Development of Reverse Transcription Loop-Mediated Isothermal Amplification Assays Targeting Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

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The coronavirus disease 2019 (COVID-19) pandemic now has >2,000,000 confirmed cases worldwide. COVID-19 is currently diagnosed using quantitative RT-PCR methods, but the capacity of quantitative RT-PCR methods is limited by their requirement of high-level facilities and instruments. We developed and evaluated reverse transcription loop-mediated isothermal amplification (RT-LAMP) assays to detect genomic RNA of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative virus of COVID-19. RT-LAMP assays reported in this study can detect as low as 100 copies of SARS-CoV-2 RNA. Cross-reactivity of RT-LAMP assays to other human coronaviruses was not observed. A colorimetric detection method was adapted for this RT-LAMP assay to enable higher throughput. *(J Mol Diagn 2020, 22: 729—735; https://doi.org/10.1016/j.jmoldx.2020.03.006)*

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative viral pathogen responsible for coronavirus disease 2019 (COVID-19), of which the outbreaks resulted in 2,245,872 confirmed cases involving 85,506 deaths worldwide as of April 20, 2020 (*https://www.who.int/emergencies/diseases/novel-coronavirus-2019*). As the name suggests, SARS-CoV-2 is closely related to a group of severe acute respiratory syndrome—related coronaviruses, namely subgenus *Sarbecovirus*, showing 96% identity to a bat coronavirus.[1,2]

COVID-19 can be diagnosed through computed tomographic scan of suspicious patients, and a confirmatory laboratory test is performed using published quantitative RT-PCR (RT-qPCR) methods[3—6] and recommendations from The World Health Organization (*https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance*, last accessed April 2, 2020). Although RT-qPCR methods are used as the gold standard for detection of pathogens because of their high sensitivity and

specificity, there are still some caveats. Briefly, laboratory-level facilities, with reliable supply of electricity, expensive instruments, and trained personnel, are required to properly perform RT-qPCR tests. These restrictions hinder use of RT-qPCR methods for various point-of-care situations, where pathogen detection might be required.[7]

To overcome such cost restriction of RT-qPCR and still detect nucleic acids from the pathogen, isothermal amplification methods have been developed.[8] Among such methods, the loop-mediated isothermal amplification (LAMP) method has some advantages to be applied for point-of-care testing.[9] Well-optimized LAMP assay shows sensitivity comparable to that of PCR, <10 copies per reaction.[10] Intercalating fluorescent dyes are compatible with LAMP reaction so that

amplification can be observed in real-time.[11] Because the amplification efficiency of LAMP reaction is high, changes in reaction mixture components make it possible to detect the result with colorimetric detection methods.[12–14] Moreover, unpurified sample can be directly used for LAMP.[15–17] This indicates that high-throughput test is possible when use of unpurified specimen is combined with noninstrumental (eg, colorimetric) detection.

In this study, one-step reverse transcription LAMP (RT-LAMP) methods to detect SARS-CoV-2 were designed and evaluated. A pair of LAMP primer sets specific to SARS-CoV-2 and accompanying optimized reaction conditions was provided. The leuco crystal violet method was applied to achieve colorimetric detection of LAMP reaction.[13]

Materials and Methods

Viral RNA Preparation

SARS-CoV-2 viral RNA was prepared, as previously described.[18] Human coronavirus (hCoV)−229E and hCoV-OC43 viral RNA were isolated from culture media of infected MRC-5 cells (ATCC, Manassas, VA; CCL-171). Middle East respiratory syndrome coronavirus (MERS-CoV) RNA was isolated from cell pellet lysate of infected Vero cells (ATCC; CCL-81).

Viral RNA Titration

In vitro transcribed standard RNA for SARS-CoV-2 was prepared, as previously described.[18] To prepare standard RNA for hCoV-229E, first amplicon of PCR (forward primer, 5′-GCTAGTGGATGATCATGCTTTG-3′; reverse primer, 5′-TGGGGCCATAAACTGTTCTATTAC-3′) was cloned to pBluescript II KS (+) plasmid with BamHI and XhoI. Then, *in vitro* transcription template was prepared by restriction enzyme cut with BglI and XhoI and purification after agarose gel electrophoresis. For hCoV-OC43 and MERS-CoV, amplicons of PCR (OC43 forward primer, 5′-AGCAAC-CAGGCTGATGTCAATACC-3′; OC43 reverse primer, 5′-AGCAGACCTTCCTGAGCCTTCAAT-3′; MERS-CoV, UpE region[19]) were synthesized and cloned to pBIC-A plasmid (Bioneer, Daejeon, Republic of Korea). *In vitro* transcription template for hCoV-OC43 and MERS-CoV was prepared by restriction enzyme cut with BamHI-XhoI or SspI-XhoI, respectively. *In vitro* transcriptions were performed with EZ T7 High Yield In Vitro Transcription kit (Enzynomics, Daejeon, Republic of Korea), per manufacturer's instructions. RNA products were then purified using Agencourt RNAClean XP (Beckman Coulter, Brea, CA). Standard RNA copy numbers were calculated from concentration measured by NanoDrop Lite (Thermo Scientific, Waltham, MA). All restriction enzymes were purchased from Enzynomics.

To evaluate genomic copy number of viral RNAs, dilutions of standard RNAs and viral RNAs in TE buffer (10 mmol/L Tris-Cl, pH 8.0, and 1 mmol/L EDTA) are subjected to one-step RT-qPCR. RT-qPCRs were performed using LightCycler 96 instrument (Roche, Basel, Switzerland) and following reagents: Luna Universal One-Step RT-qPCR Kit [New England Biolabs (NEB), Ipswich, MA] for hCoV-229E and hCoV-OC43, THUNDERBIRD Probe qPCR Master Mix (Toyobo, Osaka, Japan) for MERS-CoV, and Luna Universal Probe One-Step RT-qPCR Kit (NEB) for SARS-CoV-2.

Reverse Transcription

cDNA of SARS-CoV-2 was made using SuperScript IV Reverse Transcriptase (Invitrogen, Waltham, MA) following manufacturer's instructions with modifications. Briefly, 10 pmol of random hexamer was used as reverse transcription primer, and reaction was performed as follows: 20 minutes at 25°C, 30 minutes at 55°C, and 10 minutes at 80°C.

LAMP and RT-LAMP Reaction

LAMP reaction was performed with reaction mixture containing following components: 1.6 μmol/L forward inner primer/backward inner primer, 0.2 μmol/L F3/B3 primers, 0.4 μmol/L loop forward/loop backward primers, 1× Isothermal Amplification Buffer II [NEB; 20 mmol/L Tris-HCl, pH 8.8, 10 mmol/L $(NH_4)_2SO_4$, 150 mmol/L KCl, 2 mmol/L $MgSO_4$, and 0.1% Tween 20], 6 mmol/L $MgSO_4$ (NEB; final, 8 mmol/L Mg^{2+}), 1.4 mmol/L each dNTP (Enzynomics), 0.4 μmol/L SYTO-9 (Invitrogen), and 6 U *Bst* 3.0 DNA polymerase (NEB) in total 15-μL reaction volume. For RT-LAMP, 10 U of SuperScript IV Reverse Transcriptase (Invitrogen) was added. For end-point colorimetric detection of LAMP reaction, tweaked version of 5× stock leuco crystal violet solution[13] containing 0.5 mmol/L crystal violet (Sigma, St. Louis, MO; C0775), 60 mmol/L sodium sulfite (Sigma; S0505), and 5 mmol/L β-cyclodextrin (Sigma; C4767) was directly added to LAMP reaction mixture to 1× concentration. When using WarmStart Colorimetric LAMP 2X Master Mix (NEB), same concentrations of primers and 0.4 μmol/L SYTO-9 were added. Isothermal incubation and fluorescence signal measurement were performed using LightCycler 96 instrument at 69°C with additional heat inactivation (5 minutes at 95°C) and melting curve analysis steps. Fluorescence signals were measured for every minute during 60 (screening and optimization) or 30 [limit of detection (LoD) and cross-reactivity] minutes of incubation. Any changed conditions are specified for each experiment.

Results

LAMP Primer Design

Five SARS-CoV-2 sequences (MN908947, MN938384, MN988713, MN985325, and MN975262) and seven SARS-CoV sequences (NC_004718, AY613947,

AY313906, AY559094, AY502924, AY278491, and AY502927) were aligned by MEGA software version 7.[20] SARS-CoV-2—specific regions for LAMP primer design are manually selected: two regions from *Nsp3* (3055 to 3591 and 6172 to 7273), two regions from *Spike* (21540 to 22549 and 22890 to 23779), and one region from *Orf8* (27824 to 28396). Whole *Nucleocapsid* gene region is also included as *Nucleocapsid* is usual target of molecular diagnosis because of the abundance of its mRNA.[21] Two to five basic LAMP primer sets are designed and selected with PrimerExplorer V5 (*http://primerexplorer. jp/lampv5e/index.html*, last accessed February 22, 2020) for each target region. Loop primers are designed by PrimerExplorer V5 or manually selected. Sixteen LAMP primer sets, with loop forward primer and loop backward primer showing proper melting temperature, were selected and subjected to further screening.

LAMP Primer Screening

First round of screening was performed using WarmStart Colorimetric LAMP 2X Master Mix with cDNA, of which corresponding RNA concentration is 8.3×10^4 copies/ reaction. Of 16 primer sets, nine were selected by threshold time from the results of 40 minutes' incubation at 65°C and subjected to further screening.

Second round of screening was performed using *Bst* 3.0. The same amount of cDNA as the first round of screening is used. Two primer sets that showed relatively early non-specific amplification are discarded, and the remaining seven primer sets are subjected to further screening.

Third and fourth rounds of screenings were performed by checking sensitivity to dilutions of cDNA and RNA, respectively. Five of seven primer sets showed specific amplification for at least one replicate of duplicate, with cDNA concentration corresponding to 1.7×10^1 copies of input RNA (Supplemental Figure S1). Next, sensitivity of the five primer sets to RNA dilutions was evaluated in RT-LAMP using *Bst* 3.0 and SuperScript IV Reverse Transcriptase. Two primer sets, both targeting *Nsp3*, showed best sensitivity that showed specific amplification at 10^{-6} dilution of RNA (Figure 1). Two additional primer sets, one targeting *Spike* and the other targeting *Nucleocapsid*, were added for reaction optimization experiments as they showed fast threshold time for cDNA and to keep ranges of target genes. Primer sequences are represented in Table 1.
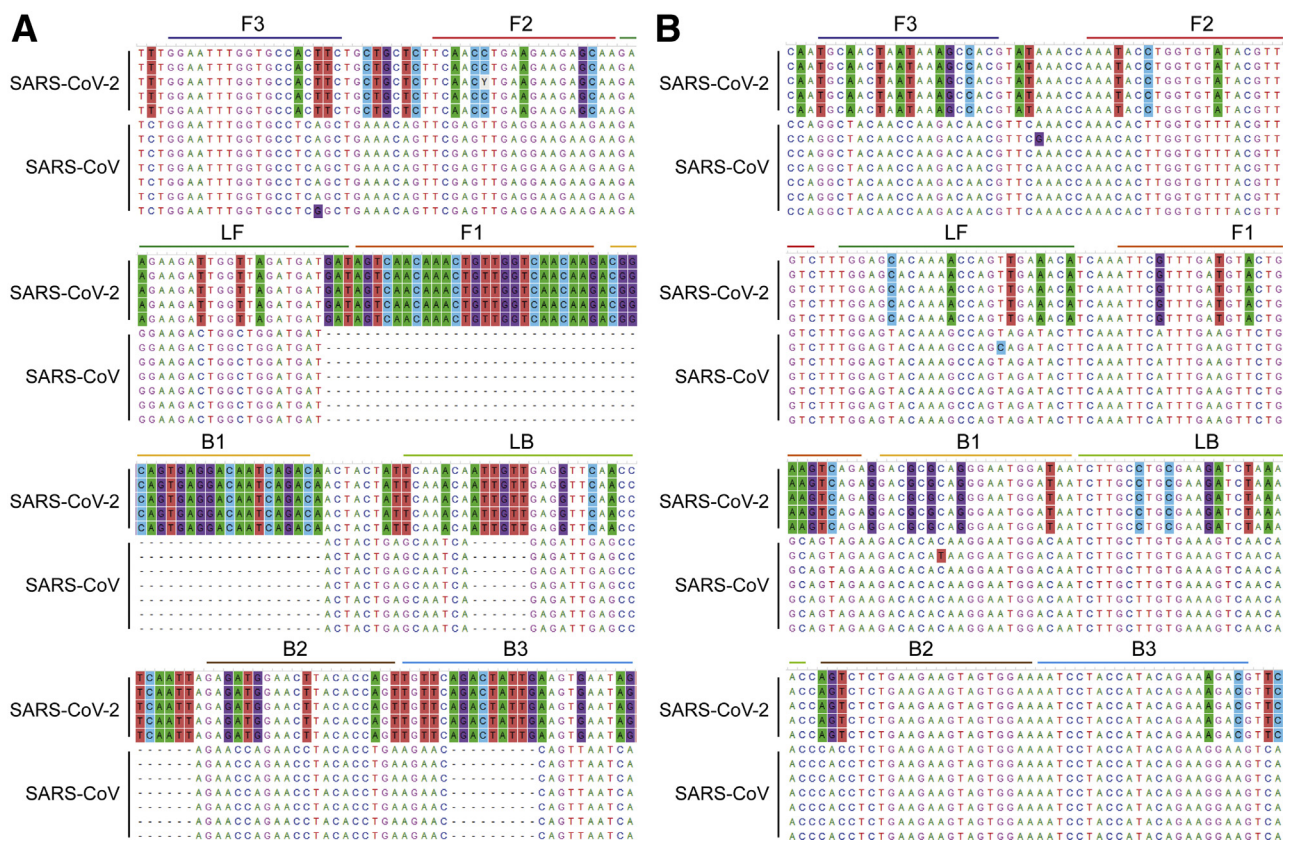

Figure 1 Loop-mediated isothermal amplification (LAMP) primer positions on aligned sequences of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and SARS-CoV. Primer binding sites of Nsp3_1-61 (**A**) and Nsp3_2-24 (**B**) LAMP primer sets are depicted on aligned sequences of five SARS-CoV-2 (from the top, MN908947, MN938384, MN988713, MN985325, and MN975262) and seven SARS-CoV (from the top, NC_004718, AY613947, AY313906, AY559094, AY502924, AY278491, and AY502927). Conserved sites are toggled at 50% level by MEGA software version 7 so that SARS-CoV-2 specific residues versus SARS-CoV are background colored. Different color bars are used as follows to distinguish binding sites: F3, blue; F2, red; F1, orange; loop forward (LF), green; loop backward (LB), light green; B1, light orange; B2, brown; and B3, light blue.

Table 1 LAMP Primers of Which Assays Were Optimized and LoD Evaluated

Set name	Primer	Sequence
Nsp3_1-61	F3	5′-GGAATTTGGTGCCACTTC-3′
	B3	5′-CTATTCACTTCAATAGTCTGAACA-3′
	FIP	5′-CTTGTTGACCAACAGTTTGTTGACTTCAACCTGAAGAAGAGCAA-3′
	BIP	5′-CGGCAGTGAGGACAATCAGACACTGGTGTAAGTTCCATCTC-3′
	LF	5′-ATCATCATCTAACCAATCTTCTTC-3′
	LB	5′-TCAAACAATTGTTGAGGTTCAACC-3′
Nsp3_2-24	F3	5′-TGCAACTAATAAAGCCACG-3′
	B3	5′-CGTCTTTCTGTATGGTAGGATT-3′
	FIP	5′-TCTGACTTCAGTACATCAAACGAATAAATACCTGGTGTATACGTTGTC-3′
	BIP	5′-GACGCGCAGGGAATGGATAATTCCACTACTTCTTCAGAGACT-3′
	LF	5′-TGTTTCAACTGGTTTTGTGCTCCA-3′
	LB	5′-TCTTGCCTGCGAAGATCTAAAAC-3′
S_1-2-2	F3	5′-CTGACAAAGTTTTCAGATCCTCAG-3′
	B3	5′-AGTACCAAAAATCCAGCCTCTT-3′
	FIP	5′-TCCCAGAGACATGTATAGCATGGAATCAACTCAGGACTTGTTCTTACC-3′
	BIP	5′-TGGTACTAAGAGGTTTGATAACCCTGTTAGACTTCTCAGTGGAAGCA-3′
	LF	5′-CCAAGTAACATTGGAAAAGAAA-3′
	LB	5′-GTCCTACCATTTAATGATGGTGTTT-3′
N_21	F3	5′-GCCAAAAGGCTTCTACGCA-3′
	B3	5′-TTGCTCTCAAGCTGGTTCAA-3′
	FIP	5′-TCCCCTACTGCTGCCTGGAGGCAGTCAAGCCTCTTCTCG-3′
	BIP	5′-TCTCCTGCTAGAATGGCTGGCATCTGTCAAGCAGCAGCAAAG-3′
	LF	5′-TGTTGCGACTACGTGATGAGGA-3′
	LB	5′-ATGGCGGTGATGCTGCTCT-3′

BIP, backward inner primer; FIP, forward inner primer; LB, loop backward; LF, loop forward; LAMP, loop-mediated isothermal amplification; LoD, limit of detection.

LAMP Reaction Optimization

To optimize RT-LAMP reaction with *Bst* 3.0, first, optimal concentrations of dNTP and Mg^{2+} were evaluated for each primer set. Three dNTP- Mg^{2+} concentration combinations are tested at 69°C: dNTP (1.4 mmol/L) and Mg^{2+} (8 mmol/L), dNTP (1.4 mmol/L) and Mg^{2+} (6 mmol/L), and dNTP (1 mmol/L) and Mg^{2+} (6 mmol/L). The copy number of RNA template was 1000 copies per reaction. Best concentration combination was selected for each primer set by whether both of duplicates show specific amplification and by threshold time. Average threshold time (Tt_av) and corresponding dNTP and Mg^{2+} concentrations are as follows: dNTP (1 mmol/L) and Mg^{2+} (6 mmol/L) for Nsp3_1-61 (Tt_av = 11.93 minutes), dNTP (1.4 mmol/L) and Mg^{2+} (8 mmol/L) for Nsp3_2-24 (Tt_av = 7.74 minutes), dNTP (1.4 mmol/L) and Mg^{2+} (6 mmol/L) for S_1-2-2 and N_21 (Tt_av = 11.08 minutes and 5.50 minutes, respectively).

Next, whether amplification is improved at lower temperature (65°C) was evaluated, because optimal reaction temperature of SuperScript IV Reverse Transcriptase is 50°C to 55°C and the manufacturer recommends 65°C to 72°C for optimal performance of *Bst* 3.0. Notably, Nsp3_1-61 and

Table 2 LoD of RT-LAMP Assays Targeting SARS-CoV-2

	SSIV, 10 U/rxn				SSIV, 20 U/rxn			
	Nsp3_1-61		Nsp3_2-24		Nsp3_1-61		Nsp3_2-24	
RNA copies	Tt_av	RPR	Tt_av	RPR	Tt_av	RPR	Tt_av	RPR
1000	13.11	3:3	9.31	3:3	10.7	3:3	7.53	3:3
500	13.47	3:3	8.16	2:3	10.63	3:3	7.59	3:3
200	13.65	2:3	11.19	2:3	11.34	2:3	7.78	3:3
100	14.62	3:3	—	—	12.26	2:3	8.47	2:3
50	—	—	—	—	—	—	—	—
20	13.88	1:3	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—
NTC	—	—	—	—	—	—	—	—

—, no amplification observed; LoD, limit of detection; RPR, ratio of positive replicates; RT-LAMP, reverse transcription loop-mediated isothermal amplification; rxn, reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SSIV, SuperScript IV Reverse Transcriptase; Tt_av, average threshold time in minutes; NTC, no template control.

S_1-2-2 primer sets show improved threshold time (Tt_av = 8.92 minutes and 8.49 minutes, respectively).

Assessing LoD and Cross-Reactivity

The LoD of optimized RT-LAMP assays was assessed through 5 to 1000 RNA copies/reaction in triplicate. Among four primer sets subjected to reaction optimization, S_1-2-2 and N_21 sets showed relatively poor sensitivity. For Nsp3_1-61 and Nsp3_2-24 primer sets, LoD was additionally evaluated with twofold SuperScript IV Reverse Transcriptase (20 U/reaction). LoD and Tt_av were improved by increasing reverse transcriptase amount (Table 2 and Supplemental Figure S2). As a result, both Nsp3_1-61 and Nsp3_2-24 RT-LAMP assays could detect as low RNA concentration as 100 copies per reaction.

The cross-reactivity of two SARS-CoV-2 RT-LAMP assays targeting *Nsp3* to other human coronaviruses was not found for hCoV-229E, hCoV-OC43, and MERS-CoV (Figure 2).

Discussion

In this study, LAMP primer sets targeting SARS-CoV-2 were designed and screened. Reaction was also optimized for selected primer sets. In summary, a pair of RT-LAMP assays for detection of SARS-CoV-2 with LoD of 100 copies per reaction was designed and evaluated. These RT-LAMP assays showed specificity to SARS-CoV-2 versus alphacoronavirus (hCoV-229E), betacoronavirus (hCoV-OC43), and MERS-CoV. Although specificity to SARS-CoV could not be tested because a proper sample was not in hand, specificity of the RT-LAMP assays is easily expectable from the mismatching bases in primer binding sites (Figure 1). Especially, both of F1/B1 sites of Nsp3_1-61 primer set are in SARS-CoV-2 specific region, of which aligning SARS-CoV sequence does not exist.

About the sensitivity of LAMP assays, note that average threshold time is not well correlated with LoD. In fact, average threshold times of S_1-2-2 and N_21 primer sets for 1000 copies were faster than that of Nsp3_1-61: 7.33 (ratio of positive replicates, 2:3) and 5.06 (ratio of positive replicates, 1:3) minutes, respectively. This disrelation is previously reported.[22] One peculiar observation is that both S_1-2-2 and N_21 showed good sensitivity to cDNA template (Supplemental Figure S1), unlike to RNA. Observed difference of sensitivity to RNA and cDNA seems significant even when accounting for slight amplification that might happen during reverse transcription. The reason may include stochastic nature of forming proper LAMP
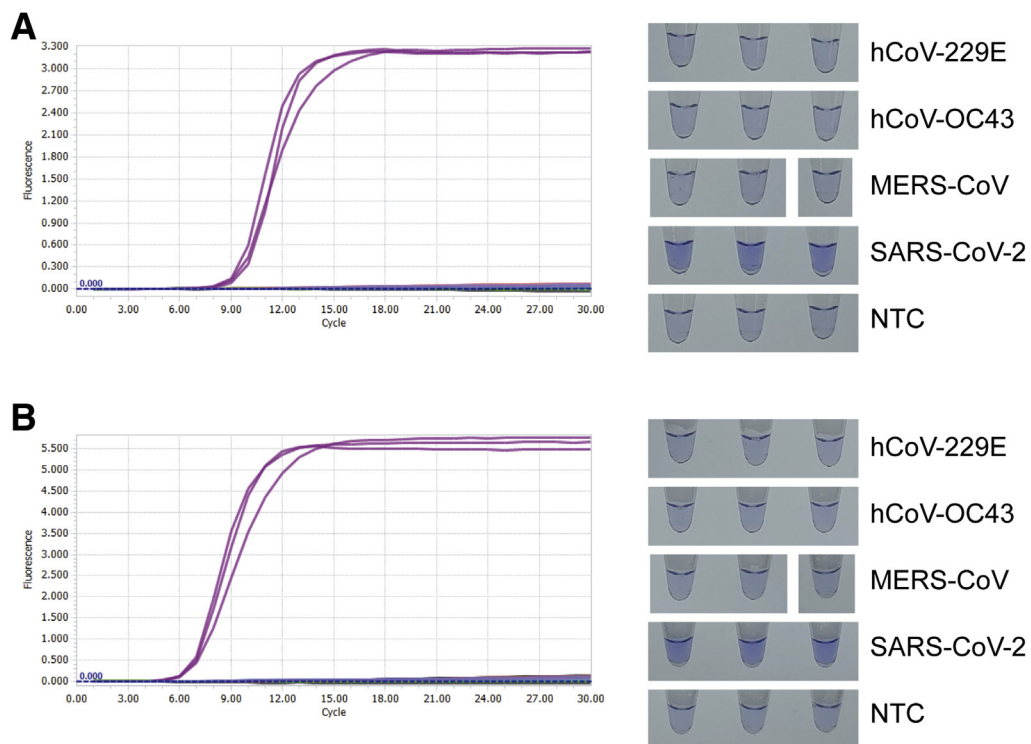

Figure 2 Cross-reactivity to other coronaviruses tested for reverse transcription loop-mediated isothermal amplification (LAMP) assay targeting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Real-time amplification fluorescence signal and end-point leuco crystal violet colorimetric detection results of cross-reactivity test for Nsp3_1-61 (**A**) and Nsp3_2-24 (**B**) LAMP primer sets. Reactions are performed with 20 U/reaction of reverse transcriptase and optimized temperature, dNTP concentration, and Mg^{2+} concentration for each primer set. RNA copy number of each viral RNA is as follows: human coronavirus (hCoV)−229E, 1.6×10^6; hCoV-OC43, 1.6×10^6; Middle East respiratory syndrome coronavirus (MERS-CoV), 4.5×10^6; and SARS-CoV-2, 2.5×10^3. NTC, no template control.

amplification intermediate-dumbbell structure,[22] higher stability of DNA/RNA double strand than DNA/DNA double strand, and more.

The LoD of RT-LAMP assays suggested in this study, 100 copies per reaction, may not be enough for sensitive screening of suspicious patients. This relatively high LoD would be from the target sequences used for primer design that are selected by specificity versus SARS-CoV. Indeed, target GC percentage and melting temperature of primers had to be adjusted to get enough number of starting sets for primer screening during LAMP primer design to give less-optimal primer sets. Therefore, there might be better target sequences for LAMP assay of SARS-CoV-2 in the viewpoint of sensitivity. However, expected high specificity of RT-LAMP assays suggested in this study would be a good feature for a confirmatory test. In addition, considering high viral load of SARS-CoV-2 at early stage after symptom onset[23] suggested RT-LAMP assays may still be useable for screening tests.

In conclusion, highly specific RT-LAMP assays for detection of SARS-CoV-2 were developed and evaluated. The results of these RT-LAMP assays can be detected within 30 minutes after amplification reaction begins. In addition, optimized reaction conditions were provided, to which leuco crystal violet colorimetric detection method is applied that can be used for point-of-care tests.

Acknowledgment

Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2020.03.006*.

References

1. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, Si HR, Zhu Y, Li B, Huang CL, Chen HD, Chen J, Luo Y, Guo H, Jiang RD, Liu MQ, Chen Y, Shen XR, Wang X, Zheng XS, Zhao K, Chen QJ, Deng F, Liu LL, Yan B, Zhan FX, Wang YY, Xiao GF, Shi ZL: A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature 2020, 579:270−273

2. Wu F, Zhao S, Yu B, Chen YM, Wang W, Song ZG, Hu Y, Tao ZW, Tian JH, Pei YY, Yuan ML, Zhang YL, Dai FH, Liu Y, Wang QM, Zheng JJ, Xu L, Holmes EC, Zhang YZ: A new coronavirus associated with human respiratory disease in China. Nature 2020, 579: 265−269

3. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, Wang D, Xu W, Wu G, Gao GF, Tan W; China Novel Coronavirus Investigating and Research Team: A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med 2020, 382:727−733

4. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y, Zhang L, Fan G, Xu J, Gu X, Cheng Z, Yu T, Xia J, Wei Y, Wu W, Xie X, Yin W, Li H, Liu M, Xiao Y, Gao H, Guo L, Xie J, Wang G, Jiang R, Gao Z, Jin Q, Wang J, Cao B: Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet 2020, 395:497−506

5. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, Bleicker T, Brunink S, Schneider J, Schmidt ML, Mulders DG, Haagmans BL, van der Veer B, van den Brink S, Wijsman L, Goderski G, Romette JL, Ellis J, Zambon M, Peiris M, Goossens H, Reusken C, Koopmans MP, Drosten C: Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill 2020, 25:2000045

6. Chu DKW, Pan Y, Cheng SMS, Hui KPY, Krishnan P, Liu Y, Ng DYM, Wan CKC, Yang P, Wang Q, Peiris M, Poon LLM: Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of pneumonia. Clin Chem 2020, 66:549−555

7. Nayak S, Blumenfeld NR, Laksanasopin T, Sia SK: Point-of-care diagnostics: recent developments in a connected age. Anal Chem 2017, 89:102−123

8. Niemz A, Ferguson TM, Boyle DS: Point-of-care nucleic acid testing for infectious diseases. Trends Biotechnol 2011, 29:240−250

9. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T: Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 2000, 28:E63

10. Shirato K, Yano T, Senba S, Akachi S, Kobayashi T, Nishinaka T, Notomi T, Matsuyama S: Detection of middle east respiratory syndrome coronavirus using reverse transcription loop-mediated isothermal amplification (RT-LAMP). Virol J 2014, 11:139

11. Oscorbin IP, Belousova EA, Zakabunin AI, Boyarskikh UA, Filipenko ML: Comparison of fluorescent intercalating dyes for quantitative loop-mediated isothermal amplification (qLAMP). Biotechniques 2016, 61:20−25

12. Goto M, Honda E, Ogura A, Nomoto A, Hanaki K: Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. Biotechniques 2009, 46:167−172

13. Miyamoto S, Sano S, Takahashi K, Jikihara T: Method for colorimetric detection of double-stranded nucleic acid using leuco triphenylmethane dyes. Anal Biochem 2015, 473:28−33

14. Tanner NA, Zhang Y, Evans TC Jr: Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. Biotechniques 2015, 58:59−68

15. Nie K, Qi SX, Zhang Y, Luo L, Xie Y, Yang MJ, Zhang Y, Li J, Shen H, Li Q, Ma XJ: Evaluation of a direct reverse transcription loop-mediated isothermal amplification method without RNA extraction for the detection of human enterovirus 71 subgenotype C4 in nasopharyngeal swab specimens. PLoS One 2012, 7: e52486

16. Nyan DC, Ulitzky LE, Cehan N, Williamson P, Winkelman V, Rios M, Taylor DR: Rapid detection of hepatitis B virus in blood plasma by a specific and sensitive loop-mediated isothermal amplification assay. Clin Infect Dis 2014, 59:16−23

17. Lee D, Shin Y, Chung S, Hwang KS, Yoon DS, Lee JH: Simple and highly sensitive molecular diagnosis of Zika virus by lateral flow assays. Anal Chem 2016, 88:12272−12278

18. Jung YJ, Park G-S, Moon JH, Ku K, Beak S-H, Kim S, Park EC, Park D, Lee J-H, Byeon CW, Lee JJ, Maeng J-S, Kim SJ, Kim SI, Kim B-T, Lee MJ, Kim HG: Comparative analysis of primer-probe sets for the laboratory confirmation of SARS-CoV-2. BioRxiv 2020

19. Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, van Boheemen S, Gopal R, Ballhause M, Bestebroer TM, Muth D, Muller MA, Drexler JF, Zambon M, Osterhaus AD, Fouchier RM, Drosten C: Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. Euro Surveill 2012, 17:20285

20. Kumar S, Stecher G, Tamura K: MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 2016, 33:1870−1874

21. Hiscox JA, Cavanagh D, Britton P: Quantification of individual sub-genomic mRNA species during replication of the coronavirus transmissible gastroenteritis virus. Virus Res 1995, 36:119−130

22. Khorosheva EM, Karymov MA, Selck DA, Ismagilov RF: Lack of correlation between reaction speed and analytical sensitivity in isothermal amplification reveals the value of digital methods for optimization: validation using digital real-time RT-LAMP. Nucleic Acids Res 2016, 44:e10

23. Zou L, Ruan F, Huang M, Liang L, Huang H, Hong Z, Yu J, Kang M, Song Y, Xia J, Guo Q, Song T, He J, Yen HL, Peiris M, Wu J: SARS-CoV-2 viral load in upper respiratory specimens of infected patients. N Engl J Med 2020, 382:1177−1179