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Review article

PCR- A Paradigm Shift with A Robust Quantum Jump

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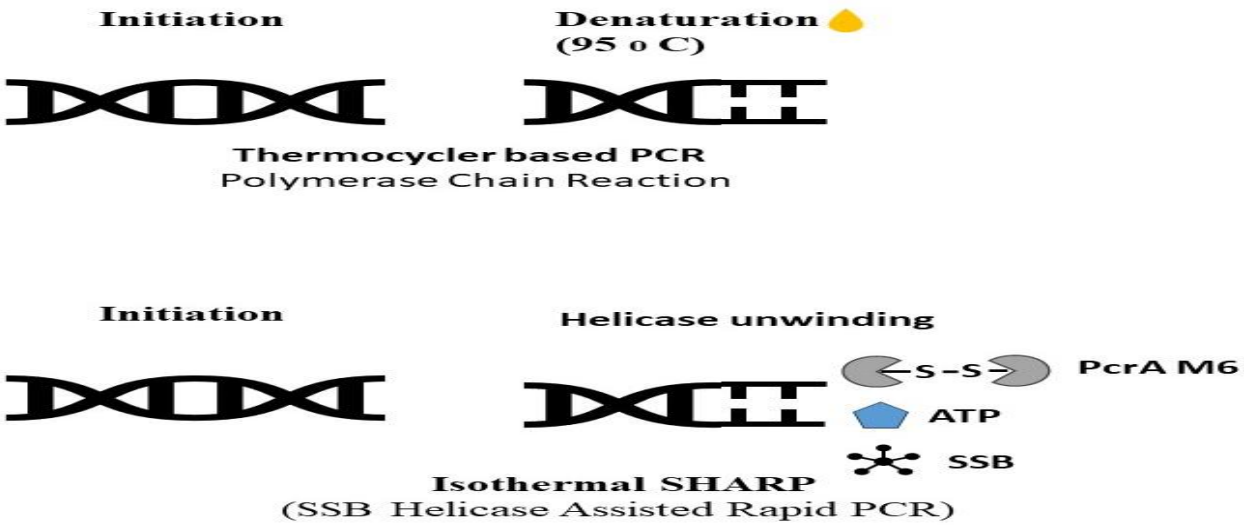
ABSTRACT

Nature Communications has recognized the article titled "Engineered Helicase Replaces Thermocycler in DNA Amplification While Retaining Desired PCR Characteristics" as one of the top 25 best papers published in 2022. Gavrilov *et al.* (2022) engineered helicase which replaces thermocycler in DNA amplification while retaining desired PCR characteristics. No experiment was so advanced that of Gavrilov et al. LAMP (Loop-Mediated Isothermal Amplification) is limited to producing only short amplicons. SHARP (SSB-Helicase Assisted Rapid PCR) was invented in 2022. We were waiting over a year for reactions from scientific community and then employed SHARP. Gavrilov et al. showed costly PCR machine is not needed for SHARP. It has huge implication for Covid-19 research and prevention in India.

Figure 1: Graphical Abstract



Keywords: Polymerase Chain Reaction, LAMP (Loop-Mediated Isothermal Amplification), SHARP (SSB-Helicase Assisted Rapid PCR).

INTRODUCTION

PCR's inception is credited to Kary Mullis. Like all transformative shifts, the utilization of PCR underwent rapid proliferation, emerging as one of the finest extensively employed methodologies in both scientific and medical realms. To date, the term

"PCR" appears in over 300,000 publications on PubMed, and its usage extends even further in publications encompassing fields such as palaeontology, forensics, and popular media. In a related article, Chung et al., in an accompanying paper, elaborated on how PCR has not only transformed the creation of medications for combating hepatitis C, facilitating the emergence of the present-day direct antiviral drugs.

Revolutionizing Nucleic Acid Amplification: Innovations and Alternatives in Molecular Biology and Medical Diagnostics

While PCR-based detection methods have become a cornerstone in molecular biology, researchers have also developed a multitude of alternative amplification methods [1,2]. Among the notable alternative amplification methods, there are nucleic acid sequence-based amplification (NASBA) [3], self-sustained sequence replication (3SR) [4], and strand displacement amplification (SDA) [5,6]. In the case of PCR, the method relies on the heat denaturation of double-stranded DNA products to initiate the next cycle of DNA synthesis. In contrast, both 3SR and NASBA terminate the need for heat denaturation by employing a series of transcription and reverse transcription reactions to amplify the target sequence.

Advancements in Nucleic Acid Amplification: Exploring the Potential of Isothermal Methods in Molecular Diagnostics

There exist over ten distinct methods for achieving isothermal amplification [2,3,7]. These diverse methods exhibit variations in their approaches to initiate each reaction cycle and facilitate the binding of primers to the template. PCR relies on the denaturation of the DNA duplex through heat, followed by cooling to trigger each replication cycle. In contrast, isothermal methods employ specialized primers or other auxiliary proteins to enable the binding of primers to the template. For instance, LAMP (Loop-Mediated Isothermal Amplification) stands out by utilizing 4 to 6 loop-forming primers, which generate extendable 3' ends through the action of Bst DNA polymerase (DNAP) [8].

Loop-Mediated Isothermal Amplification (LAMP): A Rapid and Specific Technique for DNA Amplification

LAMP is a swift, readily accessible, and exceptionally sensitive method for exponential amplification, with widespread availability [9]. Nonetheless, LAMP generates intricately structured products that pose challenges in terms of interpretation, comparison, and application in fields like cloning, gene manipulation, and sequencing. Alternatively, isothermal amplification methods center on the single-stranded DNA binding protein gp32, use a pair of primers and have the capability to produce amplicons exceeding 1 kilo base-pairs.

Exploring Helicase-Dependent Amplification (HDA) and Its Variants for DNA Amplification: Challenges and Potential Applications

HDA, or Helicase-Dependent Amplification [10,11], introduces a unique approach by employing a DNA helicase to generate single-stranded templates, facilitating primer hybridization and subsequent extension by a DNA polymerase (DNAP). This method is commercially available and employs primers similar to those used in PCR. To enhance its efficiency, Vincent et al. combined UvrD helicase, the accessory protein MutL (which boosts UvrD activity) [12], T4 gene 32 protein (SSB), and Klenow Exo- DNAP to achieve exponential amplification, typically producing 100 base-pair products [10]. Nevertheless, with an increase in amplicons length, the reaction yield significantly decreases, thereby limiting the method's suitability for certain applications.

A ground breaking advancement in Helicase-Dependent Amplification: The PcrA M6 Helicase-Enabled SHARP Isothermal Amplification Method

In a ground breaking advancement, scientists engineered a helicase PcrA M6 with increased speed and processivity, enabling the elimination of the heating step through DNA unwinding with enzymatic reaction while preserving the essential characteristics of PCR. This innovative isothermal amplification method, aptly named SHARP (SSB-Helicase Assisted Rapid PCR), incorporates the use of the designed helicase and a single-stranded DNA binding protein (SSB) in conjunction with standard PCR reagents. Remarkably, SHARP can generate amplicons with substantial 6000 base pairs of length roughly. Furthermore, it has demonstrated its capacity to produce functional DNA, including plasmids conferring antibiotic resistance to cells, and efficiently multiply desired fragments from the genomic DNA of human cells. Notably, SHARP also finds application in evaluating the outcomes of CRISPR-Cas9 genome editing at endogenous genomic sites, marking a significant stride in the realm of molecular biology and genetic research.

The PcrA M6 helicase was engineered to be replaced by a thermo cycler in DNA amplification, all while preserving the versatility and other desired characteristics of PCR. The mechanistic properties of the engineered helicase were investigated using Single-molecule Picometer Resolution Nanopore Tweezers (SPRNT) and molecular dynamic simulations. It was demonstrated that the increase in speed and processivity of the designed PcrA M6 helicase contribute to the facilitation of isothermal amplification.

All innate PCR features are retained by SHARP, with additional features being incorporated. According to eight criteria benchmarked, SHARP was found to perform equally well or even surpass PCR. These benchmarks encompassed the following: (1) the ability to generate amplicons of up to 6000 base pairs in length, (2) a rapid amplification time ranging from 5 to 30 minutes, (3) convenient and efficient primer design principles, (4) a competitive detection limit, (5) real-time detection capabilities, (6) ease of amplification-result interpretation, (7) versatility in downstream product applications, and (8) the elimination of the initial heat-denaturing step

[2, 8]. The advantages of bypassing thermal cycling, well-documented in the literature, are also highlighted, and the appropriateness of isothermal reactions for various wet lab tasks is explored. Notably, SHARP can quantitatively assess CRISPR-Cas9 genome editing within cells [13], and *E. coli* transformed with SHARP-made plasmids can replicate these plasmids and exhibit antibiotic resistance. Furthermore, SHARP's applicability extends to DNA sequences that may potentially form non-canonical structures, such as those containing (CAG) 47 repeats. The isothermal nature of SHARP allows for operation within a broad temperature range from 37 to 65°C, offering ample opportunities for future optimizations, integration with other techniques, and compatibility with primers and molecular probes of varying annealing temperatures.

The importance of certain properties of PcrA M6 for enabling SHARP is highlighted by the fact that PcrA monomers are known to be excellent ss DNA translocases but are rendered ineffective as helicases in vitro [14]. When subjected to disulfide crosslinking, PcrA M5 and M6 are transformed into highly efficient helicases that can unwind thousand base pairs after single binding to dsDNA. This transformation of the designed helicase is deemed crucial for the generation of kilobase-long amplicons in the context of SHARP. Furthermore, PcrA plays a significant role in plasmid replication and can unwind circular plasmids in the presence of the initiator protein RepD [15,16]. However, it should be noted that PcrA M5/M6, in isolation, necessitates a DNA substrate with 3′-ssDNA overhangs to initiate unwinding, rendering it unsuitable for SHARP [17,14]. Importantly, in the presence of SSB, PcrA M5/M6 can effectively unwind DNA with blunt ends and circular plasmids, and these capabilities are likely to be considered essential for initiating each replication cycle in SHARP. Additionally, it is worth mentioning that PcrA M5 exhibits reduced ATPase activity relative to PcrA[17], ensuring a consistent abundance of ATP even during prolonged amplification times typically associated with reactions involving low copy numbers of the initial template. Lastly, the thermo stability of PcrA M5/M6 and their ability to remain active over a broad temperature range are emphasized, as these characteristics open the door to numerous potential applications in molecular diagnostics, where adjustments for the annealing temperature of primers or probes can significantly enhance diagnostic specificity.

In this new discovery in Nature Communications, PcrA M6 helicase was engineered, and a novel isothermal amplification method named SHARP was introduced. SHARP entails the elimination of thermal cycling from PCR, all while retaining the attractive characteristics of PCR. It should be noted that SHARP is recognized as a highly reliable and precise molecular diagnostics technique that excels in identifying a minimal quantity of starting template molecules. Simultaneously, SHARP maintains a high degree of specificity, even when amplifying the correct product within a long and diverse genomic DNA. Notably, SHARP has the capacity to generate extended amplicons, each several kilo-base pairs in length, from a template using a pair of primers within a brief 30-minute timeframe. Beyond the substitution, SHARP technology can be harnessed for assessing CRISPR-Cas9 genome editing efficiency in human cells, using thermal cycling in PCR, a method relevant to molecular diagnostics and gene manipulation applications like cloning. Furthermore, it can detect and enhancing sequences that are inclined to create secondary structural configurations. With its potential to serve as an all-encompassing instrument for point-of-care diagnostic techniques in the field of medicine, simplifying various procedures in research laboratories, and enabling applications like environmental screening, farming pest detection, and other forthcoming consumer-focused diagnostic home tests, SHARP is positioned to be of significant utility in diverse contexts.

CONCLUSION

In conclusion, the evolution of nucleic acid amplification techniques, from the groundbreaking invention of PCR to the recent development of the SHARP isothermal amplification method, exemplifies the remarkable progress achieved through innovative thinking and relentless dedication within the scientific community. These advancements have not only transformed the landscape of molecular biology and medical diagnostics but also hold the potential to revolutionize various fields, from medicine to environmental screening. While each method has its unique advantages and limitations, they collectively expand the toolbox available to scientists, offering diverse options for nucleic acid amplification to meet the demands of an ever-evolving scientific landscape. The journey from PCR to SHARP underscores the importance of nurturing unconventional thoughts and pushing the boundaries of technology to drive transformative shifts that benefit both humanity and the world.

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