

REVIEW

Detecting amplicons of loop-mediated isothermal amplification

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Abstract

Loop-mediated isothermal amplification (LAMP) assays are used to detect diverse pathogens. Initially, LAMP amplicons were detected using electrophoresis; later, real-time monitoring based on turbidity was developed to overcome the problem of contamination with environmental DNA. Recently, real-time monitoring of fluorescence signals using a quenching primer and probe has improved the reliability of amplification signals. Here, methods of detecting LAMP amplicons are reviewed.

KEYWORDS

detection, LAMP, QPrimer, QProbe, turbidity

1 | INTRODUCTION

The loop-mediated isothermal amplification (LAMP) method was developed by Notomi *et al.*¹ and was patented by Eiken Chemical Co., Ltd (patent no. JP2000283862, WO2002024902A1). In LAMP assays, DNA is amplified by four specific primers (F3, FIP, BIP, and B3) at 60–65°C within 1 hr, although the speed of amplification can be accelerated by adding two loop primers (LF and LB).² Thus, LAMP is rapid and user-friendly; it can reduce the time required to increase and decrease the incubation temperature, facilitating the choice of incubation device, that is, LAMP can be performed using a simple isothermal device, such as heat block and water bath, and the amplification ends faster than general PCRs. The principles of LAMP are described on the manufacturer's website

(<http://loopamp.eiken.co.jp/e/lamp/principle.html>) and as an animation (<http://loopamp.eiken.co.jp/e/lamp/anim.html>). LAMP assays can also be used to detect RNA by adding a reverse transcription step (RT-LAMP).¹ LAMP uses four or six primers, which assures high sequence specificity. The primers are also tolerant of point mutation(s) other than mismatches at the 3' end of the primer and in the BIP primer.^{3–6} Therefore, LAMP enables detection of pathogen genes. LAMP assays have been developed to detect gene of bacteria,^{7–13} parasites,^{14–16} protozoa,^{14,17,18} and viruses,^{19–22} including RNA viruses such as West Nile virus,²³ dengue virus,²⁴ rubella virus,^{25,26} mumps virus,²⁷ respiratory syncytial virus (RSV),^{28,29} influenza viruses,^{30–32} severe acute respiratory syndrome coronavirus,³³ and Middle East respiratory syndrome-coronavirus (MERS-CoV).^{5,6,34,35}

Abbreviations: CoV, coronavirus; LAMP, loop-mediated isothermal amplification; MERS, Middle East respiratory syndrome; Qprimer, quenching primer; Qprobe, quenching probe; RSV, respiratory syncytial virus; RT, reverse transcription.

2 | TRANSITION OF LAMP DETECTION METHODS

2.1 | Detection by electrophoresis

LAMP amplicons can be visualized by electrophoresis in an agarose gel. The four basic LAMP primers are constructed based on six specific regions of the target sequence and form a unique dumbbell-like structure, both ends of which have stem-loop structures that each comprise three specific regions. The dumbbell-like structure is extended in a bumper-to-bumper manner; therefore, the amplicons are ladder-like in appearance because they contain various numbers of dumbbell-like structures (Figure 1). The specificity of amplification can be confirmed by incorporating restriction enzyme sites. In our RT-LAMP for RSV,²⁸ the subtype A amplicon is cleaved by *Nla*III and that of subtype B by *Xba*I. If the amplification succeeds, depending on the number of used enzymes and recognition sites in targeted sequences, several bands can be seen after digestion (Figure 1). Thus, detecting LAMP amplicons by electrophoresis and restriction enzymes can confirm the specificity of amplification. However, electrophoresis is performed under open conditions, leading to contamination of the laboratory with amplified DNA. The high sensitivity of genetic diagnostic methods increases the likelihood of false positives.^{36,37} LAMP produces a larger quantity of DNA than conventional PCR (11 vs. 0.2 μ g in a 25 μ l reaction),³⁸ suggesting that LAMP is more likely to result in contamination of the laboratory. To prevent this, separate reagent mixing and electrophoresis rooms with a one-way workflow

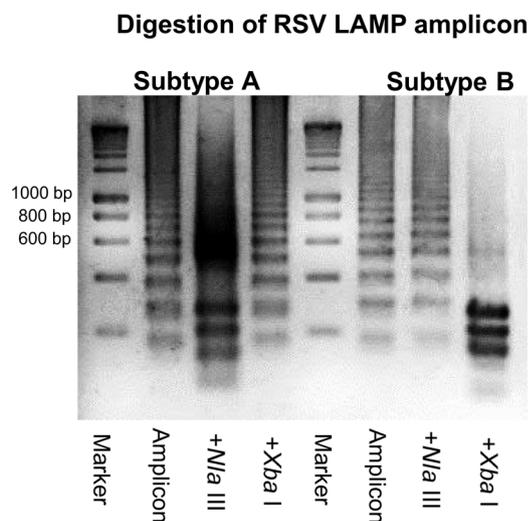


FIGURE 1 RSV RT-LAMP was performed as described previously.²⁸ The amplicons were purified using a QIAquick PCR purification kit and treated with *Nla*III and *Xba*I at 37°C for 1 hr. The resulting fragments were separated by electrophoresis on 3% agarose gels and visualized using ethidium bromide staining and ultraviolet light

are needed.³⁹ Furthermore, training of workers is needed, and the number of tests performed daily by one person should be limited to prevent contamination; however, this is time consuming and costly. The optimum means of avoiding laboratory contamination by amplified DNA is performing detection under closed conditions, that is, without opening the reaction tube.

2.2 | Detection by turbidity

Mori *et al.*³⁸ developed a turbidity-based method for amplicon detection in a closed system, based on precipitation of magnesium pyrophosphate. The pyrophosphate ion is released from deoxynucleotide triphosphate as a by-product of DNA polymerization by DNA polymerase, and precipitates with magnesium ion in the reaction buffer.^{40,41} Because LAMP produces a large quantity of DNA, the pyrophosphate ion concentration exceeds the threshold for precipitation, resulting in the formation of a visible magnesium precipitate in an amplicon concentration-dependent manner. By contrast, PCR yields a pyrophosphate concentration insufficient for magnesium pyrophosphate precipitation.³⁸ The precipitate is visible at the end of amplification (Figure 2a) and the turbidity can be monitored in real-time using a turbidimeter (Figure 2c).⁴² A fluorescence detection method using the fluorescent dye calcein has also been developed.⁴³ Calcein, known as fluorexon, is quenched by manganese ions, of which it is deprived by pyrophosphate. Therefore, it fluoresces when accompanied by pyrophosphate ion production during the LAMP reaction and is visible to the naked eye under ultraviolet light (Figure 2b). The signal can be monitored using a real-time fluorescence monitor. Turbidity monitoring enables the detection of targets under closed conditions without electrophoresis, as well as LAMP in the field and at point of care using an isothermal incubator, such as a heat block and water bath. LAMP kits for *in vitro* diagnosis are commercially available in Japan for whooping cough, tuberculosis, *Mycoplasma pneumoniae*, *Legionella pneumophila*, influenza, and severe acute respiratory syndrome.

However, the turbidity signal is derived from a by-product of DNA amplification; it is not caused by a primer's specific reaction directly. Consequently, it is not possible to completely exclude the possibility of monitoring nonspecific amplification; salt accumulation accompanying the LAMP reaction can be induced even by a nonprimer signal or primer dimers. Thus, even if host-derived DNA fragments or primer dimers form DNA amplification nonspecifically, the increased turbidity that accompanies this process can be monitored as a positive signal. Although rare, the possibility of nonprimer signals in turbidity monitoring cannot be excluded. Hence, validation of the integrity of the primer set is quite important to develop a

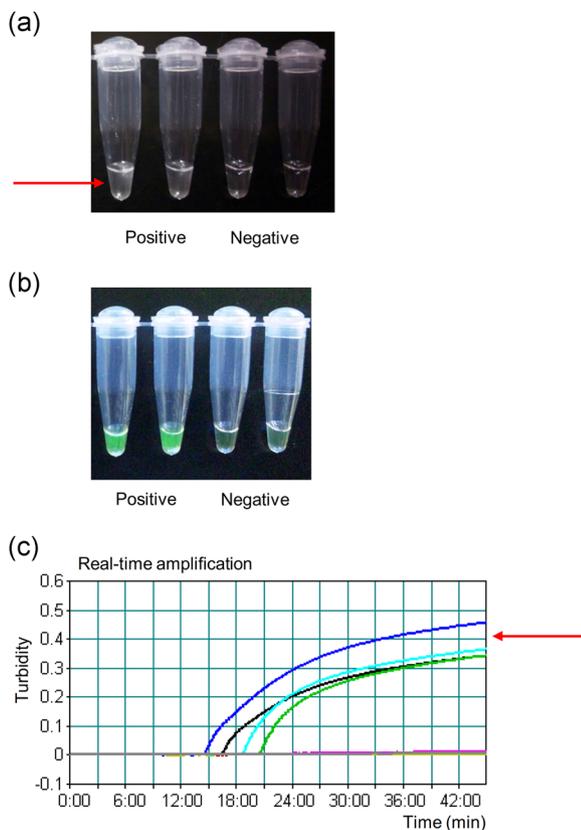


FIGURE 2 Detection of LAMP amplicons by turbidity. (a) MERS-CoV RT-LAMP was performed as described previously.⁵ A muddy white color indicates precipitation of magnesium pyrophosphate. Left two wells, positive; right two wells, negative. (b) Amplification was performed using calcein and visualized under ultraviolet light. Green fluorescence indicates positivity. (c) RSV RT-LAMP was performed as described previously,²⁸ and turbidity was monitored in real time. The signal is drawn as a power curve

LAMP system with turbidity monitoring. The primers for LAMP can be designed using online software (Primer-Explorer v. 4 or 5, Fujitsu, Tokyo, Japan; <http://primerexplorer.jp/e/>). However, the search results do not guarantee primer specificity. Candidates should be screened by strict validation tests to check whether they ever show nonspecific reactions in the presence of host and/or carrier nucleotides and the primer set itself. The primer set can be used for further validations only after it has been shown that it never causes nonspecific DNA amplification.

3 | DETECTION USING FLUORESCENT PRIMERS/PROBES

Labeling primers with a fluorescent dye ensures detection of only primer-derived signals. Takayama *et al.* developed fluorescence RT-LAMP using a quenching primer (QPrimer)⁴⁴ to detect influenza virus and RSV,⁴⁵

and Nakauchi *et al.* developed QPrimer RT-LAMP for rhinoviruses.⁴⁶ In this system, a cytosine or guanine residue at the 5' end of the loop primer is labeled with BODIPY (boron-dipyrromethene) dye and the labeled and unlabeled loop primers are mixed at a 1:10 ratio. Upon annealing to its target sequence, QPrimer fluorescence is quenched by the paired guanine or cytosine residue (Figure 3). Therefore, the positive signal can be detected as a reverse sigmoid curve using an isothermal fluorescence reader, such as a real-time PCR instrument (Figure 3). In addition, use of QPrimers reduces the LAMP detection time by several minutes relative to turbidity detection, and positive signals are typically detected within 20 min.⁴⁵ Although QPrimer can resolve concerns regarding detection of primer-derived signals directly, nonspecific DNA extension at the annealed 3' end and/or primer dimers can result in false positives, so strict screening for primer integrity is still necessary. To overcome this, LAMP using a quenching probe (QProbe)⁴⁴ was developed.⁶ In QProbe LAMP, the 3' end of one of the loop primers is labeled with BODIPY dye (Figure 3); the QProbe must be designed to be several nucleotides longer than the original loop primer toward the 3' end. Table 1 shows the sequences of QProbes used

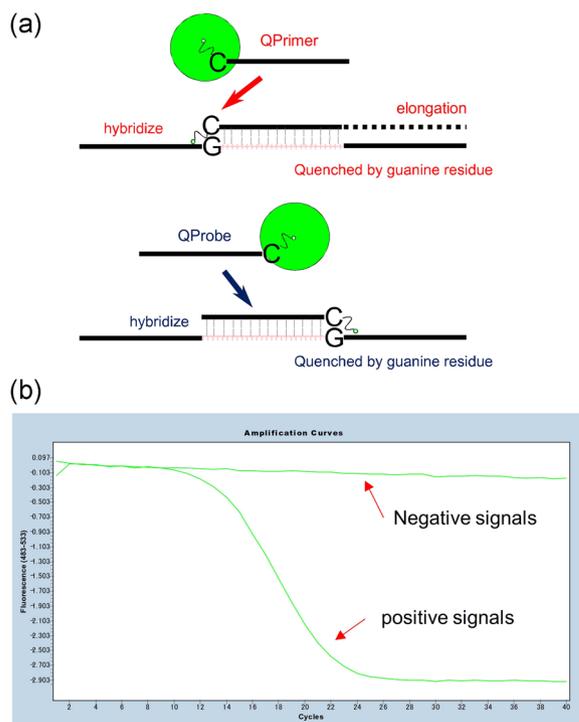


FIGURE 3 Schematic diagrams of a QPrimer and QProbe. (a) The cytosine or guanine residue is labeled with BODIPY. The QPrimer and QProbe fluoresce ordinarily. Upon annealing to their target sequence, their complementary guanine or cytosine residue quenches the fluorescence. (b) The signal can be detected as a reverse sigmoid curve using a fluorescence meter

TABLE 1 Sequences of the QProbes used to detect MERS-CoV

For N	Sequence (5'-3')
LB primer	GGAACCCTAACAATGATTCAGCT
Qprobe	GGAACCCTAACAATGATTCAGCT TATTGTTACAC
For ORF1a	Sequence (5'-3')
LB primer	GGTCACTCAAATTGCTAACATG
Qprobe	GGTCACTCAAATTGCTAACATG TTCTTGGAAACAGAC

The sequences in bold are extended relative to the loop primer.

in QProbe RT-LAMP for MERS-CoV.⁶ The sequences in bold are extended relative to the loop primer, and are responsible for the specific reaction, because the sequence exists only in the target sequence and the specific amplicon. Although this makes QProbes more difficult to design than QPrimers, the labeled dye physically blocks DNA extension at the 3' end, preventing nonspecific DNA amplification by a labeled primer and enhancing the accuracy of the signal.

Fluorescence detection requires a fluorometer, which prevents naked-eye endpoint detection. However, portable isothermal fluorometers enable fluorescence detection in the field. For example, the ESEQuant TS2 (Qiagen, <https://www.qiagen.com/jp/products/custom-solutions/automation/ese-instruments/esequant-ts2/>) is a 12 well isothermal tube scanner that has a built-in battery pack, and so can be used outdoors. Furthermore, a lyophilized RT-LAMP reaction mixture in a 12 well tube reduces the time required for MERS-CoV detection.⁶ In other words, the lyophilized reagent contains all necessary components for RT-LAMP, and all it takes is just to add extracted RNA and water, and it can reduce the time for reagent preparation. Thus, lyophilized reagents and portable fluorescence LAMP devices enable its use in field surveillance as an accurate diagnostic tool.

Although each detection method has advantages and disadvantages, overall, LAMP is a superior assay. Its high sensitivity, specificity, and rapidity are advantageous for use in clinical laboratories compared with rapid test kits based on antigen detection. Indeed, the sensitivity of LAMP is superior to that of rapid test kits based on immunochromatography.^{28,29} Thus, LAMP may replace such kits in the future.

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DISCLOSURE

The author declares that he has no competing interests.

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