Application Note

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Characterization of LAMP Assays Using a Multimode Microplate Reader

Using the Agilent BioTek Synergy Neo2 hybrid multimode reader to monitor LAMP assays kinetically

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Abstract

Over the course of the last 20 years, isothermal nucleic acid amplification tests, such as loop-mediated isothermal amplification (LAMP) have emerged as an important diagnostic tool, not only for clinical applications, but also for food quality control and environmental monitoring. LAMP is an assay technology that has gained traction for its ability to detect nucleic acid sequences under a number of different conditions without specialized equipment. While typically run as an end point reaction in PCR tubes using manual observation for positive/negative determinations, the use of colorimetric pH change detection lends itself to automated kinetic monitoring. This application note describes the adaptation of a PCR tube-based assay to microplates and the subsequent use of the Agilent BioTek Synergy Neo2 multimode reader to run LAMP assays at low volumes in 384-well microplates.

Introduction

Loop-mediated isothermal amplification (LAMP) is an auto-cycling and strand displacement DNA synthesis amplification method involving the use of the large fragment of *Bst* DNA polymerase.[1] LAMP uses 4-6 primers recognizing multiple distinct regions of target DNA for a highly specific amplification reaction. The products are generally quite long and target amplification is so extensive that a number of different detection modes are possible. Real-time fluorescence detection using intercalating dyes or direct probes, as well as agarose gel electrophoresis can be used to interpret LAMP reactions. LAMP is so prolific that the products and byproducts of these reactions can also be visualized by eye. For example, magnesium pyrophosphate produced during the reaction can be observed as a white precipitate, or added indicators like calcein or hydroxy naphthol blue can be used to signal a positive reaction.[2,3] The reaction also elicits a marked pH change that can be detected with a pH indicator dye.[4]

LAMP utilizes two outer primers (forward outer primer, F3, and backward outer primer, B3), two inner primers (forward inner primer, FIP, and backward inner primer, BIP) and a DNA polymerase with strand-displacement activity.[1] As shown in Figure 1, the inner primer, FIP (which contains two target sequences specific to two different regions in the template DNA) initially hybridizes to the target DNA and starts complementary strand synthesis (I). The outer primer, F3, starts strand displacement of the elongated FIP primer, releasing single-stranded DNA (ssDNA) that serves as a template for the backward primers (II). The BIP primer starts strand synthesis at the ssDNA and is subsequently displaced by the B3 primer (III). Both the 3′ and the 5′ end are complementary to sequences further inwards, enabling the formation of a stem-loop DNA structure (IV and V). The stem-loop structure is the starting point for exponential amplification. Self-priming and the elongation of its 3′ ends (F1 and B1) induce displacement of the 5′ ends (F1c and B1c), subsequent unfolding of the hairpin structure, and back-folding of the newly synthesized strand. Repetition of the self-priming pathway generates long amplicons with cauliflower-like structures.[1]

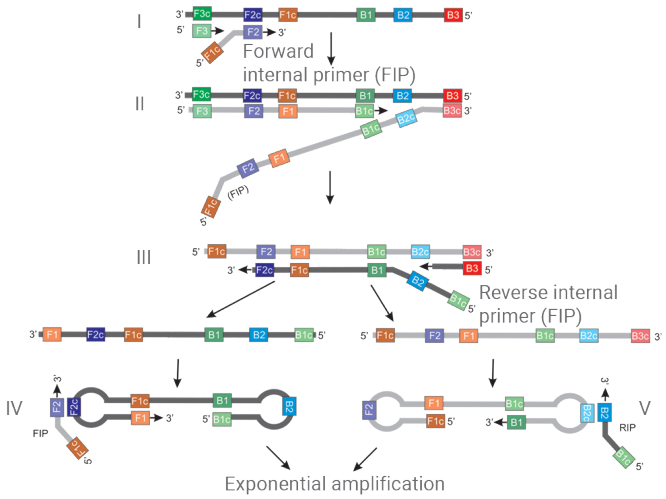

Figure 1. LAMP amplification schematic. Forward and reverse internal primers target and duplicate specific analyte DNA sequences, as well as create loop structures with primer extension. Subsequent amplification of the generated looped sequences is exponential.

When a DNA polymerase incorporates a deoxynucleoside triphosphate into the nascent DNA, as shown in Figure 2, the released byproducts include a pyrophosphate moiety and a hydrogen ion. DNA polymerization is an energetically favorable reaction as a result of the hydrolysis of pyrophosphate after the incorporation of a nucleoside into the polymer.[5] With reaction mixtures with little or no buffering capacity, the pH will eventually become acidic. *Bst* polymerase has been shown to be tolerant to changes in pH.[1]

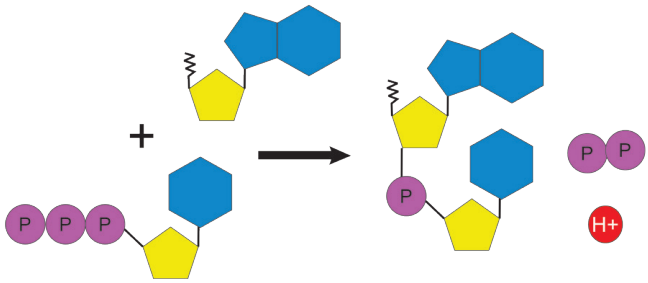

Figure 2. Polymerization of nucleotides into DNA. Nucleoside triphosphates are polymerized into DNA polymers by the action of polymerase enzymes that use the complementary strand as a template. Incorporation of the nucleotide into the polymer results in the formation of a sugar-phosphate backbone and the liberation of a pyrophosphate and a hydrogen ion.

Experimental

Agilent BioTek Synergy Neo2 hybrid multimode reader

The Agilent BioTek Synergy Neo2 hybrid multimode reader was preheated to 65 °C for 1 hour before the LAMP assay was incubated. The colorimetric LAMP reactions were performed in a final reaction volume of 5 µL. The concentrations of the LAMP assay kit components (p/n E2019S) from New England Biolabs (Ipswich, MA) were used according to the manufacturer's instructions. Samples, master mix, primers, guanidine hydrochloride, nuclease-free water and template were premixed in 1.5 ml Eppendorf tubes and transferred to a clear-bottom, black-sided 384-well plate (p/n 3542) from Corning (Corning, NY), sealed with a qPCR sealer (p/n 676040) from Greiner Bio-One North America Inc. (Monroe, NC) and centrifuged for 1 min at 300 g. After centrifugation, samples were read every 1.5 minutes using wavelength switching on the Synergy Neo2.

Results and discussion

The absorbance spectra of the LAMP assays containing the pH-sensitive dye (phenol red) changes dramatically over time, as shown in Figure 3. As the reaction progresses, a pronounced absorbance peak at 560 nm diminishes, while a smaller peak at 420 nm emerges.

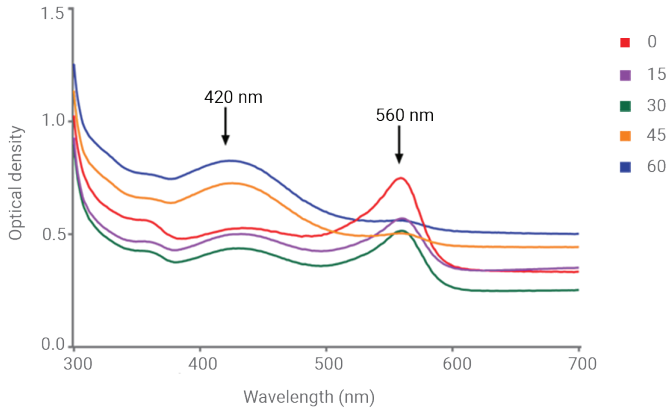

Figure 3. Absorbance spectra of LAMP assay reactions at various times. The absorbance at wavelengths 300 to 700 nm (in 1 nm increments) was determined at various times after the initiation of a LAMP assay. Data represent the mean of two separate wells.

The change in absorbance spectra for LAMP assay progress correlates with absorbance changes in spectral curves of phenol red at various pH levels. As shown in Figure 4, with acidification of phenol red solutions, the absorbance peak at 560 nm of phenol red diminishes, while a broader peak centered on 415 to 420 nm increases. The change in color of phenol red is routinely used in tissue culture media as an indicator of media integrity. As mammalian cells grow and divide in culture, consumption of media constituents and the production of waste products result in the acidification of the media, causing the media to turn yellow. The ratio of the 420 nm and 560 nm absorbance peaks has been previously used to assess the pH status for tissue cultures under a variety of conditions.[6]

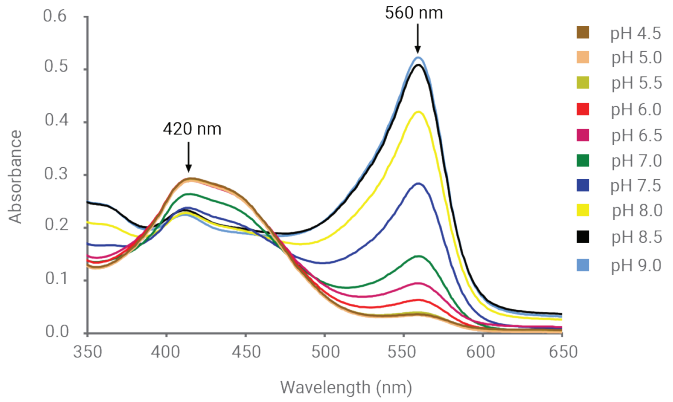

Figure 4. Absorbance spectra of phenol red at different pH levels. Phenol red was diluted to a final concentration of 15 mg/mL with a series of 100 mM phosphate buffers. Wavelengths peaks with changing values are indicated with arrows. Data represents the average of three spectral curves for each pH.

DNA polymerization produces hydrogen ions as a byproduct that results in changes in the color of phenol red-containing reactions. As such, LAMP reactions can be monitored using a 420:560 ratio. As more nucleotides are incorporated into DNA polymers, the subsequent generation of hydrogen ions results in greater acidification of the reaction mixture. Due to LAMP reaction specificity, only samples that result in DNA amplification will acidify the reaction over time. As demonstrated in Figure 5, only the positive sample results in a marked change in the 420:560 ratio over time. As the plate and samples warm, a slight increase in the ratio of all samples is observed, but at about 26 minutes, the amplification products of the reaction result in a marked increase in the 420:560 ratio, eventually reaching a steady state. Neither the negative control (which lacks a DNA target) nor the internal control (which contains the target sequences, but has inappropriate primers) generate amplification products, and have little change in the 420:560 ratio.

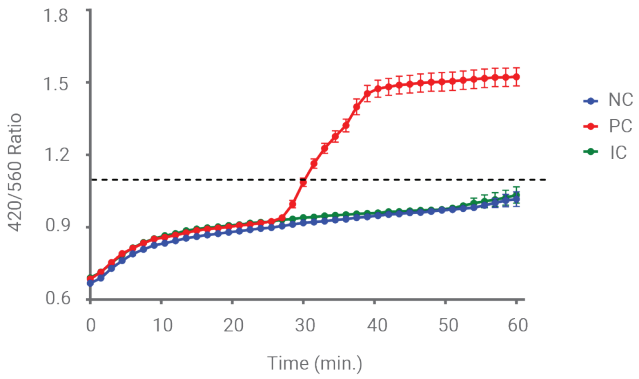

Figure 5. Kinetic measurement of the LAMP assay over 60 minutes. Data are displayed as a ratio of the 420 nm and 560 nm absorbance values (420:560). The positive reaction threshold ratio value is depicted as a dashed line. These data represent the mean and standard deviation of eight determinations.

Reactions with lower concentrations of target sequences take longer to observe significant changes in reaction pH, as monitored by a 420:560 ratio. As shown in Figure 6, when the positive control sample is diluted, the rapid increase in the ratio is delayed. Using an arbitrary cutoff value of 1.1 for the 420:560 ratio, it is apparent that the undiluted control reaches that value earlier than the 1:10 dilution. Likewise, the 1:100 and 1:1000 diluted samples require longer times to reach the threshold value.

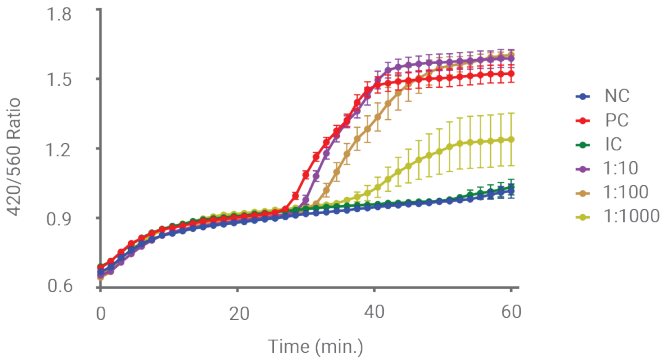

Figure 6. Effect of target concentration on LAMP assay results. Positive control sample was diluted over three orders of magnitude and assayed using a LAMP reaction. Data is displayed as a ratio of the 420 nm and 560 nm absorbance values (420:560). The positive reaction threshold ratio value is depicted as a dashed line. These data represent the mean and standard deviation of eight determinations.

Using the Agilent BioTek Gen5 microplate reader and imager software, the time to reach a defined threshold (referred to as "Time to Onset OD") can be monitored and used for further data reduction. In these reactions, the positive control was a pUC-derived DNA plasmid containing the target sequences with a known copy number. As depicted in Figure 7, when the Time to Onset OD is plotted against starting target concentration, the length of time required to reach the ratio threshold increases with a decrease in target copy number.

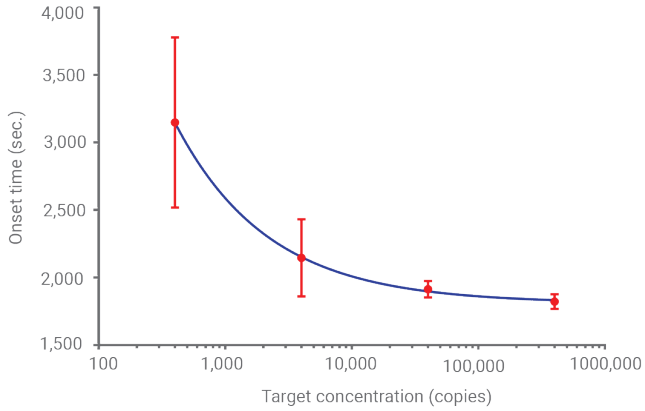

Figure 7. Effect of target concentration on onset time. The time required to reach a 420:560 ratio of 1.1 was calculated for data shown in Figure 6 and plotted as a function of the LAMP assay target copy number. These data represent the mean and standard deviation of eight determinations.

Initial results demonstrated that a change in pH can be used to monitor nucleic acid amplification using a pH-sensitive colorimetric dye such as phenol red. Spectral scans of the reaction mixture show a loss of an absorbance 560 nm peak, concurrent with an increase in absorbance centered on 420 nm. This indicates that as the LAMP reaction of a positive sample progresses, nucleotide triphosphates are incorporated into DNA polymers and the subsequent liberation of hydrogen ions results in the reaction mixture becoming acidic.

These data show that LAMP nucleic acid amplification assays can be performed and monitored using the ratio of the 420 nm and 560 nm absorbance values. Using a Synergy Neo2 multimode reader, a marked increase in the 420:560 ratio is observed about 26 minutes after initiation of the assay. Negative controls, (either lacking target DNA or having inappropriate primers) did not result in a rapid rise in ratio values. Samples that contained less starting target generally showed an increase in the 420:560 ratio, however, did so at later times than the true positive control. Plotting the time to achieve

the ratio threshold, while not truly quantitative, did provide a relative indication of target concentration. While sensitivity of LAMP has been reported to be similar to that of PCR using fluorescence detection, the use of a visual pH colorimetric change is not as efficient. Target copy numbers below 400 copies were not reliably detected using pH-based absorbance change.

Polymerase chain reaction (PCR) has long been the primary means of nucleic acid amplification. While this technology is widely used, it requires thermal cycling equipment and some mechanism of amplification detection, either through real-time monitoring (e.g., fluorescence) or post-reaction electrophoresis. In contrast to PCR, the LAMP assay is based on a *Bst* polymerase, rather than *Taq* polymerase. *Bst* polymerase has strand displacement activity and works optimally from 60 to 72 °C, meaning that the LAMP assay can be performed under isothermal conditions.[7] This scenario is ideal for plate readers, on the condition that they can achieve good temperature uniformity. Because there is no need to cycle temperature, assay runtime is reduced. The recent modifications to the *Bst* polymerase (*Bst* 2.0) have improved reverse transcriptase activity, allowing for RNA sequences to be amplified with a single enzyme.[2]

There are several ways to assess colorimetric LAMP assay progress using absorbance at 420 and 560 nm as the detection scheme. Increases in 420 nm absorbance values are indicative of the color change from red to yellow, with a decrease in pH below 7. However, the increase in peak amplitude is modest, with significant changes in the absorbance value occurring only with longer incubation. The use of the 420:560 ratio amplifies the signal change, resulting in easier and more robust detection. In this study, the use of a threshold value or a time to achieve a threshold 420:560 ratio value was more predictive than using maximal change (MaxV), or time to achieve MaxV calculation. All samples, including the negative controls, had some degree of change in their absorbance values. As a result, they also have a maximal change value, albeit generally small relative to a positive reaction, and distinguishing between a positive and negative sample is problematic.

Eventually, even negative samples will generate enough amplification products to achieve the 420:560 ratio threshold. Spurious amplification products are a well-established phenomenon in LAMP assays.[8] For this reason, along with instrumentation simplicity, LAMP assays are often run as an end point reaction, where results are determined at the end of a defined period of time. In this application note, the Synergy Neo2 was used to run the assay kinetically, as this provides more information with respect to assay performance. To that end, the assay can be performed kinetically over a limited period of time, or the kinetic analysis can be limited to a portion of the total kinetic run. The later method allows the researcher to observe more data even if it is eventually not used. The kinetic analysis data reduction features in Gen5 provide the means to set the analysis to view the entire dataset or restrict it to a limited data set within the total kinetic run.

Conclusion

The Agilent BioTek high temperature-enabled readers, such as the Synergy Neo2, are ideal platforms to run colorimetric LAMP assays at 65 °C. These readers provide high temperature incubation with uniform heating across the plate. A temperature gradient between the top and the bottom of the plate can be programmed that eliminates condensation on the underside of the plate seal. The real-time measurement of the pH-shift over time gives users confidence that the data are accurate and meaningful when developing new assays, as well as a method to understand optimal cutoff points. The Agilent BioTek Gen5 multimode reader and imager software provides reader control and automatic calculation of wavelength ratios and time to onset values, allowing users to differentiate between positive and negative controls easily. The Synergy Neo2 wavelength switching allows for closely-timed detection at 420 nm and 560 nm. Using the LAMP assay on a microplate reader, users can test samples in under 40 minutes, making this technique highly suitable as a high-throughput diagnostic tool.

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