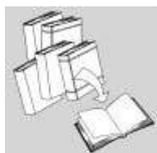


REVIEW



Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases

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SUMMARY

Loop mediated isothermal amplification (LAMP) is a powerful innovative gene amplification technique emerging as a simple rapid diagnostic tool for early detection and identification of microbial diseases. The whole procedure is very simple and rapid wherein the amplification can be completed in less than 1 h under isothermal conditions employing a set of six specially designed primers spanning eight distinct sequences of a target gene, by incubating all the reagents in a single tube. Gene amplification products can be detected by agarose gel electrophoresis as well as by real-time monitoring in an inexpensive turbidimeter. Gene copy number can also be quantified with the help of a standard curve generated from different concentrations of gene copy number plotted against time of positivity with the help of a real-time turbidimeter. Alternatively, gene amplification can be visualised by the naked eye either as turbidity or in the form of a colour change when SYBR Green I, a fluorescent dsDNA intercalating dye, is employed. LAMP does not require a thermal cyclor and can be performed simply with a heating block and/or water bath. Considering the advantages of rapid amplification, simple operation and easy detection, LAMP has potential applications for clinical diagnosis as well as surveillance of infectious diseases in developing countries without requiring sophisticated equipment or skilled personnel. Copyright © 2008 John Wiley & Sons, Ltd.

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INTRODUCTION

Nucleic acid amplification is a valuable tool for the diagnosis of infectious diseases. Several amplification methods are available including PCR, nucleic acid sequence based amplification (NASBA), self-

sustained sequence replication (3SR) and strand displacement amplification (SDA). Among these, PCR is the most widely used in various forms such as reverse transcription PCR (RT-PCR), nested PCR and multiplex PCR.

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Abbreviations used

3SR, self-sustained sequence replication; B3, backward outer primer; BIP, backward internal primer; BLP, backward loop primer; ΔG , free energy; DNA, deoxyribose nucleic acid; F3, forward outer primer; FIP, forward internal primer; FLP, forward loop primer; FMDV, foot and mouth disease virus; g-POCT, gene point-of-care testing; HPLC, high performance liquid chromatography; LAMP, Loop mediated isothermal amplification; NASBA, nucleic acid sequence based amplification; PEI, Polyethylenimine; POCT, point-of-care testing; RT-LAMP, reverse transcription loop mediated isothermal amplification; RT-PCR, reverse transcription polymerase chain reaction; SDA, strand displacement amplification; SNP, single nucleotide polymorphism; T_m , melting temperature; T_p , time of positivity; UV, ultra violet.

THE NEED FOR BETTER MOLECULAR DIAGNOSTIC TOOLS

These PCR-based methods require either high precision instruments for amplification or elaborate methods for detection of the amplified products. In addition, they are often cumbersome to adapt for routine clinical use especially in peripheral health care settings and private clinics. In addition, PCR has several intrinsic disadvantages, such as the requirement for thermal cycling, and time consuming post-PCR analysis, thereby potentially leading to laboratory contamination.

The development of real-time PCR has brought true quantitation of target nucleic acids out of the pure research laboratory and into the diagnostic laboratory, by combining PCR amplification with fluorescent-labelled virus specific probes able to detect amplified DNA during the amplification reaction. The fluorescent chemistry coupled with advanced optical detectors makes it more sensitive than conventional gel-based PCR. Several real-time PCR assays have been developed to address the need for reliable detection systems for early infection and quantification of virus load in the acute phase of illness. Real-time PCR assays used for quantitative RT-PCR combine the best attributes of both relative and competitive (end-point) RT-PCR in that they are accurate, precise, capable of high throughput and relatively easy to perform. In addition, real-time PCR automates the laborious process of amplification by quantitating reaction products for each sample in every cycle. Data analysis, including standard curve generation and copy number calculation, is performed automatically.

The real-time assays have many advantages over conventional PCR methods, including rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity and easy standardisation [1]. However, all these nucleic acid amplification methods have several intrinsic disadvantages of requiring either a high precision instrument for amplification or an elaborate complicated method for detection of amplified products. Real-time PCR requires an instrumentation platform that consists of a thermal cycler, computer, optics for fluorescence excitation and emission collection and data acquisition and analysis software. Real-time PCR machines are expensive and thus are not within purchasing reach of laboratories in developing countries. More cost-effective, sensitive and real-time based assays are, therefore needed to complement the existing PCR-based assay systems. The present review describes a new generation of novel gene amplification technique that is gaining popularity among researchers due to its simple operation, rapid reaction and easy detection.

LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP): NEW GENERATION OF GENE AMPLIFICATION ASSAY

'LAMP' is characterised by the use of six different primers specifically designed to recognise eight

distinct regions on the target gene. The amplification proceeds at a constant temperature using strand displacement reaction. Amplification and detection of a gene can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (about 63°C) [2]. Compared to PCR and real-time PCR, LAMP has the advantages of reaction simplicity and higher amplification efficiency. The LAMP reaction also yields large amounts of by-product, pyrophosphate ion, leading to a white precipitate of magnesium pyrophosphate in the reaction mixture. Since the increase in turbidity of the reaction mixture according to the production of precipitate correlates with the amount of DNA synthesised, real-time monitoring of the LAMP reaction can be achieved by real-time measurement of turbidity [3].

Design of LAMP primers

Design of a highly sensitive and specific primer set is crucial for performing LAMP amplification. The target selection for primer design can be accomplished by using the Primer Explore [LAMP primer designing support software program, Net laboratory, Japan, <http://venus.netlaboratory.com>] after considering the base composition, GC content and the formation of secondary structures. The primer set for LAMP amplification includes a set of six primers comprising two outer, two internal and two loop primers that recognise eight distinct regions on the target sequence. The two outer primers were described as forward outer primer (F3) and backward outer primer (B3) and have a role in strand displacement during the non-cyclic step only. The internal primers were described as forward internal primer (FIP) and backward internal primer (BIP) having both sense and antisense sequence in such a way that it helps in the formation of a loop. Further, two loop primers viz; forward loop primer (FLP) and backward loop primer (BLP) were designed to accelerate the amplification reaction by binding to additional sites that are not accessed by internal primers.

The design of the above mentioned six types of primers are based on the following eight distinct regions of the target gene: the F3c, F2c, F1c and FLP regions at the 3' side and the B1, B2, B3 and BLP regions at the 5' side (see Figure 1). FIP

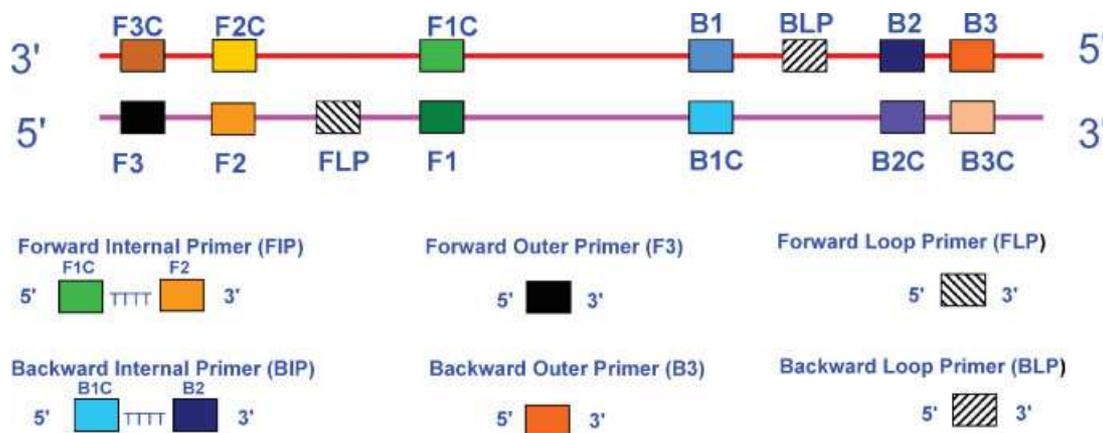


Figure 1. Schematic representation of primer design for RT-LAMP assay showing the position of the six primers spanning the target gene

consists of the F2 region (at the 3' end) that is complementary to the F2c region, and the same sequence as the F1c region at the 5' end. Forward outer primer (F3) consists of the F3 region that is complementary to the F3c region. BIP consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end. Backward outer primer (B3) consists of the B3 region that is complementary to the B3c region. FIP consists of a complementary sequence of F1 and a sense sequence of F2. BIP consists of a complementary sequence of B1 and a sense sequence of B2 (Figure 1). FIP and BIP were high performance liquid chromatography (HPLC) purified primers. The FLP and BLP primers were composed of the sequences that are complementary to the sequence between F1&F2 and B1&B2 regions respectively [2].

In addition, the following criteria needs to be considered critically for getting an ideal LAMP primer set having excellent combination of sensitivity and specificity. The GC content of the primers should be about 50–60% in the case of GC rich and about 40–50% for AT rich. The primers should be designed so as not to easily form secondary structures. 3' end sequence should not be AT rich or complementary to other primers. The distance between 5' end of F2 and B2 should be 120–180 bp, and the distance between F2 and F3 as well as B2 and B3 should be 0–20 bp. The distance for loop forming regions (5' of F2 to 3' of F1, 5' of B2 to 3' of B1) should be 40–60 bp. The melting temperature (T_m) for primer regions should be about 60–65°C in the case of GC rich and about 55–60°C

for AT rich. The stability of primer end should be established based on the change in free energy (ΔG) calculated 6 bp from the following end regions which should be less than -4kcal/mol , 5' end of F1c/B1c and 3' end of F2/B2 as well as F3/B3. If restriction enzyme sites exist on the target sequence, except the primer regions, they can be used to confirm the amplified products.

Principle of LAMP amplification

The chemistry of LAMP amplification is based on the principle of auto cyclic strand displacement reaction being performed at a constant temperature using a DNA polymerase. There are two steps of LAMP amplification comprising non-cyclic and cyclic steps [4].

Non-cyclic step

In the non-cyclic step (see Figure 2A), there is the formation of DNA with stem-loops at each end that serve as the starting structure for the amplification by LAMP cycling. Because double stranded DNA is in the condition of dynamic equilibrium at the temperature around 65°C, one of the LAMP primers can anneal to the complimentary sequence of double stranded target DNA, then initiates DNA synthesis using the DNA polymerase with strand displacement activity, displacing and releasing a single stranded DNA [2,4].

With the LAMP method, unlike with PCR, there is no need for heat denaturation of the double stranded DNA into a single strand. Through the activity of DNA polymerase with strand displace-

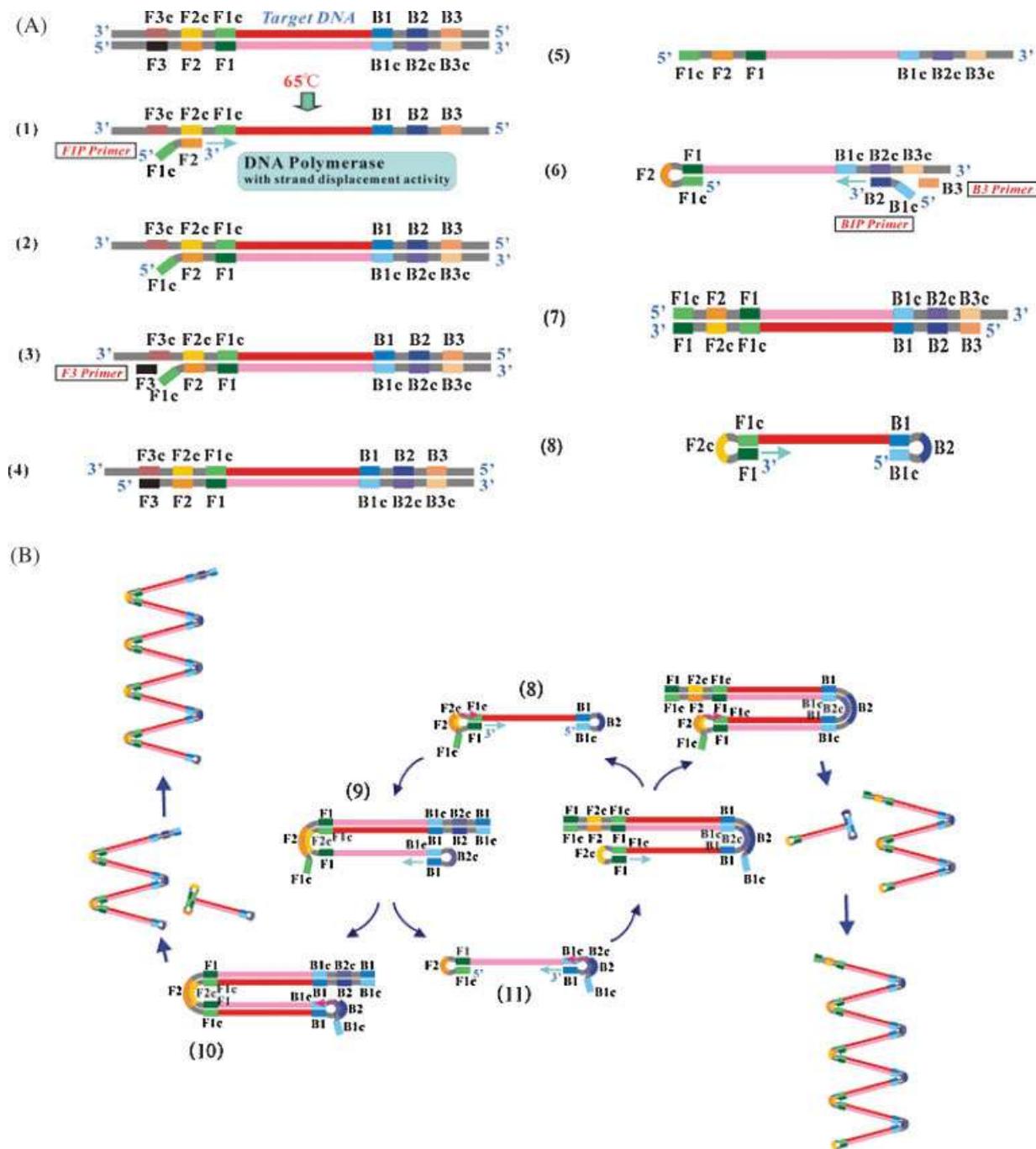


Figure 2A. Principles of LAMP amplification. Non-Cyclic Step [1–8]: generation of stem loop DNA with dumbbell-shaped structure at both ends that is ready to enter into cyclic amplification step. Initially, the strand displacement activity of *Bst* DNA polymerase helps in synthesis of a complementary DNA strand, starting with FIP. The outer primer (F3) then displaces the FIP-linked complementary strand, which forms a stem-loop structure at the 5' end. This serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The final product is a structure with stem-loops at each end. Copyright ©, 2005, Eiken Chemical Co. Ltd., Japan. (B) Principles of LAMP amplification. Cyclic Amplification Step [9–11]: exponential amplification of original dumbbell-shaped stem-loop DNA employing internal primers. The product is the differently sized structures consisting of alternately inverted repeats of the target sequence on the same strand, giving a cauliflower-like structure. Copyright ©, 2005, Eiken Chemical Co. Ltd., Japan

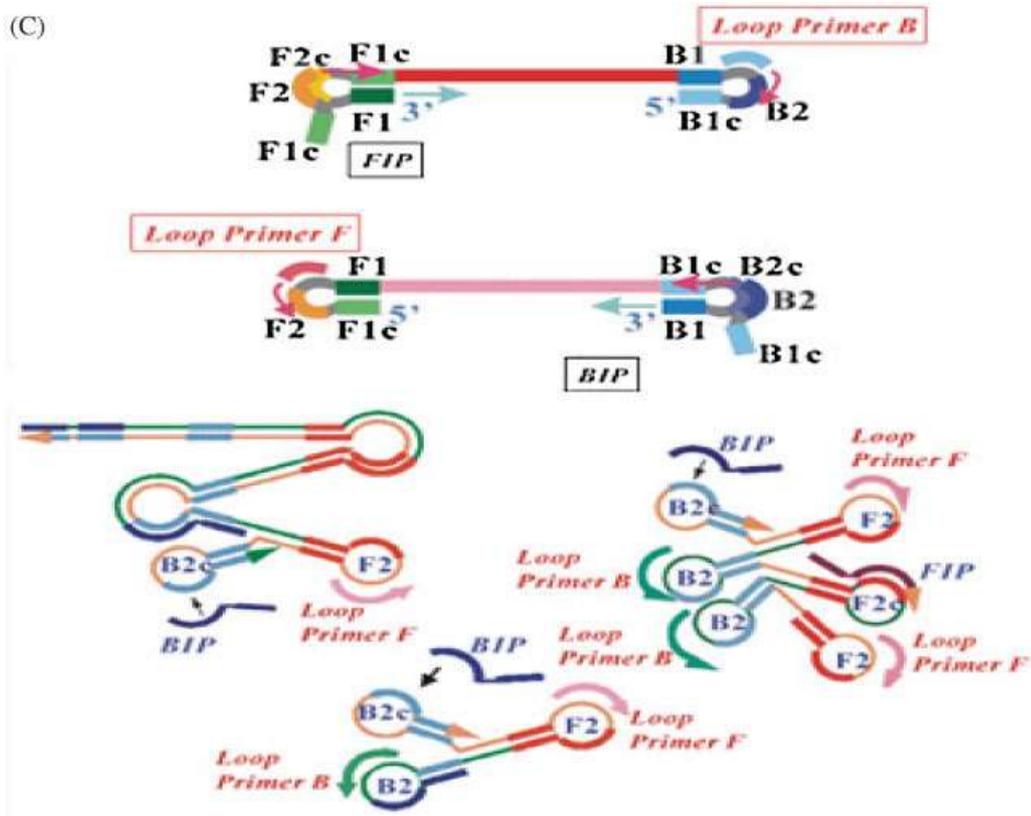


Figure 2C. Principles of LAMP amplification. Accelerated LAMP Amplification using loop primers. Loop primers are complementary to the single stranded loop region. They provide additional starting sites for DNA synthesis and accelerate the amplification thereby reducing the reaction time to less than 30 min. Copyright ©, 2005, Eiken Chemical Co. Ltd., Japan

ment activity, a DNA strand complementary to the template DNA is synthesised, starting from the 3' end of the F2 region of the FIP. The F3 primer anneals to the F3c region, outside of FIP, on the target DNA and initiates strand displacement DNA synthesis, releasing the FIP-linked complementary strand. A double strand is formed from the DNA strand synthesised from the F3 primer and the template DNA strand. The FIP-linked complementary strand is released as a single strand because of the displacement by the DNA strand synthesised from the F3 primer. Then, this released single strand forms a stem-loop structure at the 5' end because of the complementary F1c and F1 regions. This single strand DNA in turn serves as a template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The BIP anneals to the DNA strand produced by the above step. Starting from the 3' end of the BIP, synthesis of comple-

mentary DNA takes place. Through this process, the DNA reverts from a loop structure into a linear structure. The B3 primer anneals to the outside of the BIP and then, through the activity of the DNA polymerase and starting at the 3' end, the DNA synthesised from the BIP is displaced and released as a single strand before DNA synthesis from the B3 primer. The BIP-linked complementary strand displaced forms a structure with stem-loops at each end, which looks like a dumbbell structure. This dumbbell-like DNA structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. This structure serves as the starting structure for exponential amplification.

Cyclic amplification

In subsequent LAMP cycling (see Figure 2B) one internal primer hybridises to the loop on the product and initiates displacement DNA synthesis,

yielding the original stem-loop DNA and a new stem-loop DNA with a stem twice as long. Briefly the FIP anneals to the single stranded region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesised strand. This released single strand forms a stem-loop structure at the 3' end because of complementary B1c and B1 regions. Then, starting from the 3' end of the B1 region, DNA synthesis starts using self-structure as a template, and releases FIP-linked complementary strand. The released single strand then forms a dumbbell-like structure as both ends have complementary F1–F1c and B1c–B1 regions, respectively. Furthermore, BIP anneals to the B2c region and primes strand displacement DNA synthesis, releasing the B1-primed DNA strand. As a result of this process, various sized structures consisting of alternately inverted repeats of the target sequence on the same strand are formed. The cycling reaction continues leading to accumulation of 10^9 copies of target in less than an hour. The final products are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand (Figure 2B).

LAMP amplification can also be accomplished with the two outer (F3 and B3) and two internal primers (FIP and BIP) but by using the two loop primers (FLP and BLP), the amplification is accelerated thereby reducing the amplification time [5]. The investigation on how loop primers affect amplification time (original method: no loop primer; rapid method: with loop primers) revealed that the time required for amplification with loop primers is one-third to one-half of that without loop primer. With the use of loop primers, amplification can be achieved within 30 min (Figure 2C).

Assay protocol for RT-LAMP amplification

The LAMP reaction is usually carried out in a total 25 μ l reaction volume containing 50 pmol each of the primers FIP and BIP, 5 pmol each of the outer primers F3 and B3, 25 pmol each of loop primers FLP and BLP in a $2 \times$ reaction mixture having 20 mM Tris-HCl pH8.8, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 8 mM MgSO_4 , 10 mM KCl, 1.4 mM dNTPs, 0.8 M betaine, 0.1% Tween20, 8 units of the *Bst* DNA polymerase and 2 μ l of DNA template. Positive and negative

controls should be included in each run, and all precautions to prevent cross-contamination should be observed. The optimum temperature for the LAMP reaction is 63°C, which is optimum for the activity of *Bst* DNA polymerase.

The amplification of RNA template is accomplished through Reverse Transcription-Loop-mediated Isothermal Amplification (RT-LAMP) assay by employing RT for reverse transcription step in addition to the *Bst* DNA polymerase. RT-LAMP method can synthesise cDNA molecules from template RNA and apply LAMP technology to amplify and detect them. As the template is an RNA sample, in addition to the reagents of DNA amplification (primers, DNA polymerase with strand displacement activity, substrates, etc.), RT is added to the reaction mixture. After mixing and incubating at a constant temperature between 60–65°C, amplification and detection can be carried out in a single step.

Monitoring of RT-LAMP amplification

Real-time monitoring

The real-time monitoring of LAMP amplification can be accomplished through spectrophotometric analysis with the help of loop amp real-time turbidimeter (LA-200, Teramecs, Japan) that records the turbidity in the form of O.D. at 400 nm every 6 s (Figures 3 and 4A). The turbidimeter is relatively inexpensive as compared to the real-time PCR machine. Turbidity is the unique phenomenon associated with LAMP amplification and it is attributed to the higher amplification efficiency of LAMP reaction. In order to observe turbidity in the form of a white precipitate, a DNA yield of $\geq 4 \mu\text{g}$ is required so as to push the pyrophosphate ion concentration above 0.5 ppm. LAMP reaction produces a DNA yield of $\geq 10 \mu\text{g}$ compared to 0.2 μg in PCR in 25 μ l reaction scale. In addition, the low amount of pyrophosphate ion formed during PCR gets hydrolysed to phosphate due to the high temperature of $>94^\circ\text{C}$. Thus turbidity is the easiest way of monitoring gene amplification by LAMP method [6].

Agarose gel analysis

Following incubation at 63°C for 30 min, 10 μ l aliquot of LAMP amplified products are electrophoresed on 3% NuSieve 3:1 agarose gel (BMA,

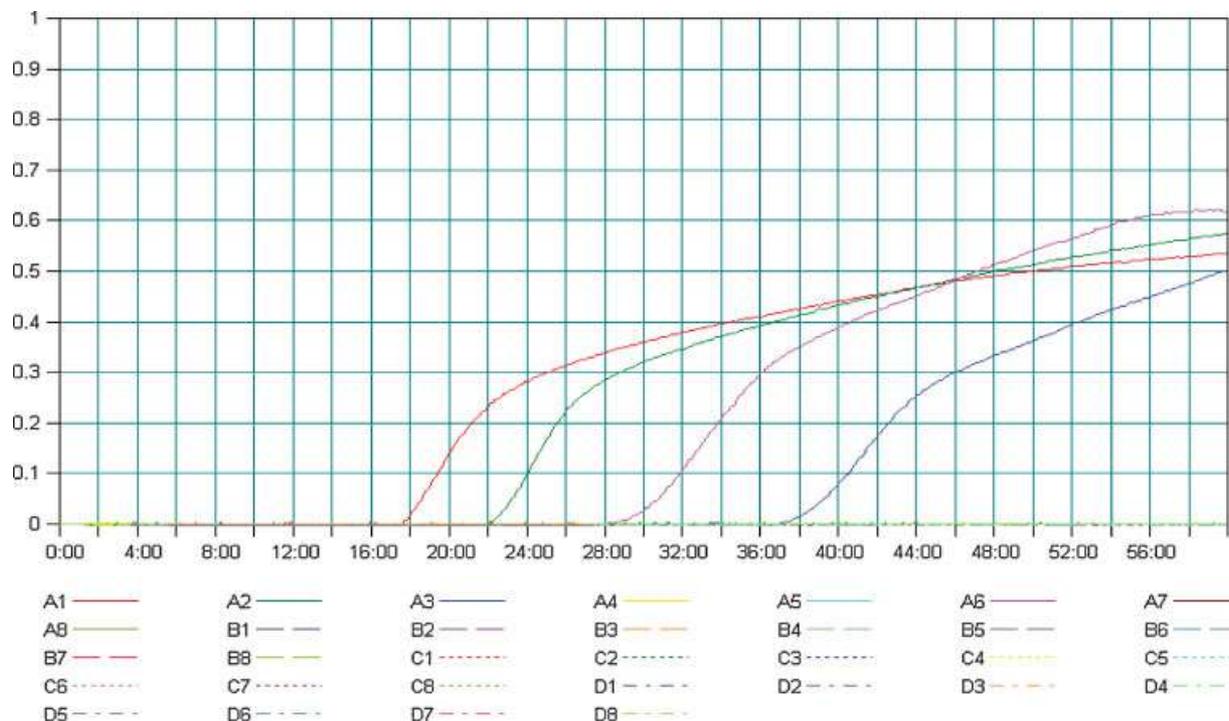


Figure 3. Real-Time monitoring of LAMP amplification showing the amplification curve. X-axis, depicts the time of positivity and Y-axis, shows the turbidity value in terms of O.D. at 400 nm

Rockland, ME, USA) in Tris-borate buffer followed by staining with ethidium bromide and visualisation on a UV transilluminator at 302 nm (Figure 4B).

Naked eye visualisation

In order to facilitate application of LAMP assay in the field, monitoring of amplification can also be carried out with naked eye inspection either in the form of visual turbidity or visual fluorescence.

Visual turbidity

The turbidity of magnesium pyrophosphate can be visually observed. Following amplification, the tubes can be inspected for white turbidity through naked eye after a pulse spin to deposit the precipitate in the bottom of the tube (Figure 4C) [3].

Visual fluorescence

The tube containing the amplified products can also be better visualised in the presence of fluorescent intercalating dye viz; ethidium bromide, SYBR Green I, Calcein, etc. by illuminating with

a UV lamp. In practice, usually the visual inspection for amplification is performed through observation of colour change following addition of 1 μ l of SYBR Green I (a fluorescent dsDNA intercalating dye) to the tube. In case of positive amplification, the original orange colour of the dye will change into green that can be judged under natural light as well as under UV light (302 nm) with the help of a hand held UV torch lamp. In case there is no amplification, the original orange colour of the dye will be retained. This change of colour is permanent and thus can be kept for record purposes (Figure 4D).

Calcein is another chelating fluorescent detection reagent that can be used in the reaction mixture prior to amplification. Calcein initially combines with manganese ions to achieve a quenching effect. The amplification generates the by-product, pyrophosphate ions, which will bind and remove manganese ions from calcein to irradiate fluorescence. The fluorescence is further intensified as calcein combines with magnesium ions. From this feature, the presence of fluorescence can indicate the presence of target gene and visual

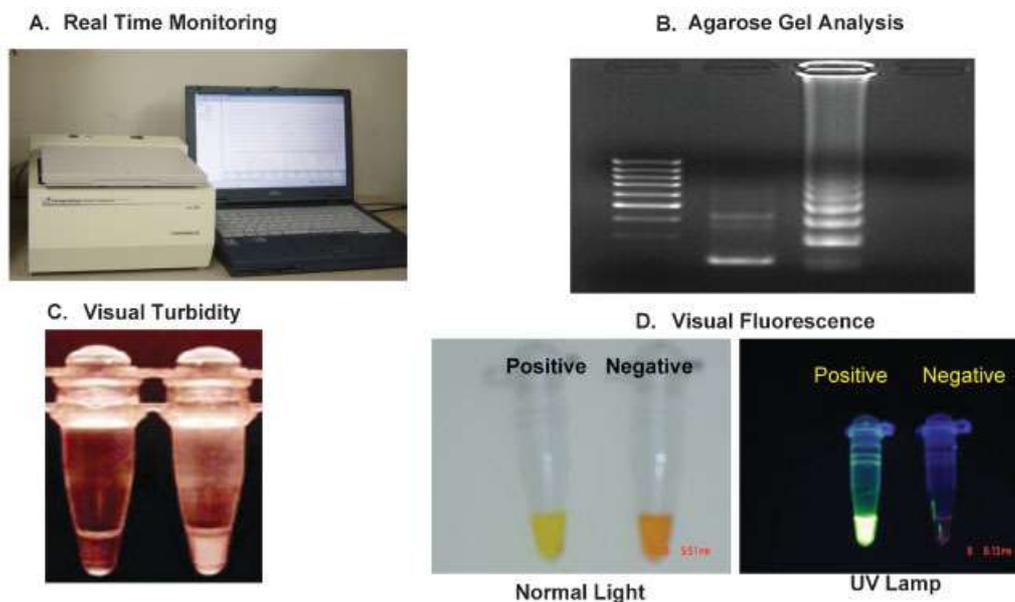


Figure 4. Monitoring of LAMP amplification. (A) The turbidity of magnesium pyrophosphate, a by-product of the reaction, can be detected real-time by a real-time turbidimeter. (B) Agarose gel analysis revealing the typical electrophoresis pattern of LAMP amplified product, which is not a single band but a ladder pattern because the LAMP method forms amplified products of various sizes consisting of alternately inverted repeats of the target sequence on the same strand. (C) Visual turbidity in the form of white precipitate as observed in positive control due to accumulation of magnesium pyrophosphate in proportion to the accumulated amplified products. (D) When the tube containing the amplified products incorporating a fluorescent intercalating dye is illuminated with a UV lamp, the fluorescence intensity increases

detection can be achieved without opening the tube, thus preventing carry-over contamination with post-amplification products.

Sequence specific visual detection of LAMP

In order to detect LAMP products in a sequence-specific manner visually, an extremely simple method was reported by adding a small amount of low-molecular weight polyethylenimine (PEI) to the LAMP reaction solution [7]. A characteristic of this technique is the ability to visually present sequence information of amplicons without using an expensive source of light or a detector. The new detection method described above utilises the unique nature of low-molecular-weight PEI, i.e. it cannot form an insoluble complex with a single-stranded anionic polymer with a low-molecular weight such as an oligo DNA probe, but it can form an insoluble complex with DNA with a high-molecular weight such as LAMP product. Since a large amount of amplification product is created by the LAMP reaction, precipitate of a size that can be easily confirmed with the eyes is generated when PEI is added to the LAMP reac-

tion solution. Moreover, the fact that the amplification is highly efficient means that the amount of labelled probe for detection that can be added is large. As a result of these characteristics, the LAMP reaction followed by addition of PEI yields precipitate with a clear colour and in a size that can be identified visually. If the 5' end of the internal primer is fluorescently labelled, the LAMP product should be visible, but this approach is not preferred, because the possibility of false positives from self-extension of the labelled primer cannot be excluded. There is no risk of false positives with the oligo DNA probes fluorescently labelled at the 3' end, so that highly accurate genetic testing can be established. It is necessary to add PEI to the LAMP reaction solution after the LAMP reaction takes place since PEI strongly inhibits the LAMP reaction. However, opening the reaction tube after amplification should generally be avoided to prevent carry-over contamination.

Interpretation of results

Unlike real-time PCR assay, where the positivity is decided on the basis of C_t value, in the case of

LAMP the criterion of positivity is based on the time of positivity (T_p). This varies from virus to virus, based on the designed primer set and nature of the selected template. The cut-off value for positivity by real time RT-LAMP assay for a particular gene can be determined by taking into account the time of positivity (in minutes) at which the turbidity increases above the threshold value fixed at 0.1, which is two times more than average turbidity value of the negative controls of several replicates. In most LAMP amplifications, it is observed after 30–40 min in the amplification cycle.

On agarose gel analysis, the LAMP amplicons reveal a ladder-like pattern in contrast to a single band as observed in PCR. This is due to the cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand.

Sensitivity and specificity of LAMP amplification

In general, the LAMP assay was found to be 10–100 fold more sensitive than PCR with a detection limit of 0.01–10 pfu of virus [8–10]. The specificity of the LAMP amplification is directly attributed to the six sets of primers spanning eight distinct sequences of the target gene that are being used for amplification. Unless all the target genes are available, amplification will not proceed. The authenticity of the LAMP amplified product can be established by digesting with a restriction enzyme cutting a site at one end of the selected target. Further confirmation of the structures of the amplified products can also be accomplished through nucleotide sequencing of the amplified products.

Quantification of gene copy numbers by LAMP assay

The quantification of gene copy number and/or concentration of the viral nucleic acid can be accomplished through generation of a standard curve by plotting known concentration of gene copy number or infectious unit of virus against time of positivity to obtain the amplification signal for that particular concentration. A linear relationship between various concentrations against time of positivity is usually obtained through the real-time monitoring of the amplification (Figure 5).

The quantification of gene copies in clinical samples can be extrapolated from a standard curve on the basis of their time of positivity [6].

Advantages of LAMP amplification

The primary characteristics of the LAMP are its ability to amplify nucleic acid under isothermal conditions in the range of 65°C; as a result it allows the use of simple and cost effective reaction equipment. The second characteristic is that LAMP has high specificity and high amplification efficiency. Its specificity is extremely high because it can amplify a specific gene from a human genome specimen discriminating a single nucleotide difference. The high amplification efficiency of LAMP is attributed to no time loss of thermal change because of its isothermal reaction. The reaction can be conducted under optimal temperature of the enzyme and the inhibition reaction at the later stage of amplification is less likely to occur compared with the PCR. It was observed that when nucleic acid is amplified by the LAMP method, the turbidity derived from the precipitate is produced according to the progress of the reaction and thus making it ideal for easy monitoring through naked eye.

In addition, both amplification and detection of gene can be completed in a single step, by incubating the mixture of gene sample, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature. It provides high amplification efficiency, with DNA being amplified 10^9 – 10^{10} times in 15–60 min. Because of its high specificity, the presence of the target gene sequence can easily be detected just by judging presence of amplified products. There is no need for a step to denature double stranded DNA into a single stranded form. LAMP assay has the great advantage of monitoring amplification by SYBR Green I dye mediated naked eye visualisation and by real-time monitoring by using an inexpensive turbid meter according to the situation. The particular importance is the substantial reduction in time required for the confirmation of results by RT-LAMP assay in 30 min as compared to 3–4 h in case of RT-PCR (Table 1).

Applications of LAMP assay

LAMP is a gene amplification method with a variety of characteristics and applications in a wide

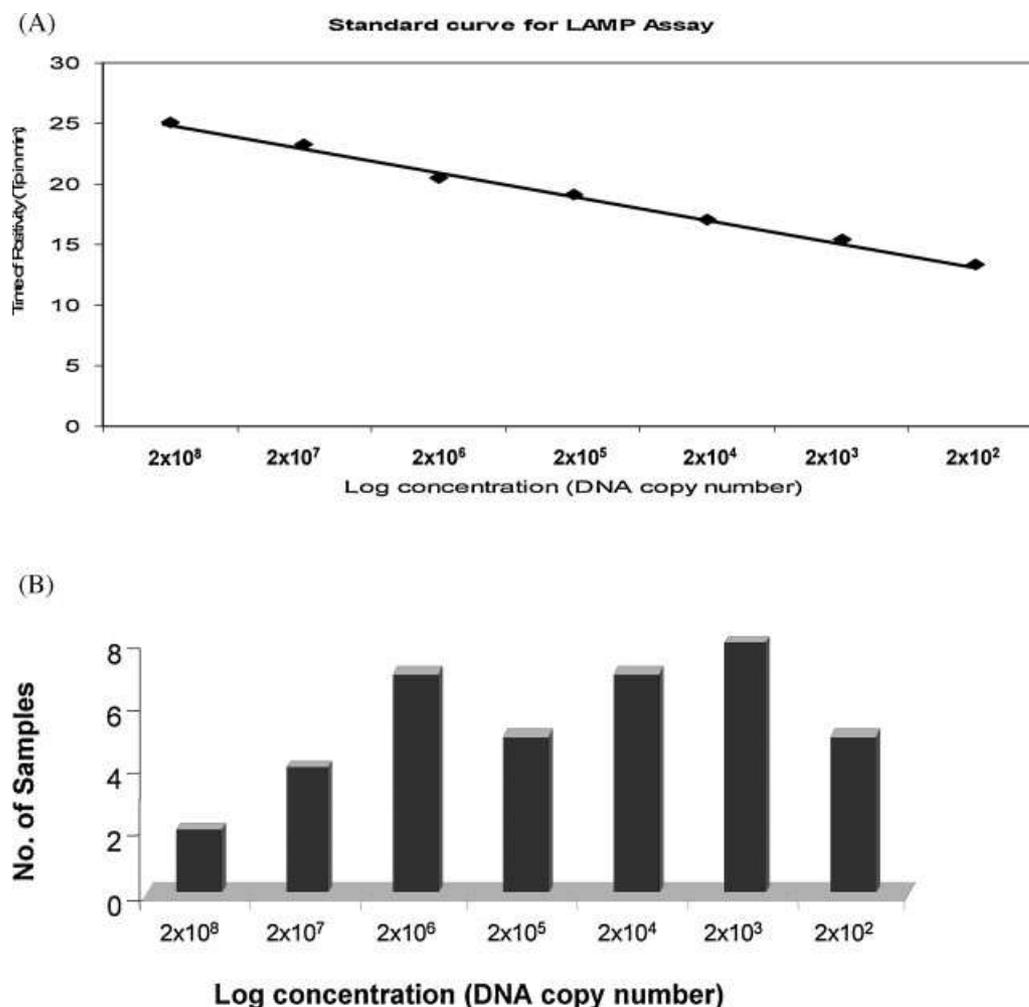


Figure 5. (A) Standard curve for LAMP assay generated from the amplification plots between 10 fold serially diluted plasmid construct and time of positivity (Tp). (B) Quantitative determination of virus concentration in clinical samples employing standard curve

range of fields, including clinical diagnosis, single nucleotide polymorphism (SNP) typing and quantification of template DNA. In particular, LAMP is considered to be effective as a gene amplification method for use in gene point-of-care testing (g-POCT) devices.

LAMP in clinical diagnosis

Although the inception of LAMP refers back to 1998, it became popular only after 2003 following the emergence of West Nile and SARS viruses. Since then LAMP assay has increasingly been adapted by researchers mostly from Japan for the clinical diagnosis of emerging diseases (Table 2).

LAMP has been successfully applied for rapid and real-time detection of both DNA and RNA viruses. However, most of the published research has been directed towards RNA viruses, maybe due to the increased incidence of RNA viruses in recent past in the form of major epidemics having significant public health importance. A one-step single tube real-time accelerated RT-LAMP assay for rapid detection of each of several recently emerged human viral pathogens viz; Dengue, Japanese Encephalitis, Chikungunya, West Nile, SARS, highly pathogenic avian influenza (HPAI) H5N1, Norwalk viruses have been developed and evaluated [8–15]. In comparison to conventional RT-PCR, RT-LAMP assay demonstrated

Table 1. Comparative advantages and disadvantages of different molecular diagnostic techniques

| | Conventional PCR | Real-time PCR | LAMP |
|--------------|--|---|---|
| Advantage | <ul style="list-style-type: none"> • Alternate gold standard for isolation in absence of live agent • Early confirmatory diagnosis • Widely used molecular diagnostic format | <ul style="list-style-type: none"> • Simultaneous amplification and detection during exponential amplification • Real-time monitoring of amplification as it happens • Quantitative, thus useful for monitoring the viral load • Lower carry over contamination due to closed tube operation • Increased sensitivity due to fluorescent chemistry • High throughput analysis due to software driven operation | <ul style="list-style-type: none"> • Isothermal field-based gene amplification without requiring thermal cycler • Amplification can be accomplished with waterbath/heating block • Real-time as well as quantitative • Higher amplification efficiency and sensitivity • Naked eye visual monitoring either through turbidity or colour change by fluorescent intercalating dye (SYBR Green I) |
| Disadvantage | <ul style="list-style-type: none"> • Qualitative (Yes or No format) • End-point detection in plateau phase with non spurious amplification • Post-PCR handling leading to carry over contaminations • Less sensitive, thereby missing borderline cases with low gene copy numbers • Time consuming (3–4 h) • Requirement for thermal cycler and gel documentation system | <ul style="list-style-type: none"> • Expensive detection equipments and consumables • Requirement for fluorescent probe • Restricted to referral laboratory with good financial support | <ul style="list-style-type: none"> • Complicated primer design (requirement for six primers) • Two long primers of HPLC grade purity • Restricted availability of reagents and equipment in some countries • Laboratory based |

10–100 fold more sensitivity with a detection limit of 0.01–10 pfu of virus in all these cases.

The usefulness of LAMP for amplification of DNA viruses was also reported for HPV (Human papillomavirus) type—6, 11, 16 and 18, HSV, VZV, CMV, Adenovirus and BK virus (Table 2) and found to be superior in terms of sensitivity, specificity, rapidity and simplicity, and can poten-

tially be a valuable tool for the detection of HPV DNA compared to PCR and real-time PCR [16–27].

Among the animal viruses, a one-step, RT-LAMP assay was reported for detection of Foot and Mouth Disease virus (FMDV) in less than 1 h in a single tube without thermal cycling [28]. A fragment of the 3D RNA polymerase gene of the virus is amplified at 65°C in the presence of a

Table 2. Applications of LAMP to the rapid detection of viral diseases of humans and animals

| Host | Virus | References | | |
|----------------------------|-------|---|--|---------|
| Human | DNA | Adenoviral keratoconjunctivitis | [26] | |
| | | Human papillomavirus type 6, 11, 16 and 18 | [16] | |
| | | Varicella-zoster virus | [24] | |
| | | Herpes simplex virus and varicella-zoster virus | [17] | |
| | | Cytomegalovirus | [25] | |
| | | Herpes simplex virus | [18,19] | |
| | | Human herpesvirus 6 | [20,21] | |
| | | Human herpesvirus 7 | [22] | |
| | | Human herpesvirus 8 | [23] | |
| | | BK virus | [27] | |
| | | RNA | Severe acute respiratory syndrome (SARS) coronavirus | [10] |
| | | | West Nile virus | [9] |
| | | | Japanese encephalitis virus | [11,12] |
| | | | Norovirus | [15] |
| | | | H5 avian influenza virus | [14] |
| Chikungunya virus | [13] | | | |
| Dengue viruses (1,2,3 & 4) | [8] | | | |
| Animal | DNA | Plum pox virus | [33] | |
| | | Newcastle disease virus | [29] | |
| | RNA | Canine distemper virus | [30] | |
| | | Canine parvovirus | [31] | |
| | | Foot-and-mouth disease virus | [28] | |
| | | Viral haemorrhagic septicaemia virus (VHS) | [32] | |

primer mixture and both reverse transcriptase and *Bst* DNA polymerase. Compared with real-time PCR, RT-LAMP was consistently faster, and ten copies of FMDV transcript were detected in 22 min. LAMP has also been utilised for diagnosis of Newcastle disease, Canine distemper, Canine parvo, Viral haemorrhagic septicemia virus and Plum pox viruses [29–33].

LAMP in SNP typing

LAMP-based SNP typing is an accurate, rapid and simple method that may be useful especially for point-of-care testing. Because of the high specificity of the LAMP method, only the target gene will be amplified from gene samples containing homologous nucleotide sequences when using LAMP-based SNP typing [34,35]. Furthermore,

because of the characteristics of its amplification reaction, the LAMP method discriminates a single nucleotide difference at each cycling step of the DNA replication, through both 'sense and anti-sense strand' reactions, and the type of SNP can easily be detected just by amplifying the DNA containing SNP in a single step. Due to the simplicity and rapidity of the LAMP method, simple detection of SNP typing can be achieved within 30 min. The products of the LAMP reaction, which was performed in the presence of an intercalating dye, were detected within 30 min without any post-reaction sample manipulation. With the use of four primers designed to recognise six distinct regions, only the target gene is strictly and specifically amplified even in coexistence with its homologous gene. The reaction is so specific as to strictly discriminate single nucleotide difference.

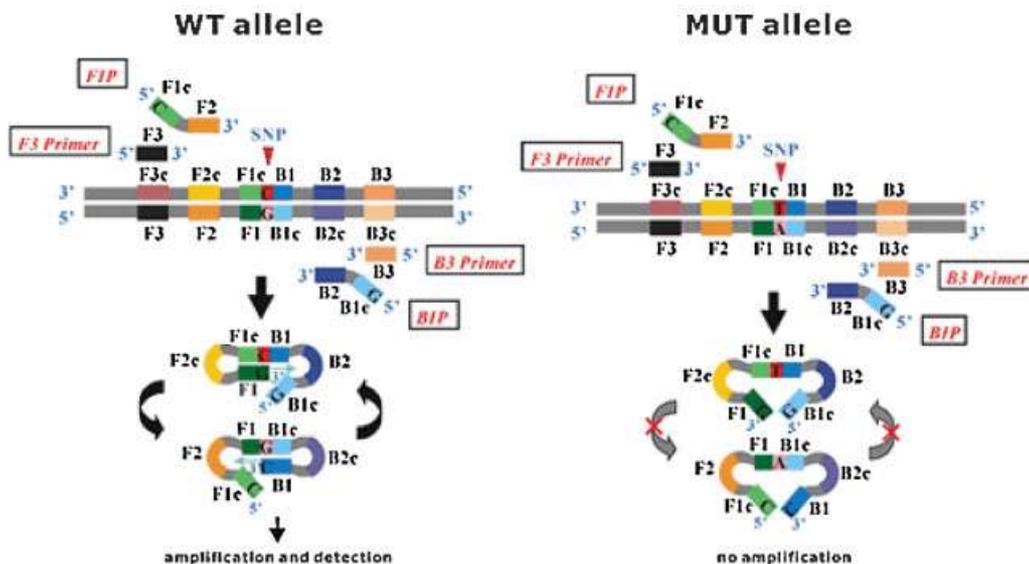


Figure 6. The basic principles of mutant detection using Wild Type (WT) primers. The FIP and BIP are designed to contain a SNP nucleotide (in this case of Wild Type allele) at 5' end, respectively. Using the WT primers, when the target gene is the WT allele, DNA synthesis from dumbbell-like starting structure proceeds and the LAMP amplification cycling continues. Copyright ©, 2005, Eiken Chemical Co. Ltd., Japan.

The basic principles of LAMP-SNP typing rely on the use of wt primers. The FIP and BIP are designed to contain a SNP nucleotide (wt allele) at 5' end. Using the wt primers, when the target gene is the wt allele, DNA synthesis from dumbbell-like starting structure proceeds and the LAMP amplification cycling continues (Figure 6). In contrast, when the target gene is the mutant (MUT) allele, no DNA synthesis proceeds from the dumbbell-like structure and the LAMP amplification cycling does not occur. Even if DNA synthesis proceeds for one step due to miscopy, the amplification reaction is either halted in other steps or is delayed since repetition of this reaction continually checks at each cycling step of the DNA replication. By simply incubating genomic DNA and reagents, including fluorescent detection reagent, at a constant temperature (60°C) for a fixed period of time, SNP typing can be achieved by determining whether amplification has taken place.

FUTURE DIRECTIONS

LAMP has all the characteristics required of real-time assays (high sensitivity, quantitative) along with simple operation for easy adaptability to field conditions. The combination of the LAMP technology and the new detection method described here

can overcome several factors that have been preventing true practical application of simple g-POCT. The integration of isothermal amplification and electrophoresis onto microchips could lead to LAMP on Chips for quick and accurate identification of disease producing genes at the patient's bed side. Thus LAMP is considered to be effective as a gene amplification method for g-POCT devices, which can be used for simple genetic testing whenever and wherever necessary. If these characteristics of the LAMP method are used effectively, it should be possible to develop simple genetic testing devices that have not been realised yet despite a strong awareness of their necessity, in a wide range of fields, including infectious disease testing, food inspection and environmental testing.

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