

# Quantification of Colorimetric Loop-mediated Isothermal Amplification Process

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Received: 4 January, 2019 / Accepted: 1 February, 2019 / Published online: 23 April, 2019  
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**Abstract** A We propose a novel quantitative assay for colorimetric loop-mediated isothermal amplification (LAMP) based on the shift of UV-vis spectral absorbance of Eriochrome black T (EBT) dye. The colorimetric LAMP assay provides on-and-off result of the LAMP reaction, not quantitative information. Since the quantification of the initial copy number of DNA is of importance for genetic diagnostics, we, for the first time, demonstrated how to quantify the DNA in the EBT mediated LAMP reaction. Due to the structural change of the EBT-Mg complexes, the color of the LAMP mixture is changed from violet to sky blue as the LAMP reaction proceeds. We noticed that the maximum absorption peak before and after the LAMP reaction is distinct, and accordingly shifted from 570 nm to 640 nm. We utilized the ratio between two particular absorbance ( $A_{640}/A_{570}$ ) of EBT as an indicator for the LAMP process, and plotted it versus the reaction time. We used serially diluted DNA samples and produced quantitative calibration curves (the threshold time versus the initial copy number of DNA). Therefore, we could quantify the copy number of template DNAs in the EBT-mediated colorimetric LAMP reaction simply by measuring the two UV-vis absorption values at 570 nm and 640 nm.

**Keywords:** Eriochrome black T (EBT), loop-mediated isothermal amplification (LAMP), quantitative analysis, the absorbance ratio ( $A_{640}/A_{570}$ ), UV-vis spectral absorbance

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## Introduction

Gene amplification is one of the most vital and invaluable techniques to study primary molecular structure and its function in various biotechnological applications such as foodborne pathogen detection, diagnosis of infectious diseases, and target gene expression, etc<sup>1-3</sup>. Polymerase chain reaction (PCR) is considered as a gold standard method for sensitive and selective gene amplification. In particular, real-time PCR, which is equipped with a fluorescence detector and a fluorescence probe, is used for the quantitative analysis of the starting copy number of nucleic acids<sup>4</sup>. However, those conventional methods rely on the bench-top thermocycler, prolonged thermal cycling process, strict thermal gradient settings, and extensive fluorescence detector<sup>5-8</sup>. These drawbacks make the conventional quantitative PCR unsuitable for the point-of-care (POC) clinical diagnostics.

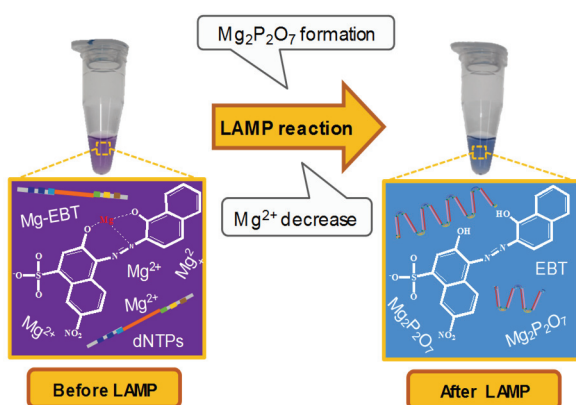
Recently, a variety of isothermal amplification methods were developed. These proceeded under a constant mild temperature with specifically designed primers and particular enzymes, thereby making them more adequate for POC molecular diagnosis. Among them, loop-mediated isothermal amplification (LAMP) reveals high sensitive and specific characteristics using four to six primer sets to recognize a target gene<sup>9,10</sup>. LAMP occurs at 60-65°C in 30-60 min through the strand displacement amplification with the support of Bst polymerase, and simple water bath or a heating block can be used for maintaining a constant temperature<sup>9,11,12</sup>. Regarding the quantitative analysis of the LAMP, fluorescence based detection using calcein<sup>13</sup>, SYTO-9<sup>14</sup>, SYBR Green I<sup>15,16</sup> or turbidity based real-

time monitoring<sup>17-19</sup> were reported. Despite high accuracy and sensitivity of quantitative analysis, the requirements of the expensive fluorescence probe and the bulky optical set-up and readout still have much room for improvement to be used for POCT.

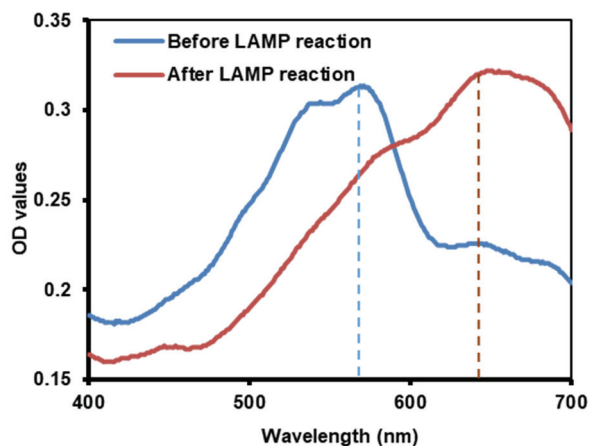
Instead of using the high-priced fluorescence probes, colorimetric detection using cheap metal indicators such as hydroxynaphthol blue (HNB)<sup>20,21</sup> or eriochrome black T (EBT)<sup>10</sup> can be employed for determining the success or failure of LAMP reaction. For example, in case of the EBT, the EBT combined with  $Mg^{2+}$  ions displays violet color. When the LAMP reaction proceeded successfully, pyrophosphates were generated as the primer is extended. The resultant pyrophosphates have strong affinity with  $Mg^{2+}$  ions, so  $Mg^{2+}$  ion in the initial Mg-EBT complexes were subtracted by the released pyrophosphates and the  $Mg_2P_2O_7$  complexes were precipitated. As a result, the initial EBT structure combined with  $Mg^{2+}$  ions was changed, making the color of the solution turned from violet to blue (Figure 1)<sup>10</sup>.

Although the colorimetric detection assay using the metal indicators is simple, cost-effective and convenient for LAMP reaction, the quantitative analysis of the colorimetric LAMP is rarely investigated. In this study, we propose a novel quantitative LAMP methodology based on the UV-vis spectral absorbance of EBT.

We note that a complexometric indicator that forms a complex with  $Mg^{2+}$  causes color change from original violet to sky blue depending on the metal ion concentration, resulting in the absorbance shift. As shown



**Figure 1.** Schematics of the EBT-based colorimetric detection of LAMP products. Initially, the high concentration of  $Mg^{2+}$  causes the purple-coloring mixture as the complexes of EBT- $Mg^{2+}$  are dominant. When pyrophosphates are released during the LAMP reaction,  $Mg^{2+}$  ions are combined with pyrophosphates to change the structure of EBT- $Mg^{2+}$ , turning the color from violet to sky blue.



**Figure 2.** The UV-vis spectrum of the LAMP mixture before and after the LAMP reaction. The initial LAMP mixture (violet color) shows the maximum peak at 570 nm, which is shifted to 640 nm after the LAMP reaction (blue color).

in Figure 2, the LAMP mixture before the LAMP reaction displays the maximum peak at 570 nm, while that after the LAMP reaction shows the maximum peak at 640 nm. The peak at the wavelength of 570 nm gradually decreases as the LAMP reaction proceeds. On the other hand, the peak at 640 nm gradually increases as the color of the LAMP mixture is converted from violet to sky blue. Therefore, we can monitor the process of the LAMP reaction and quantify the initial copy number of the LAMP product by comparing the peak intensities of the two wavelengths.

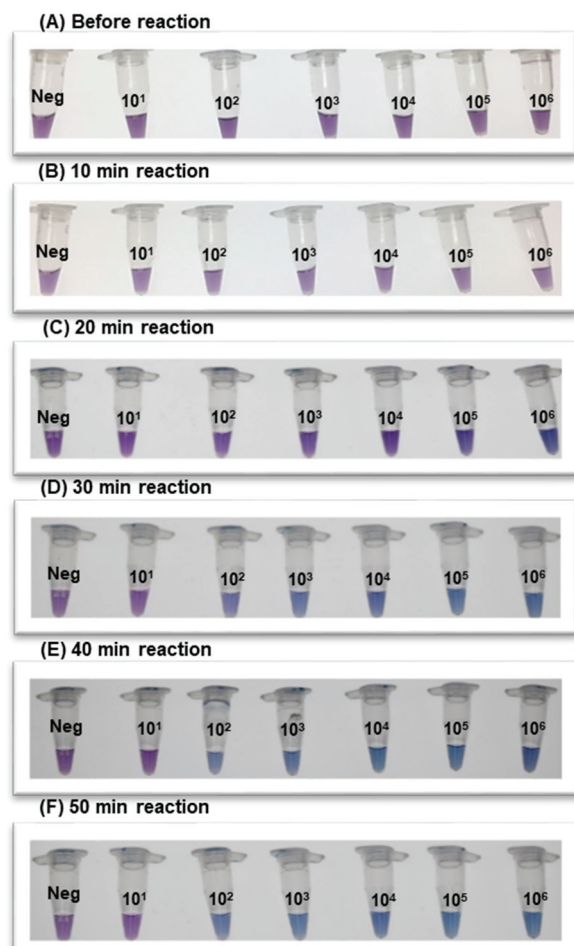
## Results and Discussion

### Limit of Detection of the Colorimetric LAMP Reaction

To demonstrate our hypothesis of the quantitative analysis of LAMP based on the UV-vis absorption at two wavelengths, we performed the LAMP reaction with a variety of the DNA copy numbers and checked the color change as well as the optical intensity. We used a template of Tim-3\_HAVCR2 gene, which is one of the biomarkers of immune-rejection of kidney transplantation, as a model, and the amount of the DNA template was serially diluted from  $10^6$  to  $10^1$  copies/ $\mu$ L. The sequence information of the template and primers is presented in Table 1. The reaction tubes were incubated at 63°C for 50 min in the water bath and the Eppendorf tubes were taken out every 10 min for digital image capture to record the color changes as shown in Figure 3.

**Table 1.** The sequence information of the LAMP primer sets for targeting Tim-3\_HAVCR2 gene.

No	Primer	Sequencing
1	F3	5'-ATACAGAGCGGAGGTCGG-3'
2	B3	5'-GGTCAGGGACACATCTCCTT-3'
3	FIP	5'-GACAGGCTCCTTTGCCCCAGATGCCTATCTGCCCTGCTT-3'
4	BIP	5'-GGCAACGTGGTGCTCAGGACTGCGGAAATCCCCATTTAGC-3'
5	LF	5'-AGACGGGCACGAGGTTC-3'
6	LP	5'-GGATGTGAATTATTGGACATCCAGA-3'

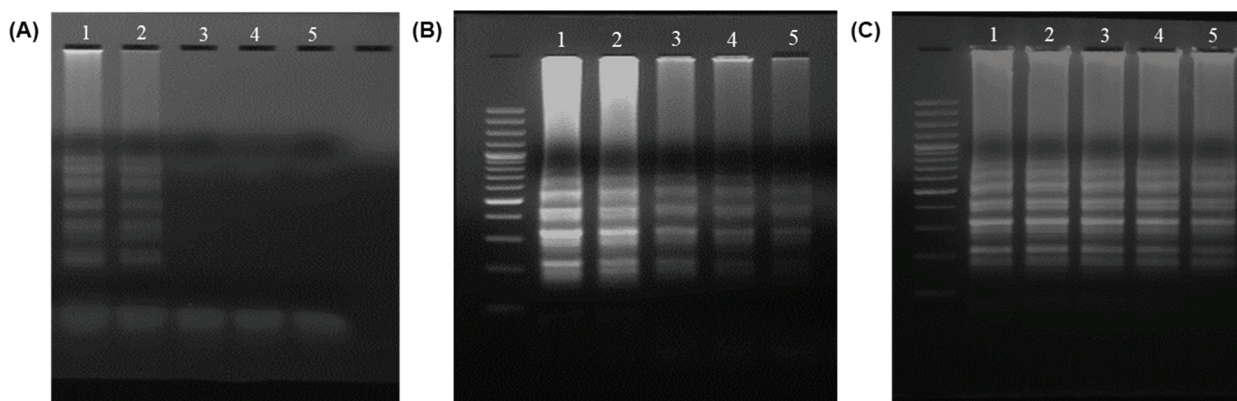
**Figure 3.** (A-F) The color change of the LAMP mixture throughout the LAMP reaction with a time interval of 10 min.

The initial color of the LAMP mixture was violet due to the excessive Mg-EBT complex formation (Figure 3A). There was no color change recorded in all tubes after 10 min reaction (Figure 3B). In the next 10 min, the tube containing  $10^6$  DNA copy number had color changed from violet to indigo, and the one having  $10^5$  DNA copy number exhibited a transition color between violet and indigo, while others were still

in violet (Figure 3C). After the reaction of 30 min, the tubes containing  $10^2$  to  $10^6$  DNA copy number completely showed sky blue color, a positive result caused by the decrease of  $Mg^{2+}$  concentration due to the reaction with  $P_2O_7^{4-}$  ions released from dNTP (Figure 3D). However, after 40 min and 50 min reaction, there was no more color change, and the tube containing 10 copy number remained violet. These results imply that the LAMP reaction using Tim-3\_HAVCR2 gene exhibited the observable color change within 30 min by EBT-based colorimetric detection and the limit of detection was  $10^2$  DNA copy number, which is comparable to the fluorescence based assay<sup>17</sup>.

### Gel Electropherogram of the LAMP Products

We also confirmed the colorimetric data of Figure 3 by the agarose gel electrophoresis (1.7% with 100V in 30 min). The gel electropherogram of the LAMP products after 10 min, 15 min and 20 min of reaction time was displayed in Figure 4A, Figure 4B, and Figure 4C, respectively. In 10 min, only two tubes having  $10^5$  and  $10^6$  DNA copy number showed slight ladder-like bands. In 15 min, the gel result revealed the clear disparity of bands in the 7 lanes (Figure 4B), although all the tubes exhibited the same visual violet color. The band pattern in the gel electropherogram was identical, but the band intensities became gradually stronger in proportional to the copy number. After 20 min, the amplification of LAMP reached the plateau status, showing similar band patterns in terms of intensity except the negative control and the case of  $10^1$  DNA copy number (Figure 4C). By comparison of the results between the EBT based colorimetric detection and the fluorescence based gel electrophoresis, it was clear that naked eyes are difficult to differentiate slight changes of color. Therefore, we utilized the UV-vis spectrophotometer to determine the transition of the color by observing the alternation of the peak intensities at 570 nm and 640 nm, which are eventually used for the quantification of the LAMP reaction.



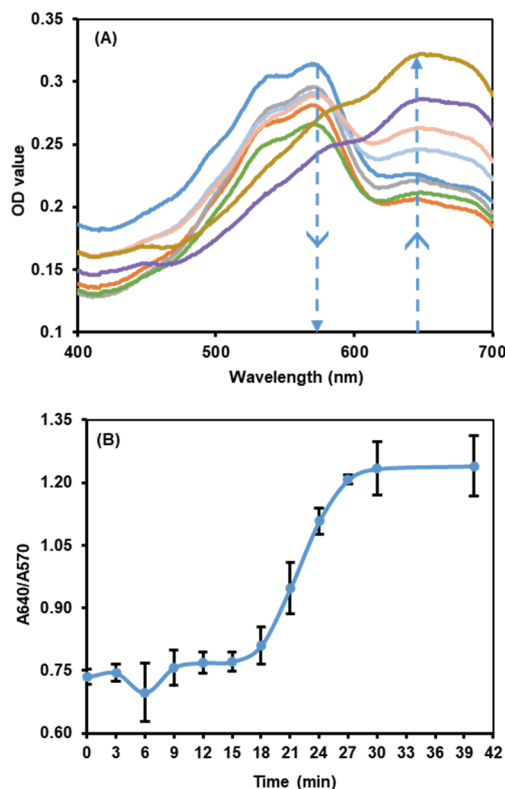
**Figure 4.** Gel electropherogram of the LAMP products after (A) 10 min, (B) 15 min and (C) 20 min running. Lane 1:  $10^6$  DNA copies number, Lane 2:  $10^5$  DNA copies number, Lane 3:  $10^4$  DNA copies number, Lane 4:  $10^3$  DNA copies number, Lane 5:  $10^2$  DNA copies number.

**Quantitative Calibration Curves by Plotting the Threshold Time Versus the Initial Copy Number of Templates**

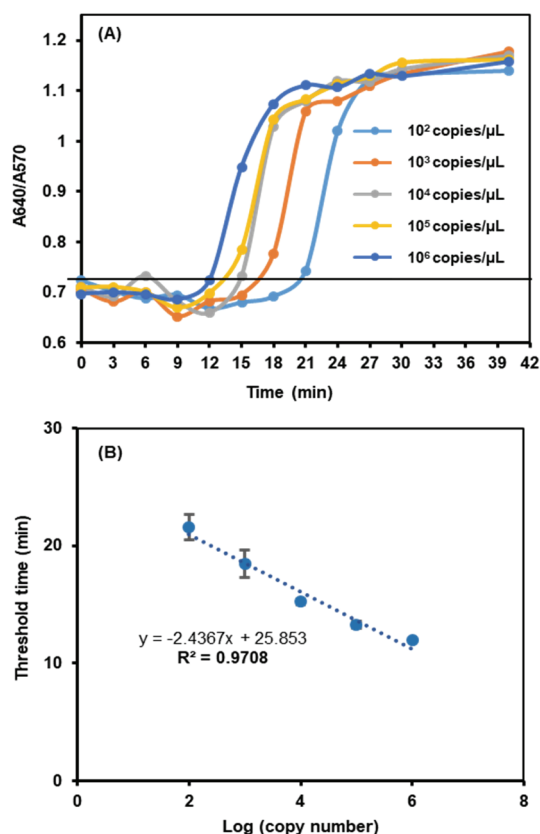
In the experiment for quantitative LAMP reaction, 70  $\mu$ L of a reaction mixture were loaded into the UV-cuvette, prior to the addition of 50  $\mu$ L vapor lock for preventing the evaporation during the LAMP reaction in the water bath at 65  $^{\circ}$ C for 40 min. The incubated cuvette was taken out for UV-vis measurement in the range of wavelength from 400 nm to 800 nm every 3 min. The EBT-based color change of the reaction mixture in the cuvette can be visually detected and recorded in the UV-vis spectrum. As the mixture color changes slowly from violet to sky blue, the  $A_{570}$  value tends to decrease, whereas the  $A_{640}$  value starts to increase (Figure 5A). Hence, the absorbance ratio between the two values ( $A_{640}/A_{570}$ ) has a tendency to rise up as the time goes on, showing the similar profile as the real-time PCR. Figure 5B shows the constant profile until the first 15 min, the exponential increase up to 27 min, and the plateau state until 40 min. Therefore, the  $A_{640}/A_{570}$  ratio-based plot versus reaction time has a potential to be used for real-time detection of LAMP reaction, because the starting point for the transition period should be different depending on the initial copy number of the DNA.

In order to obtain the quantitative calibration curves, we repeated the above experiment with serially diluted DNA solution, ranging from  $10^2$  to  $10^6$  DNA copy number. Triplicated experiments were performed and the representative graph is shown in Figure 6A. The threshold line was set at 0.724 of the  $A_{640}/A_{570}$  ratio, at which the LAMP reaction reaches the  $A_{640}/A_{570}$  ratio value above the background level. When  $10^6$  DNA copy number was used, the threshold time was

determined at 12 min. As the initial copy number increased, the threshold time became longer. In case of  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$  DNA copy number, the threshold time was around 13 min, 15 min, 19 min, and 23 min, respectively. Since we obtained the threshold time data depending on the initial copy number of DNA, we



**Figure 5.** (A) The  $A_{570}$  value decreases, whereas the  $A_{640}$  value starts to increase during LAMP reaction. (B) The plot of the ratio of  $A_{640}/A_{570}$  along with the LAMP reaction time, which is similar to that of real-time PCR.



**Figure 6.** (A) The plot of the A<sub>640</sub>/A<sub>570</sub> ratio versus the LAMP reaction time depending on the initial copy number of DNA from 10<sup>1</sup> to 10<sup>6</sup>. (B) Correlation between the logarithm of DNA copy number and the threshold reaction time, which can be used for quantifying the colorimetric LAMP reaction.

could plot the threshold time versus the logarithm of initial copy numbers. The correlation between the threshold time and the copy number of DNA templates was presented in Figure 6B. Therefore, the EBT-mediated colorimetric assay of the LAMP reaction could be quantified based on the ratio of the two absorbance values at A<sub>640</sub> nm (sky blue color) and A<sub>570</sub> nm (violet color).

## Conclusion

In summary, we, for the first time, demonstrated how the shift of specific absorbance peaks of EBT dye can be utilized for the development of EBT-based quantitative LAMP reaction. The initial state of the LAMP mixture was violet that shows the maximum peak at 570 nm, while the final color of the LAMP mixture after successful LAMP reaction was sky blue which displayed the maximum peak at 640 nm. Therefore, we could monitor the progress of the LAMP reaction

by observing the peak intensity of the two wavelengths. Based on this phenomenon, we could plot the A<sub>640</sub>/A<sub>570</sub> ratio along with the reaction time, which revealed a similar curve of real-time PCR. Thus, quantitative calibration curves could be generated depending on the initial copy number, and the plot of the threshold time versus the copy number of DNA was produced. The combination of the UV-vis spectrophotometer with the colorimetric LAMP assay would make it an ideal platform for point-of-care genetic diagnostics.

## Materials and Methods

### DNA Template and Primers Design

Tim-3\_HAVCR2 templates (500 bp) (Integrated DNA Technologies (IDT), South Korea) were synthesized and stored in a TE buffer. Serially diluted DNA samples, whose concentration ranged from 10<sup>1</sup> to 10<sup>6</sup> copies number/μL, were prepared. Six primer sets of LAMP were the forward and backward outer primers (F3 and B3), the forward and backward inner primers (FIP and BIP), and the forward and backward loop primers (LF and LP), which were designed by Primer-Explorer V5 software. Detailed sequence information is listed in Table 1.

### Preparation of the LAMP Mixture

For the eppendorf tube experiments, the total volume of the LAMP reaction mixture was 35.5 μL containing 0.85× Isothermal Amplification buffer (3 μL), 5 mM MgSO<sub>4</sub> (1.8 μL), 1.3 mM each dNTP (18 μL), 0.14 μM each of F3 and B3 primer (1 μL each), 0.28 μM each of LF and LB primer (1 μL each), 1.13 μM each of FIP and BIP primer (1 μL each), 1.2 μL of 3 mM Eriochrome Black T (EBT), DNA template (1 μL) and 4.5 μL of 8 U/mL of Bst Polymerase 2.0. For the cuvette experiments, we doubled the amount of the eppendorf tube experiments.

### Gel Electrophoresis Analysis

To investigate the LAMP product by the gel electrophoresis, we prepared the serially diluted DNA samples from 10<sup>2</sup> to 10<sup>6</sup> DNA copy number. The composition of the reaction mixture was same as above. The LAMP cocktail was incubated in the peltier thermal cyclers (Bio-Rad, ALD-1234, USA) at 63°C and then the LAMP products were analysed in the agarose gel electrophoresis. The LAMP reaction time was 10 min, 15 min and 20 min. We compared the band intensities depending on the incubation time.

### Quantitative Analysis of the LAMP Products

For the quantitative analysis of the LAMP products, serially diluted DNA samples (from  $10^1$  to  $10^6$  copies number/ $\mu\text{L}$ ) were prepared and performed for the LAMP reaction at  $65^\circ\text{C}$  for 40 min in the UV-vis cuvettes (volume 70  $\mu\text{L}$ ). During the LAMP reaction, the cuvettes were incubated in a water bath (JSWB-22T, 080526-07, Korea). We measured the UV-vis spectrum (Shimadzu UV-VIS Spectrophotometers, UV-2450, Japan) from 400 nm to 800 nm at the interval of 3 min. The raw data were obtained by an excel file and we calculated the absorption intensities at the wavelength of 570 nm and 640 nm, producing the ratio of the two values ( $A_{640}/A_{570}$ ).

**Acknowledgements** This work was supported by the Engineering Research Center of Excellence Program of Korea Ministry of Science, ICT & Future Planning (MSIP)/National Research Foundation of Korea (NRF) (2014R1A5A1009799) and by a grant of the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (grant no. HI13C1232).

**Conflict of Interest** There is no conflict of interest in this study.

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