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Loop Mediated Isothermal Amplification (LAMP) - A Robust Molecular Tool for Rapid Disease Diagnosis

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Abstract

Genetic testing is widely used method for disease diagnosis in human, animal and plant systems. Currently, PCR is a method of choice for gene amplification but requires a thermal cycler for the reaction and electrophoresis by fluorescent intercalator to detect the amplification. However, this method remains elusive only to affordable laboratories across world. Loop-mediated isothermal amplification (LAMP) is an outstanding gene amplification procedure that combines rapidity, simplicity, and high specificity. Samples are amplified at a fixed temperature through a repetition of two types of elongation reactions occurring at the loop regions. LAMP technique has wide applications, including in point-of-care testing, genetic testing in resource constrained places, and rapid testing of food products and environmental samples.

Introduction

In recent days, the variants of Polymerase Chain Reaction are widely used for the disease diagnosis. A PCR reaction necessarily operates in three different temperature regimes for denaturalization of double stranded DNA, primer annealing to the target DNA, and extension of DNA synthesis to complete a cycle. This cycle when repeated for 30-35 times, yield a detectable level of nucleic acids that aids in precise diagnostics. However, the technique has limitation with the cost factor of equipment that limits in usage in lesser accessible regions.

With the discovery of a Polymerase enzyme, which can amplify DNA, at constant temperature without a need for costly PCR machine, the diagnosis is made simple. This method of amplification in single temperature regime is referred as Isothermal PCR. Isothermal amplification methods share some commonalities and provide detection of a nucleic acid target sequence in a streamlined and exponential manner. Since the DNA strands are not heat denatured like that in conventional PCR, isothermal methods relies on alternative way to enable primer binding and initiation of the amplification reaction, simply kind of a polymerase that possess strand-displacement activity.Isothermal amplification methods has been a boon to diagnostics with great success and widely used in commercial molecular diagnostic platforms catering to the needs of mass testing centers and point-of-care markets globally.

Isothermal Amplification

Isothermal amplification includes Loop-mediated isothermal amplification (LAMP), multiple displacement amplification (MDA), Strand displacement amplification (SDA), Helicase dependent amplification, Recombinase polymerase amplification (RPA) and Nucleic acid Sequence

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Based Amplification (NASBA).

Loop-Mediated Isothermal Amplification (LAMP) Method

Loop-mediated isothermal amplification (LAMP) method deploys typically 4-6 primers that recognize 6-8 distinct regions of the target DNA for a highly specific amplification reaction. A strand-displacing DNA polymerase enzyme initiates the synthesis and two primers specifically designed, forms the Loop like structures that facilitates subsequent rounds of amplification through extension on the loops and additional annealing of primers. The protocol is originally reported by Notomi *et al*. (2000).

LAMP amplicons are very long (>20 kb) and are formed from numerous repeats of the short target sequence (80–250 bp), connected with single-stranded loop regions in long concatamers. These products are not typically appropriate for downstream manipulation, but target amplification is so extensive that numerous modes of detection are possible.

Detection or Visualization of LAMP products are possible through Real-time fluorescence detection using intercalators or probes, lateral flow and agarose gel electrophoretic techniques detection are all directly compatible with LAMP reactions.

Types of Primers used in LAMP

LAMP primers can be designed using bioinformatics tools targeting the distinct regions of the DNA to be amplified in viruses, bacteria or any specific targets, as the case may be (Table 1). It is preferred to avoid cross homologies with the database while designing the primers.

Table 1: Description of LAMP primers specific to target regions

Sl. No.	LAMP Primer	Description of primer
1	Forward Inner Primer (FIP)	The FIP consists of a F2 region at the 3'end and a F1c region at the 5'end. The F2 region is complementary to the F2c region of the template sequence. The F1c region is identical to the F1c region of the template sequence.
2	Forward Outer Primer (FOP)	The FOP (also called F3 Primer) consists of a F3 region which is complementary to the F3c region of the template sequence. This primer is shorter in length and lower in concentration than FIP.
3	Backward Inner Primer (BIP)	The BIP consists of a B2 region at the 3'end and a B1c region at the 5'end. The B2 region is complementary to the B2c region of the template sequence. The B1c region is identical to the B1c region of the template sequence.
4	Backward Outer Primer (BOP)	The BOP (also called B3 Primer) consists of a B3 region which is complementary to the B3c region of the template sequence.

Polymerase for LAMP

Isothermal methods employ unique DNA polymerases to separate duplex DNA. Some of the DNA polymerases which possess this property include Klenow exo-, *Bsu* large fragment, and phi29 for moderate temperature reactions (25–40 °C) and the large fragment of *Bst* DNA polymerase (e.g. *Bst* 3.0 demonstrates robust performance even in high concentrations of amplification inhibitors and features significantly increased reverse transcriptase activity) for higher temperature (50–65 °C) reactions. For detection of RNA species, a reverse transcriptase compatible with temperature of reaction is added to maintain the isothermal nature of the amplification.

Stages in Loop-mediated Isothermal Amplification

LAMP protocol comprises of steps with complementary strand synthesis from the DNA template (5'-3') followed by strand displacement leading to loop formation. This loop structure is extended for complementary strand synthesis in 3'-5' template (Table 2). Further formation of dumbbell structure and subsequent stem loop structures makes mixture of LAMP final products in subsequent repeat stages that can be visualized by electrophoresis or turbidity or by using fluorescing metal indicators.

Visualization Methods for Products of LAMP

LAMP Fluorescent Dye and incubation in a real-time fluorimeter can be useful for real time detection of LAMP reactions. Turbidity through salt complexation can be used to measure reaction progress. Further, LAMP products can be analyzed by agarose gel electrophoresis or addition of indicators like calcein, malachite green, or hydroxynaphthol blue. However, one needs to exercise more caution since any endpoint method requires opening the reaction vessel that may prone to high risk of chemical contamination of workspace with the LAMP products.

1. Detection of LAMP Reaction by Turbidity

An important characteristic of the LAMP method is synthesis of extremely large amount of DNA. Accordingly, large amount of the by-product, pyrophosphate ion is produced which yields white precipitate of magnesium pyrophosphate in the reaction mixture.

Table 2: Stages in Loop-mediated Isothermal Amplification

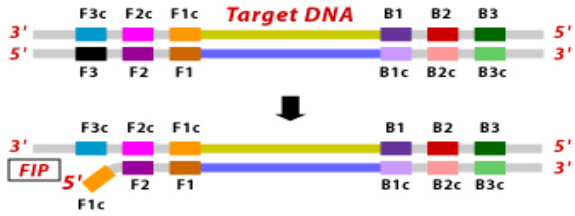
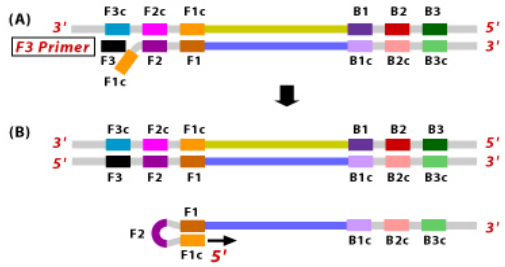
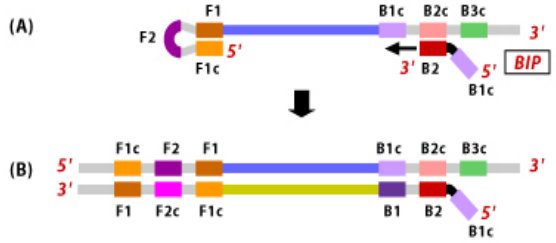
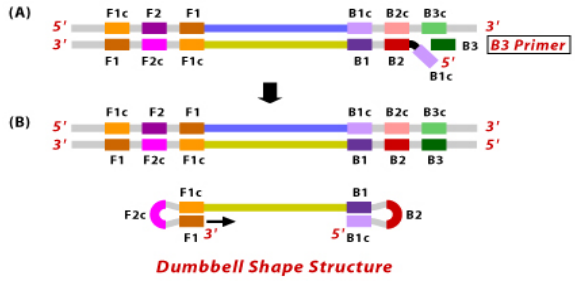
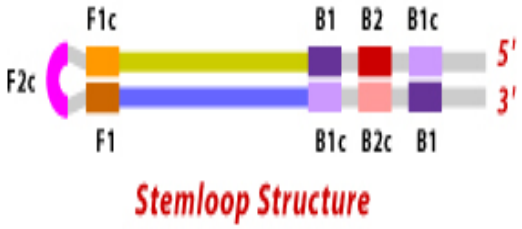
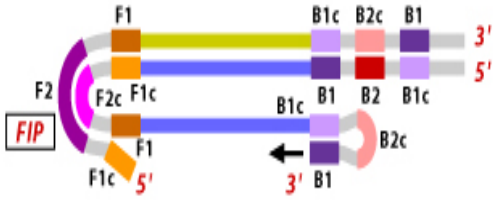
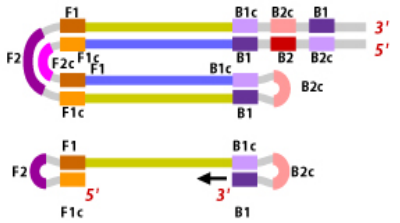
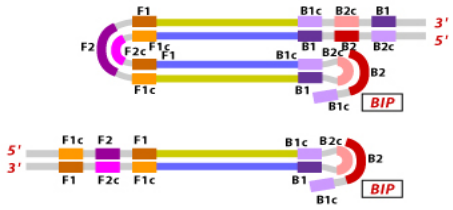
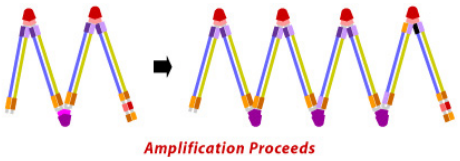
SL. No.	Sequential flow of LAMP protocol	Schematic representation of stages in LAMP
1	**Stage 1: *Complementary strand synthesis with 3'-5' template:*** F2 region of FIP hybridizes to F2c region of the target DNA and initiates complementary strand synthesis.	
2	***Strand displacement:*** Outer primer F3 hybridizes to the F3c region of the target DNA (A) and extends, displacing the FIP linked complementary strand. This displaced strand forms a loop at the 5' end (B).	
3	***Complementary strand synthesis with 5'-3' template:*** This single stranded DNA with a loop at the 5' end serves as a template for BIP (A). B2 hybridizes to B2c region of the template DNA. DNA synthesis is now initiated leading to the formation of a complementary strand and opening of the 5' end loop (B).	
4	***Formation of dumbbell structure:*** Outer primer B3 hybridizes to B3c region of the target DNA (A) and extends, displacing the BIP linked complementary strand (B). This results in the formation of a dumbbell shaped DNA.	
5	***Stem loop structure formation:*** The nucleotides are added to 3' end of F1 by DNA polymerase, which extends and opens up the loop at the 5' end. The dumbbell shaped DNA now gets converted to a stem loop structure which serves as an initiator for LAMP cycling (second stage of the LAMP reaction).	
6	**Stage 2:** To initiate LAMP cycling, the FIP hybridizes to the loop of the stem-loop DNA structure. Strand synthesis is initiated here. As the FIP hybridizes to the loop, the F1 strand is displaced and forms a new loop at the 3' end.	

Table 2: Continue..

SL. No.	Sequential flow of LAMP protocol	Schematic representation of stages in LAMP
7	Now nucleotides are added to the 3' end of B1. The extension takes place displacing the FIP strand. This displaced strand again forms a dumbbell shaped DNA. Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem loop DNA and one gap repaired stem loop DNA.	
8	Both these products then serve as template for a BIP primed strand displacement reaction in the subsequent cycles. Normally, a LAMP target sequence is amplified 13 fold every half cycle.	
9	The final products obtained are a mixture of stem loop DNA with various stems lengths and various cauliflowers like structures with multiple loops. The structures are formed by annealing between alternatively inverted repeats of the target sequence in the same strand. (Image courtesy: www.premierbiosoft.com)	

Measurement of amplicons in LAMP is achieved by real time monitoring of the increase in turbidity formation due to increased precipitation by pyrophosphate formation. This is distinct to assess the presence or absence of LAMP products.

$(DNA)_{n-1} + dNTP \longrightarrow (DNA)_n + P_2O_7^{4-}$ ..... [1]

$P_2O_7^{4-} + 2Mg^{2+} \longrightarrow Mg_2P_2O_7$ ..... [2]

During the course of amplification, polymerization of nucleic acids by DNA polymerase (1), releases pyrophosphate ion from dNTP as a byproduct. This large amount of pyrophosphate ions then react with magnesium in the LAMP reaction buffer yielding a precipitate (2).

2. Detection using a Fluorescent Metal Indicator

In the DNA amplification, pyrophosphate ions produced as a by-product from deoxyribonucleotide triphosphates (dNTPs). The calcein in the reaction mixture initially combines with manganous ion (Mn^{2+}) so as to remain quenched. When the amplification reaction proceeds, manganous ion is deprived of calcein by the generated pyrophosphate ion, which results in the emission of fluorescence (Figure 1). And the free calcein is combined with magnesium ion (Mg^{2+}) in the reaction mixture, so that it strengthens the fluorescence emission (Tomita *et al.*, 2008). The LAMP products can also be visualized by exposure to UV light (Figure 2), ascertaining the presence or absence of nucleic acids targets.

3. Detection by Electrophoresis

Agarose gel electrophoresis can be used to visualize the LAMP products. About 0.5 ml of the reaction solution is loaded onto agarose gel and electrophoresed at 100 V

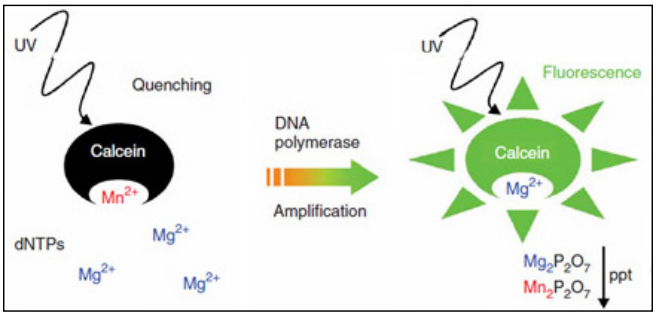
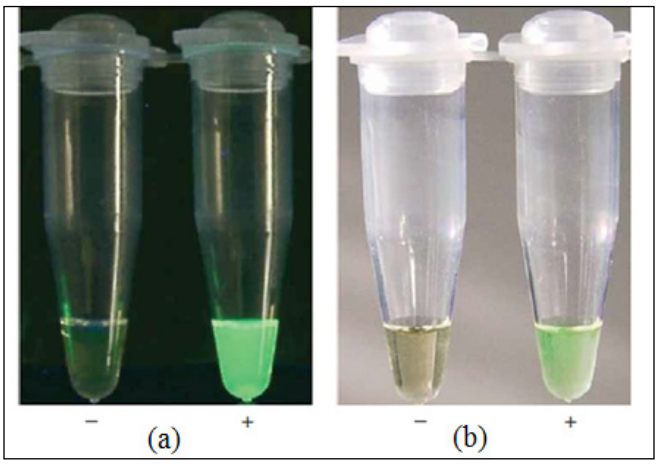


Figure 1: Principle of detection using Calcein metal indicator



(a) Visualization using a handheld-UV lamp; (b) Positive reaction with target DNA denoted as (+) sign; negative reaction (without target DNA denoted as (-) sign)

Figure 2: Visualization of the LAMP products using fluorescent metal indicator

for 40-45 min. with the 2.5-3.0 % agarose gel. The gel is then stained using 1 mg/ml of ethidium bromide and observed under UV light system.

Advantages of LAMP Method over Conventional PCR

• LAMP is an isothermal approach and does not require the thermal cycling using PCR.

• More portable or less expensive instruments, or even simple incubators or water baths is enough to carry out LAMP protocol.

• Since LAMP is not limited by a doubling-by-cycle amplification, it produces more DNA than conventional PCR in a more rapid incubation time.

• Enzymes and experimental conditions used for LAMP provide a more robust and inhibitor-tolerant amplification system for rapid detection of DNA or RNA species directly even from crude sample preparations.

Limitations of LAMP Method

• LAMP is less versatile than most widely used PCR.

• LAMP is mostly useful as a diagnostic technique, but not for cloning or many other molecular biology applications as that of PCR.

• Primer designing within a fairly small segment of the genome is subject to numerous constraints and it is difficult to design primer sets for LAMP compared to PCR.

• Multiplexing approaches for LAMP are less developed than for PCR.

• Unlike SYBR-green-based PCR assays, melt curve analysis cannot be performed in LAMP to check for the presence of primer dimers.

Applications of LAMP Method

• LAMP is used in rapid diagnosis of viral, bacterial and parasitic diseases. Examples include tests for rapid detection of coronavirus (COVID-19), SARS-CoV-2 RNA, filariasis in humans and insects and detection of the Zika virus in human samples.

• It helps in the identificationof genus and species-specific parasites.

• LAMP is gaining importance for plant disease detection in recent years.

• Rapid testing of food products and environmental samples.

Conclusion

Isothermal amplification methods offer key alternatives to lab-based protocols that demands expensive equipment for repeated cycling to amplify nucleic acids targets. Among more common isothermal amplification methods, LAMP is a novel nucleic acid amplification method that provides rapid, sensitive, and specific detection limits and has an important role in scientific research with its wide applicability in plant, animal, human disease diagnostics and in assay of environmental samples.

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