Rapid Detection of SARS-CoV-2 Virus Using Dual Reverse Transcriptional Colorimetric Loop-Mediated Isothermal Amplification

Chao Ji, Shuxia Xue, Min Yu, Jinyu Liu, Qin Zhang, Feng Zuo, Quyue Zheng, Liangjuan Zhao, Hongwei Zhang, Jijuan Cao, Ke Wang, Wei Liu, * and Wenjie Zheng *

ABSTRACT: The outbreak and pandemic of COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has developed into a public health emergency of international concern. The rapid and accurate detection of the virus is a critical means to prevent and control the disease. Herein, we provide a novel, rapid, and simple approach, named dual reverse transcriptional colorimetric loop-mediated isothermal amplification (dRT-cLAMP) assay, to accelerate the detection of the SARS-CoV-2 virus without using expensive equipment. The result of this assay is shown by color change and is easily detected by the naked eye. To improve the detection accuracy, we included two primer sets that specifically target the viral orf1ab and N genes in the same reaction mixture. Our assay can detect the synthesized SARS-CoV-2 N and orf1ab genes at a low level of 100 copies/μL. Sequence alignment analysis of the two synthesized genes and those of 9968 published SARS-CoV-2 genomes and 17 genomes of other pathogens from the same infection site or similar symptoms as COVID-19 revealed that the primers for the dRT-cLAMP assay are highly specific. Our assay of 27 clinical samples of SARS-CoV-2 virus and 27 standard-added environmental simulation samples demonstrated that compared to the commercial kits, the consistency of the positive, negative, and probable clinical samples was 100, 92.31, and 44.44%, respectively. Moreover, our results showed that the positive, but not negative, standard-added samples displayed a naked-eye-detectable color change. Together, our results demonstrate that the dRT-cLAMP assay is a feasible detection assay for SARS-CoV-2 virus and is of great significance since rapid onsite detection of the virus is urgently needed at the ports of entry, health care centers, and for internationally traded goods.

INTRODUCTION

The outbreak of COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was detected in Wuhan, China, in December 2019 and thereafter it rapidly spread globally, causing a pandemic, bringing death, illness, and disruption to our daily lives. COVID-19 has developed into a public health emergency of international concern.1–6 Coronaviruses are positive-sense single-stranded RNA viruses that are widely present in nature. The SARS-CoV-2 virus, identified in 2019 and the seventh known human coronavirus, causes disease outbreak in both humans and animals, mostly affecting the respiratory system.7,8 To date (November 14, 2020), more than 52 million confirmed cases and more than 1 million deaths have been reported in more than 191 countries since early December of 2019, according to the WHO COVID-19 report.9,10 At present, no approved drugs or vaccines have been validated for COVID-19; prompt diagnosis and quarantine management are thus the only effective methods for prevention and control of the disease.11,12 Therefore, there is still an urgent need for a highly specific and sensitive detection measure to identify infected people with SARS-CoV-2, especially asymptomatic carriers and contaminated food and food packaging.13–15
The real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) has been established as a feasible and robust detection technique, commonly used as a gold standard for gene diagnosis of COVID-19 via detection of SARS-CoV-2 virus. However, the operation of the qRT-PCR test is cumbersome and requires trained expertise, sophisticated laboratories, and sample transportation, which make the test unsuitable for low-income countries or remote areas where hospitals may not be equipped with molecular diagnostic laboratories and rapid screening/detection onsite, as such as the ports of entry. Loop-mediated isothermal amplification (LAMP), developed by Notomi and colleagues, is an efficient nucleic acid amplification technique that can be performed under constant temperature conditions. The technique relies on a DNA polymerase with strand displacement activity (Bst DNA polymerase) and four or six specific primers to recognize six or eight regions of the target gene sequences. The LAMP assay is rapid, specific, and sensitive and has low laboratory infrastructure requirements. Therefore, the assay has been widely applied for the detection of viral infections for public health emergencies of international consequence, such as Zika virus, the Middle East respiratory syndrome coronavirus (MERS-CoV), influenza virus (H7N9), West Nile virus, Ebola virus, and yellow fever virus. To accelerate clinical diagnostic testing for COVID-19, a large number of prospective techniques have been developed and validated for the detection of SARS-CoV-2 using the reverse transcription LAMP assay. These LAMP-based detection techniques are based on the detection of only a single gene of the virus; thus, once the primer site of designed genes is mutated, the detection assays will not well reflect the real situation of the sample.

At present, the prevention and control of the pandemic situation of COVID-19 cannot yet be slackened. Due to imported contaminated food, an outbreak of COVID-19 occurred in the Xinfadi wholesale market in Beijing 55 days after the last identified locally acquired case; similar events also happened in Vietnam and New Zealand. In July 2020 in Dalian, China, SARS-CoV-2 was detected on shrimp from three Ecuadorian processing factories, which caused SARS-CoV-2 infection by the contaminated shrimp and spread of COVID-19 to four provinces in China. Therefore, to prevent the spread of SARS-CoV-2 and control COVID-19, it is necessary to establish a more rapid and onsite method to detect SARS-CoV-2 in import and export products and packaging. In this work, we report the development and evaluation of a rapid and simple dual reverse transcriptional colorimetric LAMP (dRT-cLAMP) assay for the detection of SARS-CoV-2. After optimizing reaction conditions, we included two sets of designed primers that specifically target the orf1ab and N genes of SARS-CoV-2 into the same reaction mixture to improve the accuracy of screening/detection. To verify the reliability of the assay, we detected the clinical samples and standard-added environmental simulation samples, and our results demonstrated that dRT-cLAMP had higher sensitivity and cost-effectiveness, which meets the urgent needs for onsite detection at the ports of entry, health

![Figure 1](https://s3.amazonaws.com/figshare/7574541)

**Figure 1.** The primer combination ORF1ab2 + N1# is employed as the optimal primer group of the dRT-cLAMP assay. (A) Amplification curve and fitting curve of the primer sets ORF1ab1# and ORF1ab2# obtained with a turbidimeter. Inset: Fitting of the amplification curve of the primer sets ORF1ab1# and ORF1ab2#. (B) Amplification curve and fitting curve of the primer sets N1# and N2# obtained with a turbidimeter. Inset: Fitting of the amplification curve of the primer sets N1# and N2#. (C) Amplification curve of the primer groups ORF1ab2 + N1#, ORF1ab2 + N1#, and ORF1ab1 + N2# obtained with a turbidimeter. (D) Colorimetric validation of the primer groups ORF1ab2 + N2# and ORF1ab2 + N1#. The PC displayed a color change from violet to blue, whereas the NC remained violet. NC: DNase/RNase-free water; PC: plasmid containing target genes at a concentration of 10 pg/μL. Curves and photos are from a representative experiment of three independent experiments that have similar results.
care centers, remote areas, and for internationally traded goods.

■ RESULTS

The Primer Combination ORF1ab2 + N1# Is the Optimal Primer Set for the Detection of SARS-CoV-2 in the dRT-cLAMP Assay. The conserved regions of the orf1ab gene and N gene of SARS-CoV-2 with low mutation frequencies were used for the design of the dRT-cLAMP primers. Two sets of primers were designed to detect the virus N gene (N1# and N2#) and orf1ab gene (ORF1ab1# and ORF1ab2#), respectively. The pseudo-virus was used as the sample (the concentration was 10 pg/μL) to optimize the sets of primers of the LAMP using a real-time LAMP turbidimeter. Our results showed that the peak time of the target genes amplified with the primer set ORF1ab2# was faster compared to that with the primer set N2# (Figure 1A). Similarly, the peak time of the target genes amplified with the primer set ORF1ab1# (Figure 1A). The primer combination test showed that the Tt value and the Df value of the primer combination ORF1ab2 + N1# were 25:18 and 0.375, respectively, suggesting that the peak time and signal strength of the target genes amplified were better than the primer combinations ORF1ab2 + N2# (29:06 and 0.308) and ORF1ab1 + N2# (33:12 and 0.224) (Figure 1C). The same results were verified by the colorimetric assay (Figure 1D). Taken together, these results demonstrated that the primer group ORF1ab2 + N1# was feasible and was thus chosen as the optimal primers for our dRT-cLAMP detection of SARS-CoV-2.

The dRT-cLAMP Assay Is Stable and Trustworthy under Optimized Reaction Conditions. To optimize the dRT-cLAMP reaction, the concentration of Bst DNA polymerase (8, 12, and 16 U/reaction) (A), Mg\(^{2+}\) (6, 8, and 10 mM) (B), betaine (0.2, 0.4, and 0.6 M) (C), and dNTP (0.8, 1.4, and 2.0 mM) (D) with a LAMP turbidimeter. The optimized concentrations of Bst DNA polymerase, Mg\(^{2+}\), dNTP, and betaine of the dRT-cLAMP were 16 U/reaction, 8 mM, 0.4 M, and 1.4 mM/reaction, respectively. (E) The Tt value of the results of the above-mentioned four experiments was analyzed for validating the reaction stability of the dRT-cLAMP assay. Curves are from a representative experiment of three independent experiments that have similar results.
ase (8, 12, or 16 U/reaction), Mg\(^{2+}\) (6, 8, or 10 mM), dNTP (0.8, 1.4, or 2.0 mM), and betaine (0.2, 0.4, or 0.6 M) of dRT-cLAMP were evaluated by a single-factor test. The pseudo-virus (the concentration was 1 × 10^5 or 1 × 10^3 copies/μL, respectively) was used as the template to optimize the dRT-cLAMP reaction using a real-time LAMP turbidimeter. Our results showed that when the concentration of Bst DNA polymerase was 16 U/reaction, compared to 8 and 12 U, the Tt value and the Df value of the amplified target genes were earlier and stronger, especially at a lower concentration (1 × 10^3 copies/μL) of the pseudo-virus template (Figure 2A). For the concentration of Mg\(^{2+}\), we found that when the concentration of Mg\(^{2+}\) was 8 mM, compared to 6 and 10 mM, the Tt value and the Df value of the amplified target genes were faster and stronger (Figure 2B). When the concentration of betaine was 0.4 M, the Tt value and the Df value of the amplified target genes were faster and stronger compared to 0.2 and 0.6 M (Figure 2C). For the concentration of dNTP, we found that when the concentration of dNTP was 1.4 mM/reaction, the peak time and signal strength of the target genes amplified were faster and stronger compared to 0.8 and 2.0 mM (Figure 2D). To test the reaction stability of the dRT-cLAMP assay, we analyzed the results of the above-mentioned four experiments and found that when the concentration of Mg\(^{2+}\) was 8 mM, compared to 6 and 10 mM, the Tt value and the Df value of the amplified target genes were faster and stronger compared to 0.2 and 0.6 M (Figure 2C). For the concentration of dNTP, we found that when the concentration of dNTP was 1.4 mM/reaction, the peak time and signal strength of the target genes amplified were faster and stronger compared to 0.8 and 2.0 mM (Figure 2D). To test the reaction stability of the dRT-cLAMP assay, we analyzed the results of the above-mentioned four experiments and found that when the concentration of the template was 1 × 10^5 or 1 × 10^3 copies/μL, the Tt value was 21.51 ± 0.39 (CV % = 1.81%) or 28.23 ± 0.77 (CV % = 2.74%) (Figure 2E), respectively, suggesting that the reaction stability of the dRT-cLAMP assay was trustworthy under the optimal reaction conditions.

**Color Change of the HNB Indicator Is Observed and Is Suitable for the dRT-cLAMP Assay.** To optimize the dRT-cLAMP reaction dyes, calcein (a fluorescent metal ion indicator),40 hydroxynaphthol blue (HNB, a metal-ion indicator),41,42 neutral red, and m-cresol purple (two pH indicator)43 were evaluated in the dRT-cLAMP assay. The concentration of calcein, HNB, neutral red, and m-cresol purple was 1 μL, 120 μM, 100 μM, and 50 μM per reaction, respectively. Our results showed that the color change (from the negative to positive reaction) of HNB, calcein, neutral red, and m-cresol purple was from violet to blue, orange to green, yellow to red, and purple to yellow, respectively (Figure 3A). The pseudo-virus was used as the sample (the concentration was 1 × 10^3 copies/μL) to optimize dRT-cLAMP reaction dyes using a grade dry bath incubator. Our results showed that after 30 min of incubation at 63 °C, only the HNB indicator displayed a color change from violet (negative) to blue (positive), the color of other indicators did not obviously change; however, the color change of the other three indicators could also be visualized with the naked eyes at 35 min post-incubation at the same temperature condition (Figure 3A). The result indicated that the HNB indicator was more suitable and sensitive for the dRT-cLAMP reactions. We then evaluated the concentration of the HNB indicator in the dRT-cLAMP assay and found that when the concentration of the HNB indicator was 100 μM per reaction, the color change could be easily observed with the naked eyes; moreover, the color change in this reaction condition displayed earlier than that in the condition containing a higher HNB concentration (120 and 150 μM) (Figure 3B). The high concentration of HNB might interfere with the color colorimetry.

**Intraspecific and Interspecific Specificity of the Primer Sets ORF1ab2# and N1# for SARS-CoV-2 Are Stable.** To verify the specificity and trustworthiness of the dRT-cLAMP assay, we blast-searched (https://www.ncbi.nlm.nih.gov/.)
and performed a comparative sequence analysis of the complete nucleotide sequences of SARS-CoV-2. We found a total of 9968 complete nucleotide sequences of SARS-CoV-2 in the databank. The geographic regions of the virus included Africa (103), Asia (955), Europe (370), North America (7945), Oceania (568), and South America (27). Through deep sequence alignment analysis, we found that the 3′ ends of our designed primers were not located in the nucleotides of high mutation sites of N1 and orf1ab2 genes. Our results showed that the designed primers of SARS-CoV-2 are specific and trustworthy (Figures S1 and S2, Supporting Information).

To evaluate interspecific specificity of the primers, we aligned the sequences of N1 and orf1ab2 gene from pathogens with the same infection site or similar symptoms as COVID-19 via NCBI blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). This alignment analysis revealed that the gene sequences of SARS coronavirus JF292915.1, Bat SARS-like coronavirus MG772934.1, and Bat SARS coronavirus DQ022305.2 were similar with the N1 gene (NC_045512.2) of SARS-CoV-2; the identity of these genes ranged from 88.96 to 89.53% (Table 2). The comparison of the sequences of the dRT-cLAMP primers for N1 gene and similar sequences

| Table 1. Primer Sets Used for the dRT-cLAMP Assay in This Work |
| --- | --- | --- |
| primer sets | oligonucleotide sequence (5′−3′) | amplicon size (bp) |
| N1 | F3: CCCCAAATCAGCGAAATGC | 275 |
| | B3: CACCAAGAATCTGCTTGG | |
| | FIP: GTTGGTTTTGATCGCGCCCCTTTCATTACGTTTGGTGGACCCT | |
| | BIP: GCCCTTCTGCAATTTGTTTCTT | |
| | LF: TAACCACAATAGGATGCTCCAG | |
| | LB: TTCCTCTCTACTATCTCAGG | |
| N2 | F3: CACCGCGAAATCTGCTTAAC | 259 |
| | B3: TTTGCTCTCAAGGATGTTCT | |
| | FIP: CTTGCTGCTCCTCTGCTTAGATTTTATGCTGCAATCTGCTACA | |
| | BIP: CAAACAGGACAGTAAGGTTTGTCAAGCAGCAGAAAAGC | |
| | LF: GCCTTTGCGAATGTGTTTCT | |
| | LB: CTCTCCTGCTAAGGATGCTCCAG | |
| ORFlab 1 | F3: GCTGCACTTACTAACAATGT | 298 |
| | B3: GGTTGGTTAGCTGCAGTTC | |
| | FIP: TCCTCTCTTTAAGATTTTGGCATTTTTCAACGTGCTTTTCCCGG | |
| | BIP: GCAGCAGTGAGAAGGACAGG | |
| | LF: GCCGTAATCTCAATAGGACTTG | |
| | LB: GTCTAGTGTGTTTCTGCTAAGT | |
| ORFlab 2 | F3: GACATCACATACAGTAATGCC | 261 |
| | B3: TGTATACACTATGCGAGCAG | |
| | FIP: CTCATCTGAGATATTGAGTGTTGGGTACCTACACTAGTGCCACA | |
| | BIP: AAGTATTCTACACTCCAGGGACCGGTAGTAGAGAGCTAGGCC | |
| | LF: GCCAGTAATCTCAATAGGACTTG | |
| | LB: GTCTAGTGTGTTTCTGCTAAGT | |

| Table 2. Interspecific Specificity Analysis of N1 and orf1ab2 Genes |
| --- | --- | --- |
| name | GenBank ID | location | identities | location | identities |
| SARS-CoV-2 isolate Wuhan-Hu-1 | NC_045512.2 | 28,274−28,572 | 299/299 | 16,904−17,164 | 261/261 |
| SARS coronavirus | JF292915.1 | 28,080−28,387 | 266/299 | 16,794−17,054 | 238/261 |
| Bat SARS-like coronavirus | MG772934.1 | 28,110−28,408 | 268/299 | 16,825−17,085 | 237/261 |
| Bat SARS coronavirus | DQ022305.2 | 28,100−28,395 | 265/296 | 16,813−17,073 | 237/261 |
| MERS-CoV | MG987421.1 | 29,063−29,098 | 29/36 | 17,274−17,493 | 160/221 |
| influenza A virus | NC_026423.1 | no | no | no |
| human respiratory syncytial virus A | MF449863.1 | no | no | no |
| human respiratory syncytial virus B | MF449878.1 | no | no | no |
| Mycoplasma pneumoniae FH | CP010546.1 | 353,083−353,070 | 14/14 | 201,622−201,645 | 23/24 |
| Chlamydia pneumoniae AR39 | AE002161.1 | 822,538−822,521 | 17/18 | 14,790−14,807 | 17/18 |
| Streptococcus pneumoniae R6 | AE007317.1 | 452,107−452,093 | 15/15 | 1,761,219−1,761,243 | 22/25 |
| human rhinovirus 1B | MK501735.1 | no | no | no |
| human rhinovirus 1 | NC_038311.1 | no | no | no |
| human parainfluenza 3 | LC300511.1 | no | no | no |
| human parainfluenza virus 1 | MK167033.1 | no | no | no |
| human adenovirus 2 | MF044052.1 | no | no | no |

a The sequences of N1 and orf1ab2 gene were aligned from the sequences of pathogens from the same infection site or similar symptoms as COVID-19.
demonstrated that the designed primers were specific (Figure 4). The sequence identity of SARS coronavirus JF292915.1, Bat SARS-like coronavirus MG772934.1, Bat SARS coronavirus DQ022305.2, MERS-CoV MG987421.1, and SARS-CoV-2 orf1ab2 gene (NC_045512.2) ranged from 72.40 to 91.18% (Table 2). The comparison of the dRT-cLAMP primers of orf1ab2 gene and similar sequences showed that the designed primers were also specific (Figure 5). The sequences of other

Figure 4. Specificity analysis of the primer set N1# of SARS-CoV-2 N1 gene. The sequences of primer set N1# were aligned with similar sequences of MERS-CoV MG987421.1, SARS coronavirus JF292915.1, and Bat SARS coronavirus DQ022305.2 using the software Geneious. The 3’ ends of the designed inner primers (FIP) and outer primers (B3) were specific.

Figure 5. Specificity analysis of the primer set ORF1ab2# of SARS-CoV-2 orf1ab2 gene. The sequences of primer set ORF1ab2# were aligned with the similar sequences of SARS coronavirus JF292915.1, Bat SARS-like coronavirus MG772934.1, Bat SARS coronavirus DQ022305.2, and MERS-CoV MG987421.1 using the software Geneious. The 3’ ends of the designed inner primers (FIP and BIP) were specific.
The dRT-cLAMP Assay Is Sensitive to the Detection of Samples with a Low Content of SARS-CoV-2. To determine the detection limit of the dRT-cLAMP assay, we prepared serial dilutions of synthesized N1 and orf1ab2 genes (from $1 \times 10^6$ to $1 \times 10$ copies) as the assay templates and then tested their detection sensitivity. Our results indicated that 100 copies of synthesized N1 and orf1ab2 genes were detected successfully using the primer sets N1# and ORF1ab2#, respectively; the primer group ORF1ab2 + N1# also successfully detected 100 copies of the synthesized genes using a real-time LAMP turbidimeter (Figure 6A). When the tested samples contained only 10 copies of the target genes, the primer group ORF1ab2 + N1#, but not N1# or ORF1ab2# alone, could detect 50% of these tested samples, indicating that the primer combination improved the sensitivity of amplification (Figure 6A). We further validated the sensitivity of the primer group ORF1ab2 + N1# using the isothermal fluorescence and dRT-cLAMP assay methods, and our results showed that when the tested samples contained 100 copies of synthesized N1 and orf1ab2 genes, the two genes were successfully detected from the samples by both the two methods; when the samples contained only 10 copies of the genes, 75% (3/4) and 33% (1/3) of the tested samples were detected by the fluorescence isothermal method (Figure 6B) and the dRT-cLAMP assay (Figure 6C), respectively, indicating that the primer combination and reaction conditions of the dRT-cLAMP assay are sensitive and trustworthy.

The dRT-cLAMP Assay Is Effective for the Detection of SARS-CoV-2 in Environmental Samples. To test the effect of the dRT-cLAMP assay on the detection of SARS-
CoV-2 in the environmental samples, we detected the virus from a total of 27 environmental simulation samples, including water from domestic water and sea, food packaging, seafoods, and throat swabs of healthy people (Figure 8A). Our results showed that the negative samples failed to display a color change and positive signals (color changed from violet to blue) were detected in all the positive samples (Figure 8B). However, the weak positive signals appeared in some samples including seawater, salmon, chicken wings, swimming crabs, and the throat swabs of three healthy people, which might result from the sample containing high salt, fat, or protein. Overall, the dRT-cLAMP assay is powerful and sensitive enough to detect SARS-CoV-2 virus from the collected environmental samples.

### DISCUSSION

Based on the current development of the global COVID-19 pandemic, there is still a long way to go before the pandemic can be brought under control. Some viral members in the coronavirus family cause similar symptoms, known as lower respiratory tract diseases with potential fatality, such as SARS-

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Table 3. Comparison of the Detection Results of the Commercial SARS-CoV-2 RT-qPCR Kits and the dRT-cLAMP Assay

| results      | number | RT-qPCR          | dRT-cLAMP              |
|--------------|--------|------------------|------------------------|
| positive     | 5      | CT range: 11.52–33.97 (range: 20–40 min) | 100% (range: 20–40 min) |
| negative     | 13     | no signals       | 92.31% (12/13)         |
| probable     | 9      | at least one channel has a signal | 44.44% (4/9) |

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Figure 7. The dRT-cLAMP assay is sensitive enough to detect SARS-CoV-2 from clinical samples. (A) Swab samples of overseas Chinese were detected by the dRT-cLAMP assay. A total of 27 swab samples, which had been simultaneously detected by two commercial SARS-CoV-2 RT-qPCR kits, were verified by the dRT-cLAMP assay. Positive sample: 25–30 (diluted 7.5-fold) and 32–36 (diluted 15-fold); Negative sample: 3–5, 10, 15, and 17–24; Probable sample: 6–9, 11–14, and 16. NC (ddH2O): 1, 2; PC (1 × 10⁵ copies/μL): 31, 37. (B) ROC curve analysis of the results of the commercial SARS-CoV-2 RT-qPCR kits and the dRT-cLAMP assay.
A lot of efforts have been made on the research of the SARS-CoV-2 virus since the outbreak of COVID-19, and it is believed that a therapeutic or vaccine can be developed and used to prevent and/or control COVID-19, just like a flu, in the very near future. However, at present, the prevention and control of the COVID-19 pandemic cannot be neglected, especially the detection of SARS-CoV-2 virus for asymptomatic carriers and internationally traded goods.

The SARS-CoV-2 virus detection is a valuable strategy to control the pandemic of COVID-19. The current gold standard assay for the detection of SARS-CoV-2 virus is the...
multiple qRT-PCR method, in which multiple targets can be detected at one time to improve the accuracy of detection. However, it is difficult to obtain the relevant detection equipment in the grassroots community and almost impossible to achieve rapid detection onsite. Therefore, the LAMP assay can well complete the onsite screening of a large number of samples at the grassroots or the customs, and the suspected samples can be further confirmed by the detection method of qRT-PCR. In this study, we found that the detection results by different detection methods for the suspected clinical samples were greatly different, indicating that the mutual supplement of different detection techniques can improve the accuracy of detection. Moreover, compared to the screening methods for detection of a single gene, the dRT-cLAMP method developed in this work can simultaneously detect two target genes, that is, \textit{orf1ab} and \textit{N} genes, of SARS-CoV-2 virus, drastically improving the detection efficiency and accuracy.

Rapid and reliable detection of the SARS-CoV-2 virus is particularly important for the containment of the COVID-19 pandemic. In this study, we developed a simple and rapid dRT-cLAMP assay for SARS-CoV-2 detection and demonstrated that this assay has high detection sensitivity and specificity to detect the target SARS-CoV-2 virus from the clinical and standard-added environmental simulation samples. Although our developed dRT-cLAMP detection assay still needs to be further validated by testing a large number of samples, our work is sufficient to prove that the dRT-cLAMP assay is a feasible assay for SARS-CoV-2 virus detection and is of great significance for onsite detection where rapid screening is urgently needed.

**MATERIALS AND METHODS**

**Primer Design and Analysis.** A total of 424 complete genomes of SARS-CoV-2 virus were obtained from GenBank databases (https://www.uniprot.org/database/DB-0028) and were used for identification of conserved gene sequences. dRT-cLAMP primers of target gene sequences were designed by the online software Primer Explorer V5 (http://primerexplorer.jp/lamp5e/index.html). Six oligonucleotide primers targeting eight conserved regions in the \textit{orf1ab} and \textit{N} genes were used in this work. All used primers are listed in Table 1.

**Development of the dRT-cLAMP Reaction System.** To develop the dRT-cLAMP assay, the reaction mixture (25 \( \mu \)L) contained 2 \( \mu \)L of template [the pseudo-virus (plasmid containing virus target genes) or the extracted RNA of clinical samples], 40 pmol of each of FIP and BIP primers, 20 pmol of each LF and LB primers, 5 pmol of each of F3 and B3 primers, 16 U of Bst DNA polymerase and 2 U of AMV reverse transcriptase (New England Biolabs, Ipswich, MA, USA), 0.4 mol/L of betaine (Sigma-Aldrich, St. Louis, Missouri, USA), 6 mmol/L of MgSO\textsubscript{4}, 1.4 mmol/L of deoxyribonucleoside triphosphate (10 mM each), and 1X isothermal amplification buffer, which contained 20 mmol/L of Tris–HCl (pH 8.8), 10 mmol/L of KCl, 10 mmol/L of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 2 mmol/L of MgSO\textsubscript{4} and 0.1% Tween 20. For visual assessment of the dRT-cLAMP amplicons in the reaction, HNB (100 \( \mu \)mol/L; Solarbio, Beijing, China), which was selected by comparison with calcein (Eiken Chemical, Japan), neutral red, and \( m \)-cresol purple (Solarbio, Beijing, China), was added to each reaction tube and the colorimetric signals of each solution were observed using a grade dry bath incubator (Tiangen Biotech, Beijing, China). A positive control (PC, plasmid harboring the target gene) and a negative control (NC, double-distilled water, ddH\textsubscript{2}O) were also included in the dRT-cLAMP assay. The positive reactions displayed a color change from violet to blue due to the decrease of Mg\textsuperscript{2+}, which could be combined with pyrophosphoric acid produced by the reaction to produce precipitate, whereas the negative reactions remained violet. Moreover, the positive reactions could be detected with a real-time turbidimeter LAS500 (Eiken Chemical, Japan) of turbidity of the magnesium pyrophosphate that was produced in the reaction mixture. The sensitivity system of the dRT-cLAMP assay was further validated by the isothermal fluorescence method with a LightCycler 480 real-time PCR (Roche, Sweden) and the fluorescence detection reagent SYTO9 (Thermo Fisher Scientific, Hampton, NH, USA).

**Clinical Samples.** Swab samples were obtained from people via the Tianjin Customs and tested with the RT-qPCR kit (Zhijiang and BioGerm, Shanghai, China). The extracted RNA was further detected via the dRT-cLAMP assay.

**Standard-Added Environmental Simulation Samples.** The pseudo-virus (plasmid containing virus target genes) was used as the sample (the concentration was \( 1 \times 10^{5} \) copies/\( \mu \)L, 20 \( \mu \)L) that was added to other samples such as seafood or packaging. The samples without adding the pseudo-virus served as a NC. The samples were treated according to the standardized sample collection and nucleic acid extraction and then detected by the dRT-cLAMP assay.

**Ethical Statement.** The clinical samples for optimizing the detecting workflow was agreed upon under the ethical regulations of each participating partner.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05781.

**NI** gene sequence alignment analysis and \textit{orf1ab2} gene sequence alignment analysis (PDF)

**AUTHOR INFORMATION**

**Corresponding Authors**

Wei Liu — Tianjin Customs District, Tianjin 300387, P. R. China; Email: WeiLiutj@163.com

Wenjie Zheng — Laboratory for Quality Control and Traceability of Food, Tianjin Normal University, Tianjin 300387, P. R. China; Email: skyzwj@tjnu.edu.cn

**Authors**

Chao Ji — State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan and Key Laboratory for Agro-Biodiversity and Pest Control of Ministry of Education, Yunnan Agricultural University, Kunming 650201, P. R. China; Laboratory for Quality Control and Traceability of Food, Tianjin Normal University, Tianjin 300387, P. R. China

Shuxia Xue — Laboratory for Quality Control and Traceability of Food, Tianjin Normal University, Tianjin 300387, P. R. China

Min Yu — Department of Gynecologic Oncology, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Tianjin 300060, P. R. China

Jinyu Liu — Laboratory for Quality Control and Traceability of Food, Tianjin Normal University, Tianjin 300387, P. R. China
Qin Zhang — Laboratory for Quality Control and Traceability of Food, Tianjin Normal University, Tianjin 300387, P. R. China

Feng Zuo — Tianjin Customs District, Tianjin 300308, China

Quiyue Zheng — Key Laboratory of Biotechnology and Bioresources Utilization of Ministry of Education, College of Life Science, Dalian Minzu University, Dalian 116600, P. R. China

Liangjuan Zhao — Tianjin Customs District, Tianjin 300308, China

Hongwei Zhang — Tianjin Customs District, Tianjin 300308, China

Jijuan Cao — Key Laboratory of Biotechnology and Bioresources Utilization of Ministry of Education, College of Life Science, Dalian Minzu University, Dalian 116600, P. R. China

Ke Wang — Department of Gynecologic Oncology, Tianjin Medical University Cancer Institute and Hospital, National Clinical Research Center for Cancer, Tianjin 300060, P. R. China

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c05781

Author Contributions

C.J. and S.X. contributed equally to this work. W.Z. and W.L. designed the experiments; C.J., S.X., M.Y., and Q.Z. performed the experiments; C.J., S.X., Q.-L.Z., L.Z., and H.Z. performed data collection, analysis, and interpretation; W.Z., J.C., F.Z., and K.W. supervised the work; C.J., S.X., and J.L. wrote the original manuscript; C.J. and W.Z. reviewed/edited the manuscript; and W.Z. and J.C. provided the funding acquisition.

Notes

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