Rapid and real-time detection technologies for emerging viruses of biomedical importance

M M Parida

Department of Virology, Defence R & D Establishment, Gwalior 474 002, India

(Email, paridamm@rediffmail.com)

The development of technologies with rapid and sensitive detection capabilities and increased throughput have become crucial for responding to greater number threats posed by emerging and re-emerging viruses in the recent past. The conventional identification methods require time-consuming culturing, and/ or detection of antibodies, which are not very sensitive and specific. The recent advances in molecular biology techniques in the field of genomics and proteomics greatly facilitate the rapid identification with more accuracy. We have developed two real-time assays ie., SYBR green I based real time reverse transcription polymerase chain reaction (RT-PCR) and RT-loop-mediated isothermal amplification (LAMP) assay for rapid detection as well as typing of some of the emerging viruses of biomedical importance viz. dengue, Japanese encephalitis, chikungunya, west Nile, severe acute respiratory syndrome virus (SARS) etc. Both these techniques are capable of detection and differentiation as well as quantifying viral load with higher sensitivity, rapidity, specificity. One of the most important advantages of LAMP is its field applicability, without requirement of any sophisticated equipments. Both these assays have been extensively evaluated and validated with clinical samples of recent epidemics from different parts of India. The establishment of these real time molecular assays will certainly facilitate the rapid detection of viruses with high degree of precision and accuracy in future.

[Parida M M 2008 Rapid and real-time detection technologies for emerging viruses of biomedical importance; *J. Biosci.* **33** 617–628]]

1. Introduction

The rapid diagnosis of virus diseases has assumed greater significance owing to the direct benefit of patient management in absence of suitable therapeutic and prophylactic measures. With the advancement of technologies, increasingly diverse methods are available which make it possible to detect and analyze any virus, including those, which cannot be cultured. The laboratory diagnosis of virus infection can be made by the detection of specific virus, viral antigen, genomic sequences and/or antibodies. Nucleic acid amplification is one of the most valuable tools in virtually all life science fields, including application-oriented fields such as clinical medicine in which diagnosis of infectious diseases, genetic disorders and genetic traits are particularly benefited by this new technique. Now a days, several amplification methods have been invented viz. nucleic acid sequence based amplification (NASBA), Self-sustained sequence replication (3SR), Strand displacement amplification (SDA) as well as Polymerase chain reaction (PCR) (Chan and Fox 1999). These methods can amplify target nucleic acids to a similar magnitude, all with a detection limit of less than 10 copies and within a hour or so.

Although several amplification methods have been developed, PCR is the most widely used because of its apparent high simplicity and reliability. Routine use

Keywords. Emerging viruses; LAMP; rapid detection; real-time PCR

Abbreviations used: BIP, backward inner primer; BLP, backward loop primer; FIP, forward inner primer; FLP, forward loop primer; FRET, fluorescence resonance energy transfer; HBV, hepatitis B virus; HCV, hepatitis C virus; LAMP, loop-mediated isothermal amplification; NSBA, nucleic acid sequence based amplification; PCR, polymerase chain reaction; PEI, polyethylenimine; RSV, respiratory syncytial virus; RT-PCR, reverse transcription polymerase chain reaction; SDA, strand displacement amplification; 3SR, self-sustained sequence replication

of PCR as a standard approach in biotechnology and medical diagnostic laboratories has been usually practiced. During the past decade, various forms of PCRs such as reverse transcription polymerase chain reaction (RT-PCR), Nested PCR and Multiplex PCR have been developed to address the need for rapid identification of viruses to serotype level with more accuracy (Ratcliff *et al* 2007). Despite the obtainable magnitude of amplification, these PCR based methods require either high precision instruments for the amplification or elaborate methods for detection of the amplified products. In addition, these methods are often cumbersome to adapt for routine clinical use especially in peripheral health care settings and private clinics. In addition, the PCR method has several intrinsic disadvantages, such as the requirement of thermal cycling, insufficient specificity and rather low amplifications efficiency. More sensitive and real time based assays are therefore needed to complement the existing PCR based assay systems.

2. Real-time assays

The Real-time PCR assay has many advantages over conventional PCR methods, including rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity, and easy standardization. Thus, nucleic acid-based assays or real-time quantitative assay might eventually replace virus isolation and conventional RT-PCR as the new gold standard for the rapid diagnosis of virus infection in the acute-phase samples (Ratcliff *et al* 2007). Real-time PCR has enhanced wider acceptance of the PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination. 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. The majority of diagnostic PCR assays reported to date have been used in a qualitative, or 'yes/no' format. 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 (Espy *et al* 2006).

3. Real-time reporters (SYBR® Green, TaqMan®, and molecular beacons)

All real-time PCR systems rely upon the detection and quantitation of a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product in a reaction. In the simplest and most economical format, that reporter is the double-strand DNA-specific dye SYBR® Green (molecular probes).

SYBR Green binds to double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, the fluorescence increases. The advantages of SYBR Green are that it's inexpensive, easy to use, and sensitive. The disadvantage is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which results in an overestimation of the target concentration. For single PCR product reactions with well-designed primers, SYBR Green can work extremely well, with spurious non-specific background only showing up in very late cycles. The two most popular alternatives to SYBR Green are TaqMan® and molecular beacons, both of which are hybridization probes relying on fluorescence resonance energy transfer (FRET) for quantitation (figure 1)

TaqMan Probes are oligonucleotides that contain a fluorescent dye, typically on the 5' base, and a quenching dye, typically located on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a nonfluorescent substrate. TaqMan probes are designed to hybridize to an internal region of a PCR product. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes and FRET no longer occurs. Fluorescence increases in each cycle in proportion to the rate of probe cleavage figure 2).

Molecular beacons also contain fluorescent and quenching dyes, but FRET only occurs when the quenching dye is directly adjacent to the fluorescent dye. Molecular beacons are designed to adopt a hairpin structure while free in solution, bringing the fluorescent dye and quencher in close proximity. When a molecular beacon hybridizes to a target, the fluorescent dye and quenchers are separated, FRET does not occur, and the fluorescent dye emits light upon irradiation. Unlike TaqMan probes, molecular beacons are designed to remain intact during the amplification reaction, and must rebind to target in every cycle for signal measurement.

TaqMan probes and molecular beacons allow multiple DNA species to be measured in the same sample (multiplex PCR), since fluorescent dyes with different emission spectra may be attached to the different probes. Multiplex PCR allows internal controls to be co-amplified and permits allele discrimination in single-tube, homogeneous assays. These hybridization probes afford a level of discrimination impossible to obtain with SYBR Green, since they will only hybridize to true targets in a PCR and not to primer-dimers or other spurious products.

4. Application of real-time PCR in virus diagnosis

RT-PCR has become the benchmark for the detection and quantification of viruses and is being utilized increasingly

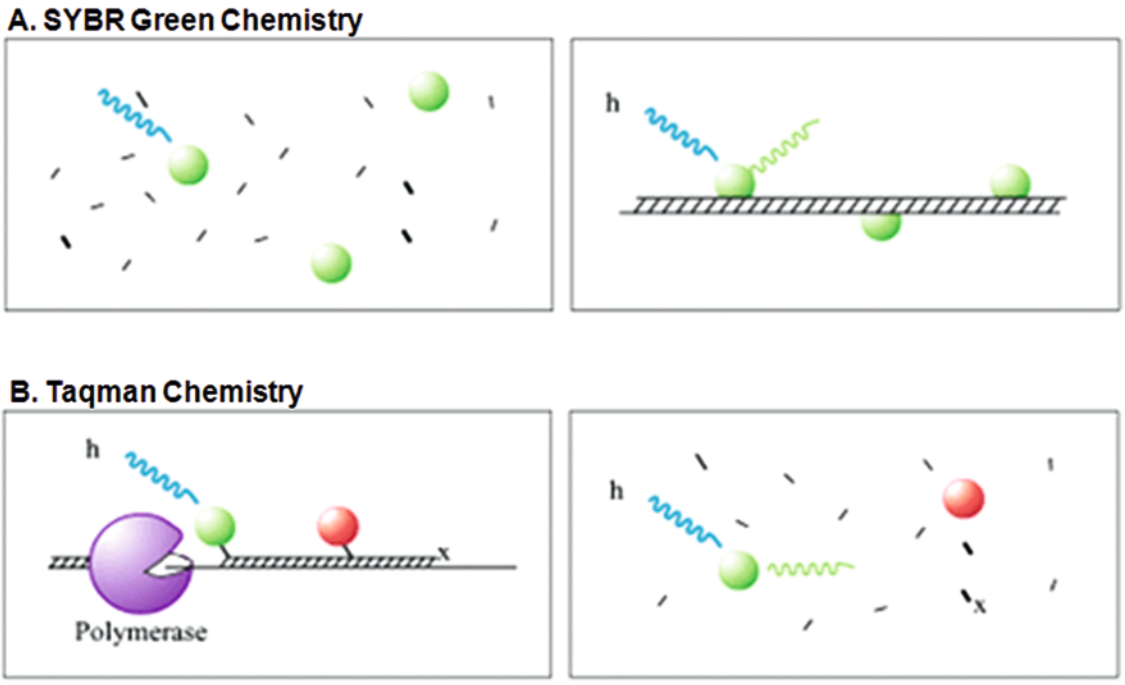

Figure 1. Principles and chemistry of SYBR Green and Taqman based real-time assays.

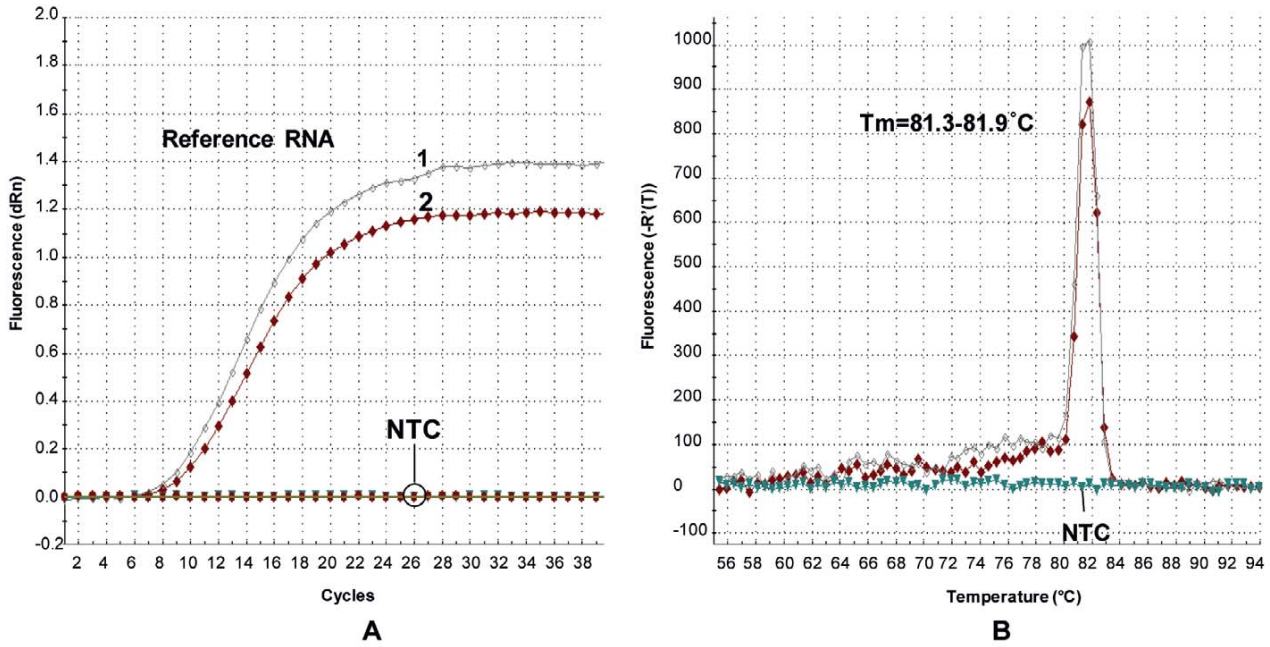

Figure 2. Real-time kinetics of SYBR Green I based real-time RT-PCR showing the amplification and Dissociation curve for the reference RNA. (A) Amplification plot. (B) Melting curve analysis depicting dissociation plot.

in novel clinical diagnostic assays. Quantitative results obtained by this technology are not only more informative than qualitative data, but simplify assay standardization and quality management. Quantitative PCR (QPCR) assays are most established for the detection of viral load and therapy monitoring (Mackay *et al* 2002). Further, nucleotide

sequence analysis of the amplification products has facilitated epidemiological studies of infectious disease outbreaks, and the monitoring of treatment outcomes for infections, in particular with viruses which mutate at high frequency.

Several investigators have reported fully automatic real-time PCR assays for the detection of viruses in acute-phase serum samples. With improved and automated nucleic acid sample isolation techniques, as well as real-time detection methods, a new generation of assays for most clinically important viruses is being developed (Niesters 2002). QPCR assays based on SYBR Green and *Taq*Man chemistries have been developed and validated and are beginning to reveal the virus's epidemiology and pathogenesis (Gunson *et al* 2006; Watzinger *et al* 2006). The QPCR assay also provides critical prognostic information for clinical management. The real-time PCR assays have been increasingly used for early and accurate detection as well as quantification of all most all human viruses including measles, mumps, herpes simplex virus (HSV), rota virus, noro virus, influenza virus types A and B, respiratory syncytial virus (RSV) and para influenza virus (PIV) types 1–4, severe acute respiratory syndrome virus (SARS) dengue, Japanese encephalitis (JE), West Nile, chikungunya, hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency virus (HIV), avian flu (H5 N1) etc. (Schwarz *et al* 2002; Poon *et al* 2003; Shu *et al* 2003; Dong-Kun *et al* 2004; Jiang *et al* 2004; Ruelle *et al* 2004; Schmid *et al* 2004; Templeton *et al* 2004; Enders *et al* 2005; Sugiyama *et al* 2005; Uchida *et al* 2005; Chien *et al* 2006; Fukuda *et al* 2006; Santhosh *et al* 2007).

More recently, real-time PCR assays have provided additional major contributions, with the inclusion of an additional fluorescent probe detection system resulting in an increase in sensitivity over conventional PCR, the ability to confirm the amplification product and to quantitate the target concentration and detection of multiple targets in a single tube. Multiplex real-time quantitative RT-PCR assays have been developed for simultaneous detection, identification and quantification of HBV, HCV and HIV-1 in plasma or serum samples. Genomic amplification of one virus was unaffected by the simultaneous amplification of the other two. Although competition between HCV and HIV-1 amplifications slightly affected the yield of HIV-1 amplification, quantification of a single virus was possible. A novel QRTPCR assay consists of two multiplex reactions, one detecting influenza A and B and RSV, the other one PIV 1–4, and can generate a result within 6 h. Other real-time assays can differentiate between influenza A subtypes and RSV A and B facilitating diagnosis, patient management and strain identification for vaccine production. QRTPCR based diagnostic assays have also been reported for the associated corona viruses and improved to give result in an assay with 100% specificity in samples collected from day 1–3 of disease onset. The assay's potential for high throughput was also of critical importance in areas with outbreaks of SARS in which large numbers of specimens had to be tested. In addition, the ability to quantify helped elucidate the pathogenesis of the disease. QRTPCR assays can now be used routinely to exclude SARS-associated coronavirus in patients hospitalized with respiratory symptoms even in the presence of other respiratory viruses. QRTPCR assays have also been developed for the clinical diagnosis of viral meningitis and to detect enteroviruses in cerebrospinal fluid and are significantly more sensitive than viral culture (Verstrepen *et al* 2001; Beuret 2004).

The ability of qPCR to generate accurate quantitative data has had a huge impact on the study of viral agents of infectious disease and is helping to clarify disputed infectious disease processes and demonstrate links between specific viral sequences and patient clinical symptoms. However, the lack of commercially available validated reagent kits for most viruses remains a major problem, as does the absence of standardization of the existing tests (Niesters 2002). Besides, 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. The high cost of instruments required for performing the real time assays restricted its use to laboratories with good financial resources.

5. Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) which stands for loop-mediated isothermal amplification is a simple, rapid, specific and cost-effective nucleic acid amplification method and is characterized by the use of 6 different primers specifically designed to recognize 8 distinct regions on the target gene (Notomi *et al* 2000). The amplification proceeds at a constant temperature using strand displacement reaction. Amplification and detection of 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 of 63°C. Compared to PCR and real-time PCR, the LAMP has the advantages of reaction simplicity and detection sensitivity. The higher sensitivity and specificity of the LAMP reaction is attributed to continuous amplification under isothermal condition employing six primers recognizing eight distinct regions of the target. Besides, the higher amplification efficiency of LAMP reaction yields large amount of by-product, pyrophosphate ion, leading to 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 synthesized, real-time monitoring of the LAMP reaction can be achieved by real-time measurement of turbidity (Mori *et*

al 2001).

Being an isothermal amplification, LAMP does not require any thermal cycler and thus can be performed even with heating block and/or water bath. Thus, LAMP method has the characteristics of not requiring special reagents and sophisticated temperature control device. Since it only requires simple equipments, cost effective point of care gene test can be achieved. Both simple detection and real-time detection of the reaction are possible. In addition, in case of LAMP, both amplification and detection occur simultaneously during the exponential phase without going through the plateau phase where the non spurious amplification leads to lower sensitivity and false positivity.

6. Designing of LAMP primers

Designing of a highly sensitive and specific primer set is crucial for performing LAMP amplification. The target selection for primer designing can be accomplished by using the Primer Explore [LAMP primer designing support software program, Net laboratory, Japan, *http://venus.net laboratory.com*)] after considering the base composition, GC contents and the formation of secondary structures. The primer set for LAMP amplification include a set of six primers comprising two outer, two inner and two loop primers that recognize 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 non-cyclic step only. The inner primers were described as forward inner primer (FIP) and backward inner primer (BIP) having both sense and antisense sequence in such a way that it helps in the formation of 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. 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 and thereby shortens amplification time by one third to one half ((Notomi *et al* 2000).

The designing of the above mentioned 6 types of primers are based on the following 8 distinct regions of the target gene: the F3c, F2c and F1c and FLP regions at the 3' side and the B1, B2, B3 and BLP regions at the 5' side. Forward Inner Primer (FIP) 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 consists of the F3 region that is complementary to the F3c region. Backward inner primer (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 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 3). 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 and F2 and B1 and B2 regions respectively ((Notomi *et al* 2000).

7. 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. This method employs a DNA polymerase and a set of six specially designed primers that recognize a total of eight distinct sequences on the target DNA. There are two steps of LAMP amplification comprising non-cyclic and cyclic steps.

7.1 *Non-cyclic step*

In the non-cyclic step, there is the formation of stem loop DNA with stem-loops at each end that serves as the starting structure for the amplification by LAMP cycling. As 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 ((Notomi *et al* 2000; Ushikubo 2004).

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 displacement activity, a DNA strand complementary to the template DNA is synthesized, 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 synthesized 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 synthesized 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

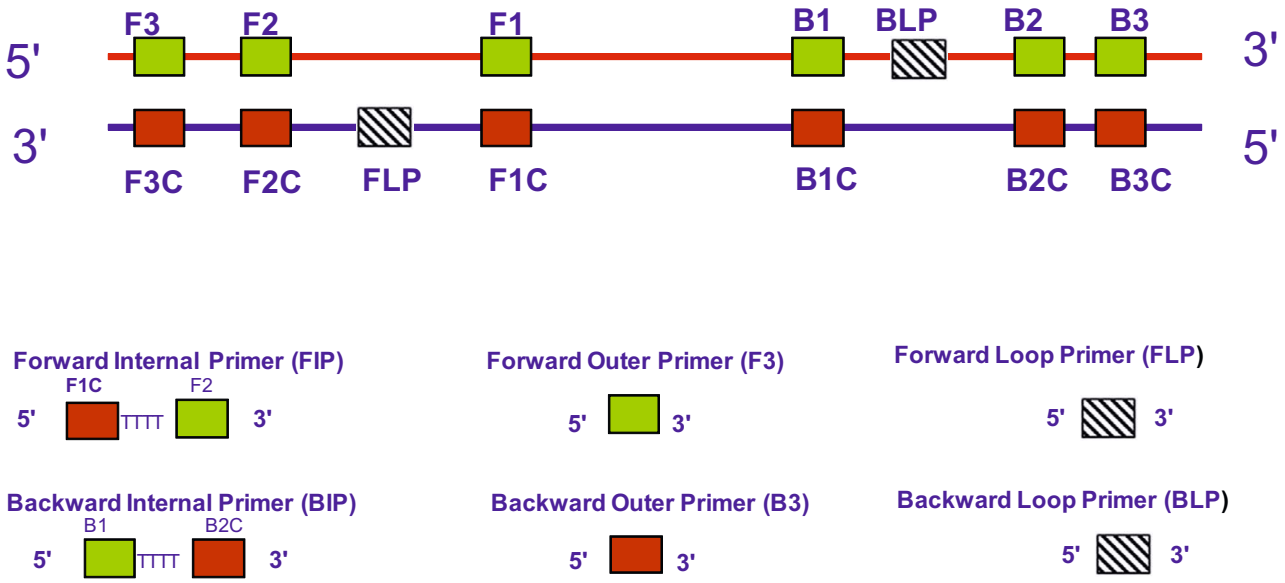

Figure 3. Schematic representation of primer designing for RT-LAMP assay showing the position of the six primers spanning eight distinct regions of the target gene.

3' end of the BIP, synthesis of complementary 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 synthesized 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. A 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 the exponential amplification in cyclic manner.

7.2 *Cyclic amplification*

In subsequent LAMP cycling one inner primer hybridizes 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, FIP anneals to the single stranded region in the stem-loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesized 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 with 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 4).

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 and thereby reducing the amplification time to almost half (Nagamine *et al* 2002).

8. Assay protocol for LAMP amplification

The LAMP reaction is usually carried out in a total 25 μl 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 2x reaction mixture having 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 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 (New England Biolabs), and 2 μl of DNA template. Positive and negative controls should be included in each run, and all precautions to prevent cross-contamination should be observed. The studies temperature optima required for efficient amplification by

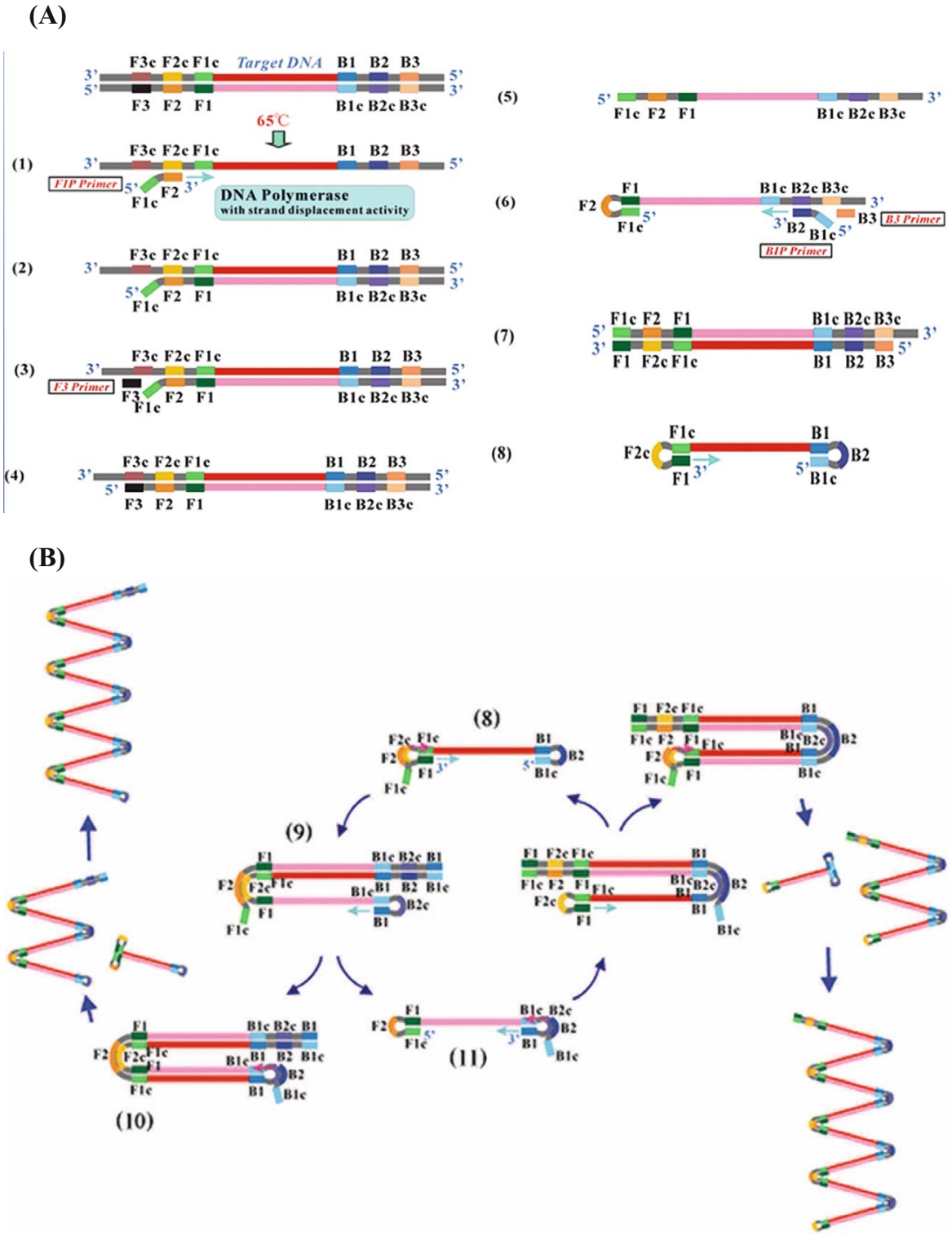

Figure 4. **(A)** Principles of LAMP amplification. NON-CYCLIC STEP (1–8) : Generation of stem loop DNA with dumb bell shaped structure at both end that is ready to enter into cyclic amplification step. Copy right ©, 2005, Eiken Chemical Co Ltd, Japan. **(B)** Principles of LAMP amplification. CYCLIC AMPLIFICATION STEP (9–11) : Exponential amplification of original dumbbell-shaped stem-loop DNA employing internal and loop 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 structures. Copy right ©, 2005, Eiken Chemical Co Ltd, Japan.

LAMP assay indicated that the optimum temperature for the LAMP reaction was 63°C, which is optimum for the activity of *Bst* DNA polymerase. The amplification of RNA template was accomplished through RT-LAMP assay by employing reverse transcriptase for reverse transcription step in addition to the Bst DNA polymerase. RT-LAMP method can synthesize cDNA from template RNA and apply LAMP technology to amplify and detect them.

9. Monitoring of LAMP amplification

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 OD at 400 nm at every 6 second (Mori *et al* 2004) (figures 5, 6A). On agarose gel analysis, the LAMP amplicons revealed ladder like pattern in contrast to a single band as observed in PCR (figure 6B). 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.

In order to facilitate the field application of LAMP assay, the monitoring of amplification can also be accomplished with naked eye inspection either in the form of visual turbidity or visual fluorescence. 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. Hence, the presence of turbidity can indicate the presence of target gene (figure 6C) .The tube containing the amplified products can also be better visualized in the presence of fluorescent intercalating dye viz. ethidium bromide, SYBR Green I and Calcein etc. by illuminating with a UV lamp, the fluorescence intensity increases. In practice, usually the visual inspection for amplification is performed through observation of color change following addition of 1 µl of SYBR Green I (a fluorescent dsDNA intercalating dye) to the tube. In case of positive amplification, the original orange color 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 color of the dye will be retained. This change of color is permanent and thus can be kept for record purpose (figure 6D).

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. The biggest feature 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 utilizes 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 (Mori *et al* 2006).

10. Quantitation of gene copy numbers by LAMP assay

Capitalizing on its exquisite sensitivity, LAMP has been designed to quantify the amount of gene copies in a person's blood (load) thereby allowing physicians to monitor their patients' disease progression and response to therapy. Assessment of the load of the organisms/pathogens before, during and after therapy has tremendous potential for improving the clinical management of diseases. The quantification of gene copy number and/or concentration of the organisms can be accomplished through generation of a standard curve by plotting a graph between known concentration of gene copy number or infectious unit of organisms and time of positivity to get the amplification signal for that particular concentration. A linear relationship between various concentrations Vs time of positivity is usually obtained through the real-time monitoring of the amplification (figure 7). The quantification of gene copies in the clinical samples can be extrapolated from the standard curve on the basis of their time of positivity.

11. LAMP in clinical diagnosis of virus infections

LAMP technology facilitates the detection of DNA or RNA of pathogenic organisms and, as such, is the basis for a broad range of clinical diagnostic tests for various infectious agents, including viruses and bacteria. These gene based tests have several advantages over traditional antibody-based diagnostic methods that measure the body's immune response to a pathogen. In particular, LAMP is capable of detecting the presence of pathogenic agents earlier than PCR even on day one of fever where the amount of gene copy number is expected to be very low due to higher sensitivity with a detection limit of about 1-2 copies. Earlier detection of infection can mean earlier treatment and an earlier return to good health.

The loop mediated isothermal amplification (LAMP) assay is emerging as a simple, rapid and powerful gene amplification technique for early detection of microbial diseases. Although the inception of LAMP refers back to 1998 but the popularity of LAMP starts only after 2003 following emergence of West Nile and SARS viruses. Since then, LAMP assay is increasingly being adapted by researchers mostly from Japan in clinical diagnosis of emerging diseases including bacteria, viruses and parasitic diseases. LAMP has been successfully applied for rapid and real-time detection

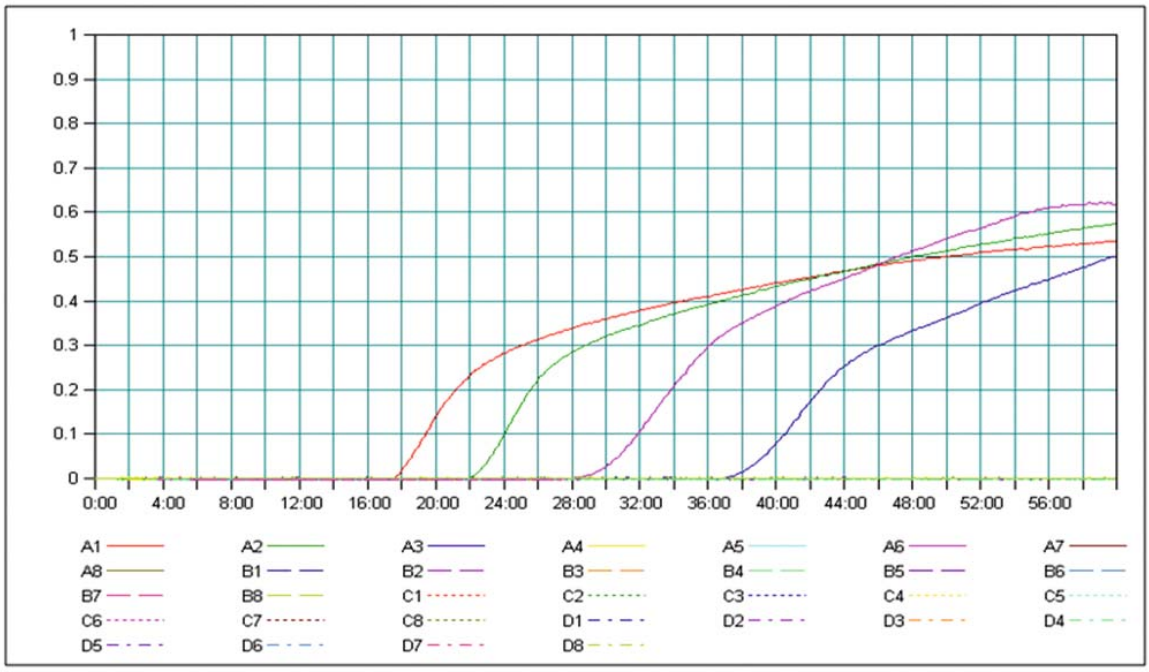

Figure 5. Real-Time Monitoring of LAMP Amplification showing the amplification curve. X- axis – depicting the time of positivity and Y- axis showing the turbidity value in terms of O. D. at 400 nm.

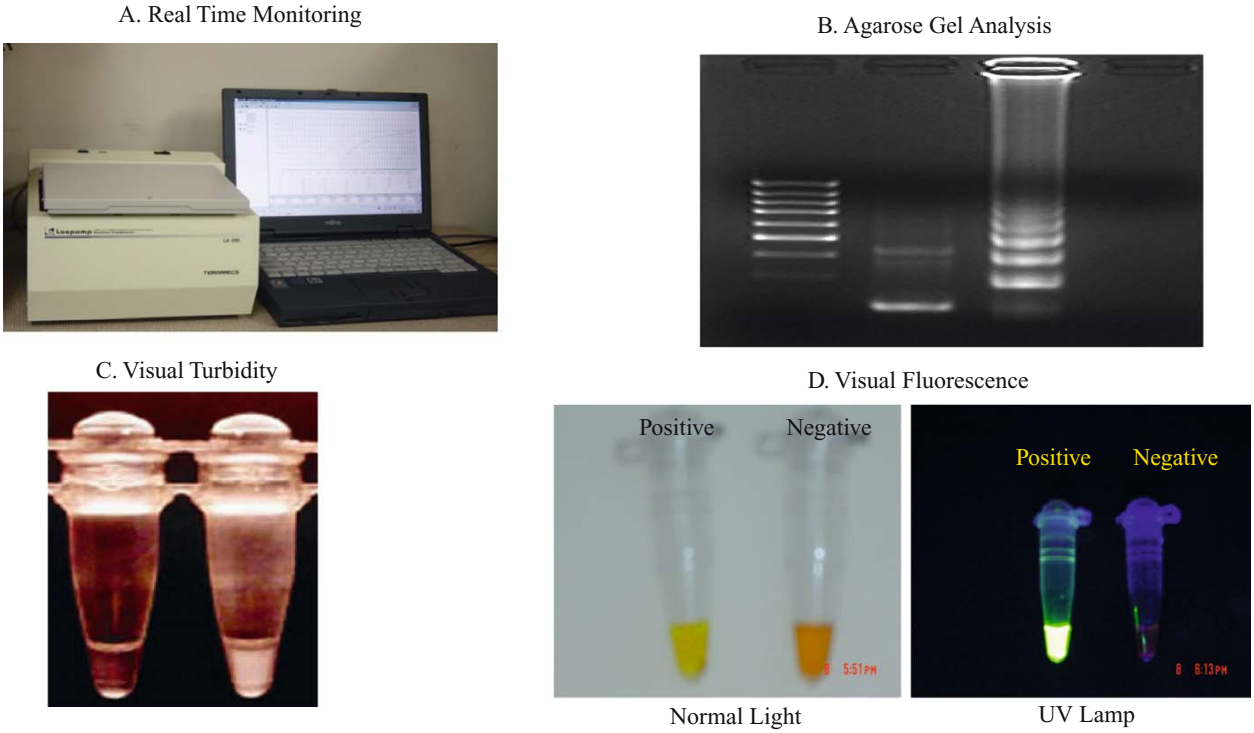

Figure 6. Monitoring of LAMP Amplification. (A).The turbidity of magnesium pyrophosphate, a by-product of the reaction, can be real-time detected by a real-time turbidimeter. (B). Agarose gel analysis revealing the typical electrophoresis pattern of LAMP amplified product, which is not a single band but a ladder pattern because LAMP method can form amplified products of various sizes consisting of alternately inverted repeats of the target sequence on the same strand. (D).The tube containing the amplified products in the presence of fluorescent intercalating dye is illuminated with a UV lamp, the fluorescence intensity increases.

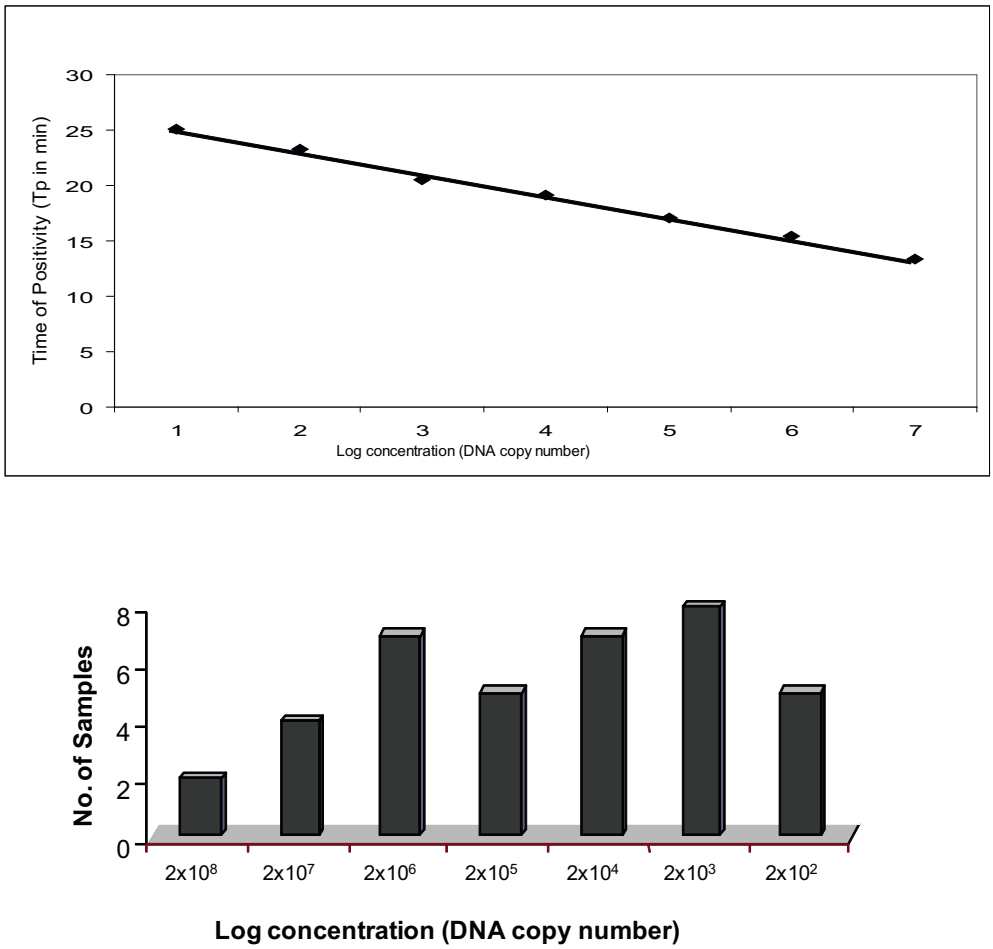

Figure 7. Standard Curve for RT-LAMP assay generated from the amplification plots between 10 fold serially diluted plasmid construct and time of positivity (Tp). **B**. Quantitative determination of virus concentration in clinical samples employing standard curve.

of both DNA and RNA viruses. However, most of the published researches have been directed for RNA viruses may be due to the increased incidence of RNA viruses in recent past in the form of major epidemic having significant public health importance. A one-step single tube real–time accelerated reverse transcription loop mediated isothermal amplification (RT-LAMP) assays for rapid detection of some of the recently emerged human viral pathogens viz. West Nile, SARS, dengue, Japanese encephalitis, chikungunya, Norwalk, H5N1 highly pathogenic avian influenza (HPAI) viruses have been developed and evaluated (Parida *et al* 2004, 2005, 2006, 2007, 2008; Hong *et al* 2005; Imai *et al* 2006; Toriniwa *et al* 2006) On comparison to conventional RT-PCR, RT-LAMP assay demonstrated 10 to 100 fold more sensitivity with a detection limit of 0.01 to 10 PFU of virus in all these cases. The usefulness of LAMP for amplification of DNA viruses was also reported for HBV, HPV (human papillomavirus) type – 6,11, 16 and 18, HSV, varivella zooster virus (VZV), cytomegalo virus (CMV) and found to be superior in terms of sensitivity, specificity, rapidity, and simplicity, and can potentially be a valuable tool for the detection of HPV DNA compared to PCR and real-time PCR (Okamoto *et al* 2004; Enomoto *et al* 2005; Kaneko *et al* 2005; Sugiyama *et al* 2005; Suzuki *et al* 2006; Hagiwara *et al* 2007)

12. Conclusion

The combination of excellent sensitivity and specificity, low contamination risk, and speed has made real-time PCR technology an appealing alternative to culture- or immunoassay-based testing methods for diagnosing many infectious diseases. The recent advances in the development of fluorophores, nucleotide labeling chemistries, and the novel applications of oligoprobe hybridization have provided real-time PCR technologies with a broad enough base to ensure their acceptance. The LAMP is emerging as a new generation of cost effective and rapid gene amplification tool

having all the characteristics of rapidity and high sensitivity of real-time assays as well as easy adaptability under field conditions due to its simple operation, rapid reaction, and easy detection. The rapidity and sensitivity of these real-time assays will assist in precise diagnosis, which is extremely useful to undertake suitable control measures and patient management at the earliest.

References

Beuret C 2004 Simultaneous detection of enteric viruses by multiplex real-time RT-PCR; *J. Virol. Methods* **115** 1–8

Chan A B and Fox J D 1999 NASBA and other transcription-based amplification methods for research and diagnostic microbiology; *Rev. Med. Microbiol.* **10** 185–196

Chien L J, Liao T L, Shu P Y *et al* 2006 Development of Real-Time Reverse Transcriptase PCR Assay To Detect and Serotype Dengue Viruses; *J. Clin. Microbiol*. **44** 1295–1304

Dong-Kun Y, Kweon C H, Kim B H, *et al* 2004 TaqMan reverse transcription polymerase chain reaction for the detection of Japanese encephalitis virus; *J. Vet. Sci*. **5** 345–351

Enders K O, Peter Ng, Cheng K C, Antia Y Y, *et al* 2005 Influenza A H5N1 Detection; *Emerg. Infect. Dis*. **11** 1303–1305

Enomoto Y, Yoshikawa T and Ihira M. 2005 Rapid diagnosis of herpes simplex virus infection by a loop-mediated isothermal amplification method; *J. Clin. Microbiol*. **43** 951–955

Espy M J, Uhl J R, Sloan L, *et al* 2006 Real-time PCR in clinical microbiology: applications for routine laboratory testing; *Clin. Microbiol. Rev*. **19** 165–256

Fukuda S, Takao S, Kuwayama M, Shimazu Y and Miyazaki K. 2006 Rapid detection of norovirus from fecal specimens by real-time reverse transcription-loop-mediated isothermal amplification assay *J. Clin. Microbiol.* **44** 1376–1381

Gunson R N, Collins T C and Carman W F 2006 Practical experience of high throughput real time PCR in the routine diagnostic virology setting; *J. Clin. Virol*. **35** 355–367

Hagiwara M, Sasaki H, Matsuo K, Honda M, Kawase M and Nakagawa H**.** 2007 Loop-mediated isothermal amplification method for detection of human papillomavirus type 6, 11, 16, and 18; *J. Med. Virol.* **79** 605–615

Hong T C, Mai Q L, Cuong D V, *et al* 2004 Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus *J. Clin. Microbiol.* **42** 1956–1961

Imai M, Ninomiya, A and Minekawa H 2006 Rapid diagnosis of H5N1 avian influenza virus infection by newly developed influenza H5 hemagglutinin gene-specific loop-mediated isothermal amplification method; *Vaccine* **24** 6679–6682

Jiang S S, Chen T C, Yang J Y, *et al* 2004 Sensitive and quantitative detection of severe acute respiratory syndrome coronavirus infection by real-time nested polymerase chain reaction; *Clin. Infect. Dis*. **38** 293–296

Kaneko H, Iida T, Aoki, K, Ohno S and Suzutani T 2005 Sensitive and rapid detection of herpes simplex virus and varicella-zoster virus DNA by loop-mediated isothermal amplification; *J. Clin. Microbiol*. **43** 3290–3296

Mackay I M, Arden K E and Nitsche A 2002 Real-time PCR in virology; *Nucleic Acids Res*. **30** 1292–1305

Mori Y, Nagamine K, Tomita N and Notomi T 2001 Detection of Loop mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation; *Biochem. Biophys. Res. Commun*. **289** 150–154

Mori Y, Kitao M, Tomita N and Notomi T 2004 Real-time turbidimetry of LAMP reaction for quantifying template DNA; *J. Biochem. Biophys. Methods* **59** 145–157

Mori Y, Hirano T and Notomi T 2006 Sequence specific visual detection of LAMP reactions by addition of cationic polymers; *BMC Biotech*. **6** 3 (1–10)

Nagamine K, Hase T and Notomi T 2002 Accelerated reaction by loop mediated isothermal amplification using loop primers; *Mol. Cell. Prob*. **16** 223–229

Niesters H G 2002 Clinical virology in real time; *J. Clin. Virol. (Suppl. 3)* S3–S12

Notomi T, Okayama H, Masubuchi H *et al* 2000 Loop-mediated isothermal amplification of DNA; *Nucleic Acids Res*. **28** e63.

Okamoto S, Yoshikawa T, Ihira M *et al*. 2004 Rapid detection of Varicella-zoster virus infection by a loop-mediated isothermal amplification method; *J. Med. Virol*. **74** 677–682

Parida M M, Guillermo P, Inoue S, Hasebe F and Morita K 2004 Real-Time Reverse Transcription Loop mediated isothermal amplification for rapid detection of West Nile Virus; *J. Clin. Microbiol*. **42** 257–263

Parida M M, Horioke K, Ishida H, *et al* 2005 Rapid detection and differentiation of dengue virus serotypes by a real-time reverse transcription-loop-mediated isothermal amplification assay; *J. Clin. Microbiol*. **43** 2895–2903

Parida M M, Santhosh S R, Dash P K, *et al* 2006 Development and evaluation of reverse transcription Loop mediated isothermal amplification assay for rapid and Real-time detection of Japanese encephalitis virus; *J. Clil. Microbiol*. **44** 4172–4178

Parida M M, Santhosh S R, Dash P K, *et al* 2007 Rapid and real-time detection of chikungunya virus by reverse transcription loop mediated isothermal amplification assay; *J. Clin. Microbiol*. **45** 351–357

Parida M M, Santhosh S R, Dash P K, Lakshmana Rao, P V and Morita K 2008 Rapid and Real-time Assays for Detection and Quantification of Chikungunya Virus; *Future Virol*. **3** 179–192

Poon L L, Chan K H, Wong O K, *et al* 2003 Early diagnosis of SARS coronavirus infection by real time RT-PCR; *J. Clin. Virol*. **28** 233–238

Ratcliff R M, Chang G, Kok T and Sloots T P 2007 Molecular diagnosis of medical viruses; *Curr. Issues Mol. Biol*. **9** 87–102

Ruelle J, Mukadi B K, Schutten M and Goubau P 2004 Quantitative real-time PCR on Lightcycler for the detection of human immunodeficiency virus type 2 (HIV-2); *J. Virol. Methods* **117** 67–74

Santhosh S R, Parida M M, Dash P K, *et al* 2007 Development and Evaluation of SYBR Green I based One Step Real time RT- PCR assay for detection and quantification of Chikungunya virus; *J. Clin. Virol*. **39** 188–193

Santhosh S R, Parida M M, Dash P K, *et al* 2007 Development and Evaluation of SYBR Green I based One Step Real time RT- PCR assay for detection and quantification of Japanese encephalitis virus; *J. Virol*. *Methods* **143** 73–80

Schmid M, Oehme R, Schalasta G, Brockmann S, Kimmig P and

Enders G 2004 Fast detection of Noroviruses using a real-time PCR assay and automated sample preparation; *BMC Infect. Dis*. **4** 15

Schwarz B A, Bange R, Vahlenkamp T W, Johne R and Muller H 2002 Detection and quantitation of group A rotaviruses by competitive and real-time reverse transcription-polymerase chain reaction; *J. Virol. Methods* **105** 277–285

Shu P Y, Chang S F, Kuo, et al 2003 Development of group- and serotypespecific one-step SYBR green I-based real-time reverse transcription- PCR assay for dengue virus; *J. Clin. Microbiol*. **41** 2408–2416

Sugiyama H, Yoshikawa T, Ihira M, Enomoto Y, Kawana T and Asano Y. 2005 Comparison of loop-mediated isothermal amplification, real-time PCR, and virus isolation for the detection of herpes simplex virus in genital lesions; *J. Med. Virol*. **75** 583–587

Suzuki R, Yoshikawa T, Ihira M. *et al* 2006 Development of the loop-mediated isothermal amplification method for rapid detection of cytomegalovirus DNA; *J. Virol. Methods* **132** 216–221

Templeton K E, Scheltinga S A, Beersma M F, Kroes A C and Claas C 2004 Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza a and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4; *J. Clin. Microbiol*. **42** 1564–1569

Toriniwa H and Komiya T 2006 Rapid detection and quantification of Japanese encephalitis virus by real-time reverse transcription loop-mediated isothermal amplification; *Microbiol. Immunol.* **50** 379–387

Uchida K, Shinohara M, Shimada S, *et al* 2005 Rapid and sensitive detection of mumps virus RNA directly from clinical samples by real-time PCR; *J. Med. Virol*. **75** 470–474

Ushikubo H 2004 Principle of LAMP method – a simple and rapid gene amplification method; *Virus* **54** 107–112

Vrstrepen W A, Kuhn S, Kockx M M, Van De Vyvere M E and Mertens A H 2001 Rapid detection of enterovirus RNA in cerebrospinal fluid specimens with a novel single-tube real-time reverse transcription-PCR assay; *J. Clin*. *Microbiol*. **39** 4093–4096

atzinger F, Ebner K, Lion T 2006 Detection and monitoring of virus infections by real-time PCR; *Mol. Aspects Med*. **27** 254–298

*e*Publication: 17 October 2008