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Development of Phenol Red Colorimetric RT-LAMP Assay in High-Buffered SARS-CoV-2 Sample

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ABSTRACT

Colorimetric RT-LAMP Assay is a diagnostic method that has attracted much attention because of its rapidity, simplicity, and accuracy compared to other disease diagnosis methods. Despite having many advantages, the RT-LAMP Colorimetric Assay has disadvantages, especially for kits that use phenol red as an indicator. The disadvantage derives from the input RNA/DNA samples containing high buffer levels, which causes no color change and false-negative results. This study aimed to develop and optimize the colorimetric RT-LAMP method on high-buffered SARS-CoV-2 RNA samples. We found that a temperature of 69°C for 50 minutes with the addition of post-treatment in the form of heating at 80°C for 10 minutes is an optimal condition for high-buffered SARS-CoV-2 samples. The condition proved effective in changing the result's color from red (negative) to yellow (positive). We also classified the analysis results based on the correlation between the Cycle threshold (Ct) value of SARS-CoV-2 viruses and the Optical Density (OD) value, which was quantified using a spectrophotometer at 415 nm (with a correlation value of -0.9084), where yellow color indicated Ct below 20, amber color indicated Ct between 20 and 30, orange color indicated Ct between 30 and 35, and red indicated Ct more than 35 (negative). In conclusion, this study successfully detects the SARS-CoV-2 virus in high-buffered samples using Phenol Red Colorimetric RT-LAMP Assay, with a sensitivity of 85% for Ct Cutoff 40.

1. Introduction

The increasing cases of the SARS-CoV-2 virus have prompted further development of science and technology to overcome it, especially in Indonesia. One of the technologies that continue to be developed is the diagnostic method. Many diagnostic methods have been developed to detect the SARS-CoV-2 virus, but only a few diagnostic methods have received authorization from the Indonesian Minister of Health. Several diagnostic methods that have been authorized include Rapid Antigen or Antibody Test, Polymerase Chain Reaction (PCR), and the recently popular Reverse Transcriptase Loop-Mediated Isothermal Amplification (RT-LAMP) (KMK 2020).

RT-LAMP, is a molecular diagnostic method first introduced by Mori and Notomi (2009). Generally, the RT-LAMP method is widely used as an alternative to RT-qPCR because the method is simple, inexpensive, easy to operate, and has a fast processing time. This diagnostic method has been widely used to detect various diseases, such as tuberculosis (Bentaleb *et al.* 2016; Iwamoto *et al.* 2003; Nagai *et al.* 2016), Zika virus (Priye *et al.* 2017; Song *et al.* 2016), SARS virus (Notomi *et al.* 2004; Poon *et al.* 2004), MERS virus (Lee *et al.* 2017; Shirato *et al.* 2018), as well as for the SARS-CoV-2 virus (Huang *et al.* 2020; Janikova *et al.* 2021; Tanner and Zhang 2021; Yang *et al.* 2020).

The use of the RT-LAMP method to detect the SARS-CoV-2 virus has been developed previously, including analyzing the results. Several analytical methods developed for the RT-LAMP method are electrophoresis, fluorometry, turbidimetry, and colorimetry (Augustine *et al.* 2020). The colorimetric RT-LAMP method is the most preferred because it is one of the fastest and simplest RT-LAMP analysis methods for detecting the presence of the SARS-CoV-2 virus. This method utilizes a color change indicator to determine whether a person is positive or negative for SARS-CoV-2 from the assay result. Many

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color indicators can be used for the colorimetric RT-LAMP method. One of the most popular indicators is a pH-dependent indicator, Phenol Red (Zhang *et al.* 2020).

Although known to be fast and simple, the Phenol Red colorimetric RT-LAMP has several problems. According to Rabe and Cepko (2020), the Phenol Red colorimetric RT-LAMP test for detecting the SARS-CoV-2 virus depends on the input sample, which often requires adjustment of the sample pH and buffer capacity before it can be applied. Excess buffer capacity in the sample can cause delays in color change in the test sample, which results in the formation of false negative results and reduces the sensitivity of the colorimetric RT-LAMP method.

This study aims to develop and optimize the RT-LAMP reaction in a high-buffered SARS-CoV-2 sample. This study will also try to quantify the Phenol Red Colorimetric RT-LAMP assay result based on the correlation between optical density (OD) with Ct value.

2. Materials and Methods

2.1. Sample Preparation and Ethical Statement

The sample used in this research is the Nasopharyngeal/Oropharyngeal archived specimens collected by the Primate Research Center (PSSP) LPPM IPB, Bogor, stored in the VTM and has been approved by the PSSP LPPM IPB to be used in this study. The sample used in this study is anonymous and cannot be directly identified or identified with the subject. Human Ethics Committee LPPM IPB, Bogor waive such informal consent for this research. Ethics approval was obtained from the Human Ethics Committee LPPM IPB, Bogor (757/IT3.KEPMSM-IPB/SK/2022)

2.2. RT-qPCR

The samples used in this study were RNA samples confirmed positive and negative for SARS-CoV-2 using the previous diagnostic technique (RT-qPCR). The RT-qPCR procedure in this study follows the procedure of the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel.

2.3. Primer for RT-LAMP Reaction

The RT-LAMP primer in this study was based on a primer previously designed by Jamwal *et al.* (2021), which is based on the N gene region of the SARS-CoV-2 viral sequence. The biotechnology company 1st Base (Malaysia) synthesized and purified this primer. The information on the primers used in this study can be seen in Table 1.

2.4. RT-LAMP Reaction for Detection of SARS-CoV-2

The RT-LAMP reaction process was modified by the manufacturer's protocol (New England Biolabs Inc.). The RT-LAMP reaction was carried out in a 25 µL reaction mixture containing 6 µL RT-LAMP Primer Mix (0.2 µM F3 primer, 0.2 µM B3 primer, 1.6 µM FIP primer, 1.6 µM BIP primer, 0.4 µM Loop F primer, 0.4 µM Loop B primer), 12.5 µL WarmStart Colorimetric RT-LAMP 2X Master Mix, 1.5 µL dH_2O, 2.5 µL Guanidine HCL, and 2.5 µL RNA sample. In this study, samples were optimized in a water bath with temperatures of 65°C, 67°C, 69°C, and 71°C at various amplification times (10 min, 20 min, 30 min, 40 min, 50 min, and 60 min). The samples were then given post-treatment by heating at a temperature of 80°C for 10 minutes.

2.5. RT-LAMP Results Analysis

The reaction results were analyzed by two qualitative methods (Xie *et al.* 2012): the colorimetric method (color change) and agarose gel electrophoresis. Analysis with the colorimetric method was carried out by observing the color changes in the tube after the RT-LAMP reaction occurred. If the results show a yellow color, the sample is declared positive for SARS-CoV-2, and if the sample is red, the sample is

Table 1. N gene primer information used in our study (Jamwal *et al.* 2021)

Gene	Protein	Genomic coordinate		Name of primer	Sequence (5'-3')
		Start	End		
N	N	28.354	28.569	F3	CCAGAATGGAGAACGCAGTG
				B3	CCGTCACCACCACGAATT
				FIP	AGCGGTGAACCAAGACGCAGGGCGCGATCAAAACAACG
				BIP	AATTCCCTCGAGGACAAGGCGAGCTCTTCGGTAGTAGCCAA
				LF	TTATTGGGTAAACCTTGGGGC
				LB	TTCCAATTAACACCAATAGCAGTCC

declared negative for SARS-CoV-2. The analysis using agarose gel electrophoresis was carried out with an electrophoresis device using 1.8% agarose, a voltage of 100 V for 45 minutes, and visualized using a UV Transilluminator. If the sample shows a band, the sample is declared positive for SARS-CoV-2, but if the sample does not, the sample is declared negative for SARS-CoV-2 (Zhang *et al.* 2020).

2.6. Colorimetric Quantification of RT-LAMP Test Results

For quantitative analysis, the results of colorimetric RT-LAMP analysis were quantified based on optical density absorption measured using a microplate reader. A total of 25 µL of the reacted sample was duplicated. The Optical Density (OD) was measured at wavelengths of 405 nm, 415 nm, 450 nm, and 490 nm with a spectrophotometer where the wavelength showed the highest OD value was chosen as a reference wavelength for a positive result (yellow color) (pH 6). From the data obtained, the sensitivity is then calculated using the following equation:

$$\text{Sensitivity} = \frac{\text{Amount of true positive}}{\text{Amount of true positive} + \text{Amount of false negative}}$$

2.7. Statistical Analysis

The results of the RT-LAMP test were processed descriptively and compared with the results of the RT-qPCR test. Standard curves are made at each wavelength using the Microsoft Excel program to interpret the test results with a spectrophotometer. Then, a regression analysis was performed to determine the wavelength with the best regression model in determining the positive and negative results of SARS-CoV-2 from the tested samples. Positive results are then categorized based on the severity of SARS-CoV-2 in the sample.

3. Results

3.1. Colorimetric RT-LAMP Temperature Optimization Results on SARS-CoV-2 Samples with High Buffer Levels

In this study, temperature optimization was carried out at temperatures between 65°C, 67°C, 69°C, and 71°C for 1 hour. Based on the colorimetric analysis, the results of the RT-LAMP optimization showed that the temperature of 69°C for 1 hour showed the best color change for positive samples from red to orange. At a temperature of 65°C, there was no color change in the positive samples of SARS-CoV-2. These results can be seen in Figure 1.

Based on the results of the electrophoresis test, it appears that the results of the RT-LAMP reaction indicated that samples that are positive for SARS-CoV-2 show the formation of a ladder-shaped band at all temperatures (65°C, 67°C, 69°C, and 71°C) while in samples that are declared negative for SARS-CoV-2 shows no band formation. These results can be seen in Figure 1.

3.2. Colorimetric RT-LAMP Test Results on SARS-CoV-2 Samples with High Buffer Levels After Post-Treatment Addition

After optimizing the reaction temperature, the effect of post-treatment addition (heating at 80°C for 10 minutes) on the results of the RT-LAMP reaction at 69°C for 1 hour was tested. The results obtained from the addition of post-treatment can be seen in Figure 2. In Figure 2, for positive SARS-CoV-2 samples, it can be seen that the color change from red to yellow is visible in the reaction product with the addition of post-treatment compared to the reaction product without the addition of post-treatment.

After obtaining the optimum conditions for the RT-LAMP reaction in the samples with high buffer content (optimum temperature with post-treatment), further optimization was carried out on the amplification time for the colorimetric RT-LAMP reaction with the reaction conditions following the conditions that have been optimized in this study. The results of the optimization of the amplification time can be seen in Figure 3. In Figure 3, it can be seen that the results began to change color to yellow at the amplification time of 50 minutes.

3.3. Quantification Results of Colorimetric RT-LAMP Test Results with Spectrophotometer

The results of the RT-LAMP test with the addition of post-treatment also showed color differences based on certain Cycle threshold (Ct) value groups. The Ct value is a value that represents the number of cycles of amplification in the RT-qPCR in which the virus can be detected; in this study, the SARS-CoV-2 virus was present in the sample. These results can be seen in Figure 4.

This study quantified the RT-LAMP colorimetric test results using a spectrophotometer at 405 nm, 415 nm, 450 nm, and 490 nm. The result is obtained

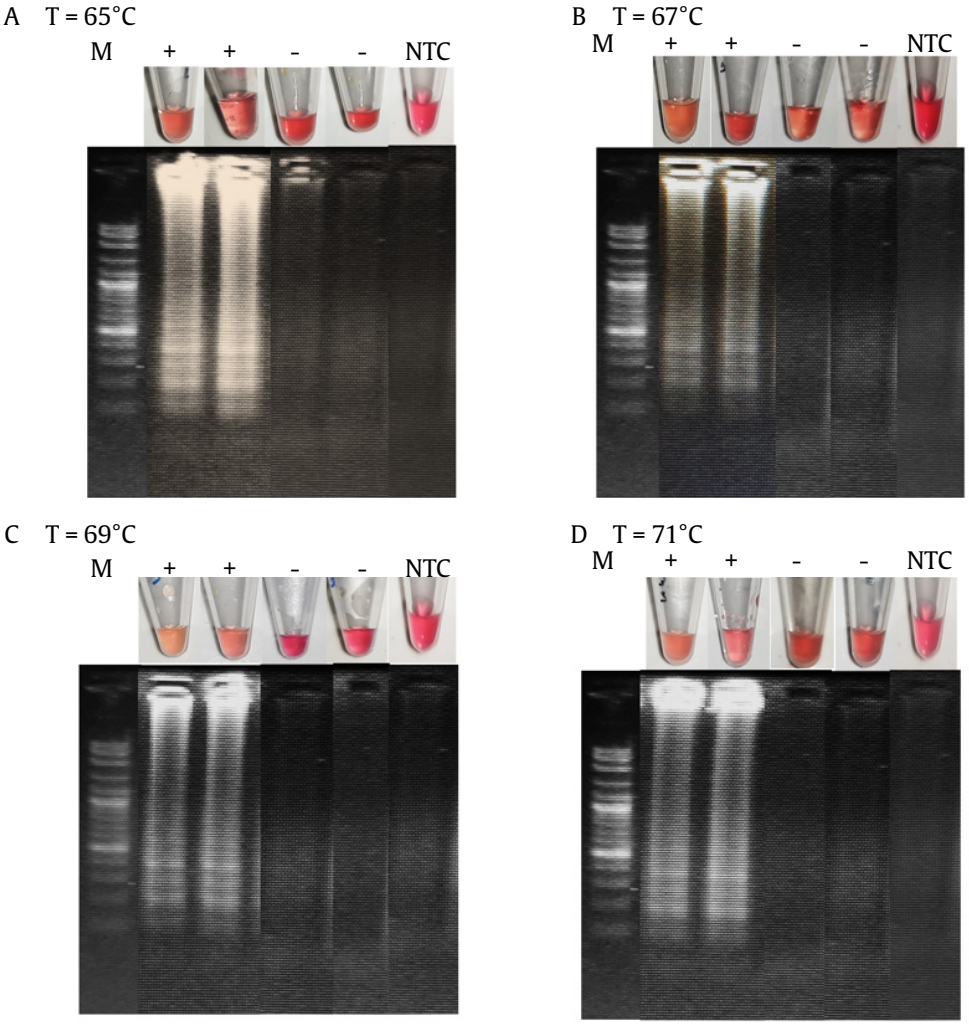

Figure 1. Comparison of RT-LAMP optimization results based on colorimetric method and electrophoresis test: (A) RT-LAMP results at 65°C, (B) RT-LAMP results at 67°C, (C) RT-LAMP optimization results at 69°C, and (D) RT-LAMP optimization results at 71°C. T: temperature (°C), +: SARS-CoV-2 positive sample, -: SARS-CoV-2 negative sample, NTC = negative control, M: DNA marker 100 bp

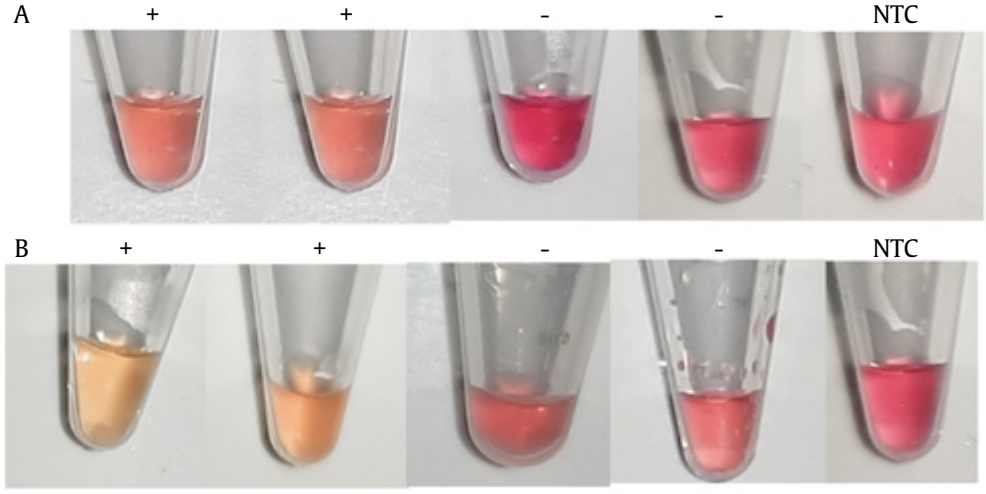

Figure 2. RT-LAMP test results. (A) Colorimetric RT-LAMP analysis results at 69°C for 1 hour without post-treatment, (B) colorimetric RT-LAMP analysis results at 69°C for 1 hour with the addition of post-treatment. +: SARS-CoV-2 positive sample, -: SARS-CoV-2 negative sample, NTC = negative control

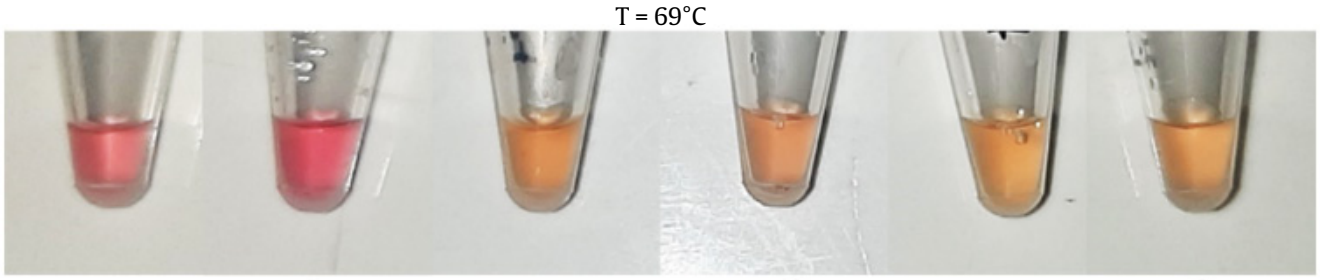

Figure 3. Comparison of colorimetric test results from optimization results of RT-LAMP reaction amplification time at (A) colorimetric test results at 10 minutes, (B) colorimetric test results at 20 minutes, (C) colorimetric test results at 30 minutes, (D) colorimetric test results at 40 minutes, (E) colorimetric test results at 50 minutes, and (F) colorimetric test results at 60 minutes. T = temperature (°C)

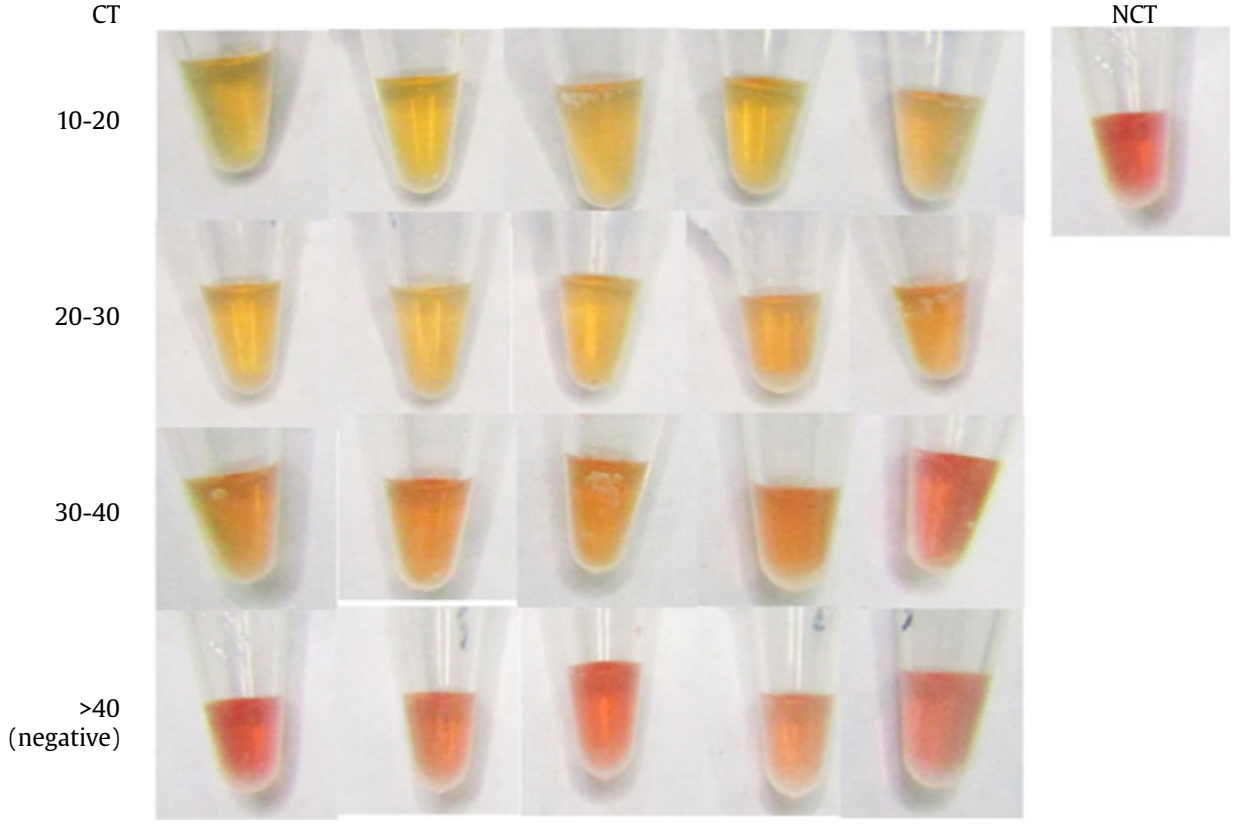

Figure 4. Colorimetric RT-LAMP test results in several CT ranges. The result shows yellow for CT ranges between 10-20, amber for CT ranges beween 20-30, orange for CT ranges between 30-40, and red for CT more than 40 (negative). CT = cycle threshold, NTC = negative control

as an Optical Density (OD) value. These samples were also tested before using RT-qPCR, and the results are grouped into Ct ranges: Ct 10-20, Ct 20-30, Ct 30-40, and Ct more than 40. The OD and Ct values are then correlated, and the standard curve was made based on this correlation. The results can be seen in Figure 5.

These results were then tested statistically with the ANOVA, correlation, and regression tests. The statistical test results can be seen in Appendix 2. Based on the results obtained, the best regression model was determined for quantifying the colorimetric RT-LAMP test results based on the correlation between OD values and Ct values. The results are then categorized based on the severity of the SARS-CoV-2 virus and are summarized in Table 2.

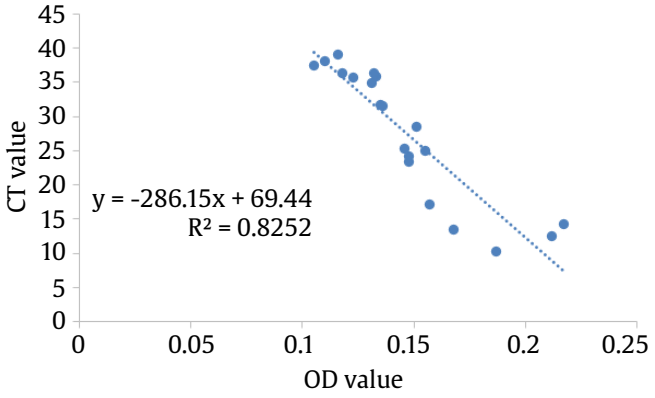

Figure 5. Graph of the relationship between Ct values and OD values on quantification results with a spectrophotometer at a wavelength of 415 nm. The figure shows that the OD value and the Ct value have a negative correlation; when the Ct value increases, the OD value will decrease

Table 2. Results of grouping colorimetric RT-LAMP test results based on severity levels of SARS-CoV-2 in the sample. This result is measured at 415 nm as the wavelength with the best regression model

Ct	Color	OD value	Interpretation
10-20	Yellow	>0.155	+++
20-30	Amber	0.155-0.146	++
30-40	Orange	0.146-0.131	+
Negative (>40)	Red	<0.123	-

4. Discussion

In this study, the colorimetric RT-LAMP method was retested at a temperature of 65°C and an amplification time of 30 minutes. The primer used in this study is a primer that targets the N gene region, which was previously developed by Jamwal *et al.* (2021). This primer was chosen because it has a much better sensitivity than other primers. This primer also has a very low mutation rate so that it can be used for any variants of the SARS-CoV-2 virus. In this study, phenol red was chosen as changing color indicator. Phenol red is used because it has a much clearer color difference than other indicators, which can reduce bias in the results visualization (Rabe and Cepko 2020).

4.1. Optimization of Colorimetric RT-LAMP Amplification Reaction on Samples with High Buffer Content

The study results found that the temperature of 65°C for 30 minutes did not change the color of the RT-LAMP reaction product. At the beginning of the study, it was suspected that this was caused by the amplification time being too short, so 60 minutes was used to increase the sensitivity of the test results (Nawatttanapaiboon *et al.* 2021). Based on the results of our tests, increasing the amplification time by up to 60 minutes did not change the indicator's color in the sample.

An electrophoresis test with 1.8% agarose, a voltage of 100 V for 45 minutes, was conducted to determine whether the enzyme was working. The electrophoresis method is one of the qualitative analysis methods, in addition to the colorimetric method, which is commonly used in analyzing RT-LAMP amplification results. In the electrophoresis test, a test result is declared positive for SARS-CoV-2 if it produces a ladder-like band on the electrophoresis gel and is declared negative for SARS-CoV-2 if there is no band on the electrophoresis gel (Augustine *et al.* 2020). Analyzing the results of RT-LAMP amplification by electrophoresis showed the formation of a ladder-shaped band in samples previously tested positive using the RT-qPCR test. No bands were found in samples previously tested negative using the RT-qPCR test. These results indicate that the enzymes in the used reagents work well, but the indicators do not.

It is assumed that this phenomenon is caused by the buffer content contained in the input sample. This study extracted RNA samples from nasopharyngeal/oropharyngeal swab samples stored in Viral Transport Medium (VTM). VTM is a culture medium designed to prevent damage to viral RNA when stored for a long time. VTM contains a high buffer content, which is strongly thought to cause a delay in color change in colorimetric RT-LAMP amplification results (Alves *et al.* 2021). In each amplification reaction, two H+ ions are generated, which causes a decrease in the pH of the test sample. The resulting H+ ion will bind to the red phenol indicator and produce a yellow color (Rabe and Cepko 2020). In the case of highly buffered samples, the resulting H+ ion binds to the red phenol indicator and the tris in the buffer (Good *et al.* 1966). From the results obtained, H+ ions tend to be bound to tris compared to phenol red, so the red phenol indicator does not work. An illustration of this process can be seen in Figure 6.

The use of Guanidine-HCL has been reported to have a significant effect on the sensitivity of the RT-LAMP assay. Based on a study from Zhang (2020), adding Guanidine-HCL increased the diagnostic

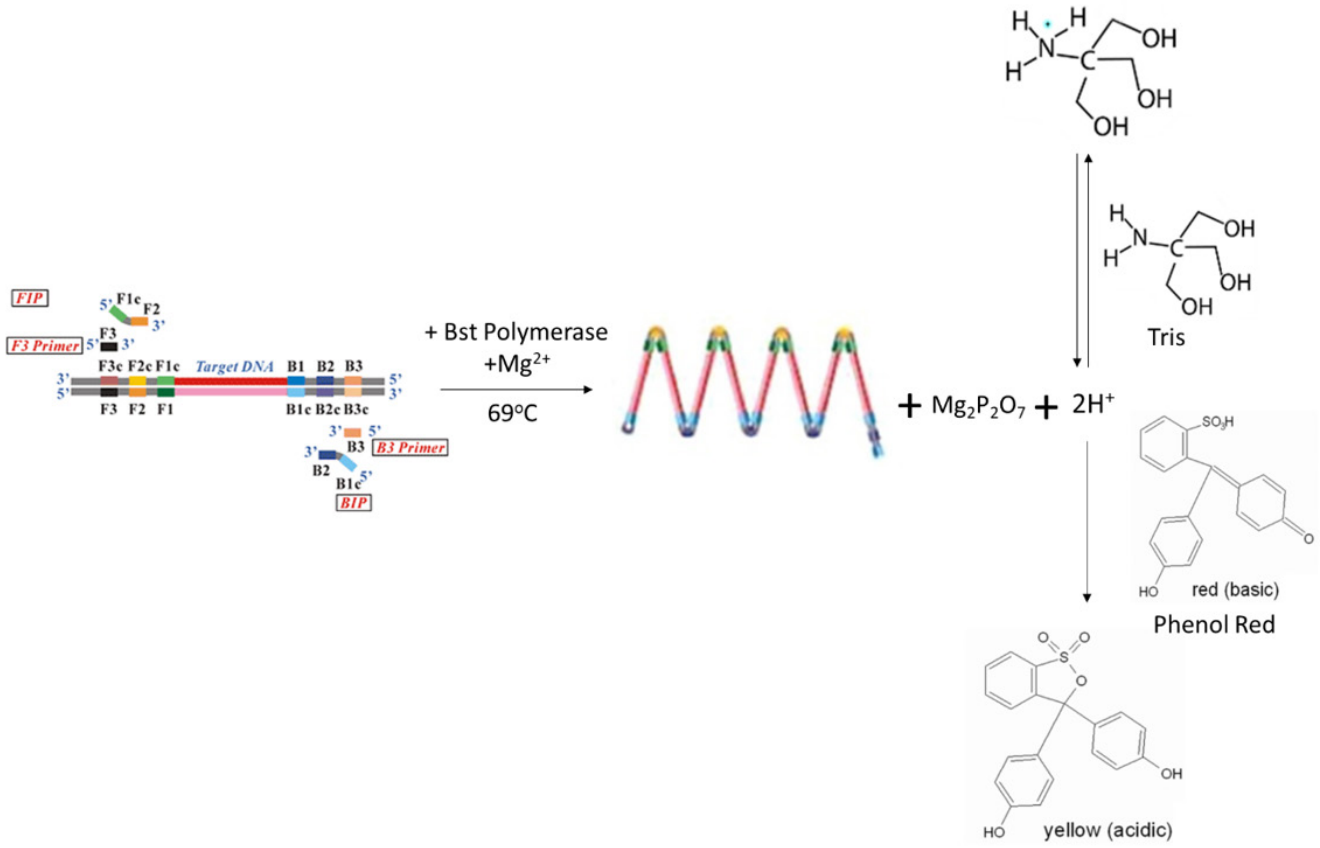

Figure 6. Illustration of the reaction for the formation of a yellow complex between phenol red and H+ ions. The illustration shows that each amplification reaction produced rod-loop-shaped DNA as a main product, $Mg_2P_2O_7$, and two H+ ions as a by-product. H+ ion will bind to the tris and form the tris-H complex or will bind phenol red to produce a complex with yellow color

effectiveness from below 50 to 90% using primers targeting the N region. In this study, Guanidine-HCL did not succeed in increasing the diagnostic effectiveness. It is suspected that this is caused by the sample used in this study. This study's samples were clinical samples that had tested positive or negative for SARS-CoV-2 before using the RT-qPCR assay. According to Dudley *et al.* (2020), adding Guanidine-HCL to increase the effectiveness of the diagnosis is only successful if the sample is synthetic and does not work if the sample is clinical.

Another solution to solve the problem is by increasing the temperature. In this study, the RT-LAMP reaction was optimized at temperatures above the optimal temperature for the RT-LAMP reaction in low buffer conditions, and there are 65°C, 67°C, 69°C, and 71°C. Held (2021) showed that increasing the temperature in the LAMP reaction could increase the annealing tightness. Thereby, it increased amplification and released larger H+ ions. However, temperatures above 72°C can cause enzyme denaturation, which causes inhibition of the BST polymerase enzyme, and consequently, the level of RT-LAMP amplification decreases (New England Biolabs 2016). Based on the optimization results, it is known that a temperature of 69°C for 1 hour is the optimal condition for the colorimetric RT-LAMP reaction with the input sample in the form of a high buffered SARS-CoV-2 RNA sample. However, this temperature only succeeded in causing a change in the color of the red phenol indicator from red to orange. Although there was a difference in color between the positive and negative samples of SARS-CoV-2, this result was not ideal. According to Aoki *et al.* (2021), the result of the colorimetric RT-LAMP reaction with phenol red in the form of orange color was categorized as a result that could not be defined as positive or negative for SARS-CoV-2 (Inconclusive). Therefore, it is necessary to retest these results using other assays, such as RT-qPCR, which reduces the efficiency of the RT-LAMP Colorimetric assay.

4.2. Addition of Post-Treatment to Colorimetric RT-LAMP Amplification Reaction Results with High-Buffered SARS-CoV-2 Samples

The results showed that the addition of post-treatment in the form of heating at a temperature of 80°C for 10 minutes significantly affected the color change of RT-LAMP Colorimetry. These results can be seen in Figure 3. According to Good *et al.* (1966), the buffer works by binding H+ ions with the help of a conjugate base in the form of tris, known as a neutralization reaction. The neutralization process can cause the opportunity for the formation of bonds between H+ ions and the pH indicator of phenol red to decrease. An increase in temperature can disrupt the neutralization process and increase the chances of forming bonds between H+ ions and the red phenol indicator, which causes the formation of complex compounds that produce a yellow color in the reaction product. This phenomenon happened because the tris neutralization process is reversible. When the temperature increases, the pKa decreases, and the dissociation process of H+ ions from tris-H will be more dominant than the binding of H+ ions by tris.

The results in the negative control of SARS-CoV-2 samples support this explanation. The RT-LAMP reaction on the negative control showed no color change after treatment in the form of heating at 80°C for 10 minutes. It indicates that the color change is not due to decreased reagent pH. Although increasing the temperature can lower the pH of the reagent, when the temperature returns to normal, the pH returns to its initial pH. The temperature increases in this study only increase the chance of binding the H+ ion with the red phenol indicator without affecting its reagent. These results were then duplicated for requirement volume in quantification with a spectrophotometer. The duplication results show the exact color as the previous result, which indicates that the method is replicative.

4.3. RT-LAMP Amplification Time Optimization

After the optimum conditions (Temperature 69°C with Post-treatment) were found, the amplification time was optimized to determine the optimum amplification time of the RT-LAMP reaction using the developed method. Based on the results obtained (Figure 3), it is known that the SARS-CoV-2 RNA sample with a high buffer content began to change color to orange at 30 minutes and began to show a yellow color at 50 minutes. These results indicate that a temperature of 69°C and an amplification time of 50 minutes is the optimum condition for the RT-LAMP colorimetric reaction on SARS-CoV-2 RNA samples with high buffer content.

4.4. Quantification of Colorimetric RT-LAMP Results by Using a Spectrophotometer

In addition to providing the best color change to distinguish positive or negative results on RT-LAMP colorimetric results in SARS-CoV-2 samples with a high buffer content, the results also show the potential for classifying test results based on the severity of SARS-CoV-2 virus exposure based on different of color. These results can be seen in Figure 5.

In Figure 5, it can be seen that the difference in Ct values can affect the color changes that occur in the final result of the colorimetric RT-LAMP test. In the RT-qPCR test, the greater the Ct value obtained, the smaller the number of viruses obtained because more cycles are needed so that the RT-qPCR machine can detect the presence of the virus. On the other hand, a small Ct value indicates a large number of viruses because it requires fewer cycles for the RT-qPCR machine to detect the presence of the virus (Pestana *et al.* 2010). In the case of the SARS-CoV-2 virus, the sample was declared positive for SARS-CoV-2 if the Ct value was less than 40. Samples with Ct ranging between $10 \leq Ct < 20$ and between $20 \leq Ct < 30$ showed significant color differences, almost similar based on the colorimetric results (Figure 5). Therefore, the spectrophotometer resulted from RT-LAMP Colorimetry in the range of $10 \leq Ct < 20$ and $20 \leq Ct < 30$.

The quantification of colorimetric RT-LAMP results using a spectrophotometer was carried out at wavelengths of 405 nm, 415 nm, 450 nm, and 490 nm. Some of these wavelengths can provide maximum absorption for the color of the positive results of SARS-CoV-2, yellow (Aoki *et al.* 2021; Thi *et al.* 2020). The SARS-CoV-2 virus exposure level was determined by the wavelengths' maximum absorption against the color of the positive result of SARS-CoV-2. The expected result is that the more positive a test result is; in this case, the smaller the Ct value, the greater the OD value, and the more negative a test result is; in this case, the red color and the greater the Ct value, the OD value will be closer to 0. The quantification results show that the difference between the OD

values for the positive and negative samples of SARS-CoV-2 is visible clearly at the wavelengths of 405 nm, 415 nm, and 450 nm. Statistical tests are performed in correlation and regression tests to determine the wavelength that gives the best regression model and color difference among the three wavelengths.

Based on the results of the correlation test, it was found that the quantification results indicated the best regression model at a wavelength of 415 nm, with a correlation value of -0.9084. The correlation value shows that the Ct value and OD value have a very strong significant correlation ($p<0.001$) and have a negative relationship. If the Ct value decreases, the OD value increases. The regression test results at this wavelength also show an R^2 value of 0.8252, which indicates that the OD value contributes 82.52% to the Ct value. The adjusted R-squared value in the results of this study shows a value of 0.8155, which indicates that the model can explain 81.55% of the variables. From these results, the wavelength of 415 nm provides maximum absorption in the positive test results for SARS-CoV-2 and provides the best regression model compared to other wavelengths.

Apart from the results obtained, several things must be considered in quantifying colorimetric RT-LAMP amplification results. In measuring the colorimetric RT-LAMP amplification, a minimum volume of 50 µL is required. Therefore, it is necessary to double the reaction that is normally used. The RT-LAMP analysis method using the strategy in this study can cause a small portion of the reaction product to evaporate, which causes the reaction volume to be less than the minimum volume required for quantification. In addition, the RT-LAMP reaction can also result in cross-contamination, which can lead to the formation of false positive results. This problem can be solved by performing the procedure sterilely, adding UDG to the sample (Kellner *et al.* 2022), and adding No-Template Control (NTC) in each reaction. Cross-contamination occurs if the NTC changes color during the reaction, and the test needs to be retested.

4.5. RT-LAMP Specificity and Sensitivity Test Results

The specificity test in this study was carried out in-silico using the NCBI BLAST bioinformatics application. In the RT-LAMP reaction, there are six types of primers designed, namely outer primers (F3 and B3), inner primers (FIP and BIP), and loop primers (LF and LB). The six types of primers detected six separate regions of SARS-CoV-2 target DNA/RNA, which caused the RT-LAMP reaction to be faster than RT-qPCR. The outer primers (F3 and B3) generally play a role at the beginning of the RT-LAMP reaction. The FIP and BIP primers were designed based on the F2/B2 and F1c/B1c region sequences. FIP and BIP primers play a role during amplification and are generally required in higher concentrations than other primers. The LF and LB primers were designed to enhance the RT-LAMP amplification reaction. This primer is designed to anneal the loop structure in the RT-LAMP amplicon. The absence of LF and LB primers generally did not affect the RT-LAMP amplification process, but it could decrease the rapidity and sensitivity of the RT-LAMP assay (Eiken 2005). Primer specificity tests were performed on primary sequences F3 and B3, F1c and B1c, F2 and B2, and LB and LF. The results of the in-silico specificity test showed that the primers were specific for the SARS-CoV-2 virus sequence.

Sensitivity tests were carried out on 40 samples of SARS-CoV-2. Based on the test results, it is found that the sensitivity of the RT-LAMP test using this method has a sensitivity of 85% for a Ct cutoff value of 40 and shows a sensitivity of 100% for a Ct cutoff value of 35. This sensitivity value is higher when compared to the sensitivity values obtained by Lu *et al.* (2022), with a Ct cutoff value of 25. It shows the same value as the sensitivity values obtained by Thi *et al.* (2020), with a Ct cutoff value of 35 for the N gene region.

In conclusion, this study successfully detected the SARS-CoV-2 virus in high-buffered samples using phenol red colorimetric RT-LAMP assay. The optimal reaction condition for phenol red RT-LAMP assay in high-buffered samples is 69°C for 50 minutes, with the addition of post-treatment in the form of heating at 80°C for 10 minutes. Apart from the optimal reaction conditions, this study also successfully correlated the RT-qPCR result, represented by the Ct value, and the phenol red colorimetric RT-LAMP result, represented by the OD value, with a correlation value of -0.9081. These results indicate that the phenol red colorimetric RT-LAMP assay can be used as an alternative diagnostic method between Rapid Antigen Test and RT-qPCR methods.

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