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RESEARCH ARTICLE

Colorimetric tetra-combo loop-mediated isothermal amplification assay for efficient SARS-CoV-2 detection

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ABSTRACT

Coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread rapidly and caused a pandemic in 2020. Both pharmaceutical approaches, such as vaccinations and non-pharmaceutical approaches such as social distancing through lockdown and international border restrictions had been implemented to control the outbreak and the transmission of SARS-CoV-2. However, the prolonged implementation of these measures negatively impacted the population and global economy, while the continuously emerging SARS-CoV-2 variants led to breakthrough infections among the vaccinated populations. Given the ongoing nature of COVID-19, an efficient detection method for SARS-CoV-2 that could enable mass screening and on-site screening is needed to manage the disease and prevent further outbreaks at large scale. Hence, we have developed a tetra-combo LAMP SARS-CoV-2 detection assay that targets four SARS-CoV-2 genes (*RdRp*, *S*, *E* and *N*), with an internal control (*RNaseP*). The LAMP assay was validated using 370 RNA samples extracted from nasopharyngeal and/or oropharyngeal swabs. The LAMP assay developed in this study has on-par performance as the qRT-PCR assay, which is the gold standard method for SARS-CoV-2 detection, with a shorter turnaround time (25 minutes). The LAMP assay possessed 98.13% sensitivity, 100% specificity, 100% positive predictive value (PPV), and 95.45% negative predictive value (NPV) for samples with Ct values ≤ 35.

Keywords: Coronavirus disease 2019; loop-mediated isothermal amplification; point-of-care testing; severe acute respiratory syndrome coronavirus 2.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is an infectious respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It was first discovered in Wuhan, China (WHO, 2020). SARS-CoV-2 can be transmitted through airborne transmission by inhalation of aerosols containing the virus particles, close contact through eyes, nose or mouth or fomite transmission (McNeill, 2022). Therefore, the virus could spread easily among the population, causing a pandemic as announced by the World Health Organisation (WHO) in 2020 (Onyeaka *et al*., 2021; NCBI, 2024).

SARS-CoV-2 carries an approximately 29,900 bases RNA genome. SARS-CoV-2 contains several open reading frames (ORFs), which are translated into 16 non-structural proteins (nsp1 – 16), including RNA-dependent RNA polymerase (RdRp), helicase, and 3'-to-5' exonuclease (Zhou *et al*., 2023). SARS-CoV-2 consists of four structural proteins, including spike (S) glycoprotein, envelope (E) protein, membrane (M) protein, and nucleocapsid (N) protein. SARS-CoV-2 demonstrates the structure of a standard *Betacoronaviruses*, where its exterior is covered with E protein and S glycoprotein embedded in the membrane (M) of the virus. E protein plays an important role in the formation of viral vesicles while acting as an ion channel (Candido *et al*., 2022). S glycoproteins on the SARS-CoV-2 surface act as the receptor binding domain (RBD) to the host receptors, which are the human angiotensin converting enzyme 2 (ACE2) receptors (McBride *et al*., 2014; Huang *et al*., 2020). SARS-CoV-2 genome is consisted of a positive sense single-stranded RNA that is bound by the N proteins to maintain its genomic stability. Besides that, N proteins also play a significant role in viral transcription and viral entry into the host cell during infection (Krammer, 2020; Wu *et al*., 2023).

Various strategies have been implemented to mitigate the COVID-19 outbreak globally. At the beginning of the COVID-19 outbreak, strategies such as lockdowns were executed to limit population movement to break the transmission chain among the population and thus minimising the number of infected individuals. Although this strategy was shown to be effective, it could not be a long-term solution to COVID-19 outbreak due to the halt in economic sectors and the burden to the mental health of the citizens. On the other hand, vaccination against SARS-CoV-2, either in the form of an inactivated viral vaccine or mRNA vaccine, has been developed and administered to the population. This approach has gained success

in providing immunity against SARS-CoV-2 infection (Onyeaka *et al*., 2021). However, the emergence of new SARS-CoV-2 variants showed vaccine breakthrough properties, which led to intermittent COVID-19 outbreaks in the communities. Therefore, there was an urge for alternative ways to combat the COVID-19 outbreak due to the shortcomings of these measures.

As for other infectious diseases, rapid detection and isolation of COVID-19 infected individuals are essential and efficient practices to halt the SARS-CoV-2 transmission (Rampal & Liew, 2021). Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR), which is the gold standard method for SARS-CoV-2 detection, has been the main method to detect SARS-CoV-2 at the beginning of the COVID-19 outbreak due to its high sensitivity and specificity (Jawerth, 2020). However, the consistent emergence of new SARS-CoV-2 variants increases the possibility of false negative SARS-CoV-2 testing, especially when new genetic mutations occur in the target region of the assay (FDA, 2023). In addition, as the number of cases increased, the qRT-PCR could not manage the high daily load due to its long test turnaround time. Hence, rapid test kit (RTK) such as antigen test, which is a point-of-care testing (POCT) method for SARS-CoV-2, has been recognised for COVID-19 diagnostic testing. However, the sensitivity and specificity of RTK are generally lower as compared to the qRT-PCR method (CDC, 2023). Therefore, there is a need for a new detection assay that is equipped with the advantages of both qRT-PCR and RTK assays.

In this study, we developed an assay based on loop-mediated isothermal amplification (LAMP) principle for SARS-CoV-2 detection, which incorporated four target genes (*RdRp, S, E, N)* and an internal control gene (*RNaseP*). LAMP assay is well-established. It has been used for the detection of various host pathogens. During Middle East Respiratory Syndrome (MERS) outbreak, RT-LAMP, which included the reverse transcriptase (RT) enzyme for reverse transcribing the single-stranded RNA into double-stranded complementary DNA (cDNA), was developed and shown comparable sensitivity and specificity as the qRT-PCR method to detect Middle East respiratory syndrome coronavirus (MERS-CoV), with a shorter turnaround time (Shirato *et al*., 2014).

Currently, RT-LAMP assays are still undergoing development and optimisation to better serve as a POCT for SARS-CoV-2 detection. To date, there is no RT-LAMP assay developed to simultaneously detect four target genes (such as *RdRp, S, E, N*) along with an internal control. Detection assays that target only a single SARS-CoV-2 gene tends to lead to gene dropout in the detection of SARS-CoV-2, as reported in some SARS-CoV-2 kits targeting the *S*, *N* and *E* genes (FDA, 2023; Park *et al*., 2023). The tetra-combo LAMP assay in this study could greatly reduce the false negative results in the detection of SARS-CoV-2, particularly for the emerging SARS-CoV-2 variants that often carry mutations in the targeted genomic regions. With multiple targets in the assay, it could mitigate the risk of gene dropout due to mutations in the target genes.

Unlike some of the existing RT-LAMP assays that rely on single gene target such as *N* or *E* genes, multi-target approach significantly enhances the robustness and reliability for SARS-CoV-2 detection. Previous studies have reported reduced sensitivity (< 90.00%) in single-target RT-LAMP assays due to variant-specific gene dropout (Supplementary Table 1) (Baba *et al*., 2021; Promlek *et al*., 2022; Erdem *et al*., 2023). Hence, the tetra-combo design in this study, which incorporates four target genes, mitigates this limitation and enhances overall assay reliability.

On the other hand, in comparison to the currently available SARS-CoV-2 LAMP detection kits in the market, majority are detecting a single target gene based on fluorescence detection method. The LAMP assay developed in this study has an advantage as a multi-target LAMP assay that operates using a colorimetric detection method without requiring specialised instrument.

METHODOLOGY

Experimental section

A flowchart that summarises the overall methodology of the study is shown in Figure 1.

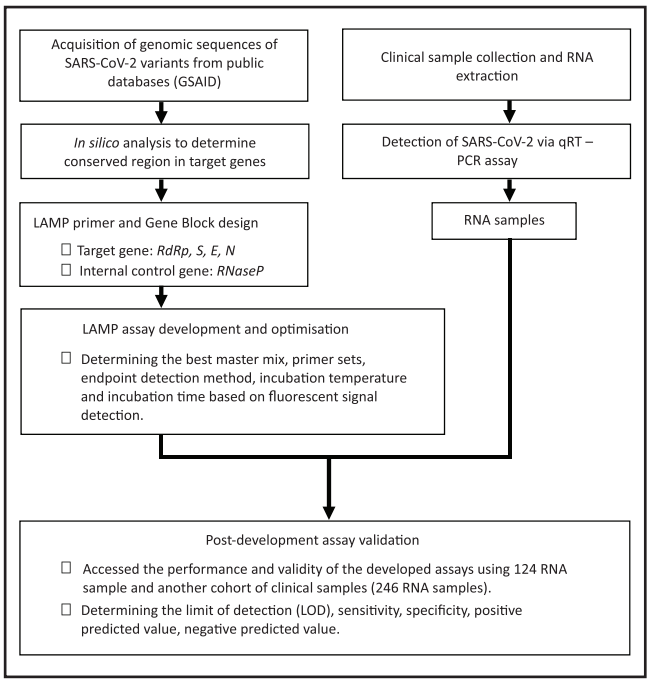

Figure 1. Flowchart of the methodology used in this study.

LAMP primers design and synthesis

NCBI severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome with the NCBI reference sequence of NC_045512.2 was used as the reference sequence for LAMP primer design. The reference sequence was aligned with the genome sequences of different SARS-CoV-2 variants (Alpha, Beta, Delta, Gamma, Omicron, Lambda, Mu and GH490R B.1640 variants), which were available at the time of primer design (December 2021) using ClustalW Multiple Alignment tool in BioEdit Sequence Alignment Editor, version 7.2.5 application. Upon the alignment, the highly conserved regions of each SARS-CoV-2 target genes (*RdRp*, *S*, *E* and *N*) were determined and subjected to the PrimerExplorer V5 software for LAMP primer design. The *RNaseP* was selected as the internal control gene of the LAMP assay. The nucleotide sequence of the *Homo sapiens* ribonuclease P/MRP subunit p30 (RPP30), transcript variant 2, mRNA, with the accession number of NM_006413.5 was subjected to the PrimerExplorer V5 software for LAMP primer design. The primer design parameters include (i) Melting temperature (Tm) for F1c, B1c and loop primers ranged from 64°C to 66°C, 59°C to 61°C for F2, B2, F3 and B3 region; (ii) 3' ends for F2, B2, F3, B3 and LF and LB and 5' ends for F1c and B1c (Refer to Figure 2) having the △G value of ≤ -4 kcal/mol for primer-template binding; (iii) GC content between 50% to 60%; (iv) Distance between ends of F2 and B2 is within 120 to 160 bases, while 5' end of F2 and 5' end of F1 is within 40 to 60 bases, F2 and F3 is within 0 to 60 bases.

For each target gene, two primer sets with the higher primer dimer change of free energy value (△G) was selected for assay development. Unlike for the individual primer sequence, the △G value, also known as the change of Gibbs Free Energy, represents the amount of energy required for the formation or disruption of spontaneous secondary structures in nucleic acid strands. Therefore,

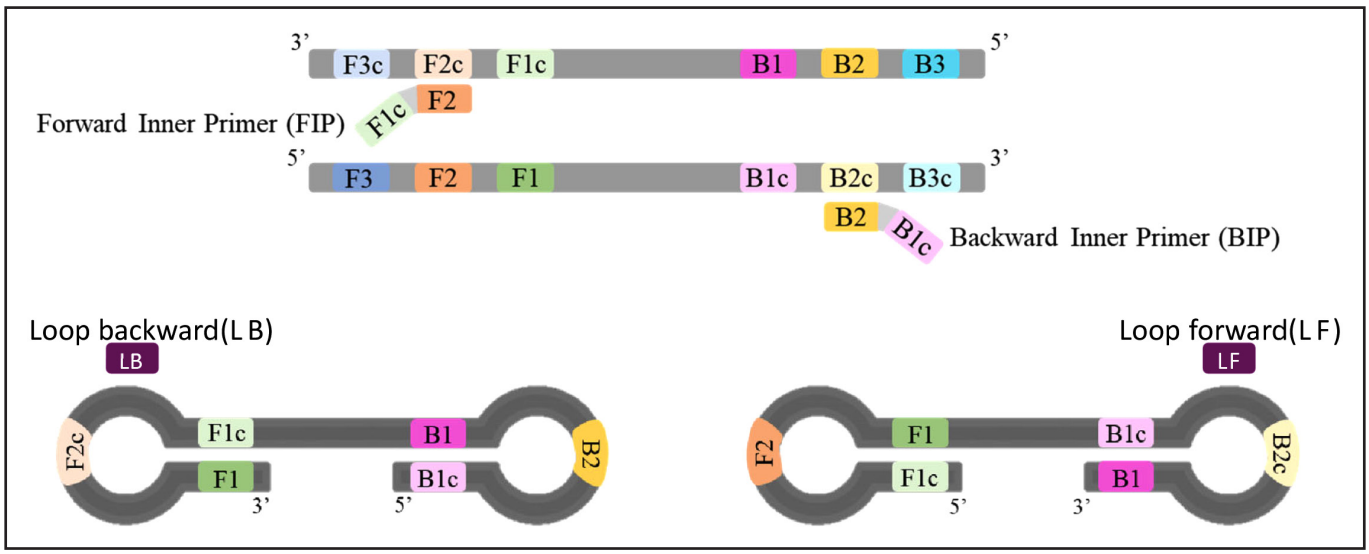

Figure 2. Region for the primer binding sites on the template.

a more positive △G value indicates that more energy is needed to form secondary structure, implying a lower likelihood of secondary structure formation to interfere with the performance of the primers (Benchling, 2024). On the other hand, the sequence regions that comprised the LAMP primer sets for each gene were subjected for gene block synthesis, which were used as the positive control during LAMP assay development. The gene blocks were synthesised in the form of double-stranded DNA.

LAMP assay development and optimisation

WarmStart Colorimetric LAMP 2X Master Mix (DNA & RNA) (New England Biolabs, United States) was used for assay development. Primer sets for each target gene were subjected to gradient temperature runs in a Genie III instrument (OptiGene, United Kingdom), which enables real-time monitoring of the LAMP amplification based on fluorescence signal. Gene blocks, as positive templates, and a non-template control (NTC) were included in the runs. The best performing primer set for each target gene was determined based on (i) the threshold time detected by Genie III (with shorter threshold time indicates better performance); (ii) the absence of primer dimers formation in the NTC that causes false positivity in the assay (indicated by amplification in the NTC reactions). The 25 µL LAMP reaction contained 1X LAMP master mix, 1.6 µM of forward inner primer (FIP) and backward inner primer (BIP), 0.4 µM of forward outer primer (F3) and backward outer primer (B3), 0.8 µM of forward loop primer (LF) and backward loop primer (LB), 10 pg of gene block, and fluorescent dye. After that, the assays were further optimised to half-reaction volume for cost efficiency. The optimal incubation time and temperature as well as the endpoint detection method suitable for the LAMP assays were determined.

Sample collection

A total of 370 nasopharyngeal and/or oropharyngeal swabs were collected from subjects who were suspected for COVID-19. These samples were used to validate the LAMP assay. Sample collection, extraction and screening were carried out in Universiti Malaysia Sabah [Ethics approval number: NMRR-20-1785-55933 (IIR)]. Viral RNA extraction was carried out on all the samples using QIAamp Viral RNA Extraction kits (Qiagen, Germany) according to the manufacturer's instructions. The extracted RNA samples were screened for SARS-CoV-2 using VIASURE Viral SARS-CoV-2 Positive Control kit (Certest Biotec S.L., Spain) or Allplex SARS-CoV-2 Assay (Seegene Inc., South Korea), depending on the availability of the kit during screening, following the recommended protocols by the manufacturers.

Assay validation with RNA samples

After completion of LAMP assay development, 124 RNA samples extracted from nasopharyngeal and/or oropharyngeal swabs were tested using the heat block incubation method and colour changes were selected as the endpoint detection method. The 12.5 µL reaction contained 1X LAMP master mix, 1.6 µM of FIP and BIP, 0.4 µM of F3 and B3, 0.8 µM of LF and LB, and 1 µL RNA sample. The reactions were incubated at 65°C on a heat block for 25 minutes and colour changes were observed after the incubation to determine the test results. The validity of the assay, in terms of sensitivity, specificity, positive and negative predictive values, was determined in comparison to the qRT-PCR results. The 95% confidence interval of all test parameters were calculated using the MedCalc software (https://www.medcalc.org/calc/diagnostic_test.php).

A limit of detection (LOD) test was carried out for the lamp combo assay. A plasmid construct (MBS-4101; 200,000 copies/µL) (Apical Scientific Sdn. Bhd., Malaysia) that contains partial sequences for *E*, *RdRp*, and *RNaseP* genes was acquired. The plasmid construct was used as the standard to determine the viral copy number of two randomly selected RNA samples via the Allplex SARS-CoV-2 assay in a real-time PCR system. Upon determination of the viral copy numbers, the RNA samples were serially diluted up to $1x10^{-6}$ times and tested with the developed LAMP assays to determine the LOD of the combo assay.

Prior to finalised the LAMP assay protocol, the final volume of the LAMP reaction was further reduced to 10 µL to ease the preparation of the master mixes. The 10-µL reaction contained 1X LAMP master mix, 1.6 µM of FIP and BIP, 0.4 µM of F3 and B3, 0.8 µM of LF and LB, and 1 µL of RNA sample. With the 10-µL reaction, apart from the reduction of the assay cost, the addition of dH_2O during master mix preparation was removed, which further simplified the assay procedure by reducing steps and materials needed during assay preparation. This also effectively reduced the risk of contamination and preparation time. A total of 246 RNA samples were then subjected to the 10-µL LAMP assay to ensure assay stability. The validity of the 10-µL assay was determined and the test performance in terms of sensitivity and specificity was not affected in samples with high viral load.

RESULTS

Assay development and optimisation

Two primer sets with the highest △G values for each target gene were synthesised and subjected to LAMP assay runs in the Genie III instrument, with the WarmStart Colorimetric LAMP Master Mix (New England Biolabs, United States) at gradient temperatures. The gene block was used to determine the efficiency of the primer sets in amplifying the target genes while the NTC was used to assess the tendency of primer dimer formation in the assay that could lead to false positive results. The best performing primer set for each target was determined (in terms of amplification threshold time and absence of amplification in NTCs) and selected for further LAMP assay optimisation (Supplementary Tables 3 and 4).

Among the tested primer sets for all target genes, amplification signal was detected in one NTC of the *E* gene primer set [29] and none in the *E* gene primer set [20]. Therefore, the *E* gene primer set [20] was selected for further optimisation. Meanwhile, the *N* gene primer set [19] was selected for the subsequent LAMP assay optimisation due to its overall shorter threshold time compared to the *N* gene primer set [5]. On the other hand, all the NTCs in the *N* gene primer set [5] exhibited amplification signal, indicating high primer dimer tendency. The *RdRp* gene primer set [14], which showed greater stability with no amplification signal detected in the NTCs, was selected for further optimisation over the primer set [37]. For *S* gene primer sets, the *S* gene primer set [38] was selected over the *S* gene primer set [1], in which amplification signal was detected in all the NTCs at various temperatures. On the other hand, false positive results were detected in NTC reactions at 61°C, 63°C, and 66°C for both *RNaseP* primer sets. However, the *RNaseP* primer set [78] demonstrated a faster amplification time (≤12 minutes) for gene block compared to the *RNaseP* primer set [24]. Therefore, the *RNaseP* primer set [78] was selected.

Based on assay optimisation, all the selected primer sets showed amplification within 30 minutes at all tested temperatures, except at 68 °C for *E* gene primer set [20] (Supplementary Tables 3 and 4). Hence, the incubation of the LAMP assay was set at 65°C for all the target gene assays. This temperature is also recommended by the manufacturer. In addition, several other studies also reported that 65°C is the optimal temperature for their LAMP assays, where clear or earlier amplification results were observed at 65°C compared to lower temperatures (Daddy Gaoh *et al*., 2021; Srisawat & Panbangred, 2015). Upon optimisation, the cut-off threshold was set to be 25 minutes for the LAMP assay.

To further enhance the cost efficiency of the LAMP assay, the reaction volume was optimized to half (12.5 µL). The performance of 25.0 µL and 12.5 µL reactions was comparable based on the threshold time (Figure 3). The threshold times were shorter after the volume reduction for the *S*, *E* and *N* genes. The reduced reaction volume consistently maintained the threshold time below 20 minutes. Thus, the 12.5 µL volume was adopted for subsequent LAMP assay validation.

Assay validation with clinical samples

A total of 370 samples (244 positive and 126 negative) were collected and screened via qRT-PCR approach. The LAMP assays for each target gene were prepared according to the optimised conditions and the cut-off threshold time for positive results was set at 25 minutes. In addition, the LAMP results were analysed in combo, as for most qRT-PCR assays, which could effectively minimise false negative results due to genetic mutations of the pathogens at the target genetic regions. A positive result of the LAMP combo assay is determined when at least two of the SARS-CoV-2 target genes are detected. The results of the combo LAMP assays are interpreted as in Table 1.

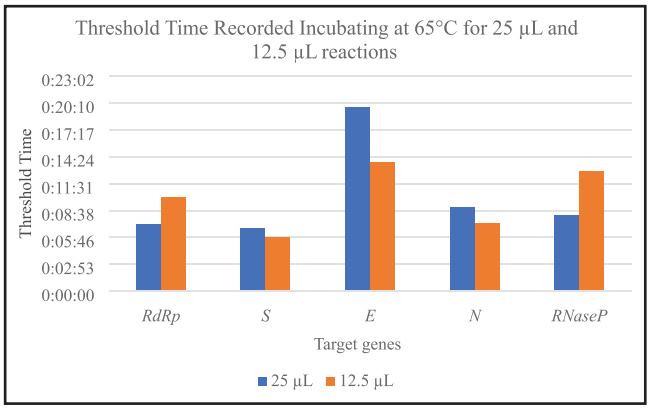

Figure 3. The threshold time for the LAMP assays incubated at 65°C recorded for each gene with the final reaction volumes of 25.0 µL and 12.5 µL.

Table 1. Combo LAMP assay interpretation for the LAMP assay

Target Gene				*RNaseP*	Interpretation
E	*N*	*RdRp*	*S*		
+	+	+	+	+	**SARS-CoV-2 Positive.** Results are valid.
-	+	+	+	+	**SARS-CoV-2 Positive.** Results are valid. Negative target gene results might due to: (a) Low sample viral load. (b) Possible mutation at the targeted gene area. (c) Other possible factors.
+	-	+	+	+	
+	+	-	+	+	
+	+	+	-	+	
+	+	-	-	+	
+	-	+	-	+	
+	-	-	+	+	
-	+	+	-	+	
-	+	-	+	+	
-	-	+	+	+	
+	-	-	-	+	**SARS-CoV-2 Negative.** Results are valid. Positive target gene results might due to non-specific amplification.
-	+	-	-	+	
-	-	+	-	+	
-	-	-	+	+	
-	-	-	-	+	
+/-	+/-	+/-	+/-	-	**Invalid test**. Suggest for resampling and retesting.

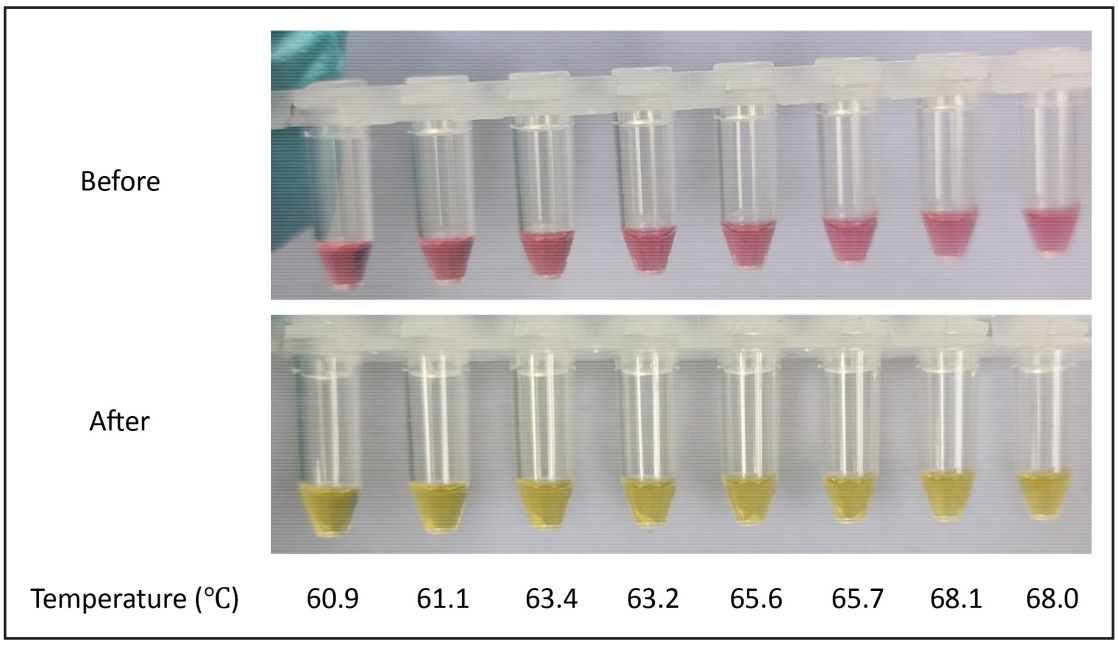

Figure 4. The colour changes of the WarmStart Colorimetric LAMP Master Mix.

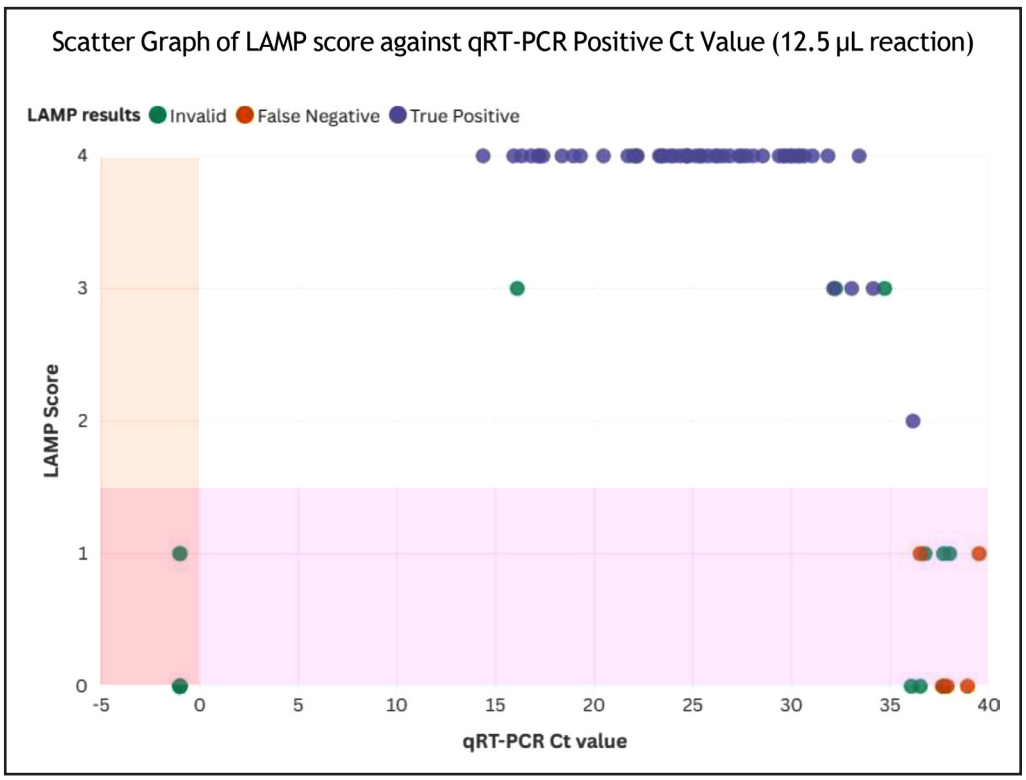

Figure 5. Scatter graph of LAMP score against Ct value of qRT-PCR for 12.5 µL reaction volume. LAMP assay score referring to the number of SARS-CoV-2 target gene being detected by the LAMP assay developed. Samples with negative Ct values indicate negative samples. Based on the scatter graph, all the false negative samples had Ct value > 35, indicating having low viral load in the samples.

A total of 124 qRT-PCR-confirmed RNA samples (66 positive and 58 negative) were subjected to the LAMP assays and incubated at 65°C on a heat block for 25 minutes for assay validation purposes. The results were observed after incubation, and positive results were indicated by a colour change from pink to yellow (Figure 4). The results were recorded and compared to the qRT-PCR results. *RNaseP* was detected in 107 out of 124 samples (86.29%). Table 2 shows the detection rate for each target gene assay, ranging from 78.79% to 84.85%. Out of the 124 samples, 17 were returned with invalid results as none of the target genes were detected. This may be due to sample degradation caused by prolonged storage between the testing period of qRT-PCR and the LAMP assay (Table 3). On the other hand, 102 samples were identified correctly by the LAMP assays. However, five positive samples were identified as negative by the combo LAMP assay. The scatter graph of the LAMP score (i.e., number of detected target genes by the LAMP assay) against Ct values of the clinical samples revealed that all the false negative had Ct value > 35, which suggest low viral load (Figure 5). The sensitivity and specificity were 91.07% (95% CI: 80.38% – 97.04%) and 100% (95% CI: 93.02% – 100%), respectively, with 100% (95%

CI: 93.02% – 100%) positive predictive value (PPV) and 91.07% (95% CI: 81.55% – 95.93%) negative predictive value (NPV).

The limit of detection (LOD) of the LAMP combo assay was determined in two random samples. The viral copy number of the samples were first determined by comparing to a standard - plasmid construct with a known copy number. Then, samples were serially diluted and subjected to the LAMP combo assay. The LOD of the assay was determined as 140 copies/µL and 155 copies/µL (Table 4).

Table 2. LAMP assay performance for each gene as compared to qRT-PCR

Gene	qRT-PCR Positive		qRT-PCR Negative		Detection Rate (%)
	LAMP Positive	LAMP Negative	LAMP Positive	LAMP Negative	
RdRp	55	11	1	57	83.33
S	56	10	3	55	84.85
E	52	14	2	56	78.79
N	54	12	3	55	81.82

Table 3. Concordance of the combo LAMP assay as compared to qRT-PCR for 124 RNA samples

Combo LAMP assay	qRT-PCR		Total
	Positive	Negative	
Positive	51 (TP)	0 (FP)	51
Negative	5 (FN)	51 (TN)	56
Invalid	10	7	17
Total	66	58	124

*TP: True positive; FP: False positive; FN: False negative; TN: True negative.

Table 4. Limit of detection of the LAMP combo assay

Sample	Dilution	Viral copies number (copies/µL)	LAMP Detection after 25-minute incubation			
			E	*N*	*RdRp*	*S*
UMLS262	$x10^{-1}$	155,000	+	+	+	+
	$x10^{-2}$	15,500	+	+	+	+
	$x10^{-3}$	1,550	+	+	+	+
	$x10^{-4}$	155	–	+	+	+
	$x10^{-5}$	15.5	–	–	+	–
	$x10^{-6}$	1.55	–	–	–	–
UMLS282	$x10^{-1}$	140,000	+	+	+	+
	$x10^{-2}$	14,000	+	+	+	+
	$x10^{-3}$	1,400	+	+	+	+
	$x10^{-4}$	140	+	-	+	+
	$x10^{-5}$	14	–	–	+	–
	$x10^{-6}$	1.4	–	–	+	–

In order to further enhance the feasibility of the LAMP assay, the reaction volume was optimised to 10.0 µL to streamline the preparation of the reaction mixture. To further validate the performance of the LAMP assay in the 10.0 µL format, another 246 qRT-PCR-confirmed RNA samples (178 positive and 68 negative) were subjected to the 10.0 µL LAMP assay. The detection rates of each SARS-CoV-2 target gene ranged from 78.09% to 91.01% (Table 5). The detection rate for *RNaseP* in these 246 RNA sample cohort had increased to 97.56% (240/246 samples). This may because these samples were recently extracted, hence highlighting the importance of sample quality for LAMP assay testing. Of note, similar to the 12.5 µL reaction assay, the scatter graph showed that the false negative samples had high CT values, which suggest low viral load in the samples (Figure 6). The sensitivity, specificity, PPV

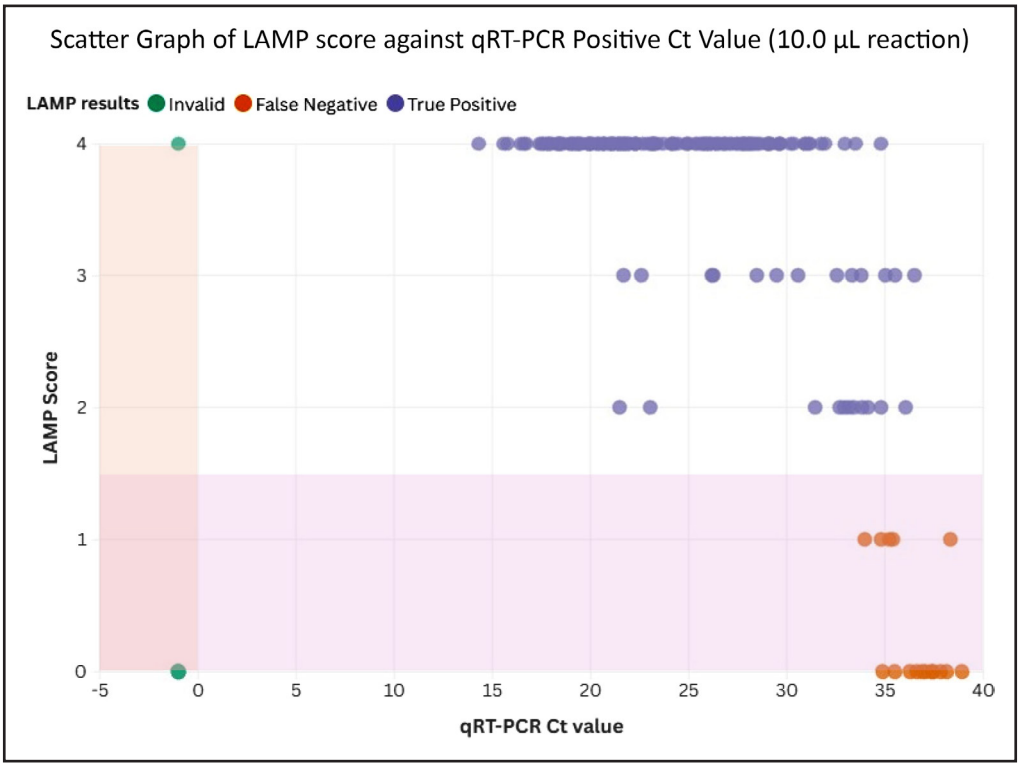

Figure 6. Scatter graph of LAMP score against Ct value of qRT-PCR for 10 µL reaction volume. LAMP assay score referring to the number of SARS-CoV-2 target gene being detected by the LAMP assay developed. Samples with negative Ct values indicate negative samples. Based on the scatter graph, majority of the false negative samples had high Ct values, indicating low viral load in the samples.

and NPV were 90.96% (95% CI: 85.74% – 94.74%), 100% (95% CI: 94.31% – 100%), 100% (95% CI: 97.73% – 100%) and 79.75% (95% CI: 71.16% – 86.27%), respectively (Table 6).

Table 5. The 10-µL LAMP assay performance for each gene as compared to qRT-PCR

Gene	qRT-PCR Positive		qRT-PCR Negative		Detection Rate (%)
	LAMP Positive	LAMP Negative	LAMP Positive	LAMP Negative	
RdRp	162	16	1	67	91.01
S	159	19	1	67	89.33
E	139	39	1	67	78.09
N	158	20	1	67	88.76

Table 6. The concordance of the 10-µL combo LAMP assay as compared to qRT-PCR for 246 RNA samples

Combo LAMP assay	qRT-PCR		Total
	Positive	Negative	
Positive	161 (TP)	0 (FP)	161
Negative	16 (FN)	63 (TN)	79
Invalid	1	5	6
Total	178	68	246

DISCUSSION

The limit of detection (LOD) of the LAMP assay developed was as low as 140 copies/µL. A previous study has reported that the relationship between the viral load in infected individuals and the risk of transmission of SARS-CoV-2. The study showed that 77.00% of the transmission occurred when the viral load in the infected individual was more than 100 copies/µL, which is close to the LOD of the LAMP assay in our study (Bhavnani *et al*., 2022). This suggests that our assay is highly useful for detecting individuals with infective viral loads, making it an effective tool for screening and transmission control.

In comparison with colorimetric LAMP assays developed in other studies, the LAMP assay in this study exhibited the shortest incubation time of 25 minutes while maintaining an optimal incubation temperature of 65°C, which is common for other LAMP assays (Supplementary Table 1). All the studies, besides Alhamid et al., had set the incubation time within 30 minutes. Prolonged incubation times exceeding 30 minutes in LAMP assays significantly increase the risk of spurious amplification, which can lead to false positive results (Dao Thi *et al*., 2020).

In addition, the viral load of the samples plays a significant role in affecting the test parameters. Table 7 shows the distribution of the samples according to the Ct value determined by qRT-PCR, with high (Ct $\leq$30), intermediate (30<Ct $\leq$35) and low (Ct >35) viral loads. The performance of the LAMP assay improved with a higher viral load of SARS-CoV-2 in the samples (Table 8). According to Table 8, we could deduce that the five false negative samples detected in the assay validation at 12.5 µL were all due to low viral load based on their Ct value, which is greater than 35 Ct value. The sensitivity of the 10-µL LAMP combo assay had been improved to 100% and 98.13% for samples with Ct value $\leq$ 30 and $\leq$ 35, respectively, while the specificity remained unchanged at 100%. The test parameters for all the samples with Ct value $\leq$ 35 at 10 µL reaction volume are 98.13% (95% CI: 94.62% – 99.61%) sensitivity, 100% (95% CI: 94.31% – 100%) specificity, 100% PPV (95% CI: 97.68% – 100%) and 95.45% (95% CI:87.25% – 98.47%) NPV.

LAMP assay is known to have poorer performance for samples with low template load, i.e., samples with low viral load or low Ct values in this case. This may be due to its simpler chemistry and set up, which is susceptible to influence by various external factors, such as interference, inhibitors, and surrounding temperature. This is also commonly reported in other studies. Amaral et al. showed that the sensitivity of the LAMP assay for samples with Ct values between 32 to 35 and 35 to 40 was 45.95% and 28.95%, respectively, while samples with Ct $\leq$ 32 showed 100% sensitivity and 96.10% specificity (Amaral *et al*., 2021). Study by Baba *et al*. also showed an increase of sensitivity from 87.00% to 98.00% for samples with Ct values < 35 and < 30 respectively, while Promlek *et al*. showed that the sensitivity of LAMP assay increased from 53.85% to 100% for samples with Ct values < 31 (Baba *et al*., 2021; Promlek *et al*., 2022). In another study by de Oliveria Coelho et al., the LAMP assay sensitivity had increased from 76.90% to 89.40% and specificity had increased from 90.40% to 94.80% for samples with Ct values < 35 and < 30 respectively (de Oliveira Coelho *et al*., 2021).

The qRT-PCR Ct values of the samples provide an insight in the performance of the LAMP assay in relation to the viral copies present in the samples. It has been shown that the LAMP assay performed better for RNA samples with high and intermediate viral loads. The invalid results of this LAMP assay were due to the absence of amplification for the internal control and all SARS-CoV-2 target genes. It may be due to sample degradation caused by prolonged or sub-optimal storage conditions prior to the LAMP assay. Other possible reasons include improper sample collection or RNA extraction process. However, due to limited volume of the extracted RNA samples, RNA quality assessment was not conducted. Therefore, sample recollection or re-extraction is recommended for samples with invalid LAMP results.

In order to ensure that the LAMP assay is able to detect the latest circulating SARS-CoV-2 variants, an *in-silico* analysis has been carried out to compare the similarity of the LAMP primers against the newly emerged SARS-CoV-2 variants and latest circulating variants as of May 2025, including a total of 11 SARS-CoV-2 variants (i.e., BA.2.86, EG.5, JN.1, XBB.1.5, XBB.1.16, BA.2.75, CH.1.1, XBB, XBB.1.9.1, XBB.1.9.2, XBB.2.3). The primer binding regions of these variants were found to be 100% identical to the LAMP primers designed for *RdRp* gene, 99.81% for *E* gene, 98.40% for *S* gene and 98.83% of *N* gene (Supplementary Table 5). Hence, it was deduced that the LAMP primers developed could also detect and amplify the latest circulating SARS-CoV-2 variants.

The LAMP assay developed in this study has greatly simplified the screening procedure of SARS-CoV-2 as compared to the qRT-PCR test. Apart from having a relatively lower cost, it would be an alternative which is beneficial for mass screening or screening of SARS-CoV-2 in low resources setting. However, the developed LAMP assay has certain limitations, as it requires extracted RNA as input and it is qualitative assay which does not allow quantification of the viral load in the tested samples.

Besides that, the LAMP assay developed in this study is currently intended for use in laboratory settings and should be carried out by trained personnel with basic laboratory knowledge. The test is only suitable as first-line screening tool for COVID-19, rather than as a diagnosis test. Positive LAMP results should be validated with qRT-PCR in the laboratories for diagnosis confirmation. The LAMP assay could be further modified by integrating a simple sample preparation method that could substitute the RNA extraction process to increase the turnaround time of the test for the ease of on-site SARS-CoV-2 screening. In addition, the primers and master mix of the LAMP assays could be lyophilised for reagent storage at room temperature, more cost-effective transportation, and easier assay preparation process.

Table 7. Distribution of samples according to the Ct value determined by qRT-PCR assays

Volume of LAMP Assay	LAMP Assay Developed	qRT-PCR				Total
		Positive			Negative	
		≤ 30	30 < Ct ≤ 35	> 35		
12.5 µL	Positive	40	10	1	0	51
	Negative	0	0	5	51	56
	Invalid	1	3	6	7	17
	Total	41	13	12	58	124
10.0 µL	Positive	134	23	4	0	161
	Negative	0	3	13	63	79
	Invalid	1	0	0	5	6
	Total	135	26	17	68	246

Table 8. Test performance of the combo LAMP assay according to the Ct values

Volume of LAMP Assay	Test Parameters	Ct value		
		≤ 30	≤ 35	≤ 40
12.5 µL	Sensitivity	100%	100%	91.07%
	Specificity	100%	100%	100%
	PPV	100%	100%	100%
	NPV	100%	100%	91.07%
10.0 µL	Sensitivity	100%	98.13%	90.96%
	Specificity	100%	100%	100%
	PPV	100%	100%	100%
	NPV	100%	95.45%	79.75%

CONCLUSION

In conclusion, the highly conserved regions found in the SARS-CoV-2 genes, i.e., *RdRp*, *S*, *E* and *N*, among the variants were used as the target region for LAMP assay design and development. *In-silico* analysis and clinical sample validation confirmed the compatibility of the designed primers for the detection of SARS-CoV-2, including the latest variants. A tetra-combo LAMP assay, with four SARS-CoV-2 target genes and one internal control gene, was developed. The LAMP reaction requires only a 25-minute incubation at 65°C for a result. The assay results can be visually interpreted based on colour change. The developed LAMP assay demonstrated 98.13% sensitivity, 100% specificity, 100% positive predictive value and 95.45% negative predictive value for RNA samples with Ct values ≤ 35. The developed LAMP assay showed comparable performance to qRT-PCR while offering several advantages that address the limitations of qRT-PCR.

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Declaration of competing interest

The authors declare no competing financial interests or personal relationships that could have influenced the work reported in this paper.

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