Application Note

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Improved LAMP Assay Performance with Increased Temperature Introduction

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Abstract

Loop-mediated isothermal amplification (LAMP) is an autocycling and strand displacement DNA synthesis amplification method involving the use of the large fragment of *Bst* DNA polymerase. While typically performed at a uniform temperature of 65 °C, there are certain advantages with the use of increased temperatures when running this assay technology. This application note describes the use of the Agilent BioTek Synergy H1 multimode reader with a reaction temperature of 69 °C to reduce time required to elicit positive LAMP assay reactions.

Introduction

Loop-mediated isothermal amplification (LAMP) is an assay technology that has gained traction for its ability to detect nucleic acid sequences under several different conditions without the need for thermocyclers. LAMP is a strand displacement DNA synthesis amplification method that uses *Bst* DNA polymerase, in conjunction with four to six primers recognizing multiple distinct regions of target DNA for a highly specific amplification reaction.[1] A strand-displacing DNA polymerase initiates synthesis and two specially designed primers form "loop" structures to facilitate subsequent rounds of amplification through extension on the loops and the additional annealing of primers. The products are generally quite long (> 20 kb) and variable, formed from numerous repeats of the short target sequence. Target amplification is so extensive that several different detection modes are possible, including fluorescence and absorbance. One commonly used method of detection is a color change by a pH-dependent dye under low buffer conditions, as the polymerization reaction elicits a marked pH change.[4] While fluorescence is generally more sensitive than absorbance with regard to DNA detecting, this method allows the rapid detection of positive reactions without agents that intercalate into DNA sequences. In addition, the degree of amplification is normally so extensive that fluorescence-based technology is not required.

The LAMP assay reaction is dependent on the faithful annealing of the designed primers to their complementary target sequences. Primer design that incorporates primer length, guanine-cytosine content and unique target sequences play a critical role in reaction fidelity. The use of 5'-tails as part of the primer design (critical for LAMP assays) also improves fidelity of primer extension reactions.[6] In addition, the stringency of the annealing process, based on the ionic strength and the temperature of the reaction mixture is equally important.[5] While the ability to alter the ionic strength of the LAMP assays is constrained by *Bst* enzyme requirements, the reaction temperature can be used to increase stringency. The initial LAMP reactions utilized *Bst* large fragment polymerase. Modifications to the wild-type enzyme (*Bst* 2.0 and *Bst* 3.0) improve LAMP performance. All three polymerases are moderately thermostable DNA polymerases with strand displacement activity and can perform isothermal amplification reactions such as LAMP.

However, *Bst* 2.0 DNA polymerase is an in silico-designed homolog of *Bst* DNA polymerase, large fragment engineered for improved amplification reaction properties. *Bst* 3.0 features further improvements in amplification speed, inhibitor tolerance, thermostability and dUTP incorporation. *Bst* 3.0 also displays significantly higher reverse transcriptase activity up to 72 °C and can be used for single-enzyme RT-LAMP reactions.[7]

Experimental

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, primers, guanidine hydrochloride, nuclease-free water and template were premixed in Eppendorf tubes in a total volume of 60 µL, then split into two 25 µL portions and kept on ice. Immediately before initiation of the assay, 25 µL of 2x Softstart Master mix was added to one portion of the assay samples, as shown in Figure 1. After mixing, 5 µL aliquots (8 replicates) were 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 minute at 300 g. After centrifugation, the microplate was placed in an Agilent BioTek Synergy H1 multimode reader preheated to 65 °C with a 1 °C gradient, and the absorbance was read every 1.5 minutes at 420 nm and 560 nm using wavelength switching on the Synergy H1. After completing a 65 °C reaction, the temperature was increased to 69 °C with a 1 °C gradient and preheated for 20 minutes. The second portion of the samples was assayed using the same reading parameters, other than temperature. A positive reaction was designated when the 420:560 ratio was 1.1 or greater. At this ratio, the reaction pH has previously been shown to be above 7.5.[8]

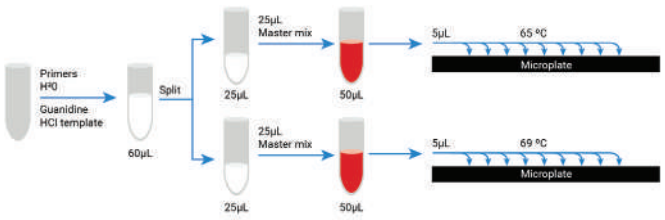

Figure 1. Lamp assay setup.

Results and discussion

The Synergy H1 can maintain elevated temperature with a high degree of accuracy. As shown in Figure 2, LAMP assays run at 65 or at 69 °C maintain the selected temperature throughout the kinetic assay. Warming from ambient to 65 °C takes place within 60 minutes. As the temperature ramps up toward the selected temperature, the thermistors are modulated to avoid overshooting the target (data not shown). At this point, equilibrium has been achieved and the assays can be run.

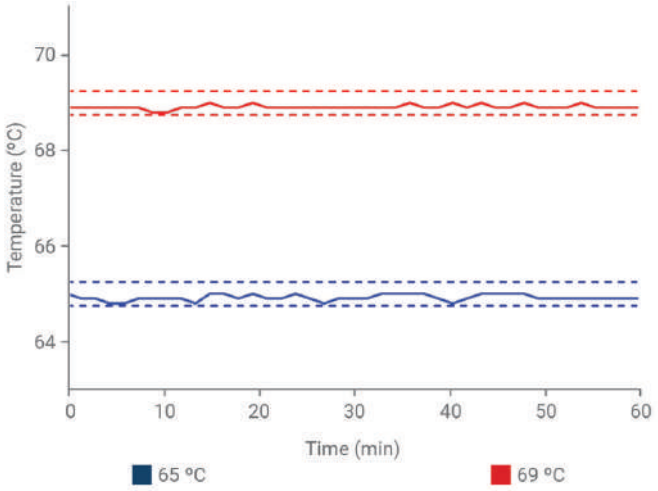

Figure 2. Temperature profiles of LAMP assays run at 65 and 69 °C. Temperature was recorded with every absorbance determination cycle during the LAMP assay kinetic runs. The solid line represents recorded temperature data, while dashed lines denote well-to-well variance specification.

The use of a slightly elevated temperature improves the assay response time, as seen in Figure 3. The positive control reaches the cutoff criteria 3.5 minutes sooner than the same sample run at 65 °C. Amplification begins almost immediately after the well samples reach temperatures at which the polymerase is active. In order to prevent spurious polymerization while setting up the assays, samples are maintained on ice. In addition, the microplate is typically at ambient room temperature. It is only after the plate with samples is in the microplate reader and warms to near 65 °C, will the primer anneal correctly and the *Bst* enzyme polymerize the DNA product. Regardless of the temperature used, the negative control did not alter the 420:560 absorbance appreciably.

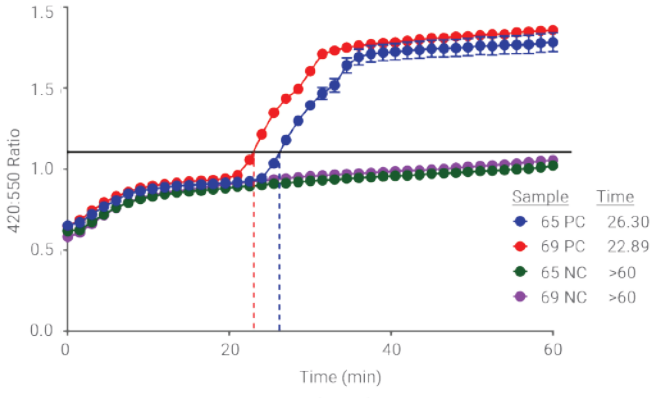

Figure 3. Comparison of kinetic LAMP assay results run at 65 and 69 °C. LAMP assays from pooled positive and negative control samples were performed at either 65 or 69 °C with a 1 °C top-to-bottom gradient to prevent condensation. The absorbances at 420 and 560 nm were ascertained at each time point and plotted as a ratio. The solid line represents a 420:560 ratio of 1.1, which was designated as a positive result. The time required to achieve a 420:560 ratio of 1.1 is also indicated. Data represent the mean and standard deviation of eight determinations.

When positive samples with known target copy number are diluted, the time necessary to achieve a 420:560 ratio greater than 1.1 increases as the target copy number decreases, as seen in Figure 3. Similar to the data observed with the undiluted positive control, running the LAMP assay at an elevated temperature reduces the time to achieve the threshold value.

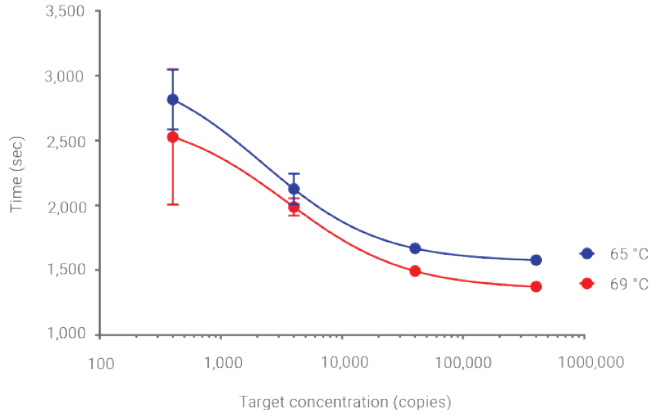

Figure 4. Comparison of onset time results for LAMP assays run at 65 and 69 °C. LAMP assays performed using positive controls were serially diluted at both 65 and 69 °C. The time necessary to achieve a 420:560 absorbance ratio was recorded and plotted as a function of starting target copy number. Each data point represents the mean and standard deviation of eight separate determinations.

These data indicate that the use of temperatures above 65 °C can improve LAMP assay performance. Elevated temperatures increase the annealing stringency of the reaction, thus reducing the likelihood of false nonspecific reactions from occurring. LAMP assays, unlike PCR, do not produce clearly defined polymerization products that can be checked using gel electrophoresis. This assay technology relies on large amounts of polymerization to elicit a visible change in the reaction tube. As such, false positive reactions can be problematic. Increasing the stringency of the reaction using reaction temperature is a method to mitigate some of this potential problem. In addition to improved assay stringency, higher reaction temperatures also warm the reactions more quickly. The reaction components and samples are generally kept on ice during assay setup; only after the reaction mixtures are complete is the microplate centrifuged and placed in the reader's incubator. Only then will the reactions warm enough for the *Bst* polymerase enzyme to have activity. As demonstrated in Figures 3 and 4, this allows for a reduction in the time necessary for positive samples to reach the positive threshold.

Conclusion

The Agilent BioTek Synergy H1 multimode reader with the extended temperature range package is a viable option to run LAMP assays at temperatures above 65 °C. The Synergy H1 uses heaters above and below the microplate, in conjunction with multiple thermistors to provide stable, uniform high temperature incubation up to 70 °C. Increasing the temperature from 65 to 69 °C increases annealing stringency and reduces the time required for a positive reaction to reach the threshold.

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