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Letter

Rapid One-Pot Detection of SARS-CoV-2 Based on a Lateral Flow Assay in Clinical Samples

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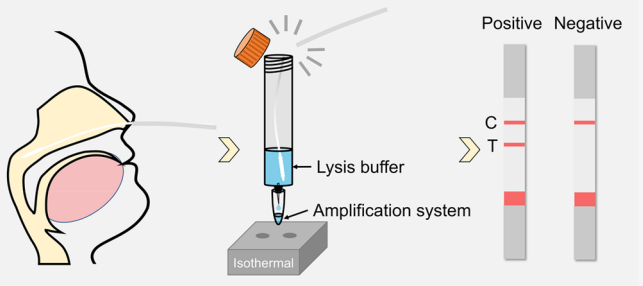
ABSTRACT: Rapid tests for pathogen identification and spread assessment are critical for infectious disease control and prevention. The control of viral outbreaks requires a nucleic acid diagnostic test that is sensitive and simple and delivers fast and reliable results. Here, we report a one-pot direct reverse transcript loop-mediated isothermal amplification (RT-LAMP) assay of SARS-CoV-2 based on a lateral flow assay in clinical samples. The entire contiguous sample-to-answer workflow takes less than 40 min from a clinical swab sample to a diagnostic result without professional instruments and technicians. The assay achieved an accuracy of 100% in 12 synthetic and 12 clinical samples compared to the data from PCR-based assays. We anticipate that our method will provide a universal platform for rapid and point-of-care detection of emerging infectious diseases.



The increasing number of infectious diseases seriously threatens human health and global security.[1,2] Some of these are recurrent large-scale epidemics from emerging pathogens such as SARS-CoV-2,[3] SARS,[4] MERS,[5] influenza H1N1,[6] and Zika virus.[7] All of these epidemics are likely caused and initiated by rapid human-to-human transmission.[8] Therefore, the establishment of an accessible and accurate point-of-care testing technology, which allows a self-check at home or rapid screen in crowded places, will provide a rapid response to emerging pathogens and reduce the cross infection and continuous spread of infectious diseases happening in hospitals and public places.

Assays based on quantitative polymerase chain reactions (qPCR) for nucleic acid detection of pathogenic microorganisms are the most commonly used methods around the world.[9−12] However, given the need to ship samples to qualified laboratories as well as the time for data analysis by experts, the typical turnaround time for detection is >24 h. As a result, their applications are limited in countries that lack sufficient resources to handle testing in specialized laboratories during a pandemic. Serological methods, on the other hand, can provide a rapid result within a few minutes, but false negative results may occur since it usually takes several days for a patient to develop a detectable antibody response after infection.[13,14] Therefore, other reliable and rapid point-of-care nucleic acid detection techniques are highly needed.

For nucleic acid detection of pathogens in clinical samples, three operational steps are generally followed: sample treatment, target nucleic acid amplification, and result readout. While each step is crucial, it should be noted that the key challenge is the integration of all these steps into a unified workflow to shorten the operating time and procedures as much as possible for the development of a point-of-care nucleic acid detection method.[15] In our opinion, several existing technical difficulties hinder the development of point-of-care nucleic acid detection. First, the conventional amplification method often requires nucleic acids with relatively high purity as the template.[16] Therefore, a time-consuming, laborious, and costly sample treatment step cannot be omitted. Second, PCR-based techniques rely on sophisticated thermal cycling instruments to ensure effective amplification, which often takes >2 h. Third, the amplicons are usually analyzed by fluorescence signals or gel electrophoresis, which leads to the need for specialized instruments and additional operational steps.

To overcome these challenges, we established an approach that can directly release the nucleic acids from the collected samples without interfering with the downstream amplification reaction. Furthermore, we employed direct reverse transcript loop-mediated isothermal amplification (RT-LAMP) to amplify the virus RNA efficiently and rapidly in the sample without the need of any specialized equipment. In addition, we

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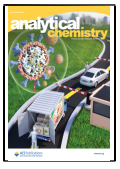

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used a lateral flow assay (LFA) that allows the interpretation of nucleic acid amplification results by direct visualization.[17−21] When these technologies are integrated into one system (RT-LAMP-LFA), the diagnosis of a clinical sample (SARS-CoV-2) can be achieved in less than 40 min from a swab sample to a diagnostic result. This sample-to-answer workflow exhibits the advantages of being simple, having less cost, and being portable for diagnosis. This technology is one step closer to achieve the on-site rapid screening of pathogenic microorganisms, which is critical for infectious disease control and prevention.

RESULTS AND DISCUSSION

Principle of the Direct RT-LAMP-LFA System. The workflow of RT-LAMP-LFA is composed of three steps, as shown in Figure 1A. First, the collected sample is treated with

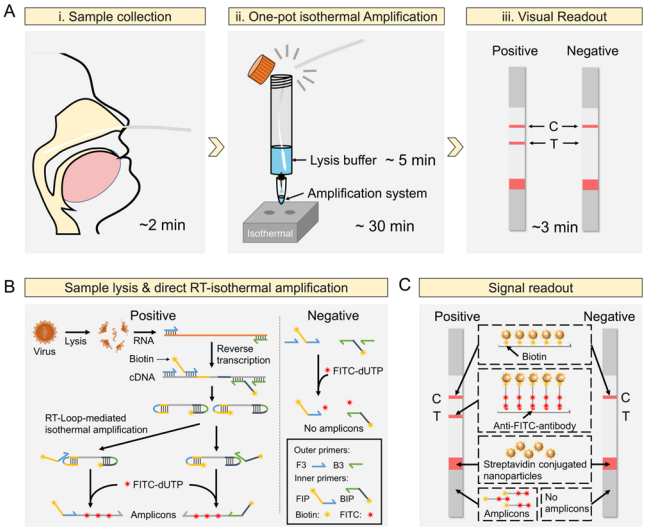

Figure 1. Schematic diagram of the RT-LAMP-LFA system. (A) The workflow for SARS-CoV-2 detection with the RT-LAMP-LFA system: (i) sample collection; (ii) lysis and direct RT-LAMP amplification; (iii) visual reading with LFA. (B) Principle of direct RT-LAMP amplification. (C) Principle of visual detection with LFA.

a lyse solution (50 mM NaOH solution) followed by direct reverse transcript loop-mediated isothermal amplification (RT-LAMP) with specifically designed primers (Table S1). To avoid contamination, a closed sample amplification tube is designed to perform sample collection and amplification in one pot. Once collected, the sample is stored in a closed system and isolated from the outside environment. The direct RT-LAMP procedure takes only 2, 5, and 30 min for sample collection, treatment, and isothermal amplification, respectively. Figure 1B elucidates the direct RT-LAMP procedure, where the rapid sample lysis, reverse transcription, and specific amplification occur with the outer primers (F3 and B3), biotin-labeled inner primers (FIP and BIP), and fluorescein (FITC)-labeled dUTP in the reaction mixture for generating amplicons with labels. Herein, we used an isothermal DNA polymerase that integrates both native reverse transcriptase and DNA polymerase activities to achieve rapid single-enzyme-based reverse transcript LAMP reactions.[22,23] In addition, DNA polymerase has also been proven to elongate on DNA template using dUTP as the reaction substrate (Figure S1).[24−26]

Second, as shown in Figure 1C, the result readout is directly visualized by a lateral flow assay. After amplification, a lateral flow strip is added into the reaction tube and the result is read with the naked eyes. As the amplified products migrate along the strip, the conjugations of amplicons and streptavidin coated particles can be captured by the anti-FITC antibody immobilized at the test line (T) because of the presence of FITC-labeled amplicons originated from FITC-dUTP, which leads to a red T line. The rest of the excess unreacted streptavidin coated particles keep moving and are captured by biotin immobilized at the control line (C). This step can help verify the correct operation of the lateral flow system.[27] It only takes 3 min to allow the completion of the operating procedure from amplicon loading to signal readout with the LFA.

Optimization of the RT-LAMP Assay. With the goal of shortening the testing time to <30 min, we selected the RT-LAMP assay due to its advantages of being rapid, simple, and sensitive.[28−31] Herein, we used an isothermal DNA polymerase that integrates both native reverse transcriptase and DNA polymerase activities.[15] Therefore, virus RNA can be reverse transcripted under the initiation of F3 and B3, while loop mediated isothermal amplification can be carried out with the joint action of F3, B3, FIP, and BIP in one step. We designed SARS-CoV-2 LAMP primers to target the nucleoprotein (N) and open reading frame (ORF1ab) genes according to the sequencing published on GenBank (MN908947.3, Figure 2A).

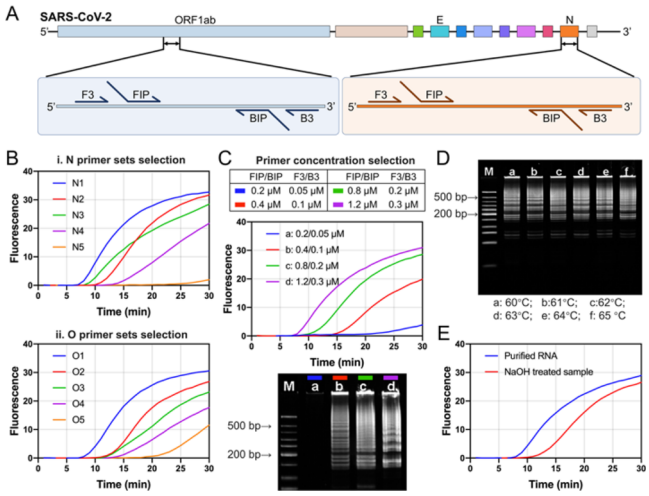

Figure 2. Optimization of RT-LAMP. (A) Detection region for the N gene and ORF1ab gene of SARS-CoV-2. (B) Fluorescence detection of SARS-CoV-2 RNA from pseudovirus (40 copies/μL) using different primer pairs for N and ORF1ab genes. (C) Fluorescence and gel electrophoresis of SARS-CoV-2 RNA from pseudovirus (40 copies/μL) using different primer concentrations. (D) Gel electrophoresis of SARS-CoV-2 RNA from pseudovirus (40 copies/μL) using N gene primer pairs under different temperatures. (E) Fluorescence detection of purified RNA (isolated from 1000 copies of pseudovirus) with commercial extraction kits and unpurified RNA (40 copies/μL) directly lysed by NaOH. The template concentrations of all reactions were unified as the final concentrations.

The detailed sequences for the pathogens are listed in Table S1. We selected the primer pairs of N1 and O1 for SARS-CoV-2 detection, respectively, due to their optimum performance in detecting the N gene and ORF1ab gene shown in Figures 2B and S2.

Our next goal was to optimize the concentrations of primers used in the RT-LAMP procedure to improve the testing specificity. According to Figure 2C, the optimized primer

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concentrations were determined to be 0.2 μM each of F3 and B3 and 0.8 μM each of FIP and BIP. In addition, we also evaluated the effect of the reaction temperature. On the basis of the optimized reaction temperature of the isothermal DNA polymerase, the amplification reaction was performed at temperatures ranging from 60 to 65 °C. No significant distinction was observed on the gel electrophoresis results (Figure 2D), indicating the isothermal amplification could be performed under temperatures of 60 to 65 °C. This proves that our method does not require any strict temperature control instrumentation and can be implemented by simple thermostatic devices.

Extraction-Free Amplification. Conventional nucleic acid detection suffers from a prerequisite to purify and isolate nucleic acids from clinical samples. Nucleic acid isolation requires specialized laboratories, which is time-consuming and costly and depends on reagents that could be material-limited during a pandemic. Alternative, noncommercial solutions for nucleic acid isolation, e.g., using silica gel matrix or magnetic beads, also require specialized knowledge and cannot be implemented easily for point-of-care decentralized screening. To enable direct amplification without nucleic acid purification, We used a simple but powerful chemical method, which did not require any costly materials and complex solutions for pathogenic microbe lysis, according to previous studies on eukaryotic cells.[32−34] Briefly, we used a NaOH solution (50 mM) to directly release target nucleic acids from the collected samples by lysing virus without additional steps such as high temperature incubation. Herein, by using the pseudovirus of SARS-CoV-2 as template (1000 copies/μL), we compared the amplification efficiency of purified RNA by commercial RNA extraction kits and samples directly treated with NaOH using the same RT-LAMP procedure. It can be seen that the NaOH-treated sample shows a comparable fluorescence signal as the purified RNA after 30 min, indicating NaOH can effectively release RNA for the amplification reaction (Figures 2E and S3).

Visual Detection with the Lateral Flow Strip. We developed an easy-to-interpret visual quantitative readout responding to the presence or absence of the pathogens using a lateral flow strip and compared it to the conventional fluorescent RT-LAMP assay. As shown in Figure 3A, if the result is negative, only a red band will appear on the C line, while if the result is positive, both a red C line and a T line will be shown. We investigated the analytic detection limit of the RT-LAMP-LFA system for SARS-CoV-2. 10-Fold serial dilutions of the pseudovirus of SARS-CoV-2 N gene and Orf1ab gene with concentrations ranging from 0.02 to 200 copies/μL were used to test the RT-LAMP-LFA system (Figure 3B). We confirmed that the assay is able to generate a visible signal when the concentrations of SARS-CoV-2 pseudovirus are ≥2 copies/μL. In comparison to the qPCR assay, our method shows a similar sensitivity but an easier-to-interpret qualitative readout for the detection of RNA from SARS-CoV-2 virus.[35−38]

Target Specificity Check of the System. Once RT-LAMP-LFA protocols were optimized, we further tested their specificity for SARS-CoV-2 diagnostics. Pseudovirus (SARS-CoV-2 N gene and SARS-CoV-2 ORF1ab gene) and 13 types of synthetic plasmids from other common pathogenic microorganisms, including synthetic SARS-CoV, MERS-CoV, HCoV-NL63, HCoV-229E, HCoV-HKU1, *Chlamydia pneumoniae*, *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*,

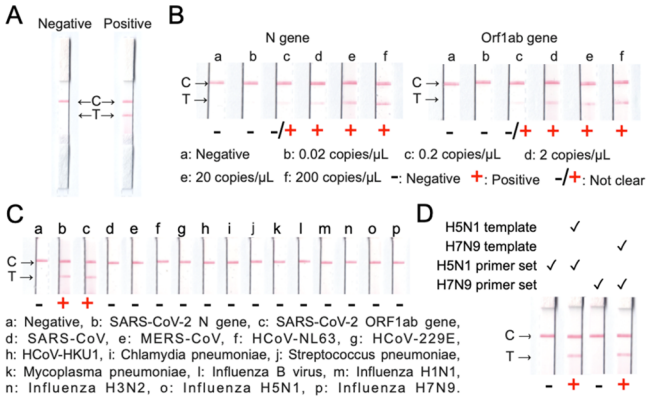

Figure 3. Optimization and verification of the lateral flow assay. (A) Scheme of the diagnostic results of SARS-CoV-2. (B) Changes of the T lines at different concentrations of pseudovirus templates of N gene and ORF1ab gene. (C) Diagnostic results of different pathogenic microorganisms using the SARS-CoV-2 primer set. The template concentrations were 40 copies/μL for SARS-CoV-2 pseudovirus and 40 copies/μL for synthetic plasmids. (D) Diagnostic results of H5N1 and H7N9 templates (40 copies/μL) using their corresponding primer sets, respectively.

Influenza B virus, Influenza H1N1, Influenza H3N2, Influenza H5N1, and Influenza H7N9, were tested on the RT-LAMP-LFA platform, and no positive signals (red T line) were observed (Figure 3C). The results showed that there is no cross-reaction of this designed system with other respiratory pathogenic microorganisms. In addition, when the primer sets were replaced with the Influenza H5N1 primer set and Influenza H7N9 primer set, positive results were obtained by using the templates of synthetic Influenza H5N1 and Influenza H7N9, respectively (Figure 3D). This indicates that the RT-LAMP-LFA system is universal and can be applied to identify other pathogenic microorganisms when specific primer sets are designed.

Clinical Applications of the RT-LAMP-LFA Platform. To evaluate the reliability of the optimized RT-LAMP-LFA system in this study, the accuracy of this system was further verified using both synthetic samples and clinical swab samples (12 synthetic positive samples, 8 positive swab samples, 4 negative swab samples from healthy individuals, and 6 negative swab samples from *Mycoplasma* infected individuals as negative controls). We first made 12 synthetic samples by adding pseudovirus that replicates the concentrations appearing in the real medical record.[39] Briefly, we prepared solutions with different concentrations of pseudovirus and dispersed them on the sidewall of the centrifuge tube by vortexing. Then, a nasopharynx swab was touched to sidewall of tube to mimic the real sample collection process. Of the 12 synthetic samples with detectable virus concentrations (Table S3), we obtained positive results from all the RT-LAMP-LFA test results (Figure 4A). The detection results provided by our method showed no discrepancies with the expected results (Figure 4B). We also calculated the time used for the entire assay and verified that all steps can be performed within ∼40 min: 2 min (sample collection) + 5 min (sample treatment) + 30 min (isothermal amplification) + 3 min (lateral flow strip detection).

Finally, we tested our RT-LAMP-LFA system with clinical RNA samples. We studied two groups of swab samples both with a standard RNA extraction procedure post-collection: (1) samples from 8 positive COVID-19 patients and (2) samples

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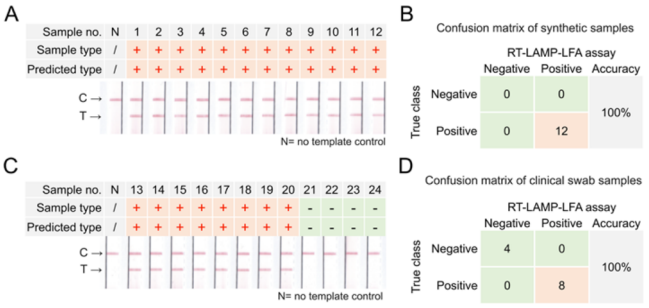

Figure 4. Diagnostic results of synthetic and clinical samples. (A) Diagnostic results of 12 synthetic positive samples. (B) Confusion matrix analysis of synthetic samples. (C) Diagnostic results of 12 clinical swab samples with RNA extraction (8 positive and 4 negative). (D) Confusion matrix analysis of clinical swab samples with RNA extraction. All the reactions were performed under optimal conditions.

from 4 negative donors both based on the RT-qPCR detection data. It should be noted that we did not directly use the extraction-free procedure due to the local sample handling restrictions. The performance of our method for detecting SARS-CoV-2 RNA is shown in Figures 4C and S4. It can be seen that all the positive and negative samples were identified accurately (Figure 4D and Table S4). The total accuracy of this method for SARS-CoV-2 diagnosis in clinical RNA samples was 100%. We also tested samples isolated from six swabs with *Mycoplasma* infections as negative controls. The results were all negative (non-SARS-CoV-2 infection, Figure S5). This indicates that our method can achieve rapid and point-of-care detection of emerging infectious diseases.

Rapid and accurate testing of pathogenic microorganisms is essential for early discovery, early reporting, early quarantine, early treatment, and efficient control of epidemic transmission. While many in vitro diagnostic companies are trying to develop new, rapid, and inexpensive nucleic acid detection kits, our RT-LAMP-LFA approach provides an alternative route for rapid diagnosis by the integration of direct extraction of nucleic acids, rapid amplification, and a convenient result readout by a lateral flow assay. Compared to the PCR-based diagnostic methods that rely on the use of purified nucleic acids, our RT-LAMP-LFA system is capable of detecting the target nucleic acid directly by using a NaOH-based lysis to release the targets for cascading isothermal amplification without undergoing the conventional nucleic acid isolation step, which shortened the sample preparation time from 2 h to 5 min.[40]

Compared with previously studied pathogenic microorganism detection methods, our assay has significant advantages.[28−30,37,41−44] The major advantage of this assay is its rapid and qualitative visual output of "Yes" or "No" without professional instruments. Specifically, the previously developed assays often involve complex operational procedures and costly instrumentation that may not be accessible in many situations. Our method is simple to operate and interpret; thus, it can be extended to future studies that focus on the development of point-of-care diagnostics for pathogenic microorganisms.

In summary, we designed and tested a RT-LAMP-LFA-based pathogenic microorganism detection method that enables one to rapidly, accurately, and visually interpret the diagnostic results from clinical samples without professional instruments and expertise. We envision that the power of RT-LAMP-LFA could inspire more clinical applications toward family self-examination or screening in resource deficient areas.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c05059.

Experimental details; detailed sequences; details of synthetic samples; RT-qPCR and RT-LAMP-LFA results; RT-LAMP reaction; illustrations of SARS-CoV-2 Orf1ab and N gene; pH of RT-LAMP system; confusion matrix and ROC analysis of the RT-LAMP-LFA method; diagnostic results of *Mycoplasma* infection samples (PDF)

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Notes

The authors declare no competing financial interest.

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