

## RESEARCH ARTICLE

# A quantitative RT-qLAMP for the detection of SARS-CoV-2 and human gene in clinical application

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## Abstract

Reverse transcription (RT) – loop-mediated isothermal amplification (LAMP) assay is a rapid and one-step method to detect SARS-CoV-2 in the pandemic. Quantitative estimation of the viral load of SARS-CoV-2 in patient samples could help physicians make decisions on clinical treatment and patient management. Here, we propose to use a quantitative LAMP (qLAMP) method to evaluate the viral load of SARS-CoV-2 in samples. We used threshold time (TT) values of qLAMP, the isothermal incubation time required for the fluorescent or colorimetric signal to reach the threshold, to indicate the viral load of clinical samples. Similar to the cycle threshold ( $C_t$ ) values in conventional qPCR, TT values of qLAMP show a linear relationship to the copy numbers of SARS-CoV-2. The higher the viral loadings, the lower qLAMP TT values are. The RT-qLAMP assay was demonstrated to quantify the viral loads of synthesized full-length RNA, inactivated viral particles (BBIBP-CorV), and clinical samples within 15 min by fluorescent reading and 25 min by colorimetric reading. The RT-qLAMP has been applied to detect Alpha, Beta, Kappa, Delta, and Omicron variants of SARS-CoV-2, as well as the human beta-actin gene, and their TT values showed the linear patterns. The RT-qLAMP assays were evaluated by 64 clinical samples (25 positives and 39 negatives) for the assessment of viral loads, and it was also used to quantify the human beta-actin gene, which was used as a control and an indicator of sampling quality in clinical swab samples. The result of RT-qLAMP was in good agreement with the result of RT-qPCR. The RT-qLAMP assay detected all clinical samples, including those with  $C_t = 35$ , within 10 min using fluorescent reading.

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## INTRODUCTION

The SARS-CoV-2 pandemic has had a significant impact on the human society (Canas et al., 2021; Rogers et al., 2020; Tangcharoensathien et al., 2021; Yang et al., 2020), and adversely damaged the economies of many countries (Borio, 2020; Gu et al., 2020; Ozili, 2020; Swinnen & McDermott, 2020; Ye et al., 2020). To control the spread of the virus, identifying asymptomatic carriers and infected individuals at an early stage, even before symptoms begin, is of utmost importance (Huff, 2021; Olalekan et al., 2020). Early diagnosis and rapid isolation of infected individuals and monitoring their contacts could prevent further transmission and assist governments in implementing effective public health measures (Brouard et al., 2020; Huff, 2021; Jones et al., 2021; Peck, 2020; Walsh et al., 2020). A nucleic acid amplification test (NAAT) based on reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR) was promptly developed, as a 'golden standard' method, to detect the viral RNA of SARS-CoV-2 once the genetic sequence was published (Peck, 2020). Although RT-qPCR assays are characterized by high sensitivity and specificity, they require a long reaction time (around 2 h), high purity samples, sophisticated instruments for thermal cycling, and highly trained personnel (Babiker et al., 2020; Yu et al., 2020), limiting its application in low-resource settings and point-of-care test (POCT).

We have previously developed reverse transcription loop-mediated isothermal amplification (RT-LAMP), to detect SARS-CoV-2 (Cui et al., 2020; Huang et al., 2020). The RT-LAMP only needs a single temperature, normally 65°C, to detect the virus, which could be achieved using a heat block or thermocup (Ben-Assa et al., 2020; Chow et al., 2020; Thompson & Lei, 2020). Moreover, the high robustness of the enzyme in the RT-LAMP assay allows the viral detection without nucleic acid extraction, which considerably simplifies the testing process. The duration of amplification requires 30min or less. The LAMP assay for SARS-CoV-2 has been widely used in POCT (Anurup et al., 2020; Garcia-Venzor et al., 2021; Huang et al., 2022; Marino et al., 2022; Wei et al., 2021). Six primers in the LAMP assay targeting eight distinct sites of the sequence ensure sensitivity and specificity (Hardinge & Murray, 2019; Jiang et al., 2020; Wang et al., 2015). To date, FDA-EUA has approved nine diagnostic devices using RT-LAMP to detect SARS-CoV-2 (Oh et al., 2021), and US FDA has approved the home-test product Lucira (USA FDA, 2020). The results of the LAMP assay could be read by both fluorescent reading (Garcia-Venzor et al., 2021; Woo et al., 2020), and naked eyes (Cui et al., 2020; Huang et al., 2020; Lim et al., 2021). Since hydrogen ions were released during the nucleic acid amplification, decreasing the pH of the solution, the colour change of the pH indicator, such as phenol red, could be used to indicate the results, pink at high pH values for negative samples, and yellow at low pH values

for positive samples (Alves et al., 2021; Aoki et al., 2021; Huang et al., 2020; Lim et al., 2021; Loan et al., 2020).

In our RT-LAMP assay, a temperature-dependent 'molecular switch' primer, was designed and added to the O117 primer set to eliminate false positive problems (Lim et al., 2021). This RT-LAMP assay with QO117 primer set (O117 primers with molecular switch) has been validated by 198 clinical samples and demonstrated an overall sensitivity of 90.2% (95% CI 83.8–94.7%) and specificity of 92.4% (95% CI 83.2–97.5%) (Lim et al., 2021). However, the standard LAMP assay only detects the presence or absence of the virus in a sample but is unable to estimate the viral loads.

The cycle threshold ( $C_t$ ) values of RT-qPCR are correlated with the viral load of the sample (Han et al., 2021; Vogels et al., 2020). The lower  $C_t$  values indicate the higher viral load. The individuals with  $C_t$  values less than 25 were correlated to mortality in an investigation containing 875 clinical samples (Faico-Filho et al., 2020). By contrast, samples with  $C_t$  values above 35 were regarded as non-infectious, as epidemiological data showed a low risk of transmission. The Chinese government has recently used this criterion  $C_t > 35$  for the infectivity of patients (Chinese Government, 2022; Gilad et al., 2021; Jones et al., 2021; Raveh et al., 2021; Rueda-Garrido et al., 2020; Walsh et al., 2020). The  $C_t$  values could be used to assess the patient's risk and help the physicians make decisions on clinical treatment and patient management (Faico-Filho et al., 2020; Rao et al., 2020).

Similar to qPCR, presumably a quantitative LAMP, termed as 'qLAMP', could also be used to evaluate the viral load of SARS-CoV-2. qLAMP has been used to assess different viruses and pathogens in previous works using fluorescent reading (Cao et al., 2017; Ongerth & Danielson, 2021; Soares-Santos et al., 2018; Yang et al., 2017) and colorimetric reading (Nguyen et al., 2020). In this work, the RT-qLAMP assay was developed to quantify RNA of SARS-CoV-2 based on the threshold time (TT) value. RT-qLAMP was used to test different variants of synthesized full-length SARS-CoV-2 RNA and inactivated viral particles. It shows that there was a linear relationship between the TT values of RT-qLAMP and the copy numbers of SARS-CoV-2. The RT-qLAMP assay was also applied to quantify SARS-CoV-2 in 64 clinical samples.

## EXPERIMENTAL PROCEDURES

### Sample collection

The 64 clinical samples obtained from the Oxford University Hospital NHS Foundation Trust (OUH), Department of Microbiology, were accessed for the qLAMP assay. All samples were collected as nasal and/or throat swabs in viral transport media during

routine clinical care, and the extract was stored at  $-80^{\circ}\text{C}$ . Samples were provided to the LAMP laboratory, with no identifiers nor means of linking them back to the patients.

## Ethics

The protocol for the use of residual clinical samples at OUH John Radcliffe Hospital, as described in this manuscript, was reviewed by the Research Governance Team of OUH and determined to constitute service evaluation and development. As such, this study was deemed to not require a research ethics review.

## Primer design and DNA synthesis for RT-LAMP

Viral primer sets, QO117, N1, and N15 primer sets, were designed as previously described in Huang et al. (Huang et al., 2020; Lim et al., 2021). Each primer was synthesized by Integrated DNA Technologies (IDT, UK). The 10x N1 and N15 primer mix were prepared with RNase/DNase-free water (11538646, Invitrogen) in the concentration of  $16\ \mu\text{M}$  for FIP and BIP,  $2\ \mu\text{M}$  for F3 and B3, and  $4\ \mu\text{M}$  for LB and LF. The additional  $24\ \mu\text{M}$  temperature-dependent switch was added for QO117 primers (Lim et al., 2021).

A set of ACTB-n primers, including loop primers, were designed to target the human ACTB gene. Primers were designed using LAMP primer designing software, PrimerExplorer (<http://primerexplorer.jp/e/>) (Tomita et al., 2008). The 10x ACTB primer mix was prepared with RNase/DNase-free water in the concentration of  $16\ \mu\text{M}$  for FIP and BIP,  $2\ \mu\text{M}$  for F3 and B3, and  $4\ \mu\text{M}$  for LB and LF. The sequences of each primer of ACTB-n are listed in Table S2.

## RT-qLAMP assay for fluorescent reading and colorimetric reading

The volumes of LAMP reaction components are listed in Table 1. Reverse transcriptase was included in the

master mix for all the tests in the work. The mixed reagents were placed on ice before running the reaction. For the reactions, Bio-Rad CFX OPUS 96 Real-Time PCR machine was applied for fluorescence acquiring while using the thermostatic photometer (Sinopharm) for colorimetric reading.

After placing the PCR plate into the qPCR machine, the PCR cycle conditions were set to 20s at  $65^{\circ}\text{C}$  for 90 cycles with fluorescence acquiring (FAM channel). The qLAMP TT values were given by qPCR machines based on Cq Values.

For colorimetric reading, the image of the samples was taken every 20s by a thermostatic photometer (Sinopharm). The colour on the image was digitized based on RGB. The average of the first five readings was set to the initial average value. Then, the colour index, the difference between the real-time reading and the initial average value, was recorded along with the reaction. When the colour index was greater than 2, this time point was set to the qLAMP TT values by colorimetric reading.

## Sample preparation

### Full-length RNA

The full-length RNA of all SARS-CoV-2 variants (Twist Bioscience) were serially diluted six-fold with RNase/DNase-free water.  $5\ \mu\text{l}$  of RNA solution was added as the template while RNase/DNase-free water was used as a negative control. Each test was performed in triplicate.

### Inactivated viral particle

Inactivated SARS-CoV-2 viral particles BBIBP-CoV (Wang et al., 2020) were used for spiking. The viral particle was serially diluted with RNase/DNase-free water five-fold and split into three sets. The first set was measured directly without any treatment. The second set was measured after heat inactivation at  $95^{\circ}\text{C}$  for 5 min on a Dry Bath incubator (MVS-200; Scottech Medical co.).

TABLE 1 RT-LAMP reaction components

LAMP reaction components for each reaction	Volume	
	Fluorescent reading	Colorimetric reading
WamStart Master Mix (M1800; New England BioLabs)	$12.5\ \mu\text{l}$	$12.5\ \mu\text{l}$
10X Primer Mix	$2.5\ \mu\text{l}$	$2.5\ \mu\text{l}$
Fluorescent Dye, SYTO9 (S34854; ThermoFisher)	$0.5\ \mu\text{l}$ with the concentration of $25\ \mu\text{M}$	–
Template	$5\ \mu\text{l}$	$5\ \mu\text{l}$
Total Reaction volume	Add RNase/DNase to $25\ \mu\text{l}$	Add RNase/DNase to $25\ \mu\text{l}$

The third set was measured after RNA extraction with Qiagen QIAamp Viral RNA Mini Kit (52904; Qiagen). The RNA extraction procedure followed the manufacturer's protocol. Since the volume difference between the sample (140  $\mu$ l) and RNA extract (60  $\mu$ l), 80  $\mu$ l RNase/DNase-Free water to maintain the concentration. A 5  $\mu$ l of the template solution was added to the RT-LAMP reagents. All the measurements were performed in triplicate.

## Clinical samples

Nucleic acid was extracted from the clinical samples using MagMAX Viral/Pathogen Nucleic Acid Isolation Kit (A42352; Thermo Fisher) to minimize the impurity effect in the qPCR assay. All the nucleic acid extraction processes followed the manufacturer's protocol. After nucleic acid extraction, 5  $\mu$ l of the eluted nucleic acid were added to LAMP reagents in the 96-well plate. At the same time, 5  $\mu$ l of the eluted nucleic acid were added to qPCR reagents. After following the qPCR assay protocol,  $C_t$  values of the sample were obtained.

## qPCR assay and digital PCR assay

We used the GoTaq Probe one-step RT-qPCR kit (A6121; Promega) with a 1.5  $\mu$ l primer mix to assess the  $C_t$  values of the samples. CDC N1 primer set (1006770; IDT) was used for SARS-CoV-2 detection, while CDC RNase P primer set was used for human gene detection. Both primer sets contain 100  $\mu$ M forward and backward primers. After adding 5  $\mu$ l of the eluted solution, the final reaction volume was increased to 20  $\mu$ l by adding RNase/DNase Free water.

The PCR cycle conditions were set according to the manufacturer's instructions. The solutions were reverse transcribed for 15 min at 45°C and initial denaturation for 2 min at 95°C. Then, 45 cycles of 3 s at 95°C and 30 s at 55°C were processed.

Digital PCR assay (QX200 Droplet Digital PCR System; Bio-Rad) was used to acquire the template copy number in the samples. The whole procedure was performed using the manufacturer's protocol. Briefly, 20  $\mu$ l of the sample (RNA solution or CDC standard positive control plasmid) was mixed with 70  $\mu$ l DG oil (Bio-Rad) and placed into a droplet generator. After conducting PCR on a thermal cycler, droplets were analysed by QX200 Droplet Reader. Meanwhile, the sample was run qPCR assay simultaneously to obtain the  $C_t$  values.

## qLAMP assay with human genomic DNA and cell lines

TaqMan Control Genomic DNA, purchased from Thermo Fisher, was used as the positive control. The

human bronchial epithelium cell line (BEAS-2B) and the human A549 adenocarcinoma cell line obtained from Sigma–Aldrich were dissociated with Trypsin and resuspended in sterile sodium chloride solution (S8776; Sigma–Aldrich). The cells were counted and diluted into 2000, 1500, 1000, 500, 100, 50, 10, 5, and 1 cell/ $\mu$ l. The same batch of cell samples was split into two groups, treated with and without heat inactivation (95°C for 5 min). 5  $\mu$ l of the template solution was added to the RT-LAMP reagents. All the measurements were performed in triplicate.

## RESULTS

### The linear relationship between the copy number and threshold time in RT-qLAMP assay

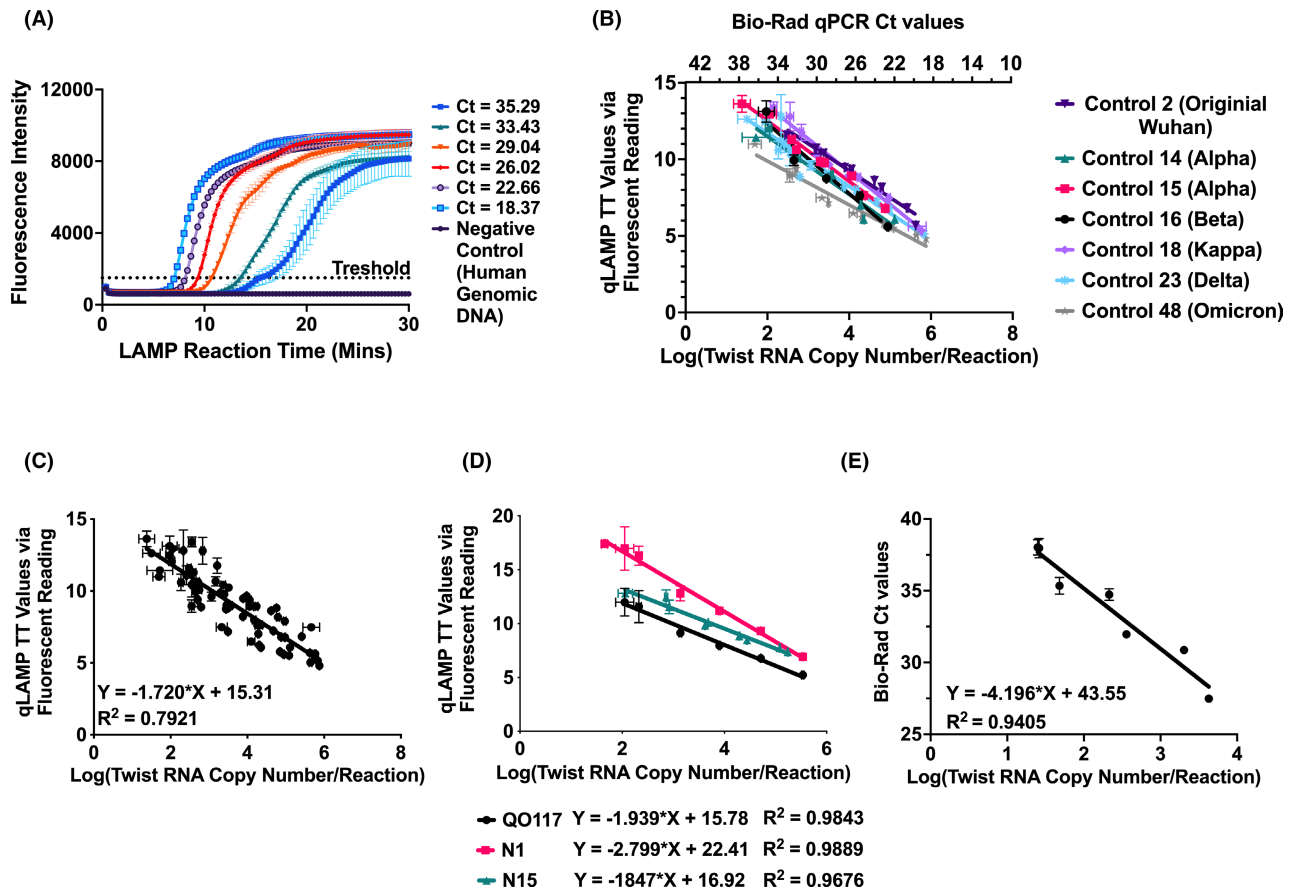
The components of the RT-qLAMP assay are listed in Table 1. Using SYTO 9 as a fluorescent dye, the real-time amplification curves of RT-LAMP assays with the QO117 primer set (Huang et al., 2020; Lim et al., 2021) were plotted in Figure 1. The RT-LAMP reaction shows a similar pattern as RT-qPCR: the lower concentration of SARS-CoV-2 RNA, the longer time to reach the threshold. RT-qPCR was run in parallel with RT-qLAMP using the same batch of RNA samples, to compare  $C_t$  values of qPCR with TT values of qLAMP. To quantify the copy number of SARS-CoV-2, digital PCR and qPCR were used to establish a calibration curve of the copy number vs  $C_t$  values of qPCR (Figure 1E).

We propose that the time of fluorescence signal reaching the threshold is the TT value of qLAMP, which showed a linear relationship with the concentrations of full-length RNA (Twist Bioscience Ltd, USA) (Figure 1B). The synthesized full-length RNAs for seven different variants of SARS-CoV-2 have been studied (Table 2). This linear relationship was observed to all of these different RNAs of SARS-CoV-2, including the original virus (Twist Control 2), Alpha (Twist Control 14 & 15), Beta (Twist Control 16), Kappa (Twist Control 18), Delta (Twist Control 23), and Omicron variants (Twist Control 48). The equations of calibration curves for these variants are listed in Table 2.

In spite of many mutations in the variants, the results show that the QO117 primer set can detect all the variants of SARS-CoV-2, consistent with the in-silico analysis in Table S1. The RT-qLAMP assay was especially sensitive to Twist Control 15 (Alpha) and Twist Control 23 (Delta), which detected the samples with  $C_t = 37.77$  and  $C_t = 37.75$ , respectively. Remarkably, all the amplification of these variants was detected within 15 min via fluorescent reading.

We combined all the data from different variants of SARS-CoV-2 to give an overall standard curve (Figure 1C), which could estimate the viral loads of





**FIGURE 1** Standard curves for the qLAMP assay detecting synthesized full-length RNA of SARS-CoV-2. (A) LAMP amplification curves were based on the fluorescent intensity in 30 min with a full-length SARS-CoV-2 RNA template (control 2). The RNA concentrations were measured by qPCR assay. (B) Standard curves for the qLAMP TT value against the twist RNA copy number. The copy number was converted from the qPCR  $C_t$  values (secondary x-axis) based on the standard curve presented in (E). The different variants of SARS-CoV-2 in full-length RNA were tested. (C) Standard curve for qLAMP assay after combining all the data in (b) from different SARS-CoV-2 variants. (D) Standard curves for qLAMP assay using different COVID-19 primer sets. Twist RNA control 2 was used as the positive control. (E) the standard curve between bio-rad qPCR  $C_t$  values and twist RNA copy numbers. Twist RNA control 2 was used as a positive control. The copy number was determined by digital droplet PCR. All the results (A–E) were presented as *MEAN* ± *SEM*.

**TABLE 2** The linear relationship between the copy number and LAMP  $C_t$  values

Control	GISAID nAME	Regression equation <sup>a</sup>	$R^2$
Control 2	Wuhan-Hu-1	$Y = -1.758 \cdot X + 16.29$	0.9658
Control 14 Alpha	England/205041766/2020	$Y = -1.893 \cdot X + 15.28$	0.9445
Control 15 Alpha	England/MILK-9E05B3/2020	$Y = -1.987 \cdot X + 16.48$	0.9803
Control 16 Beta (B.1.351)	South Africa/KRISP-EC-K005299/2020	$Y = -2.282 \cdot X + 16.89$	0.9473
Control 18 Kappa (B.1.617.1)	India/CT-ILSGS00361/2021	$Y = -2.138 \cdot X + 17.75$	0.9796
Control 23 Delta (B.1.617.2)	India/MH-NCCS P1162000182735/2021	$Y = -1.750 \cdot X + 15.23$	0.9121
Control 48 Omicron (B.1.1.529)	Hong Kong/HKU-211129-001/2021	$Y = -1.432 \cdot X + 12.76$	0.9499

<sup>a</sup>Y represents the qLAMP TT values and X stands for the logarithm of the template copy number.

clinical samples. The deviation of RT-qLAMP TT values was increased with the decrease of the viral RNA concentrations. When  $C_t$  values of qPCR assays with the RNA samples were greater than 32, the variation of RT-qLAMP TT values was greater than 1, which is about 2  $C_t$  value in qPCR.

Apart from the QO117 primer set, the other two primer sets, N1 and N15 primer sets, were designed

for detecting SARS-CoV-2 in the previous work (Huang et al., 2020). According to Figure 1D and Table S1, these three primer sets were all capable of detecting the original SARS-CoV-2 (Twist RNA control 2) within 20 min and presented a linear relationship between the qLAMP TT values and qPCR  $C_t$  values. Among these three primer sets, using QO117 resulted in the shortest incubation for the same RNA concentration. The

confidence of RT-qLAMP can be estimated by comparing R values with RT-qPCR in the calibration curves, when the same set of samples (different concentrations of Twist RNA control 2) were tested. RT-qLAMP has R values greater than 0.96, whilst RT-qPCR has an R-value of 0.94 (Figure 1D, E).

## Fluorescent and colorimetric RT-qLAMP assay

In our previous work (Huang et al., 2020; Lim et al., 2021), we have employed a colorimetric assay for the detection of SARS-CoV-2. The test result of RT-LAMP could be read by the colour change, yellow for positive and pink for negative. The time-course of colour change was plotted against the reaction time (Figure 2). Similar to the curves of fluorescent reading, the colorimetric signal entered the exponential phase when the amplification accelerated, and the TT value of RT-qLAMP is related to the viral RNA concentration.

After setting the threshold for colorimetric reading, the TT values of qLAMP for the detection of Twist Control 2 (Original) and Twist Control 23 (Delta variant) were linear to the copy numbers of the viral RNA, as well as the corresponding qPCR  $C_t$  values (Figure 2B). Compared to fluorescent reading, the colorimetric assay had a longer incubation time but could reach the same sensitivity within 30 min.

## RT-qLAMP assay with inactivated viral particles

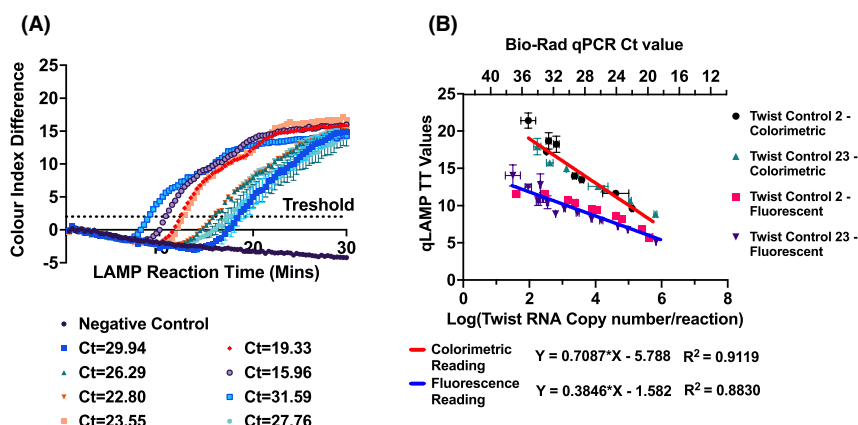
Since RNA would be quickly degraded by the RNase in clinical samples, this work also evaluated qLAMP

assays using serially diluted inactivated SARS-CoV-2 viral particles – BBIBP-CorV (Wang et al., 2020; Xia et al., 2021). The inactivated viral particles were diluted in RNase/DNase-free water with three different treatment conditions for RT-qLAMP running, with and without heat inactivation, and extracted RNA.

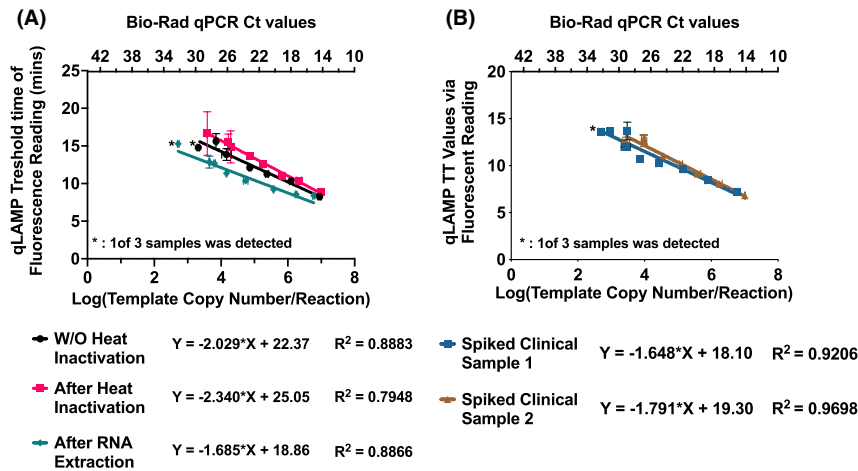
The linear relationship between the TT values of qLAMP and the corresponding qPCR  $C_t$  values was established for these three different pre-treatments (Figure 3A). The RT-qLAMP assay for RNA extracts had a shorter reaction time and higher sensitivity than those assays for the heat-inactivated viral particles. All samples were detected within 15 min (Figure 3A). The deviation of TT values in RT-qLAMP was large for the heat-inactivated samples, especially for the sample with high  $C_t$  values (Figure 3A). Although RNA should be released from viral particles after heat inactivation, the TT values of RT-qLAMP were even higher than in the same samples without heat inactivation. However, in practice, it is safer to heat the samples at 95°C for 5 min to inactivate the virus before the RT-qLAMP assay. Negative swab samples were spiked with BBIBP-CorV and heated to 95°C for 5 min to assess the potential of qLAMP application to clinical samples. The linear relationship was also observed for the spiked clinical samples vs the TT values of RT-qLAMP (Figure 3B).

## qLAMP assay with human gene

In the COVID-19 screening, LAMP primers for the beta-actin gene (ACTB) in human samples were also used as an internal control to ensure sampling quality. Previously, only four primers were included in ACTB primer set to detect ACTB gene, which prolonged the



**FIGURE 2** Standard curves for the qLAMP assay using synthesized full-length RNA of SARS-CoV-2 in six-fold serial dilution via colorimetric reading. (A) LAMP amplification curves were generated by colorimetric reading in 30 min with a full-length SARS-CoV-2 RNA template in different concentrations. The RNA concentrations were measured by qPCR assays. (B) Standard curves for the qLAMP TT value against the template concentration via colorimetric and fluorescent readings. The copy number was converted from the qPCR  $C_t$  values (secondary x-axis) based on the standard curve presented in Figure 1E. Twist RNA control 2 (original) and twist RNA control 23 (Delta variant) were used as the positive control. The individual standard curves were presented in Figure S1. All the results were presented as  $MEAN \pm SEM$ .



**FIGURE 3** qLAMP assay using viral particles in serial dilution with water (A) and clinical negative samples (B). In (A), the copy number in the logarithm was calculated qPCR  $C_t$  values in the secondary x-axis. The calibration between qPCR  $C_t$  values and copy number was presented in (C). The regression equations below the figure describe the relationship between qLAMP TT values and template copy numbers in logarithm. In (B), inactivated viral particles were spiked into three clinical samples. The qLAMP assay was run after heat inactivation. The copy number was converted from  $C_t$  values of qPCR assays, shown in the secondary x-axis.

reaction (Huang et al., 2020; Poon et al., 2005). The reaction time was significantly shortened by adding loop primers (Avelar, 2019; Jamwal et al., 2021; Moore et al., 2021; Zhang et al., 2020). Therefore, we designed a new set of primers, ACTB-n, which also detected the ACTB sequence with the additional loop primers.

Using human genomic DNA as positive controls, the qLAMP TT values by fluorescent reading were decreased linearly with the increase of the masses of human genomic DNA (Figure 4A). The original ACTB required a much longer incubation time and could only detect the samples with  $C_t$  values less than 25 within 30 min. By contrast, the ACTB-n primer set was able to detect samples with  $C_t$  value >35 or 10 pg of human genomic DNA within 20 min based on the fluorescent reading (Figure 4A).

Figure 4B illustrated the difference in TT values of qLAMP by the fluorescence and colorimetric readings. ACTB-n primer set was able to detect 10 pg human genomic DNA per reaction within 30 min and had a relatively small deviation in TT values of qLAMP. The time for fluorescence reading was much shorter than that of colorimetric reading. For example, in the reaction containing 50 ng human genomic DNA, there was around a 6-min gap between these two readings (Figure 4B).

To evaluate the potential of estimating the concentration of human genomic DNA in the clinical sample, we tested the performance of ACTB-n with three different human cell lines (Figure 4C, D), which were the human bronchial epithelium cell line (BEAS-2B), human lung adenocarcinoma cell line (A549), and human mesenchymal stem cells (hMSC). The qLAMP assay was conducted with two different pre-treatments, with and without heat inactivation.

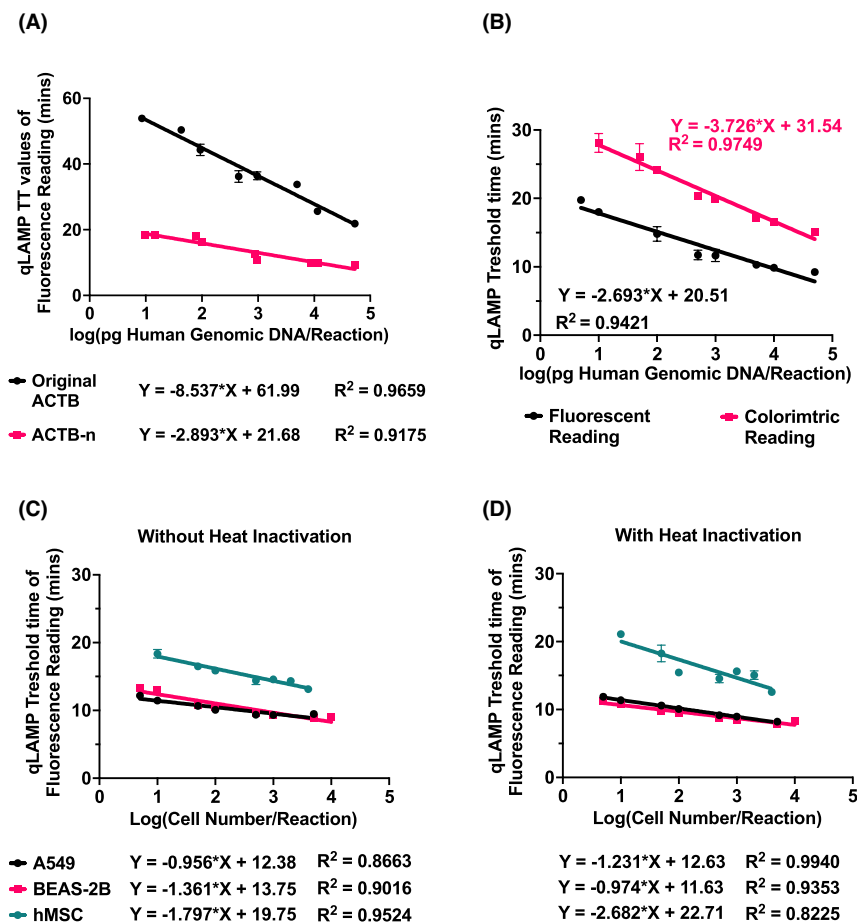
The qLAMP TT values of fluorescent reading were linearly increased with the decrease of the cell numbers

(Figure 4C, D). There was no significant difference in qLAMP TT values for detecting the ACTB gene in the samples with and without heat inactivation. It showed that the ACTB-n primer set could detect all those three cell lines down to 10 cells per reaction within 20 min. The qLAMP TT values for hMSC were relatively higher than the other two cell lines, BEAS-2B and A549 (Figure 4C, D). Since reverse transcriptase was included in the testing kit, RNA containing the ACTB gene could also be detected. Therefore, the different ACTB gene expression levels of different cell lines could result in different qLAMP TT values, even with the same cell amount.

## RT-qLAMP assay for clinical samples

Sixty-four nucleic-acid extracted clinical samples, including 25 positives and 39 negatives, were applied to validate the qLAMP assays. As presented in Figure 5A, the RT-qLAMP reaction detected all the positive samples by fluorescent reading within 10 min, even to the samples with  $C_t = 35$  (RT-qPCR assay). The RT-qLAMP TT values of positive samples were linear to the logarithm of the template copy number (Figure 5A). For negative samples, the ACTB gene, both on human DNA and mRNA, were all detected with ACTB-n primers (Figure 5B). The RT-qLAMP TT values for ACTB primers also had a linear relationship with the mass of the human genomic gene, with higher variations than the RT-qLAMP assay with QO117 primers (Figure 5B).

To assess the quality of sampling, 13 positive samples were run RT-qLAMP assays with QO117 and ACTB primers simultaneously (Figure 5C). The RT-qLAMP TT values of the sample from both the RT-qLAMP assay using QO117 primers and ACTB-n primers were plotted against the  $C_t$  values from RT-qPCR assay using the



**FIGURE 4** qLAMP assay using human primers to detect human genome and cell lines. (A) Standard curves for the qLAMP TT values against human genomic DNA. Human genomic DNA was used as the positive control. The secondary x-axis presents the corresponding  $C_t$  values of qPCR using RNase P primers. Different human primers were used. (B) Standard curve for qLAMP assay detecting human genomic DNA by human primers (ACTB-n) via fluorescence and colorimetric readings. (C) Standard curve for qLAMP assay detecting human genomic DNA in human cells without heat inactivation. Human bronchial epithelium cell line (BEAS-2B), human lung adenocarcinoma cell line (A549), and human mesenchymal stem cells (hMSC) were tested. (D) Standard curve for qLAMP assay detecting human genomic DNA in human cells after heat inactivation. Human bronchial epithelium cell line (BEAS-2B), human lung adenocarcinoma cell line (A549), and human mesenchymal stem cells (hMSC) were tested. All the results were presented as  $MEAN \pm SEM$ .

same sample with N1 primers to detect SARS-CoV-2. The RT-qLAMP TT values for detecting SARS-CoV-2 were linear to the  $C_t$  values of RT-qPCR, while the RT-qLAMP TT values for detecting human ACTB in the positive samples were maintained at around 11.05. It indicates that the number of human cells in the swab samples was consistent, and the sampling process was reliable.

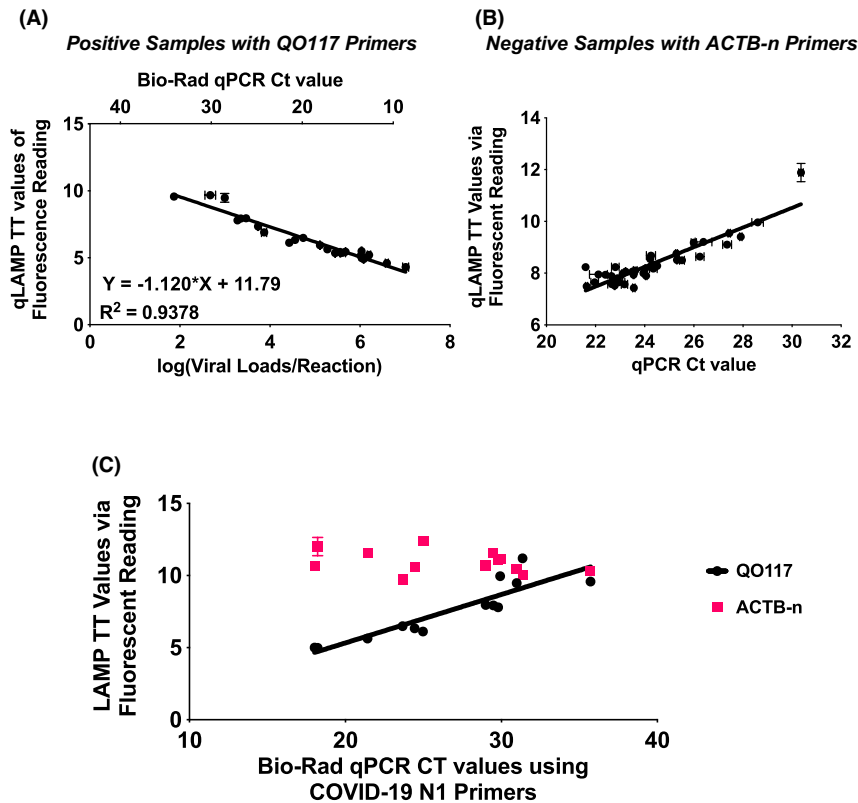
## DISCUSSIONS

This work demonstrates that RT-qLAMP can be applied to quantitatively evaluate the viral loads and assess the sampling quality. This work used fluorescent dye, SYTO 9, to acquire the real-time amplification curve. qLAMP TT values were linear with the template concentrations, either in RNA or DNA. This linear relationship was validated by two different targeted sequences,

SARS-CoV-2 and the human ACTB gene. In practice, a low TT value of qLAMP can be generally considered to indicate a higher viral load in a patient specimen and a high TT value can be considered to indicate a lower viral load in a patient specimen. The TT values of qLAMP should be calibrated by comparing with  $C_t$  values of authorized molecular diagnostic qPCR tests. Accordingly, the standardization of qLAMP instrument should be developed in the future, as we did for qPCR.

The qLAMP assay using QO117 primers was able to detect the samples containing the original SARS-CoV-2 strain as well as its variants (Alpha, Beta, Kappa, Delta, and Omicron). Although there was no mutation in the QO117 primer targeting region (Table S1), the mutations of different variants might change the RNA secondary structures, which will affect the efficiency of reverse transcription, leading to the variation of standard curves for different variants in RT-qLAMP. To achieve a better estimation, it is probably good to use





**FIGURE 5** Application of qLAMP assay with clinical samples. (A) Standard curves for qLAMP assay using QO117 primers to detect positive clinical samples. The qLAMP TT values were plotted against template copy numbers in logarithm, calculated from qPCR  $C_t$  values based on the calibration curve in Figure 1E. (B) Standard curves for qLAMP assay using ACTB-n primers to detect negative clinical samples. The qLAMP TT values were plotted against template copy numbers in logarithm, calculated from qPCR  $C_t$  values based on the calibration curve in Figure 1SB. (C) qLAMP assays with QO117 and ACTB-n primers tested the viral load and the human gene of the same sample. The qLAMP TT values of both qLAMP assays were plotted against the  $C_t$  values of qPCR using N1 primers to detect SARS-CoV-2. A total of 13 samples were tested. All the results were presented as *MEAN* ± *SEM*.

the different calibration curves for different variants. The RNA concentration of 200 copies per reaction, or  $C_t < 34$  can be detected within 15min by fluorescent reading in the RT-qLAMP assay. The colorimetric reading had the same sensitivity but required a longer incubation time (Figures 1B and 2B). Since the colorimetric assay was based on the colour changing of the pH indicator, the accumulation of hydrogen ions, released from the amplification, was required to overcome the low concentration of pH buffer in the reagents (Tanner et al., 2015).

The design of primers could affect the amplification efficiency. The addition of loop primers could significantly reduce the amplification time (Figure 4A). The targeting region's selection may also be essential for amplification (Figure 1D). With the same RNA template concentration, using the QO117 primer set, targeting RNA encoding Orf1ab, exhibited lower qLAMP TT values than using N1 and N15 primer sets, targeting two regions of nucleocapsid protein (Table S1). The primer binding could be much easier if the target region was exposed, shortening the amplification time.

Since RNase quickly digested full-length Twist RNA in the real samples, the inactivated BBIBP-CorV

SARS-CoV-2 viral parties, were spiked to evaluate the feasibility of applying qLAMP assay for clinical samples (Figure 3). Detecting the heat-inactivated samples took even longer than those without heat treatment, and after RNA extraction, which might be caused by the RNA degradation over the heat inactivation (Brisco & Morley, 2012) or the interaction between the RNA template and the protein shell when the sample cooled back to room temperature (Sanchez de Groot et al., 2019). Despite this result, it is still the most practical way to inactivate the virus and releases the RNA for testing.

The RT-qLAMP assay was run for spiked clinical samples after heat inactivation and cooling back to room temperature. The qLAMP TT values showed a linear relationship with the RNA concentrations in each clinical sample. The RT-LAMP reaction after RNA extraction gave a higher sensitivity and smaller qLAMP TT values than the sample with and without heat inactivation. Even with RNA extraction, the overall detection time using qLAMP could be completed within 1 h, much quicker than qPCR. The additional requirements associated with extraction may limit its application in the point-of-care setting.

A new set of ACTB primers was designed to quantify the human genes in the samples to access the quality of sampling. For detecting the ACTB gene in cell lines, there was no significant difference in the qLAMP TT values and sensitivity between the sample with and without heat treatment. The reason for this might be that the reagents do not maintain the osmosis pressure of cells. The cells burst after being added to the reagents, which would be a similar result to heat inactivation. Since reverse transcription was included in the RT-qLAMP assay, mRNA of ACTB genes in cells could also be detected and amplified. Therefore, the qLAMP TT values were slightly inconsistent in different cell lines.

Since RT-qLAMP can detect both RNA and DNA in cells, the deviation of qLAMP TT values for detecting cells was always greater than using human genomic DNA (Figure 4). The same trend was found for the qLAMP assay with QO117 primers (Figure S2). The R-squared values of standard curves with fragment DNA sequence were always relatively higher than using the RNA template. The efficiency of reverse transcription could affect the following LAMP amplification.

The RT-qLAMP assays with QO117 and ACTB-n primers were both validated by 64 nucleic acid extracted clinical samples, including 25 positive samples. The linearity of qLAMP TT values against template concentrations was maintained for both qLAMP assays. In addition, the human gene and SARS-CoV-2 for 13 positive clinical samples were detected simultaneously. Similar to qPCR assay, the TT value of RT-qLAMP could be used to estimate the concentration of RNA/DNA template. This RT-qLAMP requires a much shorter reaction time (<20min) compared to the RT-qPCR assay (around 2.5 h). In practice, the RT-qLAMP assay using QO117 and ACTB-n primers can be used to estimate the viral load of SARS-CoV-2, and also indicate sampling quality for clinical samples.

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## CONFLICT OF INTEREST

This project is academic research funded by Prenetics. There is no patent nor commercial interest related to this research work.

## DATA AVAILABILITY STATEMENT

The other data are available in supplementary information.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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