Emerging Alternatives to PCR for Efficient Nucleic Acid Amplification

Asim Debnath[1*], Md Abu Bakar Siddique[1], Nabil Deb Nath1, Md Abdur Rahman Biswash[2], Tufael[2]

Abstract

Nucleic acid amplification is a vital part of molecular biology and biotechnology, and it serves a wide range of purposes, including clinical diagnostics, infectious disease detection, gene cloning, and quality control. While the polymerase chain reaction (PCR) has been the traditional go-to method for quite some time, it does come with some limitations. For instance, it requires thermal cycling, can be tricky with certain inhibitors, and relies on pricey equipment. To tackle these challenges, scientists have been excitedly working on alternative methods that can amplify nucleic acids without the need for complicated temperature changes. These isothermal techniques are not only easier and more cost-effective but also speed up the detection process. Some popular options include loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), multiple displacement amplification (MDA), rolling circle amplification (RCA), and ligase chain reaction (LCR). Each of these methods brings something special to the table! For instance, LAMP is known for its awesome ability to provide visual results, while NASBA shines when it comes to detecting RNA. SDA and MDA are fantastic for amplifying small amounts of DNA, with MDA being particularly great for whole-genome amplification. RCA is super specific and resistant to contamination, and LCR is a go-to for spotting single-nucleotide variations. Even though these innovative methods have a lot of potential, many are still being fine-tuned for broader use in clinical and industrial settings. Luckily, advancements in real-time detection technologies, like molecular beacons and electrochemiluminescence (ECL), are making these methods even more reliable. As research continues to progress, these alternatives are set to complement or even take the place of PCR, offering faster, simpler, and more accessible solutions for a wide range of applications.

Keywords: Isothermal amplification, sensitivity, cost-effective, rapid detection, diagnostics, polymerase chain reaction alternatives.

Significance | Emerging PCR alternatives offer faster, cost-effective, and sensitive nucleic acid amplification, enhancing diagnostics, research, and point-of-care testing.

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Introduction

Nucleic acid amplification is an important technique in the fields of biotechnology and molecular biology, with a wide range of applications in research, medicine, agriculture, and forensics. The polymerase chain reaction (PCR), invented by Kary Mullis in the 1980s, has become the go-to method for amplifying DNA and RNA because it's straightforward, reliable, and comes with well-established protocols (Mullis et al.,1990). Its popularity is due in part to the easy availability of the necessary reagents and equipment, along with its effectiveness in diagnosing infectious diseases, identifying genetic mutations, and allowing for gene cloning. However, PCR is not without its challenges. It requires expensive thermal cyclers, can easily be contaminated, and is sensitive to various inhibitors found in biological samples (Fakruddin et al.,2011). Moreover, the thermal cycling process can be slow and consume a lot of energy. To tackle these issues, researchers have been exploring alternative methods for nucleic acid amplification, many of which work at a constant temperature eliminating the need for the thermal cycling that makes PCR

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cumbersome. These isothermal methods offer quicker, simpler, and more cost-effective workflows (Notomi et al.,2000). Some of the notable techniques include loop-mediated isothermal amplification (LAMP), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), rolling circle amplification (RCA), multiple displacement amplification (MDA), and ligase chain reaction (LCR) (Guatelli et al.,1990). Each has its own strengths, like enhanced sensitivity, rapid results, lower equipment costs, and better suitability for point-of-care testing. LAMP, in particular, stands out due to its fast amplification, high specificity, and the ability to visually detect results, making it ideal for settings with limited resources (Compton et al.,1991). NASBA, originally focused on amplifying RNA, is excellent at detecting live pathogens while correctly identifying non-viable ones, improving diagnostic accuracy. SDA and MDA are effective at amplifying small DNA samples, with MDA being beneficial for whole-genome amplification from very little starting material (Walker et al.,1993). RCA is renowned for its specificity and low risk of contamination, making it suitable for detecting specific genetic markers, while LCR is exceptional for identifying single-nucleotide polymorphisms (SNPs) and genetic mutations (Lizardi et al.,1998). Despite their potential, many of these alternative amplification methods are still being validated to ensure they are reliable, reproducible, and scalable for clinical and industrial use (Wiedmann et al.,1994). As research continues to improve their performance, reduce costs, and simplify the processes, we can expect their use to grow. Coupling these methods with real-time detection technologies, such as molecular beacons and electrochemiluminescence (ECL), will enhance their diagnostic capabilities even further.

This review focuses on understanding the principles, benefits, and applications of these promising alternatives to PCR, showcasing how they could address the limitations of traditional PCR and transform nucleic acid amplification in both laboratory environments and point-of-care situations.

Alternative Methods of Polymerase Chain Reaction

Over the years, researchers have come up with various alternative methods to amplify genetic material (Nagamine et al.,2002). Some of these include LAMP (Loop-Mediated Isothermal Amplification), 3SR (Self-Sustained Sequence Replication), NASBA (Nucleic Acid Sequence-Based Amplification), SDA (Strand Displacement Amplification), RCA (Rolling Circle Amplification), and LCR (Ligase Chain Reaction) (Fakruddin et al.,2012). Each of these methods has its own unique approach and advantages, and here's a brief overview of how they work.

Loop-Mediated Isothermal Amplification

LAMP, which stands for Loop-Mediated Isothermal Amplification, is a straightforward and efficient method for rapidly amplifying nucleic acids (Figure 1). What sets LAMP apart is its easy-to-use detection system for visualizing the results (Fakruddin et al.,2012). The process takes place at a steady temperature of 60-65°C for about 45-60 minutes and uses a special enzyme from the bacteria Bacillus stearothermophilus, along with specific primers and the target DNA (Gill et al.,2008). The method involves a unique approach where four specially designed primers two inner and two outer target six specific sequences in the DNA to ensure precise amplification. The LAMP reaction progresses through three main steps: producing the starting material, cycling to amplify the DNA, and elongating the DNA strands (Sooknanan et al.,1995). One of the great advantages of LAMP is that it can achieve high accuracy without the need for expensive equipment. Compared to traditional PCR methods, LAMP simplifies sample preparation and makes it easier to visualize changes in fluorescence in the reaction tube without requiring specialized tools since the detection system is very sensitive (Deiman et al.,2002). It's a quick one-step process that takes only 30-60 minutes, and it handles various inhibitors commonly found in clinical samples better than PCR, which means you won't have to go through extensive DNA purification (Sergentet et al.,2008).

Additionally, when combined with reverse transcription, LAMP can efficiently amplify RNA sequences as well. It's highly sensitive and capable of detecting as few as six copies of DNA in the mixture (Manojkumar et al.,2006). This makes LAMP a promising tool not just in medical and pharmaceutical research, but also in areas like environmental hygiene, point-of-care testing, and cost-effective diagnosis of infectious diseases. Finally, LAMP can be used for DNA sequencing similarly to PCR, whether you're looking at Sanger sequencing or Pyrosequencing.

Nucleic Acid Sequence Based Amplification

NASBA, which stands for Nucleic Acid Sequence Based Amplification, is a specialized tool used for detecting RNA (Fakruddin et al.,2012). It works best at a steady temperature of 41°C, ensuring that every step of the amplification process happens smoothly as new RNA copies are formed (Walker et al.,1992). Unlike traditional DNA amplification methods that only double the amount of DNA each cycle, NASBA can produce multiple RNA copies from a single DNA template, making it a more efficient choice (Figure 2). The system relies on a combination of three key enzymes: avian myeloblastosis virus reverse transcriptase, RNase H, and a specific RNA polymerase (McHugh et al.,2004). This unique setup primarily generates single-stranded RNA products. One of the appealing aspects of NASBA is that it doesn't require costly equipment to achieve high precision in amplifying RNA sequences, typically between 100 and 250 nucleotides in length (Nadeau et al.,1999).

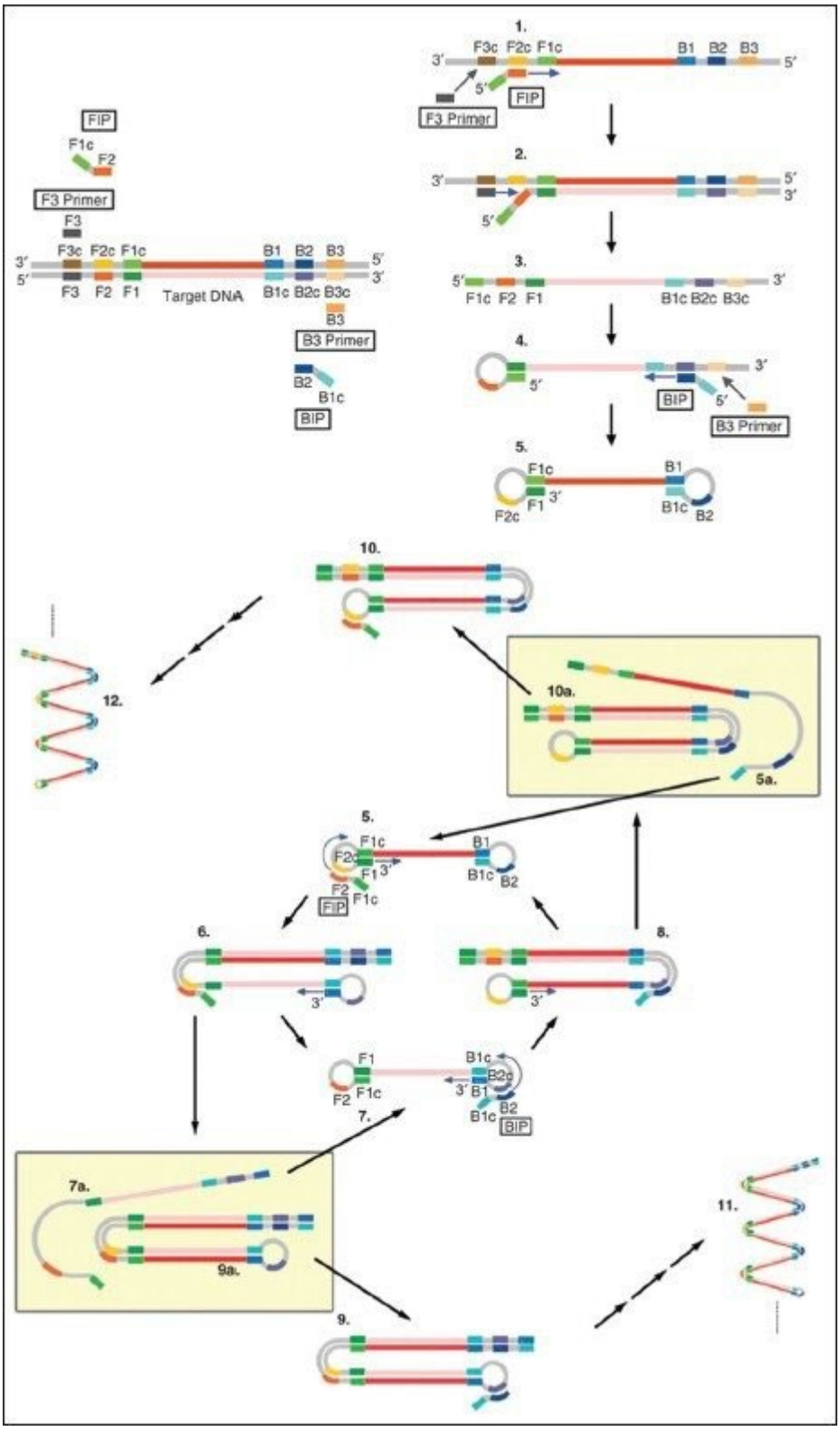

Figure 1. Schematic description of loop mediated isothermal amplification assay (Courtesy of image from Fakruddin et al., 2013).

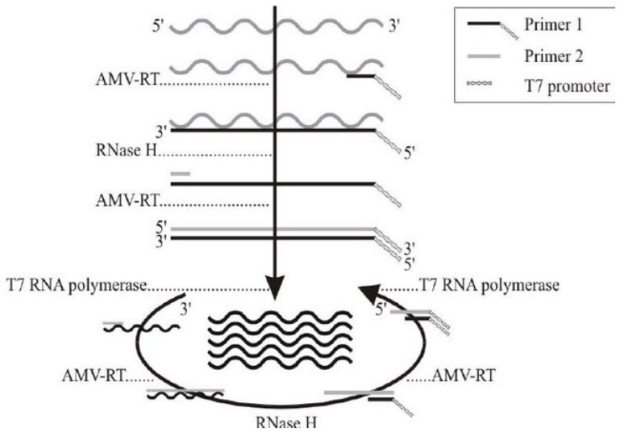

Figure 2. Principles of nucleic acid sequence-based amplification (Courtesy of image from Fakruddin et al., 2013).

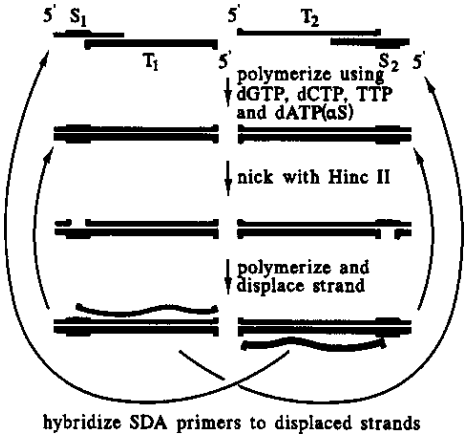

Figure 3. Target generation scheme for strand displacement amplification (Courtesy of image from Fakruddin et al., 2013).

Over the years, the detection methods for NASBA products have advanced significantly. New techniques like enzyme-linked gel assays, enzymatic bead-based detection, electrochemiluminescent (ECL) detection, molecular beacon technology, and fluorescent correlation spectroscopy have all enhanced the way we can identify NASBA amplicons. In clinical settings, NASBA is becoming an established diagnostic tool, especially in pathogen detection. It has the potential to offer greater sensitivity compared to traditional reverse transcription-polymerase chain reaction (RT-PCR) methods (Hawkins et al.,2002). This makes it particularly useful for detecting and differentiating viable cells by amplifying messenger RNA, all while effectively distinguishing it from genomic DNA.

Strand Displacement Amplification

SDA, or Strand Displacement Amplification, was first introduced in 1992 (). It's a method that allows for the rapid amplification of DNA using four different primers. One of these primers includes a specific restriction site that is recognized by the HincII exonuclease (Hughes et al.,2005). The process begins with the primer binding to a specific section of the DNA template. Then, an exonuclease-deficient version of the E. coli DNA polymerase 1, known as exo-Klenow, extends this primer (Figure 3). Each cycle of the SDA process involves several steps. First, the primer binds to the target DNA fragment (Dean et al.,2001). Next, exo-Klenow extends this primer/target complex. After that, a nick is created at the hemiphosphothioate HincII site, leading to the dissociation of HincII from the nicked area (Morisset et al.,2008). Finally, exo-Klenow extends the nick, causing the downstream strand to be displaced. This method can work efficiently at higher temperatures and can produce up to 100 million copies of target DNA in less than an hour (Lasken et al.,2003). However, like other amplification methods such as PCR and LCR, SDA only allows for semi-quantitative analysis. One notable drawback is its difficulty in amplifying longer DNA sequences effectively (Dean et al.,2002). SDA is the foundation for several commercial tests, like BDProbeTec from Becton Dickinson, and is currently being evaluated for its ability to identify Mycobacterium tuberculosis in clinical samples (Schweitzer et al.,2000). Despite its promise, further research and large-scale studies are needed to confirm its efficiency and reliability. Recent advancements have also led to real-

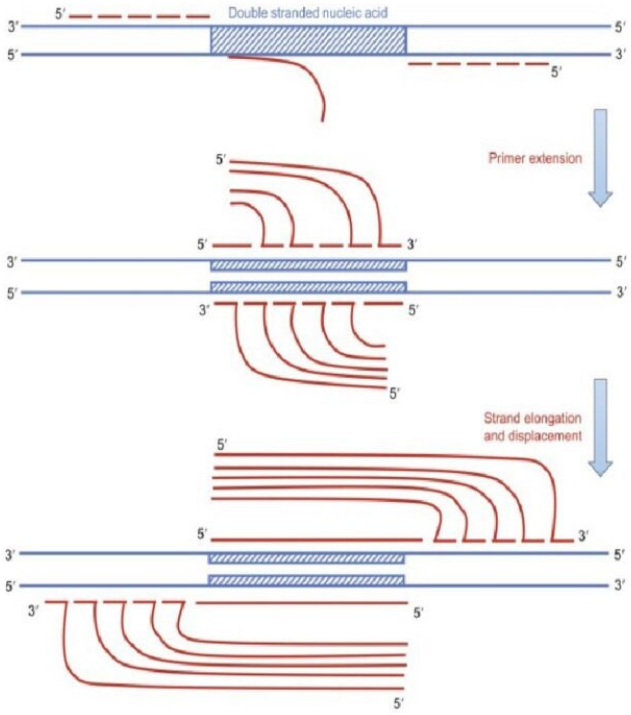

Figure 4. Schematic representation of multiple displacement amplification mechanism (Courtesy of image from Fakruddin et al., 2013).

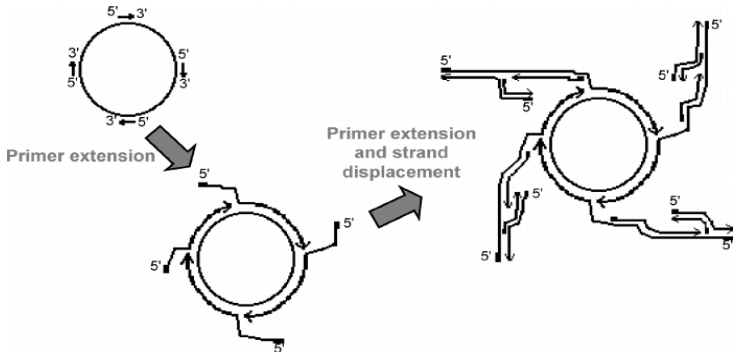

Figure 5. Scheme for multiply-primed rolling circle amplification (Courtesy of image from Fakruddin et al., 2013).

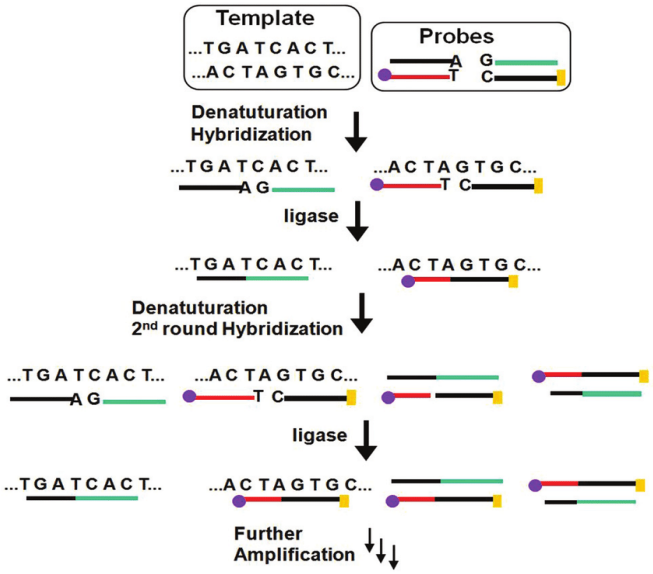

Figure 6. Ligase chain reaction (Courtesy of image from Fakruddin et al., 2013).

time detection of specific DNA targets using fluorogenic reporter probes (Wiltshire et al.,2000).

Multiple displacement amplification

The MDA (Multiple Displacement Amplification) technique is a powerful method for amplifying DNA, particularly useful when working with very small samples (Schweitzer et al.,2001). It relies on a special enzyme from the bacteriophage Ø29, which is known for its ability to efficiently create copies of DNA strands (Figure 4). During the process, random primers are introduced along with the DNA and essential building blocks called dNTPs to amplify the entire genome with impressive accuracy (Cho et al.,2005). The procedure involves mixing these components and incubating them at a warm temperature of 30°C for about 16 to 18 hours. After this period, the enzyme is inactivated by heating it to 65°C for 10 minutes, and the resulting DNA can be used in various applications right away (Demidov et al.,2002). Unlike traditional PCR methods that require multiple cycles of heating and cooling, MDA just needs a brief initial step to separate the DNA strands followed by the long amplification step. This makes it particularly streamlined (Zhong et al.,2001). This technique is great for generating DNA for microarrays or for obtaining highly pure samples, and it shines in situations where you only have a limited amount of starting material. Remarkably, you can extract around 20 to 30 micrograms of DNA from just 1 to 10 copies of human genomic DNA (Mothershed et al.,2006). Moreover, MDA works directly with biological samples, including fresh whole blood and cultured cells. Its unique characteristics make it an exciting option for applications like multiplex detection and identification in microarrays (). However, its effectiveness in areas such as forensics, sample archiving, and single-cell clinical diagnostics is still being explored (Lievens et al.,2005). Overall, MDA offers a promising and versatile approach to DNA amplification.

Rolling Circle Amplification

RCA, or Rolling Circle Amplification, is an innovative method for amplifying nucleic acids under isothermal conditions, meaning it operates at a constant temperature (Gusev et al.,2001). This technology can boost the amount of specific DNA sequences by over a billion times, whether in a solution or attached to a solid surface (Wu et al.,1989). One of its standout features is its remarkable sensitivity, allowing it to detect even a few specialized circular probes within a sample (Figure 5). In the RCA process, an enzyme called Ø29 DNA polymerase plays a key role. It works by continuously extending a primer that has attached to a circular DNA probe, effectively replicating the DNA sequence multiple times. This method has several advantages compared to traditional techniques like PCR. For instance, RCA is less susceptible to contamination and typically requires minimal optimization of the assay conditions (Barany et al.,1991).

Additionally, RCA yields products that can remain bound to the target molecules, making it particularly useful for in situ applications or microarray assays. It's especially effective for analyzing cells and tissues, enabling researchers to track multiple markers at once while preserving important morphological details (Lisby et al.,1999). The technique allows for precise localization of signals, which is valuable for identifying specific genetic traits or biochemical characteristics. RCA also stands out for its excellent sequence specificity, which is crucial for accurately identifying DNA markers and conducting tasks like genotyping or mutation detection (oCsak et al.,2006). Compared to PCR, RCA can handle a greater number of targets simultaneously and reduces the likelihood of amplification errors, making it a robust choice for contamination-free detection in various testing scenarios. The simplicity and efficiency of RCA make it a strong candidate for miniaturization and automation, particularly in high-throughput analyses (Barany et al.,1991).

Ligase Chain Reaction

LCR (Ligation Chain Reaction) is an innovative way to amplify DNA using a cyclic template. While it shares some similarities with PCR (Polymerase Chain Reaction), LCR stands out because it focuses on amplifying probe molecules instead of creating amplicons through nucleotide polymerization (Khanna et al.,1999). This method employs both a DNA polymerase and a DNA ligase to facilitate the reaction (Figure 6). In LCR, two pairs of complementary oligonucleotides come together near the target DNA fragment (Gerry et al.,1999). When these oligonucleotides precisely attach to the target sequence, a DNA ligase then links them by sealing the gap between them, effectively generating a new fragment that combines the sequences of both oligonucleotides (Dean et al.,1998). This ligated product can then serve as a template for further rounds of annealing and ligation. Like PCR, LCR requires a thermal cycler to regulate the temperature, with each cycle doubling the amount of target nucleic acid (Prasad et al.,2009).

One of the notable advantages of LCR is its greater specificity compared to PCR. This makes it an excellent choice for multiplex reactions, allowing simultaneous detection of various targets, especially in microarray applications. However, LCR does have some limitations. Its specificity is mainly tied to the ligation junction, which can restrict detection (Kolbehdari et al.,2007). Additionally, LCR has the drawback of being able to detect DNA from deceased organisms, which can lead to misleading results.

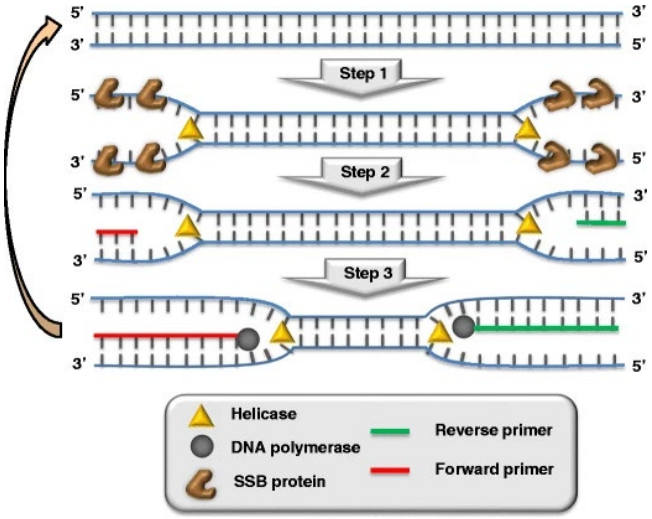

Figure 7. Helicase-dependent amplification process. (1) Unwinding dsDNA by helicase and stabilization of ssDNA by SSB; (2) annealing of primers, and (3) elongation of primers by DNA polymerase (Courtesy of image from

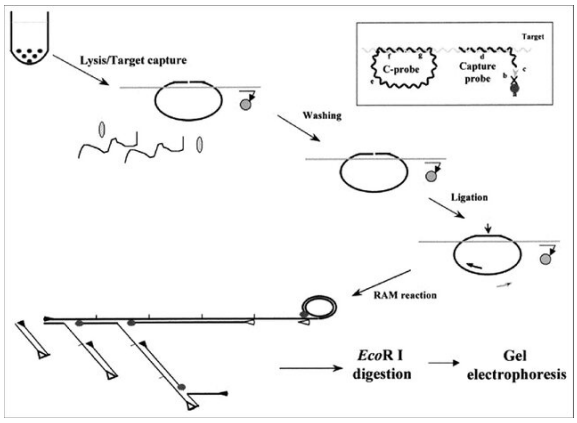

Figure 8. Schematic representation of ramification amplification of ligated circular probe (Courtesy of image from Fakruddin et al., 2013).

There's also a risk of contamination and limited sensitivity, particularly in situations where rare target DNA needs to be distinguished from a large background of wild-type DNA (Andras et al.,2001). LCR has proven effective in diagnosing various pathogens, such as Neisseria gonorrhoeae, Chlamydia trachomatis, and M. tuberculosis, in human samples. It is also useful for identifying point mutations in various DNA sequences (Vincent et al.,2004). On the other hand, there's HDA (Helicase Dependent Amplification), which works at a constant temperature, using the natural replication mechanisms of DNA. The technique capitalizes on the unwinding activity of DNA helicase, which separates the two strands of DNA, creating single-stranded templates necessary for amplification. Specific primers then bind to these templates, and specialized DNA polymerases extend the primers to generate double-stranded DNA. One impressive aspect of HDA is that it enables multiple cycles of amplification at a single temperature, making traditional thermal cycling equipment unnecessary.

Detection of HDA amplicons can be done using methods like gel electrophoresis, real-time monitoring, or ELISA (An et al.,2005). HDA boasts several benefits, such as rapid amplification rates and the ability to produce high yields with relatively simple procedures. Its straightforward approach allows it to be implemented in portable DNA diagnostic devices, which can be incredibly valuable for field applications and point-of-care testing.

Ramification amplification method

RAM is an innovative method for amplifying nucleic acids at a constant temperature, which is why it's called "isothermal amplification." The name RAM stands for the way it boosts amplification through a combination of primer extension, strand displacement, and branching points (Eisenstein et al.,2004). In this technique, a special circular probe known as a C-probe is used. It's designed so that its two ends can come together by binding to a target DNA sequence, creating a closed circle. This closure is achieved with a specific ligase that connects the ends in a way that

Table 1. Comparison of nucleic acid amplification methods (Courtesy of table from Fakruddin et al., 2013).

Parameter	PCR	LAMP	3SR	SDA	LCR	NASBA	RCA
High sensitivity	<10	<10	10	10	<10	10	<10
High specificity	+	+	+	+	+	+	+
Allow quantification	+	+	±	±	—	±	—
Live versus dead microorganisms	+	+	+	—	—	±	—
Commercial availability	+	—	±	±	—	—	—
Linear dynamic range	6-7	6	7	ND	ND	7	ND
Multiplexity	+	—	+	—	+	+	+
No. of enzymes	1	1	2-3	2	2	2-3	1
Primer design	Simple	Complex	Simple	Complex	Simple	Simple	Complex
Tolerance to biological compounds	—	+	—	—	—	—	—
Need to template denaturation	+	—	+	+	+	+	—
Denaturing agents	Heat	Betaine	Rnase H	Restriction enzymes; bumper primers	Helicase	Rnase H	Ø29 DNA polymerase
Product detection method	Gel electrophoresis, ELISA, real time	Gel electrophoresis, turbidity, real time	Gel electrophoresis, ELISA, real time, ECL	Gel electrophoresis, real time	Gel electrophoresis, real time	Gel electrophoresis, ELISA, real time, ECL	Gel electrophoresis, real time

PCR – Polymerase Chain Reaction, LAMP – Loop-Mediated Isothermal Amplification, SR – Sequence Replication, SDA – Strand Displacement Amplification, LCR – Ligase Chain Reaction, NASBA – Nucleic Acid Sequence-Based Amplification, RCA – Rolling Circle Amplification, DNA – Deoxyribonucleic Acid, ELISA – Enzyme-Linked Immunosorbent Assay, ECL – Electrochemiluminescence.

depends on the target. Once this circle is formed, an abundant amount of forward and reverse primers is added (Gill et al.,2007). A unique enzyme called bacteriophage Ø29 DNA polymerase then extends the forward primer, displacing the downstream strand and effectively rolling over the circular DNA. This process produces long strands of single-stranded DNA (ssDNA) similar to how certain viruses replicate. As the amplification continues, multiple reverse primers attach to the ssDNA, extending and displacing more downstream strands, resulting in a complex web of branched DNA (Figure 7). This branching continues until all the ssDNA is converted into double-stranded DNA, leading to exponential amplification a notable distinction from older methods that amplify just in a linear fashion (Jeong et al.,2009). Thanks to the high efficiency of Ø29 DNA polymerase, significant amplification can happen quickly, within just an hour at 35°C. Because RAM operates at a constant temperature and creates large DNA products, it preserves the morphology of cells while providing targeted amplification, making it especially useful for in situ applications (Zhang et al.,1998).

The RAM assay brings several key benefits over traditional amplification techniques (Figure 8). First, it allows primers to easily attach to the displaced ssDNA, meaning it doesn't require the complex cycling temperatures of thermocyclers (Beals et al.,2010). The generic nature of the primers means they amplify various probes equally well, enhancing multiplexing capabilities compared to conventional PCR methods. Furthermore, both ends of the probe can be connected regardless of whether the target is DNA or RNA, which streamlines the detection process and does not necessitate reverse transcription for RNA detection (Hsuih et al.,1996). It also uniquely ensures that both ends of the probe must perfectly match the target for ligation to occur, allowing for the detection of subtle genetic differences, such as a single-nucleotide polymorphism

(Zhang et al.,2001). Overall, RAM has great potential for use in clinical laboratories for identifying genes and infectious agents across various fields including hematology, oncology, infectious diseases, pathology, forensics, blood banking, and genetic disorders. Its straightforward and isothermal format also makes it suitable for use in field testing and at doctor's offices.

Comparison of nucleic acid amplification methods

Each nucleic acid amplification method has its own unique features, while also sharing some common characteristics (Fakruddin et al.,2013). A brief comparison of these key properties can be found in Table 1.

Conclusion

The future of nucleic acid amplification lies in the development of rapid, accurate, and accessible alternatives to traditional PCR. Emerging isothermal techniques such as LAMP, NASBA, and RCA offer promising advantages operating without thermal cycling, reducing costs, and enhancing usability in diverse settings. With improved sensitivity and specificity, these methods are especially valuable for point-of-care diagnostics and use in low-resource environments. While further validation is needed, advances in detection technologies are accelerating their adoption. Continued innovation could see these techniques not only complementing but potentially surpassing PCR in diagnostics, research, and clinical applications worldwide.

Author contributions

A.D and T. conceptualized the research idea, designed the study framework, and supervised the overall project. M.B.S. conducted the literature review, analyzed the digital marketing strategies, and drafted major sections of the manuscript. N.D.N. and M.A.R.B. performed data collection, interpreted artificial intelligence applications in healthcare, and contributed to manuscript editing and reviewed related studies, visualized data, and assisted in refining the final draft. All authors read and approved the final manuscript.

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Competing financial interests

The authors have no conflict of interest.

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