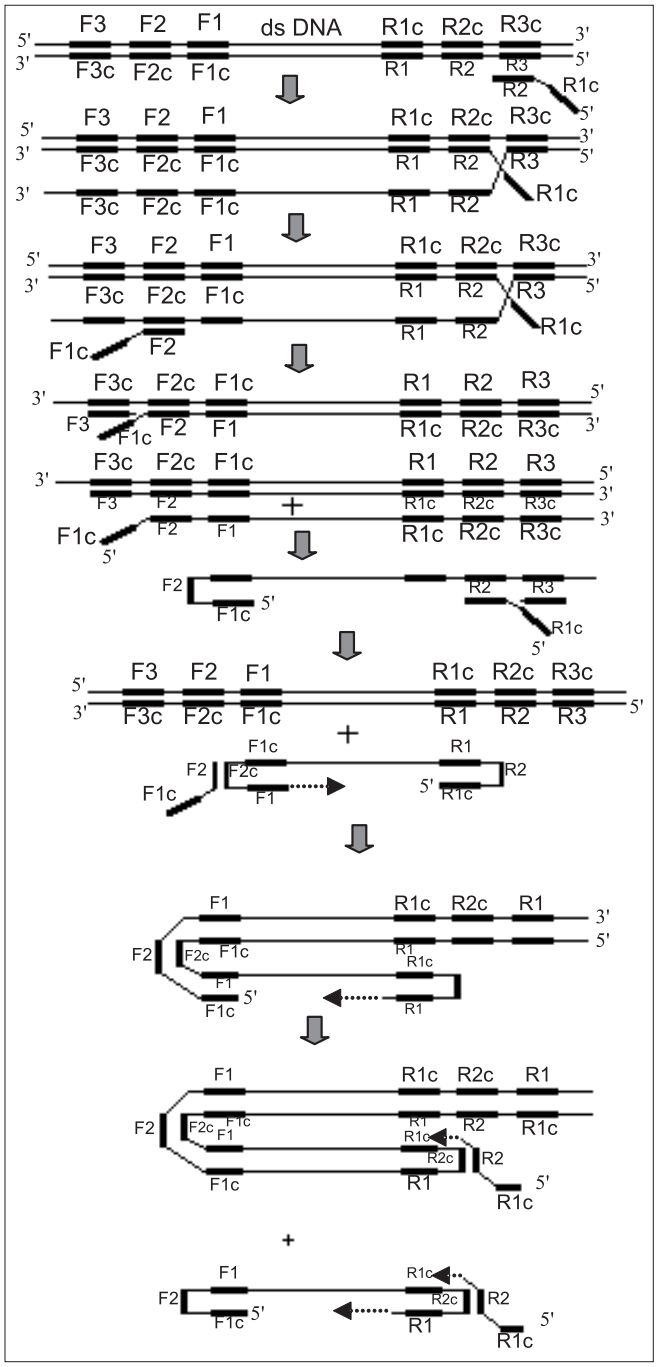

Figure 6: Schematic representation of the loop-mediated isothermal amplification of DNA (LAMP) mechanism. Steps in the original LAMP reaction. In the first step, in which the starting material is produced, the dumbbell-like DNA form is generated. Then, in the cycling amplification step, DNAs of this form are generated continuously. The elongation reactions are started from the sub-products of the cycling amplification step, generating various sizes of the products.

IMDA

This technique is based on strand displacement replication of the nucleic acid sequences by multiple primers.[29] 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 7]. The primers in the right set are complementary to one strand of the nucleic acid molecule to be amplified and

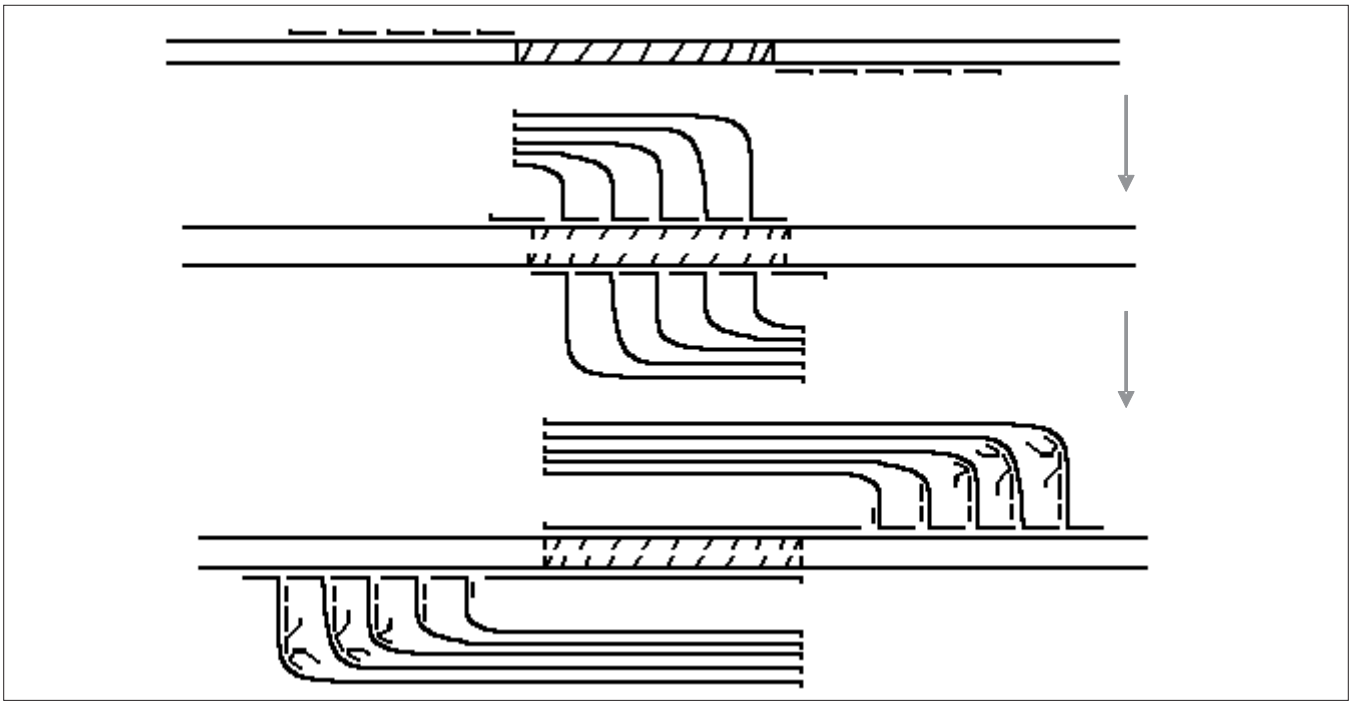

Figure 7: Schematic representation of the isothermal multiple displacement amplification mechanism. This is a diagram of an example of isothermal multiple strand displacement amplification. Diagramed at the top is a double-stranded nucleic acid molecule that contains a nucleic acid of interest (hatched 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 that 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

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, 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.[30,31] Amplification proceeds by replication with a highly processive polymerase initiated at each primer and continuing until spontaneous termination. A key feature of this method is the displacement of the intervening primers during replication by the polymerase. In this way, multiple overlapping copies of the entire genome to be synthesized are made in a short period of time.

HDA

HDA is based on the unwinding activity of a DNA helicase.[32] This process uses a helicase rather than heat to separate the two strands of a DNA duplex, generating

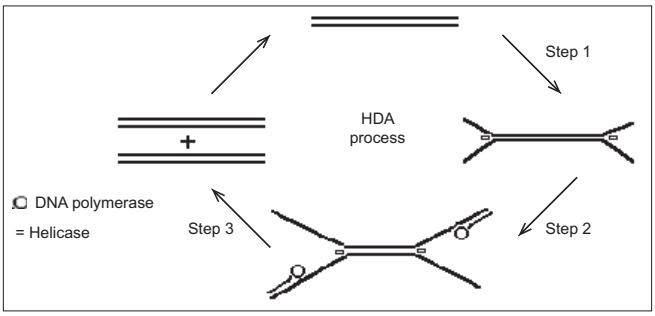

Figure 8: The figure shows the helicase-dependent amplification process. Step 1: the helicase unwinds DNA duplexes. Step 2: the primers anneal to the ssDNA. Step 3: DNA polymerase extends the primers; one duplex is amplified to two duplexes. The dsDNAs are separated by helicase and this chain reaction repeats itself

single-stranded templates for the purpose of *in vitro* amplification of a target nucleic acid.[33] 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 temperature [Figure 8]. This process allows multiple cycles of replication to be performed at a single incubation temperature, completely eliminating the need for the thermocycling equipment.[2] The HDA amplicons can be detected using gel electrophoresis, real-time format and ELISA.[15,34,35]

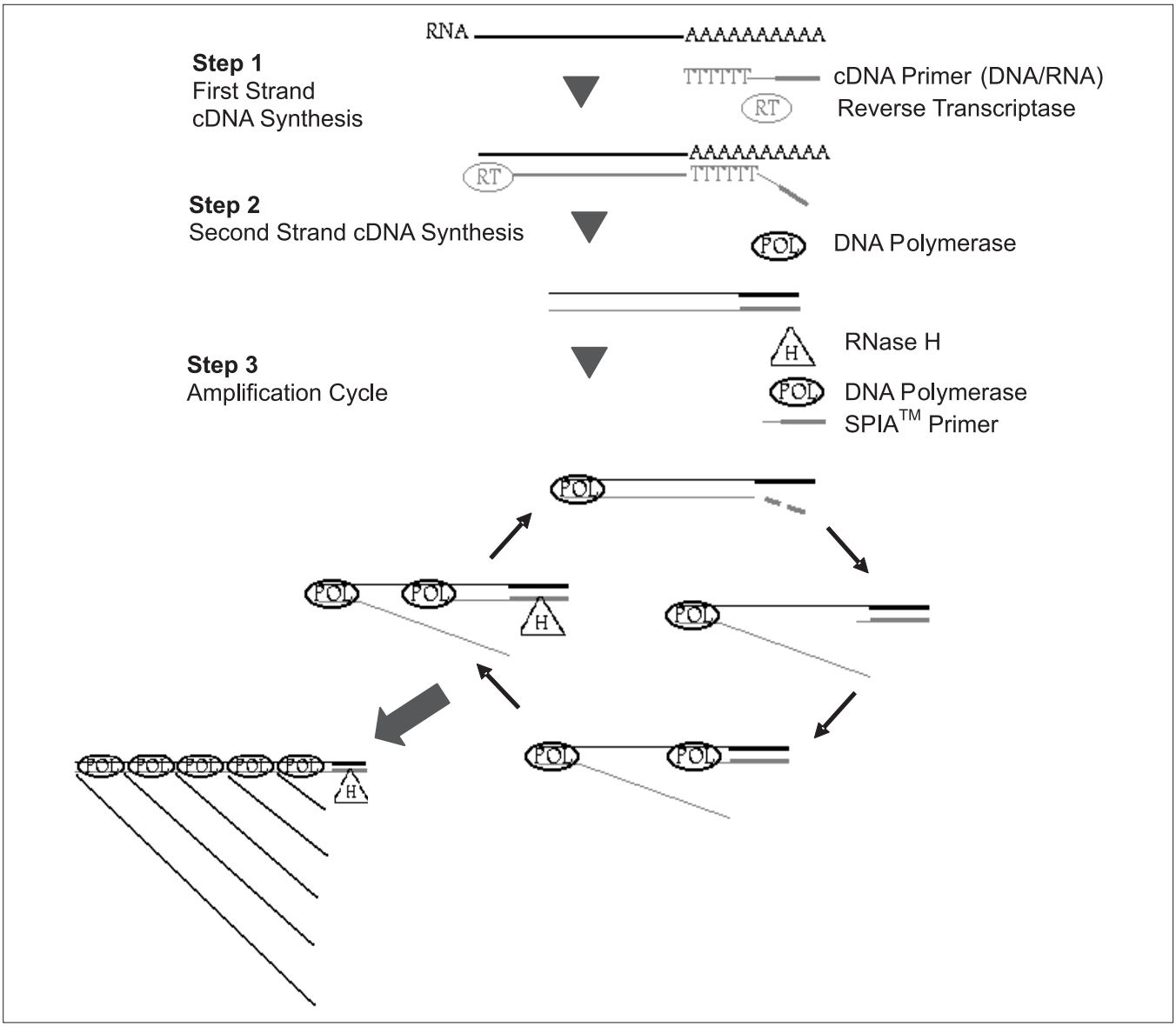

Figure 9: The single primer isothermal amplification (SPIA) process. The schematic representation of the 3'-initiated Ribo-SPIA process

SPIA

This amplification method uses a single chimeric primer for the amplification of DNA (SPIA) and RNA (Ribo-SPIA).[36] The process needs DNA polymerase with strand displacement activity and RNaseH [Figure 9]. 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.[4,37,38] In addition, this process can be used for the amplification of large populations of nucleic acid species, which are limited in biological samples, as are commonly encountered in clinical research.[36]

cHDA

The cHDA is used for amplifying nucleic acids from a circular DNA template. This system combines a DNA polymerase and a helicase preparation to amplify a target sequence as well as the entire circular DNA template containing the target sequence.[39] 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 protein. After the duplex DNA template is unwounded 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

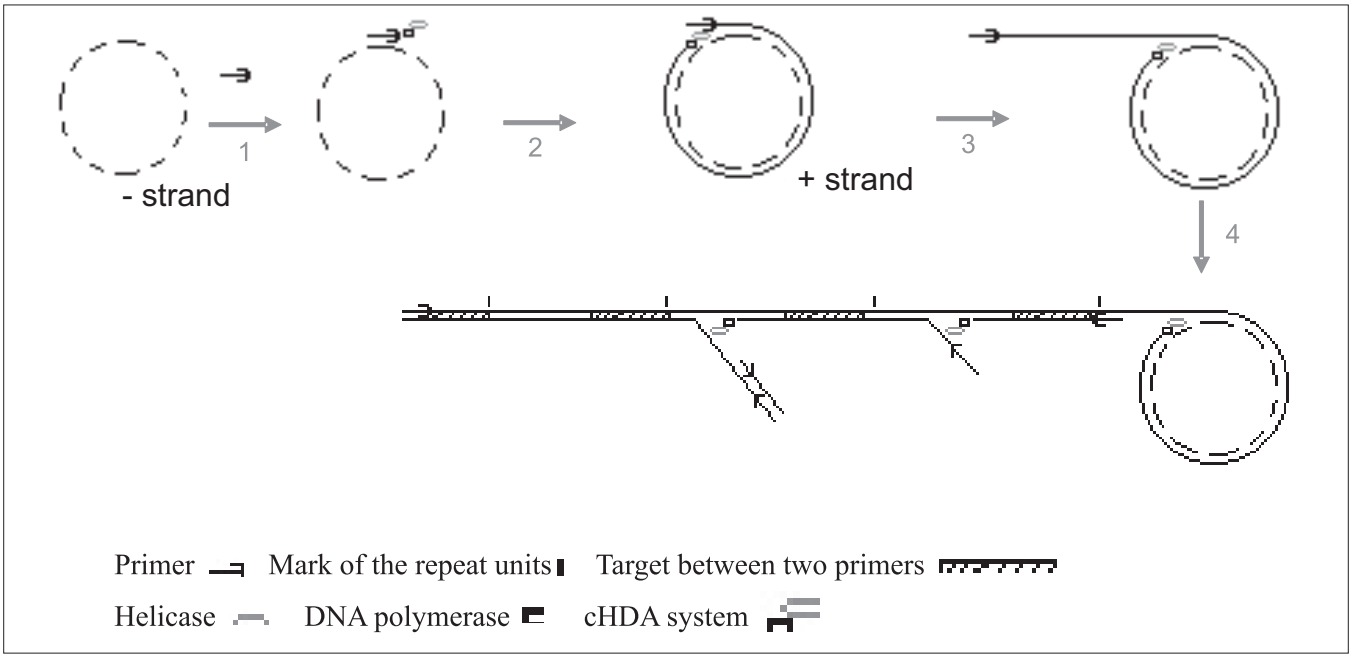

Figure 10: Circular helicase-dependent amplification mechanism. This figure shows an antisense primer annealing to the template. Primer extension produces a concatemer of the template. Multiple sense primers anneal to the concatemer and are extended by the DNA polymerase. As the polymerization reaches the downstream primer extension product, the helicase/DNA polymerase complex displaces the nontemplate strand. Multiple rounds of displacement and polymerization produce a specific target DNA defined by two primers and multimers of the DNA template

Table 1: Properties of various isothermal amplification methods

Property	PCR	NASBA	SMART	SDA	RCA	LAMP	HDA	SPIA
DNA amplification	+	+	+	+	+	+	+	+
RNA amplification	+ (RT*-PCR)	+	+	+ (RT*-SDA)	+ (RT*-RCA)	+ (RT*-LAMP)	+ (RT*-HDA)	+
Temperature(s)ºC	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	Gel electrophoresis, turbidity, real-time	Gel electrophoresis, ELISA, real-time	Bioanalyzer
Portable test designing	-	+	+	+	-	+	+	+
Tolerance to biological components	-	-	-	-	-	+	+	-
Need to template denaturation	+	+	+	+	-	-	-	+
Denaturing agent(s)	Heat	RnaseH, DMSO	RNaseH	Restriction enzymes, bumper primers	Strand-displacement property of Φ29 DNA polymerase	Btaine	Helicase	RNaseH

*RT=reverse transcriptase; **Room=22–24°C; ELISA=enzyme-linked immunosorbent assay; ELOSA=enzyme-linked oligosorbent assay; ECL=electrochemiluminescence

continuous concatemers of the template [Figure 10]. The process can be carried out at one temperature (25ºC) for the entire process. Amplification can be performed using purified plasmid DNA; crude cell lysate can amplify inserts as large as 10 kilo base pairs.

In this study, we reviewed the best known isothermal techniques for DNA/RNA amplification that offer several advantages over PCR, namely that they eliminate the need for an expensive and power-hungry thermocycler. However, these isothermal amplification methods have weaknesses that limit their use in some aspects of molecular biology [Table 1], like PCR. Some isothermal DNA amplification techniques have complicated reaction mechanisms and experimental designs. SDA uses four primers to generate initial amplicons and modified deoxynucleotides to provide strand-specific nicking.[14,32] LAMP requires four to six specific primers and thus their designs are complicated for the new user.[26] NASBA needs three different enzymatic steps (transcription/cDNA synthesis/RNA degradation)

to accomplish an isothermal RNA amplification.[20]

Because of its robustness and simplicity, RCA holds a pole 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 the transcription-based system, strand-displacement approach or loop-mediated techniques, are rather complicated and, in most cases, require prior assay optimization. The RCA-based approaches have recently been attracting attention of diagnostics-oriented biotech companies and research centers for gene tests and immunoassay, SNP scoring, sequencing, template preparation, single cell analysis and gene expression analysis.

On the other hand, some of these methods, such as had, have a simple reaction scheme, in which a target sequence can be amplified by two flanking primers, similar to PCR.[15,33]

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.[40] 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.[15] This demonstrates that HDA can be performed on crude samples and has the potential to be used as a diagnostic tool.

Another important advantage of the isothermal amplification techniques is that there is no need for initial heat denaturation at a high temperature followed by amplification at a lower temperature. This property has been reported in 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.[41] 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.[15]

CONCLUSION

We reviewed the best-known isothermal techniques for DNA/RNA amplification. The above information can be used for the application of valuable molecular diagnostic techniques for the detection of infectious agents in small-scale hospital laboratories in the field. Therefore, with regards these properties of isothermal DNA/RNA amplification techniques, the design and development of novel rapid molecular tests for application in field may be possible.

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