Original Article

A Simple and Multiplex Loop-Mediated Isothermal Amplification (LAMP) Assay for Rapid Detection of SARS-CoV

Jin Hwa Kim[1,†], Minhee Kang [iD][1,2,†,*], Eunkyoung Park[1,2], Doo Ryeon Chung [iD][3,4,5,*],
Jiyeon Kim[6,7] & Eung Soo Hwang[6,7]

Abstract The current diagnosis of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) relies on laboratory-based tests since its clinical features are nonspecific, unlike other respiratory pathogens. Therefore, the development of a rapid and simple method for on-site detection of SARS-CoV is crucial for the identification and prevention of future SARS outbreaks. In this study, a simple colorimetric and multiplex loop-mediated isothermal amplification (LAMP) assay was developed to rapid screening of severe acute respiratory syndrome-associated coronavirus (SARS-CoV). It can be visually detected based on color change and monitored in real-time with fluorescent signals. The performance of this assay, based on six primers targeting open reading frame (ORF1b) and nucleocapsid (N) genes located in different regions of the SARS-CoV, was compared with real-time RT-PCR assay using various concentrations of target genes. The detection limit of the LAMP assay was comparable to that of real-time RT-PCR assay and therefore a few target RNA to 10^4-10^5 copies could be detected within a short period of time (20-25 min). In addition, we established a multiplex real-time LAMP assay to simultaneously detect two target regions within the SARS-CoV genome. Two target sequences were amplified by specific primers in the same reaction tube and revealed that it was able to detect down to 10^5 copies. The standard curve had a linear relationship with similar amplification efficiencies. The LAMP assay results in shorter "sample-to-answer" time than conventional PCR method. Therefore, it is suitable not only for diagnosis of clinical test, but also for surveillance of SARS virus in developing countries.

Keywords: SARS-CoV, Loop-mediated isothermal amplification, Colorimetric detection, Point-of-care test

[1]Biomedical Engineering Research Center, Smart Healthcare Research Institute, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea
[2]Department of Medical Device Management and Research, SAIHST, Sungkyunkwan University, Seoul, Republic of Korea
[3]Center for Infection Prevention and Control, Samsung Medical Center, Seoul, Republic of Korea
[4]Asia Pacific Foundation for Infectious Diseases (APFID), Seoul, Republic of Korea
[5]Division of Infectious Diseases, Department of Internal Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Republic of Korea
[6]Department of Microbiology and Immunology, Seoul National University College of Medicine, Seoul, South Korea
[7]Institute of Endemic Diseases, Seoul National University Medical Research Center, Seoul, South Korea
[†]These authors contrilbuted equally.
*Correspondence and requests for materials should be addressed to
M.H. Kang (✉ minhee.kang@samsung.com) and
D.R. Chung (✉ minikang@skku.edu)

Introduction

Severe acute respiratory syndrome (SARS) caused by a novel coronavirus (CoV) is a highly contagious respiratory illness[1]. Its initial systemic symptoms include muscle pain, headache, and fever. Onset of respiratory symptoms such as cough, dyspnea, and pneumonia is then followed in 2~14 days[1,2]. The SARS

epidemic in July 2003 was one of the worst global epidemics. It affected 30 countries with 8,096 cases and 774 deaths[3-7]. Since the end of its global epidemic, SARS has reemerged four times according to statistics of the World Health Organization (WHO): three times from laboratory accidents (Singapore and Taipei) and once in southern China where the source of infection remains undetermined although there is circumstantial evidence of animal-to-human transmission[8]. Nevertheless, considering the recent outbreaks of SARS-like disease caused by the newly emerged Middle East respiratory syndrome (MERS-CoV) that is highly related to SARS-CoV[9], the possibility of re-emergence of SARS may still remains[10,11]. Hence, the development of a rapid and simple method for on-site screening of SARS-CoV is important for the determination and prevention of future outbreaks. SARS-CoV could be detected at sera, throat washes and stool after the onset of illness with viral load ranging from 10^4 to 10^9 copies/ml[12] and the presence viral RNA differ from different anatomical site[13]. Current diagnosis of SARS-CoV infection depends on laboratory-based tests (*i.e.*, virus isolation in cell culture, serological, and molecular tests) because its clinical features are nonspecific particularly at the early stage of disease, unlike other respiratory illnesses. Virus isolation by inoculating cell cultures has long served as the gold standard to identify the presence of infection. However, this method needs considerable expertise and it is usually time consuming. Serological detection of viral antibodies (IgG, IgM) by enzyme linked immunosorbent assay (ELISA) and immunofluorescence (IFA) have been found to be sensitive (98.2% for ELISA and 99.1% for the IFA; 33.6%, respectively)[14]. However, antibodies are often undetectable in the first few days after the onset of infection, making early diagnosis difficult. On the other hand, conventional reverse transcriptase polymerase chain reactions (RT-PCR) and their quantitative approaches can allow direct detection of viral RNA. However, they require complex analytical instruments, expensive reagents, and considerable expertise[15]. In addition, these techniques have limitations in developing countries and field situations because of poor resources and the lack of specialized personnel.

Loop-mediated isothermal amplification (LAMP) is a nucleotide acid amplification method that features high sensitivity, specificity, and rapidity under isothermal conditions[16]. LAMP requires a set of four specific primers that recognize six distinct sequences on the target. Primers used for LAMP assays contain a forward outer primer (F3), a backward outer primer (B3), a forward inner primer (FIP, comprised of two binding domains, F1c and F2), and a backward inner primer (BIP, comprised of two binding domains, B1 and B2c). FIP and BIP, of which both the 3′ region and 5′ region match the target, can interact with upstream external primers, resulting in the displacement of strands containing self-complementary regions that form stem-loop structures[17]. The reaction can be conspicuously accelerated by adding two loop primers (LF and LB), thus reducing the total reaction time[18]. Amplification products can be detected at the end-point by using several methods, including gel electrophoresis, measuring turbidity derived from magnesium pyrophosphate precipitation[19], and fluorescent signal generated by DNA intercalators such as calcein and SYBR green I[20]. LAMP does not need any special devices for processing. Thus, it is widely used for the detection of a wide range of pathogens such as bacteria[21,22], parasites[23], and viruses[24,25] including SARS-CoV[26,27]. For instance, H. Thai *et al.*[26]. had been validated reverse transcription (RT) LAMP assay using SARS-positive clinical specimens with high sensitivity. By the way, in the detection method based on turbidity monitoring and DNA intercalators, the detection of unexpected signals derived from primer dimer and/or non-primer reactions can lead to false-positive results[28]. Apart from that, colorimetric detection methods using pH indicator dyes that monitor a significant pH change during LAMP amplification have been recently developed[29]. Its results can be simply and readily visualized with naked eyes based on color change. Recently, fluorescent RT-LAMP assays using quenching probes have also been developed to specifically monitor only primer-derived signals[30,31]. However, most reported detection methods for LAMP are end-point techniques that can only detect a gene in a single reaction[32,33]. Multiplex LAMP assays that can detect and discriminate two or more target genes in a single reaction are limited.

In the present study, a simple colorimetric and multiplex loop-mediated isothermal amplification (LAMP) assay was developed to rapid screening of severe acute respiratory syndrome-associated coronavirus (SARS-CoV). The structural information of SARS-CoV and sequence alignment for LAMP assay are described in Figure 1. A set of primers comprising an inner pair (FIP, BIP), an outer pair (F3, B3), and a loop pair (LF, LB) and primer sequences for the open reading frame 1b (ORF1b) and N (nucleocapsid) genes are lised in Table 1. We optimized, and validated our LAMP assay by color change and agarose gel electrophoresis.
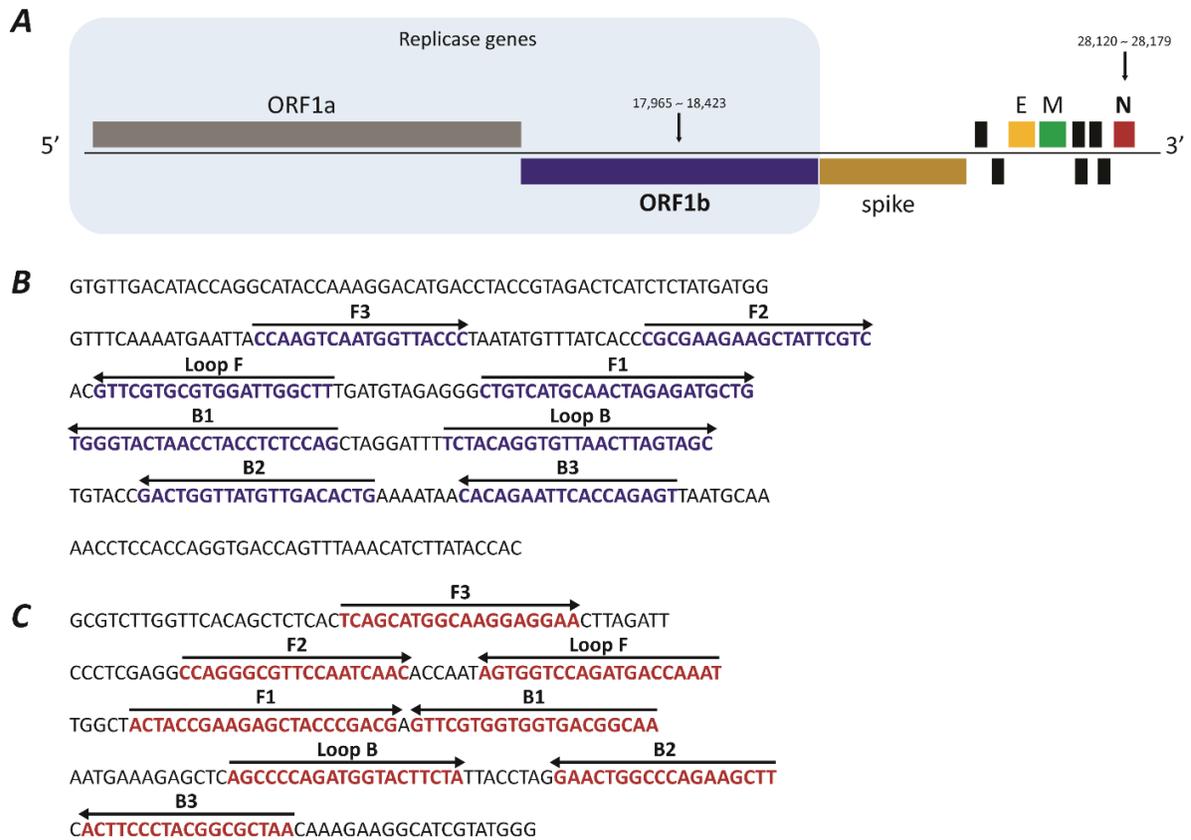
Figure 1. Structural information of SARS-CoV and sequence alignment for LAMP assay. A. Structure information of SARS-CoV (Genbank accession number: AY274119.3). Partial sequence was used for designing LAMP primers (ORF1b; 17,965~18,423, 459bp, N; 28,120~28,179, 600bp). B-C. Design of LAMP primers (B for ORF1b gene, C for N gene). Positions of designed primers are indicated by arrows.

Table 1. Primers used for LAMP assay.

Primer	Sequence (5'-3')	Length (bp)
ORF1b - F3	CCAAGTCAATGGTTACCC	18
ORF1b - B3	ACTCTGGTGAATTCTGTG	18
ORF1b – LF	AAGCCAATCCACGCACGAAC	20
ORF1b – LB	TCTACAGGTGTTAACTTAGTAGC	23
ORF1b – FIP*	[HEX]TGCAATGCAGCATCTCT[BHQ1-dT]AGTTGCATGACAGCGCGAAGAAGCTATTCGTC	42
ORF1b – FIP	CAGCATCTCTAGTTGCATGACAGCGCGAAGAAGCTATTCGTC	42
ORF1b – BIP	TGGGTACTAACCTACCTCTCCAGCAGTGTCAACATAACCAGTC	43
N - F3	TCAGCATGGCAAGGAGGAA	19
N - B3	TTAGCGCCGTAGGGAAGT	18
N – LF	ATTTGGTCATCTGGACCACT	20
N – LB	AGCCCCAGATGGTACTTCTA	20
N – FIP	CGTCGGGTAGCTCTTCGGTAGTCCAGGGCGTTCCAATCAAC	41
N – FIP*	[FAM]TGCAATGCGTCGGGT[BHQ1-dT]AGCTCTTCGGTAGTCCAGGGCGTTCCAATCAAC	41
N – BIP	GTTCGTGGTGGTGACGGCAAGAAGCTTCTGGGCCAGTTC	39

In addition, performance of the LAMP assay was compared with real-time PCR assay using fluorescence- modified FIP primers. Finally, we also have established a multiplex LAMP assays that can be detected simultaneously two target regions in a single reaction.

Results and Discussion

As a preliminary experiment, LAMP assays using either four or six primers targeting N gene of SARS-CoV were performed. To determine the optimal reaction temperature, LAMP assay was conducted using a temperature gradient ranging from 50 °C to 72 °C for 30 min. When the tube was examined before gel electrophoresis, a positive LAMP reaction was indicated by an orange color while negative reactions remained a color of pink (see Supplementary information S1 Figure). After tubes were visually assessed for color change, samples were subjected to agarose gel electrophoresis. Characteristic bands were evident in the gel if the product was present. Based on agarose gel electrophoresis, the reaction with four primers produced a detectable LAMP product at 62 °C (see Supplementary information 2S Figure). However, non-specific amplification occurred. For the reaction with six primers, amplifications occurred anywhere between 57 °C and 68 °C for N gene and between 52 °C

and 65 °C for ORF1b gene. Based on these results, all LAMP assays conducted later were performed using a mixture II containing loop primers LF and LB.

To confirm that our primer sets were suitable for LAMP analysis, gradient assay was performed using temperature from 50 °C to 72 °C with both cDNAs as templates. After reaction for 30 minutes, 20 μl reaction containing cDNA showed color change from pink to yellow whereas the reaction containing distilled water as a negative sample did not show such color change. As a result of electrophoresis, smear band appeared at 62°C in a control containing only sterile water without a template. This non-specific amplification was attributed to sets of primers under certain assay conditions because LAMP relies on six different primers independently recognizing eight regions on the target sequences[34]. It is important therefore to determine optimal assay condition without non-specific amplification associated with primers. In Figure 2, the reaction at 65°C of a control sample did not show a smear band. Therefore, the detection of
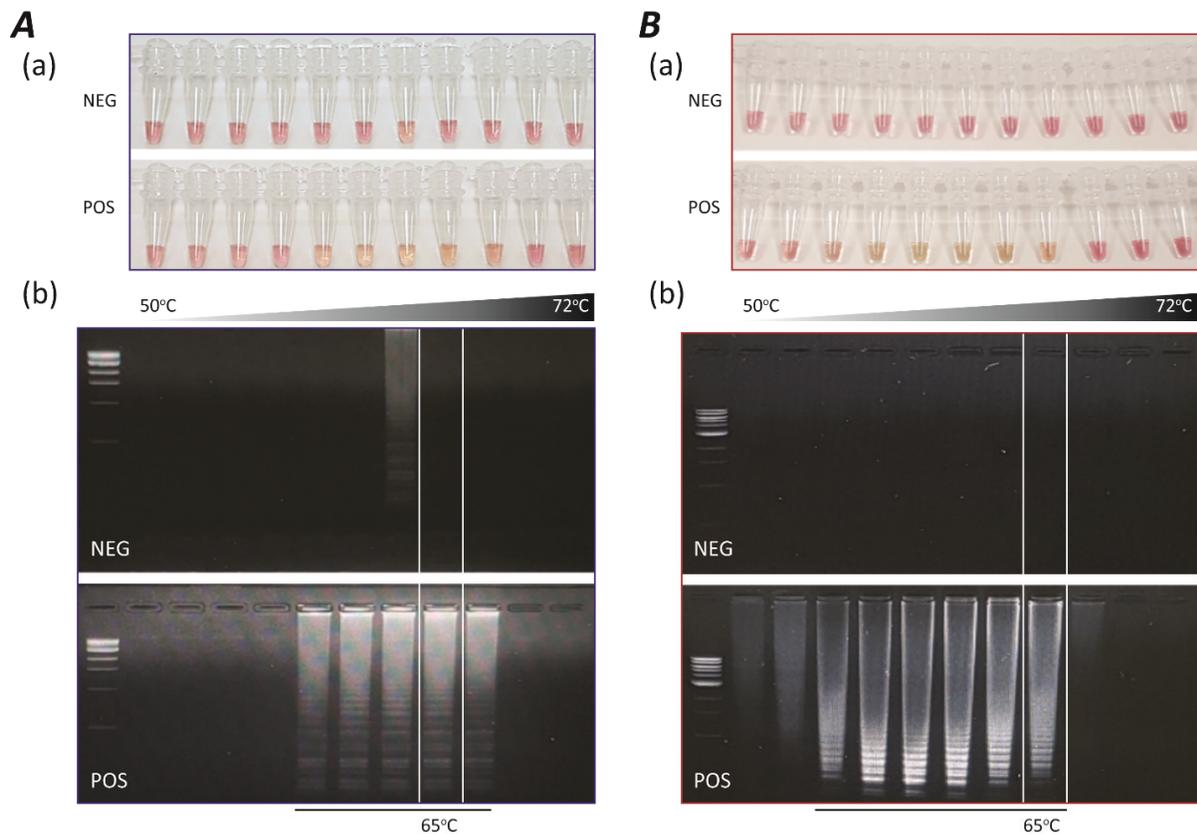

Figure 2. Optimization of LAMP assay. Detection of LAMP products. A, N gene; B, ORF1b gene (NEG, negative (non-template control, NTC); POS, positive with 50 ng of genomic DNA). (a) Naked eye detection at the end of assay (30 min). An orange color indicates a positive reaction and a neutral pink color indicates a negative reaction. (b) Agarose electrophoresis results of LAMP assay (temperature gradient from 50°C (lane 2) to 72°C (lane 11); lane 1, 1kb DNA ladder). The optimal temperature was determined to be 65°C for both targets.
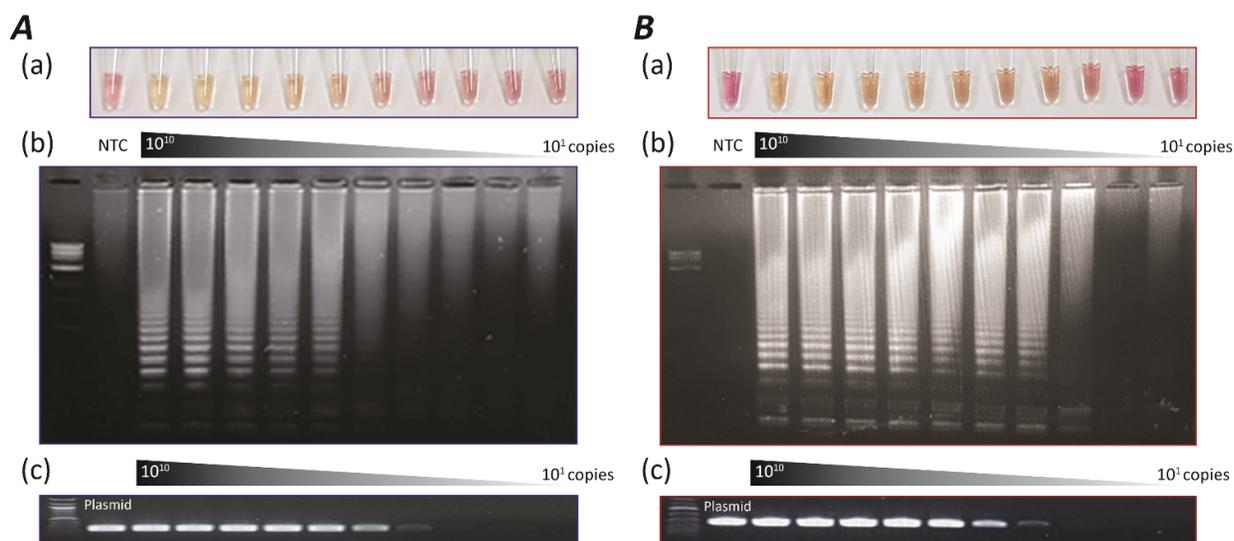
Figure 3. Sensitivity of LAMP assay. 10-fold serial dilution starting from ~ 10^{10} copies of genomic DNA (lane 3) down to ~10^1 copies (lane 12). A, ORF1b gene (from $4.2 * 10^{10}$ to $4.2 * 10^1$ copies), and B N gene (from $3.2 * 10^{10}$ to $3.2 * 10^1$ copies); (a) Naked eye detection at the end of assay (30 min). An orange color indicates a positive reaction and a neutral pink color indicates a negative reaction. (b) Agarose electrophoresis results of LAMP assay (lane 1, 1kb DNA ladder; lane 2, non-template control (NTC)). The positive reaction shows a characteristic ladder of multiple bands in electrophoresis analysis. (c) Agarose electrophoresis results of RT-PCR assay (lane 1, 1kb DNA ladder; lane 2, 50 ng of plasmid DNA).

ORF1b and N genes could be performed at this temperature. Sensitivities of LAMP and conventional PCR were then assessed by using 10-fold serial dilution of target (10^{10}~10^1 copies per reactions). The limit-of-detection under optimal conditions (using six primers at 65 °C for 30 min) are shown in Figure 3. Results of 1.5 % agarose gel electrophoresis for ORF1b gene and N gene are shown in Figures 3A and 3B. The detection limit of LAMP assay was estimated to be 10^5 copies per reaction for ORF1b gene and 10^4 copies per reaction for N gene. This indicates that visual detection results are correlated with results from agarose gel electrophoresis. When the same amount of RNA was used in conventional RT-PCR, a similar level of amplification was observed. The detection limit of conventional RT-PCR was estimated to be about 10^5 copies for ORF1b gene and 10^4 copies for N gene.

The performance of real-time LAMP assay was also compared with that of real-time PCR assay (Figure 4). Real-time LAMP reaction was carried out at 65 °C for 30 min and fluorescence signals were collected at 30 sec intervals. During real-time LAMP reaction, fluorescence data were obtained from HEX channel for ORF1b gene and FAM channel for N gene. Threshold time (T_t*) was calculated as the time at which the fluorescence signal was equal to the threshold value. In the plot, the Y-axis denotes fluorescence and the X-axis shows time in minutes. Standard curves of LAMP assays were established. They were linear over at least five orders of magnitude (R^2 > 0.98 for ORF1b gene and R^2 > 0.99 for N gene). Meanwhile, standard curves produced by real-time PCR revealed a good linearity within the detection limit, showing high correlations between Ct and DNA quantities (R^2 > 0.99 for both genes). The detection limit of the LAMP assay was slightly lower than or comparable to that of real-time PCR assay. However, target DNA with 10^5-10^6 copies can be detected within a short period of time (20-25 min).

Loop-mediated isothermal amplification (LAMP) assay is attractive for molecular diagnostic assays, especially for POC use. LAMP assay enables autocycling amplification under isothermal conditions within a short period of time without requiring expensive thermocycling equipment. In addition, its detection limit is comparable to that of conventional PCR. In this study, a LAMP assay was developed for rapid and quantitative detection of SARS-CoV. LAMP reaction performed at 65 °C within 30 min using six primers was able to detect up to 10^5 copies. There were no significant differences between results obtained by using gel electrophoresis and those obtained by visual detection based on color. Additionally, real-time LAMP assay using fluorescence dye and their quencher decorated in either 5' end and internal of FIP primers were established and compared with real-time PCR assay. LAMP assay can detect
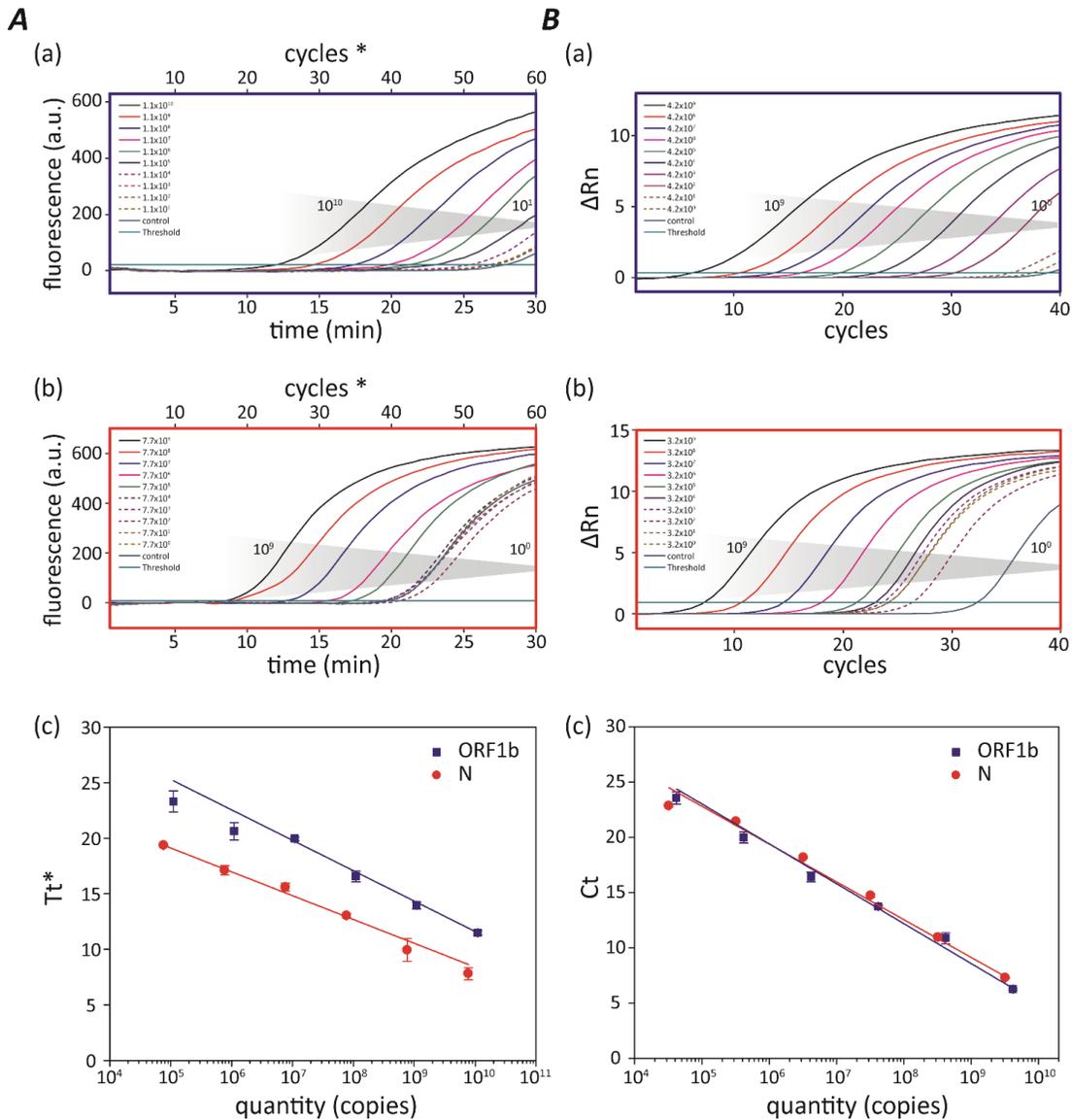
Figure 4. Sensitivity comparison of real-time LAMP and real-time RT-PCR assay using 10-fold serially diluted RNA containing about 10^{10}-10^0 copies. A, Real-time LAMP assay, and B, Real-time RT-PCR assay. (a-b) Amplification plots for (a) ORF1b gene and (b) N gene. (c) Standard curve derived from amplification plots. The standard curve was generated using Log_{10}-transformed concentrations of a 10-fold serially diluted RNA with corresponding threshold cycle (C_t) and threshold time (T_t*). Threshold time (T_t*) represents the time at which the fluorescence was equal to the threshold value during LAMP cycles. Error bars represent standard deviations from triplicate reactions.

target RNA within a few minutes (20-25 min). Its limit of detection was 10^5 copies, similar to real-time PCR.

On the other hand, multiplex assay is more attractive than performing multiple single-plex reactions. It also reduces the time and effort spent in the assay. In multiplex assay, two or more target sequences are simultaneously amplified in the same assay[35]. Complicating matters is that multiplex requires two or more sets of primers that do not interfere with each

other in a single reaction. Since four or six primers are contained in a single LAMP reaction, optimization and validation of multiplex PCR need considerable time and effort. In this work, multiplex real-time LAMP assays have been validated as shown in Figure 5. Our multiplex LAMP assay with adjustment of primer concentration could detect down to 10^5 copies based on RNA standards. The standard curve had a linear relationship ($R^2 > 0.99$) with similar amplification efficiencies. These results indicate that there is

Table 2. Detection limit of Colorimetric, LAMP, and RT-PCR assays.

Target gene	Assay	Detection Copy Number (copies)									
		10^{10}	10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10^1
ORF1b gene	Colorimetric	+	+	+	+	+	+	-	-	-	-
	LAMP	+	+	+	+	+	+	-	-	-	-
	RT-PCR	+	+	+	+	+	+	+	-	-	-
	Real-time LAMP	+	+	+	+	+	+	-	-	-	-
	qRT-PCR	+	+	+	+	+	+	-	-	-	-
N gene	Colorimetric	+	+	+	+	+	+	+	-	-	-
	LAMP	+	+	+	+	+	+	+	-	-	-
	RT-PCR	+	+	+	+	+	+	+	-	-	-
	Real-time LAMP	+	+	+	+	+	+	-	-	-	-
	qRT-PCR	+	+	+	+	+	+	-	-	-	-

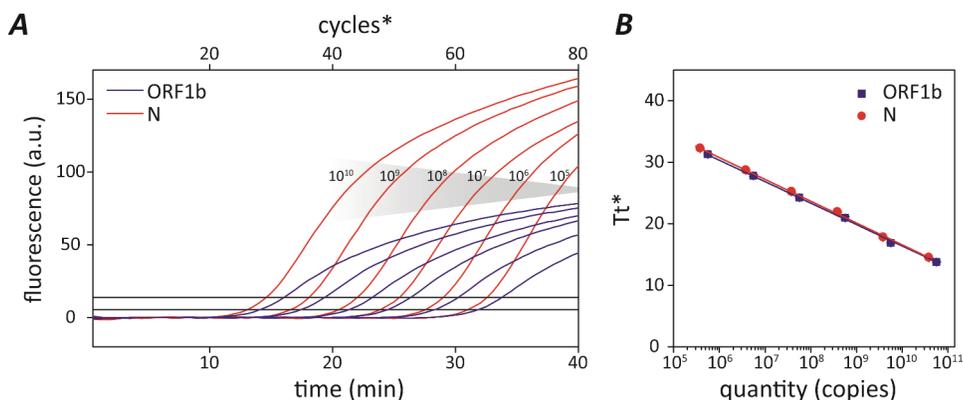+, Amplification occurred; -, amplification did not occur.

Figure 5. Multiplex capability of LAMP assay. Representative amplification plots and standard curves for 10-fold serially diluted RNA containing about 10^{10}-10^5 copies of ORF1b gene and N gene. A Amplification plots and B standard curve derived from amplification plots. Threshold time (Tt*) represents the time at which the fluorescence was equal to the threshold value during LAMP cycles. Error bars represent standard deviations from triplicate reactions. Multiplex LAMP assay could detect down to 10^5 copies. The standard curve had a linear relationship ($R^2 > 0.99$) with similar amplification efficiencies. There was no amplification bias.

no preferential amplification of one target sequence over another (*i.e.*, amplification bias) when equal primer concentrations were used.

Conclusion

In conclusion, a simple colorimetric LAMP assay for SARS-CoV was designed, optimized, and validated. We also demonstrate a multiplex LAMP assay in a single reaction for the specific detection of two target regions within the SARS-CoV genome. The LAMP assay has a shorter "sample-to-answer" time with detection performance comparable to conventional RT-PCR. It can be easily adapted to different laboratory settings without expensive thermo-cycling equipment, particularly in developing countries.

Materials and Methods

Virus Information

We used nucleotide sequence of SARS-coronavirus Tor2 (accession number AY274119.3 from the NCBI nucleotide database) isolated from patients with severe acute respiratory syndrome (Figure 1A). Coronavirus (CoV) genome is typically arranged in the order of 5' -polymerase (ORF1ab), spike (S), envelope (E), membrane (M), and nucleocapsid (N)-3' and short regions at both termini[36,37]. Generation of CoV full-length infectious cDNA clones has long been hampered due to the large size of the genome (around 30 kb) and the instability of some CoV replicase gene sequences during its propagation in bacteria[38]. Therefore, partial SARS-

CoV cDNAs were selected as target regions for amplification. (see Supplementary information 1S Table).

Linearization and Extraction of Synthetic DNA

Single-stranded DNA oligonucleotides covering target regions open reading frame (ORF1b) and nucleocapsid (N) genes of SARS-CoV were cloned into pBIC-A vector (Bioneer Inc.) to be downstream of T7 promoter region for RNA transcription. Recombinant plasmid was linearized by digestion with BamHI and XhoI (New England Biolabs) for ORF1b and BamHI and PciI (New England Biolabs) for N gene. These reactions were carried out with 20 μg DNA, 5 μl of 10X CutSmart buffer (New England Biolabs), and 3 μl of an appropriately diluted BamHI and XhoI or BamHI and PciI. They were then incubated at 37 ℃ for 4 hours. Detected cDNA fragments were separated on 1% agarose gel and purified using Wizard® DNA Clean-Up system (Promega).

In Vitro RNA Transcription and cDNA Synthesis

An in vitro RNA reverse transcription and DNA fragment removal were carried out using RiboMaxTM Large Scale RNA Production System (Promega) and T7 polymerase (Promega). Transcripts were produced in 20 μl reaction buffer containing 1 μg linearized DNA template, 10 μl 2x RiboMAX express T7 buffer, 2 μl T7 polymerase, and nuclease-free water followed by incubation at 37°C for 30 min. These transcripts were purified using RNase-Free DNase Set (Qiagen) which included DNase to remove unwanted DNA from RNA preparation according to the manufacturer's protocol. Total RNA was quantified by using ultraviolet (UV) spectroscopy (NanoDrop ND-1000, Wilmington). cDNA synthesis was performed with 100 ng RNA and 20 pmol of specific reverse primer incubated at 70°C for 5 min prior to reaction with 10 μl AccuPower® RT PreMix (Bioneer). For cDNA synthesis, the reaction was performed at 42 °C for 60 min and then incubated at 94 °C for 5 min for RTase inactivation. Total cDNA was quantified by ultraviolet (UV) spectroscopy (NanoDrop ND-1000, Wilmington). The copy number per μl was calculated according to DNA/RNA Copy Number Calculator (http://www.endmemo.com/bio/dnacopynum.php). The concentration of the synthesized cDNA was 20 ng/μl as measured with a spectrophotometer, which corresponded to $4.2 * 10^{10}$ copies/μl for ORF1b and $3.2 * 10^{10}$ copies/μl, and 10-fold serial dilutions of the cDNA raging from copies were prepared. The synthesized cDNA were stored at -70°C.

Design of Primer Sets for LAMP Reaction

Primers for LAMP amplification targeting the open reading frame 1b (ORF1b) and N (nucleocapsid) genes, respectively, were designed. A set of six primers corresponding to inner (FIP and BIP), outer (F3 and B3) primers, and internal loop primers (LF and LB) were designed using Primer expression 4.0 (http://primer explorer.jp/e/). They included an inner pair (FIP, BIP), an outer pair (F3, B3), and a loop pair (LF, LB). Inner primers included a forward inner primer (FIP) consisting of a complementary sequence of F1 and a sense sequence of F2 and a backward inner primer (BIP) consisting of a complementary sequence B1 and a sense sequence of B2. Two outer primers included a forward outer primer (F3) and a backward outer primer (B3) for initiation of LAMP reaction. Internal loop primers (LF and LB) were designed to accelerate the reaction. Amplicon sizes of ORF1b and N genes were 214 and 207 bps, respectively. A pair of primers (named F3 and B3) were also used for RT-PCR to amplify target genes.

For real-time monitoring, fluorescence dye including FAM and HEX and their quencher dye (Black Hole Quencher®) were tagged in the 5' end and internal of FIP primers, respectively. Using each primer set containing modified FIP primers, real-time LAMP analysis was carried out with a Quantstudio 6 Flex Real-Time PCR system (Applied Biosystems). All primers were synthesized and purified by Bioneer. Sequence alignment and details of these primers with regard to their positions in genomic sequences are shown in Figures 1B and 1C and Table 1.

LAMP Assay

LAMP assay was performed with a WarmStart® LAMP 2X Master mix (New England Biolabs) according to the manufacturer's protocol. As a preliminary experiment, we compared two primer sets with or without loop primer and confirmed that the target was amplified more efficiently by the loop primer. Briefly, a LAMP reaction mixture (20 μl) consisted of 10 μl of 2 x reaction mixture, 10 x LAMP primer set (I or II), template (cDNA or RNA), and sterile water. The 10 x LAMP Primer set I consisted of outer primers F3 and B3 (0.6 μM/each) and inner primers FIP and BIP (1.4 μM/each). The 10 x LAMP Primer set II consisted of outer primers F3 and B3 (0.2 μM/each), loop primers LF and LB (0.4 μM/each), and inner primers FIB and BIP (1.6 μM/each). LAMP assays were performed with 10 μl of 2x reaction mixture, 10 x LAMP primer set, template (cDNA or

RNA), 0.8 mmol/L FIP and modified FIP (FIP*), 1.6 mmol/L BIP, 0.8 mmol/L each of LF and LB primers, and 0.4 mmol/L each of F3 and B3 primers. Each reaction was performed with a thermal cycler (TaKaRa PCR Thermal Cycler Dice® Touch) under temperature control. Amplified products from the LAMP assay were analysed by electrophoresis with stained agarose gel as well as by naked eye using Warm-Start® Colorimetric LAMP 2X Master mix (New England Biolabs). Positive reaction showed a characteristic ladder of multiple bands in electrophoresis analysis and colour change from pink (prior to amplification) to yellow. All LAMP assays reported in this work were observed either by color change or agarose gel electrophoresis or both.

Real-time LAMP assay was carried out at 65 °C for 30 min using an QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) which was set to collect fluorescence signals at 30 s intervals. During real-time amplification, fluorescence data were obtained on HEX (ORF1b gene) or FAM (N gene) channel. Threshold time (T_t*) was calculated as the time at which the fluorescence equaled the threshold value[39].

Optimization of LAMP Assay

To determine the optimal reaction temperature, LAMP assay was conducted using cDNA of targets with a temperature gradient ranging from 50 °C to 72 °C for 30 min. Amplified products from the LAMP assays were visualized by agarose gel electrophoresis and colour change.

Sensitivity of LAMP Assay

The sensitivity of LAMP assay compared to RT-PCR was assessed using 10-fold serially diluted purified genomic RNA, whose concentration ranging from $1.1*10^{10}$ copies to $1.1*10^1$ copies for ORF1b gene and from $7.7*10^9$ copies to $7.7*10^1$ copies for N gene. Non-template control (NTC, sterile water) was include in each LAMP run.

RT-PCR Assay

Primer pairs F3 and B3 for N gene (5'-TCAGCATG GCAAGGAGGAA-3', 5'-TTAGCGCCGTAGGGAA GT-3') and F3 and B3 for ORF1b gene (5'- CCAAGT CAATGGTTACCC-3', 5'- ACTCTGGTGAATTCTG TG-3') were used for RT-PCR. RT-PCR assays using ORF1b and N genes were also performed for detection using a RT-PCR kit (AccuPower® HotStart PCR PreMix) and a thermal cycler (Applied Biosystems)

following manufacturers' protocols. Reactions were carried out in a total volume of 25 µL containing PCR premix, 20 pmol of each primer, 1 µL of template cDNA, and distilled water. Thermal cycling conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Reaction products were also subjected to 1.5 % agarose gel electrophoresis followed by visualization. All PCR experiments were repeated at least three times.

Real time PCR reactions were performed in a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) using Power SYBR PCR Master Mix (Applied Biosystems) under the same condition of RT-PCR. Standard curves were analysed by calculating the log of target DNA concentration against cycle threshold (Ct) value. A standard curve was generated by using log10-transformed concentrations of 10-fold serially diluted RNA and corresponding Ct value. Reactions were repeated at least three times.

Conflict of Interests The authors declare no competing financial interests.

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