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Brief review of monitoring methods for loop-mediated isothermal amplification (LAMP)

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ABSTRACT

The loop-mediated isothermal amplification (LAMP) technique has the potential to revolutionize molecular biology because it allows DNA amplification under isothermal conditions and is highly compatible with point-of-care analysis. To achieve efficient genetic analysis of samples, the method of real-time or endpoint determination selected to monitor the biochemical reaction is of great importance. In this paper we briefly review progress in the development of monitoring methods for LAMP.

1. Introduction

Loop-mediated isothermal amplification (LAMP), which amplifies DNA under isothermal conditions, requires a set of four specially designed primers that recognize six distinct regions of the target, and relies on autocycling strand displacing DNA synthesis by the *Bst* DNA polymerase large fragment (Notomi et al., 2000; Abdul-Ghani et al., 2012; Tanner and Evans, 2014). It has the potential to revolutionize molecular biology by reducing the need for highly sophisticated equipment, and by having low running costs and short turnaround times compared with many amplification methods such as polymerase chain reaction (PCR). Hence, LAMP has been advocated as a low cost genetic analysis tool for resource poor settings (Abdul-Ghani et al., 2012; Mori and Notomi, 2009; Parida et al., 2008; Neonakis et al., 2011; Poon et al., 2006).

The sensitivity of LAMP does not appear to be affected by the presence of non-target DNA in samples (Notomi et al., 2000; Inacio et al., 2008), and the method is also more tolerant to well-known PCR inhibitors such as blood, serum and food ingredients (Inacio et al., 2008; Wang et al., 2008; Kaneko et al., 2007; Kiddle et al., 2012). The LAMP reaction can be implemented even when the DNA extraction step is eliminated (Poon et al., 2006; Kaneko et al., 2007; Hill et al., 2008; Dugan et al., 2012). Meanwhile, its sensitivity and amplification efficiency are much higher than that of PCR (including real time quantitative PCR, RT-qPCR) (Tanner and Evans, 2014; Mori and Notomi, 2009; Parida et al., 2008; Hara-Kudo et al., 2007; Chen et al., 2014; Siljo and Bhat, 2014; Hong et al., 2004). Moreover, the amplification specificity of LAMP is considered extremely high because the primers must bind six distinct regions on the target DNA (Notomi et al., 2000; Tanner and Evans, 2014; Goto et al., 2009; Asiello and Baeumner, 2011). These attractive properties have motivated researchers to explore the use of LAMP for genetic analysis in diverse fields.

Achieving efficient genetic analysis with LAMP depends not only on the performance of DNA amplification, but is also highly dependent on the method selected for monitoring the reaction (Parida et al., 2008). Many researchers have contributed to the search for sensitive, accurate, stable and simple monitoring methods. In recent years there has been rapid development in this line of research. This review is devoted to addressing advances in monitoring methods for LAMP and their possible future prospects.

2. Advances in mainstream monitoring methods

In this section, advances in mainstream monitoring methods for LAMP are discussed. The ordering of this section is designed to explain the advances in the field and does not necessarily reflect the relative capabilities of the respective techniques.

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2.1. Naked eye monitoring

2.1.1. Naked eye monitoring by observing precipitate

Eq. (1) represents the DNA polymerization reaction mediated by *Bst* polymerase in LAMP, which releases pyrophosphate ions from deoxyribonucleotide triphosphates (dNTPs) as a by-product (Mori et al., 2001). When a large amount of these pyrophosphate ions are produced, they react with magnesium ions in the reaction buffer (Eq. (2)), yielding a white precipitate (Abdul-Ghani et al., 2012; Tanner and Evans, 2014; Mori and Notomi, 2009; Mori et al., 2001). In a successful LAMP reaction, a large amount of white precipitate is produced, which can be visualized with (Le Roux et al., 2009) or without (Karanis et al., 2007; Le et al., 2012; Kubota et al., 2008) the help of centrifugation. The presence or absence of this white precipitate as an endpoint measurement allows easy distinction of whether or not nucleic acid has been amplified by the LAMP reaction (Kubota et al., 2008).

$$(DNA)_{n-1} + dNTP \rightarrow (DNA)_n + P_2O_7^{4-} \quad (1)$$

$$P_2O_7^{4-} + 2Mg^{2+} \rightarrow Mg_2P_2O_7 \quad (2)$$

There is no instrumental cost in monitoring LAMP using this method. Furthermore, there is no risk of contamination of amplicons because the contents of the reaction tube are not exposed to the atmosphere. However, Le et al. (2012) found that it was difficult to determine sensitivity and variability between tubes with the naked eye under sunlight illumination. Kubota et al. (2008) also found that the detection limit is rather high (6×10^4 colony forming unit (CFU)/ml). Notably, the turbidity of the positive samples is stable for only a short time, meaning that the monitoring must be performed as soon as possible after the reaction (Almasi et al., 2013).

Although there are risks of subjective error and high detection limit, this method is sufficient for a preliminary determination of the LAMP reaction, especially for rapid point-of-care testing.

2.1.2. Naked eye monitoring employing DNA-binding dyes

DNA-binding dyes possess specific molecular structures that allow them to bind selectively to double-stranded DNA (dsDNA). Typically, formation of the dye–dsDNA complex causes a visible color change of the dye. Thus, these dyes can be used to monitor the products of the LAMP reaction. The sensitivity of the detection using DNA-binding dyes is considerably higher than that obtained by turbidity alone (Le et al., 2012). At present, a number of fluorescent dyes have been used for qualitative monitoring.

In the presence of sufficient dsDNA, the color of the fluorescent dye SYBR Green I turns from orange to green. The color change is apparent under natural light (Zhang et al., 2012b) and under UV light (Le et al., 2012). Thus the result of the LAMP reaction can be monitored by the naked eye by the addition of SYBR Green I dye at the endpoint (Le et al., 2012; Zhang et al., 2012b; Iwamoto et al., 2003; Balbin et al., 2014; Wang et al., 2014; Soli et al., 2013; Shan et al., 2012). The employment of SYBR Green I leads to increased sensitivity when compared with visual turbidity measurements (Soli et al., 2013). According to Zhang et al. (2012b) and Soli et al. (2013), the detection limits can be as low as 13 CFU/reaction and < 3.2 CFU/reaction, respectively. On the other hand, the increase in sensitivity by adding DNA-binding dyes is associated with higher running costs. In addition, the risk of contamination of amplicons is increased because of the need to open the reaction tube in order to add the dye. To overcome the latter shortcoming, Honget al. (2012) reported a two-step method to avoid having to open the tubes: SYBR Green I dye was suspended on tinfoil within the tube; after the LAMP reaction the tube was centrifuged, causing the dye to drop into the LAMP reaction mixture. Using this method the products of LAMP could be detected when the minimal template concentration was 1 copy/μl. Similarly, Quant-iT PicoGreen (Wastling et al., 2010), GeneFinder (Almasi et al., 2013; Zhang et al., 2009, 2011) and ethidium bromide (Wastling et al., 2010) have also been employed to monitor the result of the LAMP reaction.

Polyethylenimine is also employed to enhance the detection of dye-labeled LAMP products (Mori et al., 2006). In the polyethylenimine-enhanced procedure, fluorescently-labeled oligo DNA probes were used to bind to the LAMP product. Subsequent addition of polyethylenimine neutralized the charged dye-labeled LAMP products, yielding a precipitate with a clear color and in an amount that could be identified visually. There is no risk of false positives as long as the oligo DNA fluorescent probes are labeled at the 3′ end, and because the polyethylenimine does not interact with the short oligo DNA probes. This procedure allows for highly accurate genetic testing but does require extra reagent costs and labor.

Drawbacks of the DNA-binding dyes include inhibition of the LAMP biochemical amplification process (for example, polyethylenimine strongly inhibits the LAMP reaction (Mori et al., 2006)), meaning that the reagents must be added at the endpoint, post-LAMP reaction. Additionally, some of these dyes, ethidium bromide (Tomita et al., 2008), for instance, may be mutagens, carcinogens, or teratogens, although this depends on the organism exposed and the circumstances of exposure.

This kind of method requires two steps: amplification and addition of dye. Note that opening the reaction tube after amplification should generally be done with care to prevent carry-over contamination.

2.1.3. Naked eye monitoring employing colorimetric indicators

Another class of naked eye methods utilizes indirect colorimetric indicators. The indicators can be added directly during the LAMP reaction mixture preparation, allowing for a single-step assay. Hence, compared with the two-step DNA-binding dye approach, the risk of cross-contamination is much lower because the tube is not opened after the reaction (Parida et al., 2008; Tomita et al., 2008).

One example of a colorimetric indicator is calcein, which indicates the result of the LAMP reaction indirectly. As shown in the schematic drawing (Fig. 1), before the amplification reaction, calcein molecules combine with manganous ions, quenching calcein fluorescence; at this stage the LAMP reaction solution appears orange. As the LAMP reaction proceeds in the presence of target DNA, it is suggested that calcein molecules give up manganous ions to newly generated pyrophosphate ions, thereby recovering their green fluorescence. In addition, the calcein molecules combine with residual magnesium ions, enhancing the green fluorescent signal (Tomita et al., 2008). Positive results can be determined from the change of color by the naked eye (Le Roux et al., 2009; Hong et al., 2012; Tomita et al., 2008; Liang et al., 2009). Le Roux et al. (2009) found that the detection limit was ≥100 copies, which is relatively high. Wastling et al. (2010) also found that the inclusion of calcein and $MnCl_2$ seemed to reduce the absolute sensitivity of LAMP as compared to results seen without extra reagents added to the reaction mixure. There are two possible cautions. One is that in fact the presence of calcein and $MnCl_2$ inhibits the LAMP reaction to some extent (Goto et al., 2009; Wastling et al., 2010). The other is that the interaction between calcein and dsDNA (Yu et al., 2008; Zhang et al., 2012a) brings about decrease of the sensitivity.

Another dye with a similar mode of action is hydroxy naphthol blue (HNB) which develops a purple color in the presence of Mg^{2+}. In the amplification process, a significant amount of insoluble magnesium pyrophosphate is produced, causing a major decrease of Mg^{2+} concentration in the solution. This reduction in concentration causes the color of the HNB solution to change from purple to blue (Goto et al., 2009). Wastling et al. (2010) reported that this reagent does not inhibit the LAMP reaction, and is better than calcein as an indicator. Thus it is employed widely to monitor

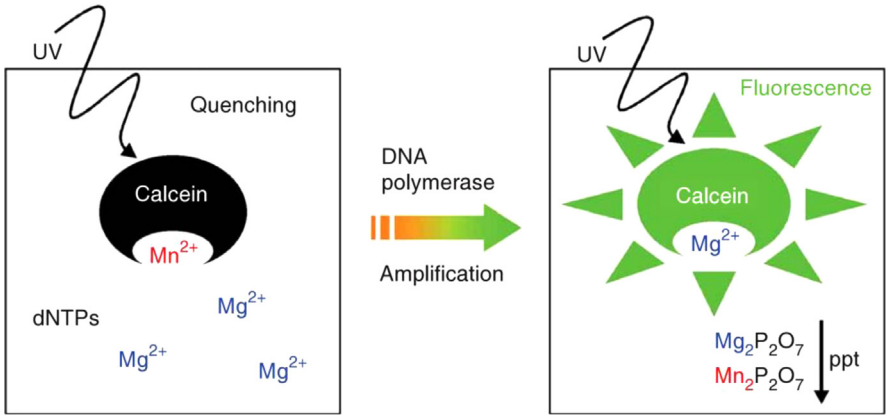

Fig. 1. Principle of monitoring using a colorimetric indicator (calcein). In the DNA amplification process by *Bst* polymerase, pyrophosphate ions are produced as a by-product from the reaction substrate dNTPs. The calcein in the reaction mixture initially combines with Mn^{2+} so remaining quenched. As the amplification reaction proceeds, Mn^{2+} is displaced from calcein by the generated $P_2O_7^{4-}$, which results in the emission of fluorescence. Additionally, the free calcein is apt to combine with Mg^{2+} in the reaction mixture, enhancing the fluorescence emission.
Source: Reprinted with permission.

the LAMP reaction in single-step methods (Goto et al., 2009; Hong et al., 2012; Wastling et al., 2010; Ma et al., 2010; Safavieh et al., 2014; Luo et al., 2011; Ahmadi et al., 2013). Using HNB, a detection limit of 60 copies (Ma et al., 2010) or 30 CFU/ml (Safavieh et al., 2014) can be obtained, which is a little lower than that found by using calcein. The detection limit for LAMP using HNB was found to be at a ten-fold higher concentration compared with assays without any additional reagents in the reaction mixture (Wastling et al., 2010). Thus, further studies should be carried out to determine the inhibitory potential of HNB.

To perform monitoring with colorimetry successfully, as well as with white precipitate and fluorescent DNA dyes, a "trained-eye" is needed (Wastling et al., 2010).

2.2. Gel electrophoresis

Gel electrophoresis is a method for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. It is a conventional method for monitoring the LAMP amplicons directly (Notomi et al., 2000; Abdul-Ghani et al., 2012; Tanner and Evans, 2014; Mori and Notomi, 2009; Parida et al., 2008), even as a "gold standard" in many situations.

Ethidium bromide is a polycyclic fluorescent dye that binds to double-stranded DNA molecules by intercalating a planar group between the stacked base pairs of the nucleic acid, resulting in enhanced fluorescence emission. In addition to being used in naked eye monitoring (see above), it is an extremely common dye used for gel electrophoresis monitoring of the LAMP reaction (Mori and Notomi, 2009; Chen et al., 2014; Le et al., 2012; Zhang et al., 2012a; Tomita et al., 2008). SYBR Green dye is also used for these applications (Notomi et al., 2000; Iwamoto et al., 2003). After staining with fluorescent dyes, the positive post-LAMP reaction mixture subjected to gel electrophoresis produces many bands of different sizes in a reproducible ladder-like pattern (Fig. 2) (Le et al., 2012). Techniques relying on indirect detection methods, i.e. turbidity and colorimetry, may not be able to distinguish between real and false positives under some unexpected cases when non-specific amplification occurs (Lee et al., 2009a). Using gel electrophoresis the length of amplicons can be recognized directly and hence the risk of non-specific detection is reduced. Moreover, gel electrophoresis gives slightly more sensitive than inspection of color change by the naked eye with SYBR Green (Zhang et al., 2012b). In particular, microchip-based electrophoresis (Iseki et al., 2007) and ultrafast electrophoresis (Hataoka et al., 2004) have been reported recently. These developments accelerate the advance of monitoring methods for LAMP by reducing the turnaround time considerably.

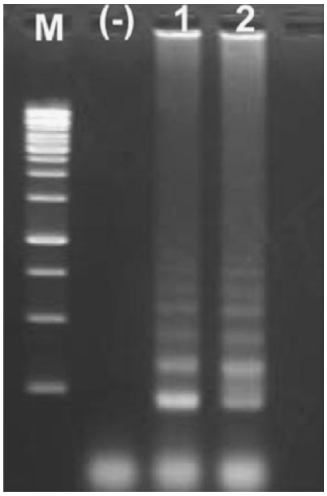

Fig. 2. Products of LAMP monitored using gel electrophoresis after staining with ethidium bromide. Lanes: M, 1-kb ladder marker; (-), negative control (no DNA); 1 and 2, LAMP products from positive template DNA.
Source: Adapted with permission.

As an endpoint monitoring method, gel electrophoresis works well. However, because it works on the principle of separation of LAMP products, it is only suitable for qualitative analysis at the endpoint. In addition, the method possesses a risk of cross-contamination and also relatively long turnaround times (Zhang et al., 2011). The requirement for electrophoresis apparatus and UV detection limits the suitability for field applications.

2.3. Real-time turbidity

Based on the large amount of magnesium pyrophosphate precipitate by-product generated (Tomita et al., 2008; Boehme et al., 2007), the result of the LAMP reaction can be monitored in real-time with optical instruments, including turbidimeters (Le Roux et al., 2009; Mori et al., 2004; Han et al., 2011; Wang et al., 2012a,b ; Denschlag et al., 2013), optical fibers (Fang et al., 2010, 2011) and spectrophotometers (Mori et al., 2001). Gene copy number can also be quantified with the help of a standard curve generated from different concentrations of gene copy number

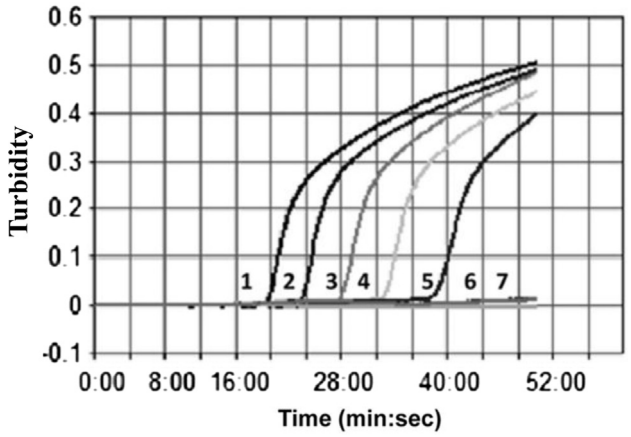

Fig. 3. Typical LAMP amplification kinetics monitored with a real time turbidimeter. Samples 1 to 6 correspond to 10-fold serial dilutions of *Escherichia coli* O26:H11 strain 97–3250 cells ranging from 1×10^5 to 1 CFU/reaction mixture; sample 7 is water.
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plotted against time to positive signal (threshold time) (Parida et al., 2008). For example, Mori et al. (2004) reported that real-time turbidity measurements of the LAMP reaction permitted the quantitative analysis of minute amounts of nucleic acids present in a sample by observing the luminescence intensity of light emitting diodes. Plotting the log of initial copy number of template DNA versus threshold time generated a linear relationship from 2×10^3 copies (0.01 pg/tube) to 2×10^9 copies (100 ng/tube) of template DNA. Le Roux et al. (2009) used a real-time turbidimeter to monitor the accumulation of magnesium pyrophosphate and analyzed results using the LA-200E software package, allowing for the monitoring of amplification products as early as 16 min after initiation of the reaction. Shan et al. (2012) utilized a Loopamp real-time turbidimeter to record the optical density of LAMP reaction mixture and determine the threshold time. They found a detection limit of 6 CFU/tube. A typical kinetics graph is shown in Fig. 3 (Wang et al., 2012b). Notably, Triton X-100 included in some kinds of *Bst* DNA polymerase buffers can interfere with the performance of real time monitoring turbidity (Tanner and Evans, 2014).

The real time turbidity method is capable of quantitative monitoring with high automation, not requiring special probes/indicators or any other auxiliary regents. There are no running costs except for the turbidimeter or spectrophotometer. Moreover, the risk of amplicon contamination is eliminated (Le Roux et al., 2009; Mori et al., 2004; Han et al., 2011; Wang et al., 2012a,b; Denschlag et al., 2013; Fang et al., 2011). It has even been considered as the easiest way of monitoring gene amplification for LAMP (Mori et al., 2004). However, monitoring the LAMP reaction by turbidimetry is limited by inhomogeneity of particle size, uneven spatial distribution, re-dissolution of magnesium pyrophosphate particles and samples possessing high turbidity prior to LAMP; these factors result in relatively low sensitivity (Chuang et al., 2012).

2.4. Real-time fluorescence

The dyes employed in the naked eye endpoint determination of the LAMP reaction can also be applied in real-time monitoring. Some types of fluorescent dye can bind to the double-stranded structure as soon as the dsDNA products are synthesized, yielding a sensitive change in optical signal. Hence, these intercalated indicators have been employed to monitor the process of the LAMP reaction in real time (Tanner and Evans, 2014). The threshold of detection of amplified product can be defined as the time at which a positive fluorescent signal is recorded. Using calibration standards, the initial concentration of a sample can be extrapolated (Cai et al., 2008). After recording of the fluorescence intensity with optical readers, the data are analyzed to obtain quantitative information about the initial template DNA.

Because of its wide availability and its property of quick integration with dsDNA, SYBR Green I is often used as a real-time monitoring dye. Typical examples are listed in this section. Cai et al. (2008) reported that combining the robust LAMP technology with the SYBR Green signal produced an assay with a detection limit of 210 copies/ml. Maeda et al. (2005) developed a real time monitoring method for quantifying target genes, whereby accumulation of LAMP products was detected by monitoring the increase in fluorescence of dsDNA-binding SYBR Green, followed by analysis of the data with the GeneAmp 5700 SDS software. Lucchi et al. (2010) combined a portable amplification platform and fluorescent monitoring device for performing real time LAMP using SYBR Green dye. A detection limit of 125 parasites/ml was obtained (Patel et al., 2013). Yi et al. (2014) developed an ESE Quant tube scanner system using SYBR Green as the real-time monitoring dye. The detection limit of the assay was determined to be as low as 7 CFU/tube. Nevertheless, they considered that the method needed to be evaluated with many environmental samples to check its efficacy.

Some other types of fluorescent dyes have also been employed as real time indicators. Nagamine et al. (2002) reported that ethidium bromide could also be used to indicate the progression of the LAMP reaction in real time for quantitative monitoring, using the ABI PRISM 7700 sequence monitoring system (Fig. 4). Ahmad et al. (2011) used SYTO-82 dye for real-time, rapid detection and quantification of the LAMP reaction with a charge-coupled device (CCD)-based fluorescence imaging system. A single DNA copy (Ahmad et al., 2011) or 10 genes (Tourlousse et al., 2012) could be detected within 1 h. Other groups have also used YO-PRO-1 iodide (Ohtsuki et al., 2008) and SYTO-81 (Stedtfeld et al., 2012) as real-time monitoring dyes.

Normally, probe based monitoring systems such as TaqMan are not applicable to the LAMP reaction since the amplification principle of the LAMP reaction is based on strand displacement DNA synthesis. However, Kouguchi et al. (2010) developed a completely homogeneous duplex method to detect the result of the LAMP reaction by using a single 6-carboxyfluorescein (FAM)-labeled primers and the indicator ethidium bromide. By this method the sequence-specific LAMP reaction products could be

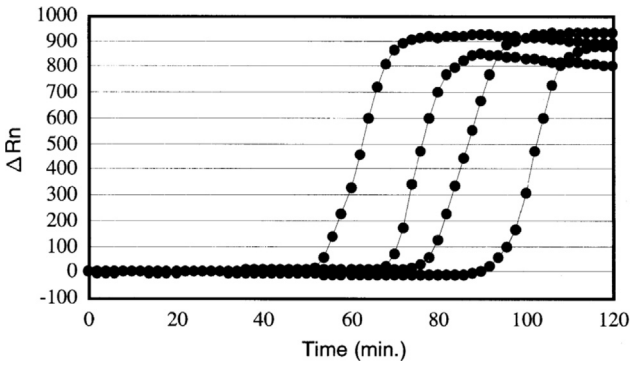

Fig. 4. Real-time monitoring of the LAMP amplification signals via ethidium bromide fluorescence using the ABI PRISM 7700 sequence detection system. The signal reaches a plateau after a few minutes, presumably because the free ethidium bromide is depleted by binding to amplified DNA. ΔR_n is the normalized fluorescence emission at 615 nm.
Source: Reprinted with permission

distinguished quantitatively with a detection limit of 100 CFU. Tanner et al. (2012) adapted standard LAMP primers to contain a quencher–fluorophore duplex region that, upon strand separation, generated an increase of fluorescent signal. This approach permitted the real-time monitoring of 1–4 target sequences in a single LAMP reaction tube utilizing a standard real-time fluorimeter, detecting less than 100 copies of human genomic DNA. In the two cases mentioned above, the utilization of the fluorescent probe helps to improve the specificity of detection. However, bulky apparatus such as optical analyzers with complicated setups and sophisticated chemical synthesis of dye-labeled primers were required.

Generally, the fluorescence-based real-time monitoring of LAMP reaction is considerably faster ($>50\%$) than that performed by a real-time turbidity (Denschlag et al., 2013). Besides, compared to the real time turbidity method, the real time fluorescence method possesses two further merits at the expense of higher running cost. The first is higher sensitivity. The second is that the sensitivity is less affected by the presence of opaque substances in the mixture, such as plasmids and protein (Francois et al., 2011). However, although the inhibitory potential of the indicators, such as SYBR Green I (Cai et al., 2008; Maeda et al., 2005) and SYTO-81 (Stedtfeld et al., 2012), are considered to be marginal, it should not be neglected (Denschlag et al., 2013).

2.5. Electrochemical methods

Electrochemical methods are faster, lower cost, simpler and can be applied in a miniaturized format more readily than optical methods (Ferguson et al., 2009). These prominent advantages make electrochemical monitoring a reliable and robust method for analyzing DNA amplification (Defever et al., 2011). The bulk of studies focus on the employment of voltammetry for monitoring the LAMP reaction, by utilizing electrochemical sensors/chips and electrochemical biosensors.

2.5.1. Electrochemical sensors/chips

2.5.1.1. Endpoint. Binding of electrochemically active species with dsDNA can cause a change in the measured current. For instance, DNA aggregation and minor groove binding with Hoechst 33258 redox molecule in solution causes a significant drop in the current response. Safavieh et al. (2012) and Ahmed et al. (2010) employed this molecule as electroactive indicator to detect the products of the LAMP reaction at the endpoint, and obtained a detection limit of 8.6 fg/μl DNA (24 CFU/ml) with this probe-immobilization-free method (Safavieh et al., 2012). To minimize the risk of cross-contamination, they developed a platform to allow the amplification and detection in a single device. Using this device and the Hoechst 33258 indicator, a detection limit of 300 copies was obtained (Ahmed et al., 2009).

2.5.1.2. Real time. Real-time monitoring of the LAMP reaction is achievable through *in situ* electrochemical interrogation and relies on two mechanisms: redox electron transfer between methylene blue (MB) molecules and the working electrode, and the intercalation of MB with dsDNA (Yang et al., 2002; Kerman et al., 2002). As the reaction progresses, intercalation of the MB with LAMP amplicons reduces the free MB concentration and thus decreases this redox current (Nagatani et al., 2011; Hsieh et al., 2012; Xie et al., 2014). Using MB as the indicator, Hsieh et al. (2012) and Xie et al. (2014) obtained a detection limit of 16 copies (4 fg/μl) and 0.3 pM, respectively. However, the DNA binding affinity of MB is 10^4–$10^5\ M^{-1}$ (Baranovskii et al., 2008), which predicts a low-efficiency interaction of MB with dsDNA in solution with LAMP amplicons (Ahmed et al., 2013), suggesting that lower detection limits could be achieved with other indicators. Ruthenium hexaamine lacks intercalating ligands and binds electrostatically with the anionic dsDNA backbone (Steel et al., 1999). Ahmed et al. (2013) employed it as an indicator to monitor LAMP amplicons quantitatively, with a detection limit of 20 copies/ml in less than 30 min. Both the *in vitro* amplification and real-time monitoring are performed in a single polypropylene tube using a single biochip; this approach could avoid all risks of potential cross-contamination throughout the entire procedure. In the ideal situation, the electro-active indicator should be chemically stable, should preferentially bind to the dsDNA amplicons, and should not inhibit the amplification during the monitoring process (Ahmed et al., 2013). Nevertheless, it is believed that almost all kinds of redox probes exhibit inhibitory effects towards DNA amplification (Safavieh et al., 2012). For example, Hoechst 33258 significantly inhibits polymerase enzyme activity, limiting DNA amplification and sensing in the solution phase. To overcome this issue, Zhang et al. (2011) developed a voltammeric mode for monitoring the biochemical process of DNA amplification by testing the oxidative response of free 2′-deoxyguanosine 5′-triphosphate (dGTP), which is one of the reactants in the LAMP reaction mixture. With this strategy, inhibition is not a concern because there are no auxiliary indicators used. Unfortunately, the need to place electrodes into the reaction mixture increases the potential for cross-contamination.

2.5.2. Electrochemical biosensors

Electrochemical biosensors incorporate some form of biorecognition into the detection method. Several electrochemical biosensor/biochip-based methods have been reported for monitoring the LAMP reaction. Sun et al. (2010) fabricated an electrochemical DNA biosensor by immobilizing sequence-specific ssDNA probes onto an ionic liquid modified basal electrode. Then they used the biosensor and methylene blue electrochemical indicator to monitor LAMP amplicons based on hybridization. Nakamura et al. (2007) developed an electrochemical DNA biochip to monitor six LAMP products simultaneously using Hoechst 33258 as the hybridization indicator. The same group (Nakamura et al., 2010) also successfully obtained the copy number of a specific gene by combining LAMP and an electrochemical DNA biochip based on hybridization, consisting of 1 h for DNA amplification and 0.5 h for DNA detection by the Genelyze system. Commonly, biosensor/biochip-based methods are used to monitor sequence-specific target DNA directly, and are carried out after the LAMP reaction for qualitative detection. On the one hand, the specificity of the monitoring is higher than that by indirect measurement. On the other hand, it is costly and time-consuming to prepare DNA biosensors/chips and in particular, it is not easy to implement in parallel with the LAMP reaction for real-time monitoring.

There have been interesting developments in the monitoring of the LAMP reaction via the simple and cost-effective voltammetric mode. But there is no detailed explanation offered about the potential fouling of working electrodes by the biological components of the LAMP mixture; though Ahmed et al. (2013) observed that there were no thin films or stains on the working and counter electrode surfaces even after repeated measurements.

2.6. Lateral flow dipstick (LFD)

The LFD is an immunochromatographic technique utilizing antibody capture followed by secondary antibody labeling. On the LFD strip, an antibody specific to biotin is immobilized at the test line. At the test line, these strips capture biotin-labeled LAMP products that have been hybridized with FITC-labeled DNA probes. In order to develop a readable output, gold-labeled anti-FITC

antibodies are introduced. In the presence of LAMP products, the gold anti-FITC antibodies are trapped at the test line as a triple complex with dsDNA. Non-hybridized FITC probes are bound by the gold-labeled anti-FITC to form a double complex without biotin and move through the test line to be trapped at the control line (Nimitphak et al., 2008; Jaroenram et al., 2009; Khunthong et al., 2013; Diribe et al., 2014; Njiru, 2011; Wang et al., 2013). The LFD test is highly specific because the probe targets a specific complementary sequence within the LAMP product as opposed to nonspecific binding of dsDNA by intercalating dye (Diribe et al., 2014).

LAMP combined with a LFD for highly specific, and simple, visual monitoring of amplicons was reported by Jaroenram et al. (2009, 2013). Using this protocol, a 30 min amplification step followed by 5 min hybridization with a FITC-labeled DNA probe and 5 min LFD step resulted in visualization of amplicons trapped at the LFD test line. Thus, 10 min for rapid DNA extraction followed by LAMP combined with LFD detection resulted in a total assay time of approximately 50 min. In the detection of human infective trypanosome DNA from clinical samples, the LAMP-LFD showed analytical sensitivity equivalent to 0.01 trypanosomes/ml, levels that are identical to that using gel electrophoresis and SYBR Green I dye. In addition, LAMP-LFD exhibits superior specificity to SYBR Green I (Njiru, 2011). By this method, detection limits of 0.039 fg/μl (Diribe et al., 2014), 2.4 copies (Wang et al., 2013), 3 copies (Roskos et al., 2013) and 100 copies (Chowdry et al., 2014) were obtained, respectively.

The LFD method has particular advantages in specificity and sensitivity, and does not require any specialist instrumentation since the user simply dips the LFD into an appropriately buffered LAMP mixture. Thus it is a competitive candidate for point of care qualitative tests as an endpoint format. Nevertheless, the preparation of the strips and the operation of detection are not only time-consuming, but also costly. Moreover, to eliminate the risk of contamination, a single step reaction that will allow direct monitoring of product with the strips without necessity of tube opening would need to be advanced further (Njiru, 2011; Roskos et al., 2013).

2.7. Enzyme-linked immunosorbent assay (ELISA)

The ELISA method involves direct incorporation of antigen-labeled nucleotides into amplicons during the LAMP-amplification process; subsequent hybridization to specific immobilized oligonucleotide probes, and finally detection of the captured amplicons by immunoassay technology. Concerning the assay time, the LAMP-ELISA is performed in a few hours and is highly flexible with the ability to process simultaneously up to several hundred samples (Ravan and Yazdanparast, 2012; Ravan and Yazdanparast 2013). The detection of target amplicons depend on biomolecular recognition. Hence, its specificity is superior to that by indirect monitoring methods. As a qualitative monitoring method, its sensitivity is outstanding. Ravan and Yazdanparast (2012) and Lee et al. (2009b) reported that detection limits of 4 CFU and 1 copy could be obtained, respectively. This technique does not require expensive equipment, and thus it can be performed in poorly equipped laboratories. However, it would require trained staff, unless expensive ELISA kits are purchased, and the necessity of tube opening carries a high risk of carry-over contamination.

2.8. Comparative features of different methods

All the methods presented above can be used to achieve monitoring of the LAMP reaction in a variety of situations and experimental conditions. Their distinct features are summarized in Table 1.

3. Development of other monitoring methods

A few niche methods for monitoring the LAMP reaction have been developed. We have ordered the section based on the amount of literature published per technique.

3.1. NanoAu probe

The principle of this combined nanoparticle-LAMP assay relies on stabilising Au nanoparticles (nanoAu) against salt-induced aggregation. In this assay, DNA-functionalized nanoAu probes are introduced to hybridize with the LAMP product. Upon addition of salt, and if the LAMP product is not complementary to the ssDNA probes, the nanoAu probes will aggregate due to the screening effect of salt, resulting in the change of solution color from red to blue/gray and a shift of the surface plasmon peak to a longer wavelength. When the DNA-functionalized nanoAu probes are perfectly matched to the LAMP product, the color of solution remains red and no surface plasmon spectral shift is observed. This assay provides a simple technique, is time-saving and its results could be achieved qualitatively and quantitatively by visualization using the naked eye due to the colorimetric change and by measurement using UV spectroscopy due to the surface plasmon spectral shift, respectively. By this method, detection limits of 0.02 fg (Suebsing et al., 2013) and 0.003 μg (2000 copies) (Seetang-Nun et al., 2013) respectively were obtained after the LAMP reaction. It could be a useful tool in field conditions for diagnosis or surveillance programs.

3.2. Field effect sensor

Veigas et al. (2014) developed a label-free method for real-time monitoring of LAMP amplicons based on a field effect sensor. The monitoring of LAMP amplification was performed by following the increase of free protons in the reaction solution. In comparison with the real-time SYBR Green fluorescence method, it has a slightly lower sensitivity (response range of 1×10^{8}–1×10^{11} copies of target DNA). However, there is almost no running cost.

3.3. Surface plasmon resonance (SPR) sensor

Because the numbers and sizes of solute molecules change in the LAMP reaction, the refractive index of the solution also changes. Thus the LAMP reaction can be monitored directly by measuring refractive index changes of the bulk solution with SPR sensing methods using an inexpensive and disposable sensing cartridge. The advantages of the SPR method compared to turbidimetry are relatively high sensitivity (detection limit of 2 fg/ml), rapid monitoring, and low sample volume used (Chuang et al., 2012). At present the precision could be improved further.

3.4. Absorbance of amplicons

The LAMP reaction yields a large amount of dsDNA, which possesses a high extinction coefficient at a wavelength of 260 nm (OD_{260}). Based on the optical absorbance, Wang et al. (2011) developed a semi-quantitative method for monitoring LAMP amplicons with a detection limit of 10 fg/μl. To implement the operation, high transparency of both the reaction mixture and the reaction vessel is required.

3.5. Bioluminescence

The bioluminescence real time assay allows dynamic changes in pyrophosphate levels to be monitored continuously over extended periods of up to 2 h. During the reaction, the time taken

Table 1
Comparative features of monitoring methods for LAMP.

	Principle	Advantages	Disadvantages	Quantitative/ qualitative	Specificity	Direct/ indirect	Response	Portability	Duration	Cost	Detection limit
Naked eye (white precipitate)	Magnesium pyrophosphate precipitate	Simplicity, free from contamination	Subjective error, medium sensitivity	Qualitative	–	Indirect	Endpoint	Field/lab	0	No	6×10^4 CFU/ml[a]
Naked eye (DNA dye)	Fluorescent dye (add after reaction)	Simplicity, high sensitivity	Cross-contamination	Qualitative	–	Indirect	Endpoint	Field/lab	~2 min	Low	1 copy/μl[b]
Naked eye (colorimetry)	Colorimetric indicator (add before reaction)	Simplicity, free from contamination, relatively high sensitivity	Inhibition of amplification	Qualitative	–	Indirect	Endpoint/ real time	Field/lab	0	Low	30 CFU/ml[c]
Electrophoresis	Gel electrophoretic separation, DNA dye stain	Reporting length of amplicons, high sensitivity	Labor-intensive post-amplification process, risk of contamination	Qualitative	Yes	Direct	Endpoint	Lab	~1 h	Device: medium; running: medium	100 fg[d]
Real time turbidity	Magnesium pyrophosphate precipitate	Automation, free from contamination, probe/indicator-free	Homogeneity and high transparency demand	Quantitative	–	Indirect	Real time	Field/lab	0	Device: medium; running: low	6 CFU[e]
Real time fluorescence	Fluorescent dye binding to amplicons	Automation, fast response, free from contamination	Inhibition of amplification	Quantitative	–	Indirect/ direct	Real time	Field/lab	0	Device: medium; running: low	7 CFU[f]; 1 copy[g]
Electrochemical sensor	Electroactive indicator interacting with amplicons	Automation, simple sensor, unaffected by sample opacity	Inhibition of amplification/cross-contamination	Qualitative/ quantitative	–	Indirect	Endpoint/ real time	Field/lab	~10 min/ 0	Device: medium; running: low	24 CFU[h]
Electrochemical biosensor	Electro-active indicator, hybridization	Automation, unaffected by sample opacity, high specificity	Abor-intensive post-amplification process, cross-contamination	Qualitative	Yes	Direct	Endpoint	Lab	~1 h	Device: medium; running: medium	–
LFD	Chromatography, hybridization, immunorecognition	High specificity, special equipment not required	Cross-contamination, labor-intensive process	Qualitative	Yes	Direct	Endpoint	Field/lab	~20 min	Running: high	2.4 copies[i]
ELISA	Hybridization, immunorecognition	High specificity, special equipment not required	Cross-contamination, labor-intensive process	Qualitative	Yes	Direct	Endpoint	Field/lab	~20 min	Running: high	1 copy[j]

[a] Kubota et al. (2008).
[b] Hong et al. (2012).
[c] Safavieh et al. (2014).
[d] Le et al. (2012).
[e] Shan et al. (2012).
[f] Yi et al. (2014).
[g] Ahmad et al. (2011).
[h] Safavieh et al. (2012).
[i] Wang et al. (2013).
[j] Lee et al. (2009b).

to attain peak bioluminescence is dependent on the starting concentration of target DNA and can be calibrated against a control of known concentration. Quantification of real-time bioluminescence reactions is achieved by measuring the time taken to reach peak light output and is not dependent on absolute light intensity produced, which greatly simplifies data interpretation and the hardware requirements. Kiddle et al. (2012) showed the applicability of this method combined with LAMP for determination of levels of contamination of reference maize samples by genetically modified (GM) maize DNA, at low levels of contamination (0.1–5.0% GM).

3.6. Giant magnetoresistive (GMR) sensor

Zhi et al. (2014) employed a GMR sensor to monitor amplicons qualitatively after the LAMP reaction. In brief, oligonucleotide probes immobilized on the bottom of a microchannel were allowed to hybridize with biotinylated target DNA fragments produced by LAMP. Following hybridization, a solution of streptavidin conjugated magnetic nanoclusters was injected into the microchannel, magnetically labeling the target DNA *via* streptavidin–biotin binding. The resistance values were measured with a GMR sensor before and after amplification. The difference between the resistance values was used to determine the positive samples, allowing a detection limit of 10 copies/ml. Note, the sensor can be repeatedly used. However, the device cost and running cost are both non-competitive.

4. Conclusion and prospects

By using alternating extension and strand displacement reactions, the entire LAMP process can continuously yield long DNA concatamers. Its efficiency is outstanding, as upward of $\sim 10^9$ copies accumulate from less than 10 copies of input template within an hour or two (Abdul-Ghani et al., 2012; Tanner and Evans, 2014). In combination with an appropriate monitoring method, LAMP will be an extremely powerful tool. The monitoring of the LAMP reaction is critical and as such monitoring methods are currently a rapidly developing field because of the significance for practical application and commercial value. The methods listed above have succeeded in meeting various requirements for different situations. However, as concluded in some of the literature published previously, monitoring technologies for the LAMP reaction are still in their infancy (Dugan et al., 2012; Xie et al., 2014; Njiru, 2011; Li et al., 2012).

An ideal method for monitoring the LAMP reaction should be highly sensitive and fast-response, should be of low cost and non-labor-intensive, should be user-friendly and environmentally-friendly, should be capable of obtaining quantitative information automatically (so it can be operated by untrained personnel, minimizing human error), should be high throughput, and should be practicable both in the laboratory and at the point of care. At the present time, it is exciting to see these goals being combined into integrated systems, particularly with the employment of miniaturized equipment and platforms.

The feasibility of achieving ideal LAMP monitoring depends on the development of miniaturized detection components, which are easily integrated to miniaturized platforms for amplification, in particular, lab-on-a-chip devices (Tourlousse et al., 2012; Stedtfeld et al., 2012; Daw and Finkelstein, 2006; Mori et al., 2013). Amongst the methods reviewed above, those based on optical and electrochemical techniques have been in the forefront. For example, a device for point-of-care genetic testing combining LAMP and fluorescence monitoring on a microfluidic chip (state-of-the-art in miniaturization of conventional laboratory apparatuses (Lee, 2013)), has been developed (Stedtfeld et al., 2012; Duarte et al., 2013; Chang et al., 2013). As for portable devices, the amplification-monitoring methods established possess fast response, are environmentally-friendly, high-throughput, automated and highly sensitive. Hence, they are approaching the goal of the ideal LAMP detector. However, the requirement of high transparency of both the reaction mixture and the reaction vessel has not been fully addressed. In theory, electrochemical techniques and other electronic sensors are superior to optical techniques, due to their higher sensitivity and also non-necessity of optical-electrical signal transferring components in the equipment, thus can be miniaturized more easily. For example, Stedtfeld et al. (2012) and Satoh et al. (2012) have succeeded in this approach for monitoring the LAMP reaction with voltammetry on a micro-fluidic chip. Their systems are time-saving and cost-effective compared with fluorescence based monitoring, and have the potential to be a simple method for point-of-care amplification. However, for continuously monitoring the process of the LAMP reaction in real time with voltammetry, there is still a critical challenge: low reproducibility due to the fouling of working electrode. To solve this problem, researchers may turn to employing the anti-fouling electrodes advocated by Gui et al. (2013), or employ a contactless impedance format advocated by Fang et al. (2013). Moreover, other kinds of contactless electrochemical sensors or electronic sensors also show promise as ideal detectors for LAMP.

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