SCIENCE

A New Auto-RPA-Fluorescence Detection Platform for SARS-CoV-2

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Abbreviations: qRT-PCR, quantitative reverse transcription–polymerase chain reaction; RPA, recombinase polymerase amplification; CDC, Centers for Disease Control and Prevention; LAMP, loop-mediated isothermal amplification; CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated; STC, Southern Theater Command; S, spike, N, nucleocapsid; BLAST, basic local alignment search tool; FAM, carboxyfluorescein; HEX, hexachlorofluorescein; Ct, cycle threshold.

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ABSTRACT

Objective: The outbreak of COVID-19 caused by SARS-CoV-2 has led to a serious worldwide pandemic. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR)–based methods were recommended for routine detection of SARS-CoV-2 RNA. Because the reaction time and analytical sensitivity of qRT-PCR limits the diagnosis of SARS-CoV-2, development of a quick process of SARS-CoV-2 detection technology with high analytical sensitivity remains urgent.

Methods: We combined isothermal amplification and fluorescence detection technology to develop a new auto-recombinase polymerase amplification (RPA)-fluorescence platform that could be used in the diagnosis of SARS-CoV-2.

Results: By optimization of primers and probes, the RPA platform could detect SARS-CoV-2 nucleotides within 15 min. The limits of detection and specificity of the auto-RPA-fluorescence platform were 5 copies/μL and 100%, respectively. The accuracy of detection of the auto-RPA-fluorescence platform in the 16 positive samples was 100%.

Conclusion: The RPA platform is a potential technology for the diagnosis of SARS-CoV-2 infection.

Since the outbreak of COVID-19, caused by SARS-CoV-2, this infectious disease has become a global pandemic that affects many countries in the world. According to the World Health Organization homepage (https://www.who.int), more than 500 million people have been infected with COVID-19, and more than 6 million people have died as of April 2022. Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) is a common approach for the detection of SARS-CoV-2 infection. It is routinely performed worldwide, including in the Centers for Disease Control and Prevention (CDC) and other clinical laboratories.[1,2] Globally, qRT-PCR has been defined as a standard method for the detection of COVID-19. However, this method is time-consuming (the process takes more than 2 h), and requires high purity samples, sophisticated equipment, and well-trained personnel, which can delay the diagnosis of COVID-19. In addition, this method has relatively low analytical sensitivity and poor-quality nucleotide extraction, which causes false-negative results.[3,4] Therefore, more rapid, sensitive, and accurate diagnostic methods are greatly needed to deal with the COVID-19 pandemic.

To detect COVID-19 infection rapidly, scientists have made various efforts to develop a series of novel detection methods with great potential.[5] For example, loop-mediated isothermal amplification (LAMP) starts amplification by using *Bst* DNA polymerase at a constant temperature of approximately 65°C. The LAMP can cycle, elongate, and continue subsequent rounds of amplification with 6 specially designed primers and has high reaction efficiency. The reaction generates magnesium pyrophosphate and results in color change, which can be judged by the naked eye under blue light.[6,7] However, such a method is mediated by detecting the by-products of loop amplification, which may cause false-positive results due to nonspecific reactions.[8–11] Clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) nuclease-based methods have properties that can be used for ultrasensitive diagnostic tests. The Cas nucleases, such as RNA-guided RNase Cas13a and DNase Cas12a, cause collateral cleavage when activated by the recognition of target nucleotide sequences.[5] Recently,

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researchers have developed exciting (CRISPR)-based methods by integrating PCR, LAMP, or recombinase polymerase amplification (RPA) with Cas-mediated collateral cleavage,[6,9,12–15] which were amplified by diverse reaction systems.[5] However, current CRISPR strategies have some disadvantages, including multiple-step operations and long incubation times. A new approach that is more rapid, accurate, and simple for detecting SARS-CoV-2 is urgently needed.

To achieve more rapid and accurate detection of SARS-CoV-2 infection, we applied RPA and a fluorescence detection system to develop an auto-RPA–fluorescence assay for detecting SARS-CoV-2 RNA. The results show that the new assay described here is more rapid and simple than the standard qRT-PCR used currently to detect SARS-CoV-2 and has potential to detect other major pathogenic microorganisms as well.

Materials and Methods

RNA Extraction

Oropharyngeal swabs were used to collect samples from participants and qRT-PCR was used to determine whether the samples were positive or negative for SARS-CoV-2. Briefly, RNA was extracted using the SSNP-9600A Thermo Fisher Scientific nucleic acid extraction workstation in the P3 laboratory of the Center for Disease Control and Prevention of Southern Theater Command (CDC-STC). Prior to the experiment, the purity and concentration of each virus RNA sample was determined using Qubit 3.0 fluorometer (Thermo Fisher Scientific). Subsequent experiments were performed at Jining No. 1 People's Hospital. All samples were frozen at −80°C or transferred on dry ice and kept for less than 6 mo.

Design and Analysis of Primers and Probes

The complete genomic sequence of SARS-CoV-2 was obtained from GenBank (MN985325.1) and used as reference sequence for the design of primers and probes. To establish the auto-RPA-fluorescence assay, we first designed RPA-specific primers and probes for targeting the spike (S) gene and nucleocapsid (N) gene sequences of SARS-CoV-2 using the PrimedRPA online bioinformatic tool.[16,17] The panel of primers and probes used in the RPA reaction are listed in **TABLE 1**. We used Primer-basic local alignment search tool (BLAST) to determine whether the primer and probe sequences were specific for the SARS-CoV-2 genome. The primer region sequence was then compared with the genomes of other viruses using BLAST to calculate homology. Fluorescence and modifications were then labeled following principles that were suggested previously.[17] The S gene and N gene fragments of SARS-CoV-2 were synthesized and cloned in pcDNA3 vectors (Sangon Biothech). According to the criteria suggested in the TwistAmp amplification guidelines (TwistDx), the primers/probes with better amplification efficiency and fluorescent quantity were used for subsequent detection.

Construction of a New Auto-RPA–Fluorescence Platform

We designed a new auto-RPA-fluorescence platform based on the RPA principle and a fluorescence detection system. Briefly, tetrahydrofuran is added to the probe between two adjacent thymine residues that are separated by 1, 3, or 5 intervening nucleotides when synthesized. In this study, a fluorescent dye—carboxyfluorescein (FAM) or hexachlorofluorescein (HEX)—was added to the upstream thymine, and Black Hole Quencher dye was added to the downstream thymine in the probe (Genomics).

TABLE 1. Primers and Probes Used in the Auto-RPA–Fluorescence Platform[a]

Primer/Probe	Sequence 5'→ 3'
N256For	ACTACCGAAGAGCTACCAGACGAAT
N540Rev	CGTGATGAGGAACGAGAAGAGGCTTG
N540For	CAAGCCTCTTCTCGTTCCTCATCAC
N689Rev	GCCTTTACCAGACATTTTGCTCTCA
N860For	GGACCAGGAACTAATCAGACAAGGAA
N1167Rev	GCAGCAGGAAGAAGAGTCACAGTTTG
S3053For	CAGAGCTTCTGCTAATCTTGCTGCTA
S3202Rev	TTGTGAAGTTCTTTTCTTGTGCAGGG
S2531For	TGCTGCTAGAGACCTCATTTGTGCAC
S2850Rev	AAAGCTTGTGCATTTTGGTTGACCAC
Probe N645 exo	GGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAGATT GAACCA
Probe N408 exo	GAGGGAGCCTTGAATACACCAAAAGATCACATTGGCAC CCGCAAT
Probe S2560 exo	AACGGCCTTACTGTTTTGCCACCTTTGTCACAGATGAA ATGA
Probe S2760 exo	CTATTGGCAAAATTCAAGACTCATTTCTTCCACAGCAA
Probe S3135 exo	GCTATCATCTTATGTCCTTCCCTCGTCAGCACCTCATGG

[a]*Primers and probes were designed and selected by the online tool PrimedRPA (see Materials and Methods). All primers and probes were tested by BLAST to detect specificity for SARS-CoV-2 virus genome and subsequently tested in the auto-RPA-fluorescence platform.*

During detection with the auto-RPA-fluorescence platform, we first used the RPA exo kit (Weifang Amp-Future Biotech) to perform reverse-transcribed isothermal amplification in a 50 μL reaction volume. The 50 μL reaction mixture contained 2 μL primer mix (10 μmol/L each), 1 μL probe (10 μmol/L), and 5 μL RNA sample. For the positive control group, we used synthesized gene fragments that covered the amplification regions. For the negative control group, the template DNA was replaced with scrambled synthesized DNA. The reactions were performed in a SLAN-96P fluorescence qPCR detection system (Shanghai Hongshi Medical Technology), and the reaction mixtures were incubated at 42°C for 2 min followed by 40 cycles. Since the temperature was consistent in RPA reaction and there were no real cycles, the program automatically defined 42°C/40 sec/step as a cycle. The fluorescence signal was monitored for each cycle using a fluorescence qPCR detection system. For reactions with cycle threshold (Ct) values of less than 38, the results were determined as positive and otherwise determined as negative.

Limit of Detection and Specificity of the Auto-RPA–Fluorescence Platform in Detecting SARS-CoV-2

To assess the limit of detection of the auto-RPA–fluorescence platform for detecting SARS-CoV-2, the virus gene template was diluted from 50,000,000 to 5 copies/μL with double-distilled water and applied to the platform. The specificity of the new platform was evaluated using RNA extractions from influenza, HCoV-229E, measles, mumps, HIV, and coxsackie viruses.

Application and Assessment of the Auto-RPA–Fluorescence Platform for Detecting SARS-CoV-2-Infected Samples

A total of 16 positive samples and 2 negative samples were collected from the CDC-STC (NBJJ-2020-007), Guangzhou, China. Specimen

FIGURE 1. The optimization of primer pairs and probes for auto RPA–fluorescence-based SARS-CoV-2 detection. A, Agarose gel electrophoresis analysis of amplification products by different primer pairs as indicated. B, Schematic representation of probe modification for SARS-CoV-2 detection in the auto-RPA–fluorescence detection platform. C, Fluorescent signal generated from S2531For/S2850Rev/ProbeS2560 in the platform. D, Fluorescent signal generated from S2531For/S2850Rev/ProbeS2760 in the platform.

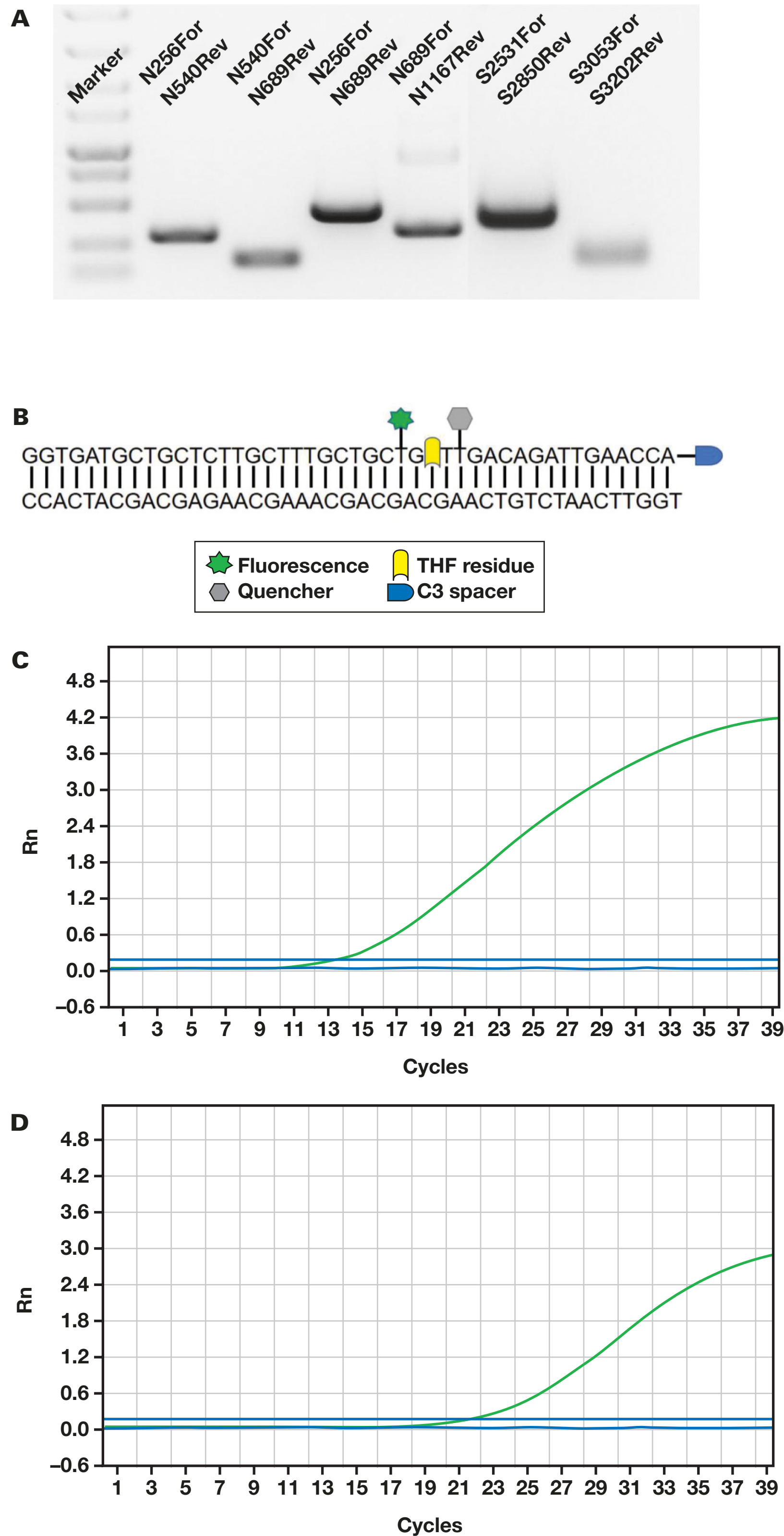
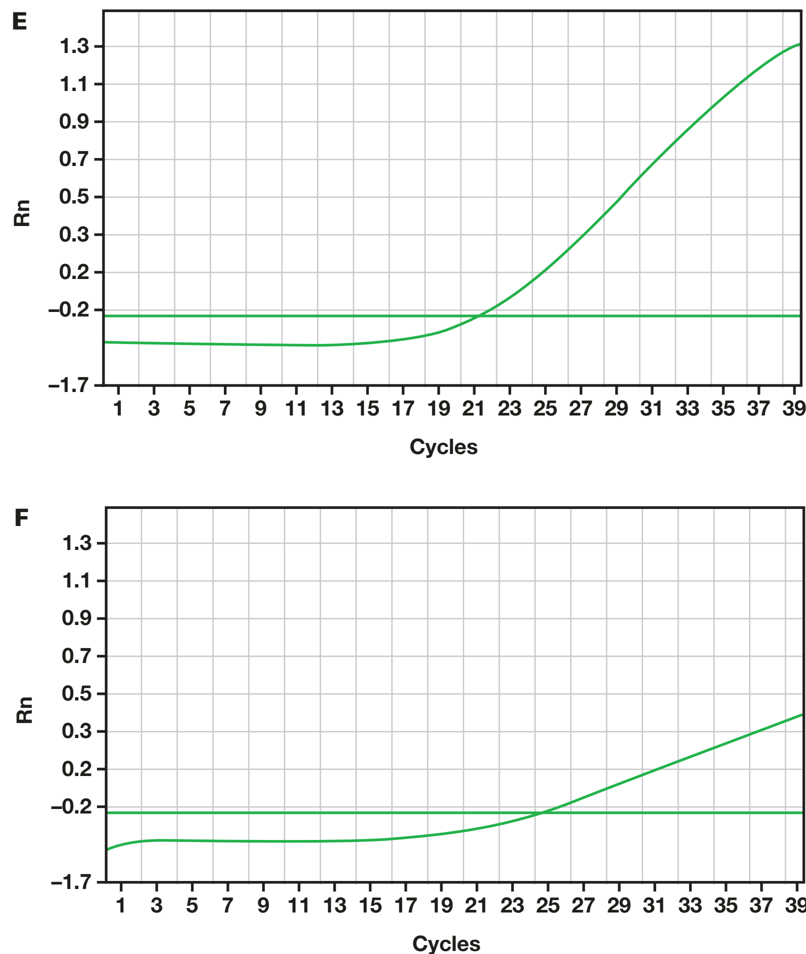


FIGURE 1. **(cont) E, Fluorescent generated from N256For/N689Rev/ProbeN645 in the platform. F, Fluorescent signal generated from N256For/N689Rev/ProbeN408 in the platform. Signals are presented as normalized reporter (Rn).**



RNA was subsequently extracted and evaluated using the new auto-RPA–fluorescence platform.

Ethical Statement

Sample collection and analysis were approved by the CDC-STC. The internal use of samples accorded with the medical and ethical rules pertaining to participating individuals.

Results

Efficiency of the Auto-RPA–Fluorescence Assay for SARS-CoV-2 Detection

As described in Materials and Methods, we designed RPA-specific primers and probes for targeting the N and S gene sequences of SARS-CoV-2 using PrimedRPA. After the analysis of specificity, we selected 10 primers and 5 probes with their genomic locations, as shown in **TABLE 1**.

To select the primer with optimal efficiency, we compared the sequences of the primers with those of other virus genomes (including 7 similar coronaviruses, 2 influenza viruses, and 2 other coronaviruses) using BLAST. We found that the sequences were not similar to most of the viral sequences we chose. The amplification efficiency of different pairs of primers was then tested by normal PCR with synthesized gene fragments, as suggested by TwistDx. Agarose gel electrophoresis revealed that N256For/N689Rev and S2531For/S2850Rev were the best primer pairs for amplification with the highest efficiency (**FIGURE 1A**). We modified the probes for fluorescence detection, as indicated in the Materials and Methods section (**FIGURE 1B**). To compare the efficiency of probes N408, N645, S2560, and S2760—which are located in the region confined by the selected primer pairs—we performed RPA reactions and obtained quantified real-time fluorescence signals using the SLAN-96S fluorescence detection system. The results showed that although all the primer sets generated fluorescence, the N256For/N689Rev/ProbeN645 and S2531For/S2850Rev/ProbeS2560 sets produced the strongest signals with lower Ct values (**FIGURE 1C–1F**).

The Auto-RPA–Fluorescence Platform Was Sensitive in the Detection of SARS-CoV-2

Next, to explore the limits of the single auto-RPA-fluorescence assay in the detection of SARS-CoV-2, we prepared reaction mixtures containing serially 10-fold-diluted S and N gene fragments (from 50,000,000 to 5 copies). In the established reaction system, we applied FAM-labeled N256For/N689Rev/ProbeN645 for detecting the N gene and HEX-labeled S2531For/S2850Rev/ProbeS2560 sets for detecting the S gene and monitored the resulting real-time fluorescence signals. As indicated in **FIGURE 2**, the auto-RPA-fluorescence platform was able to detect

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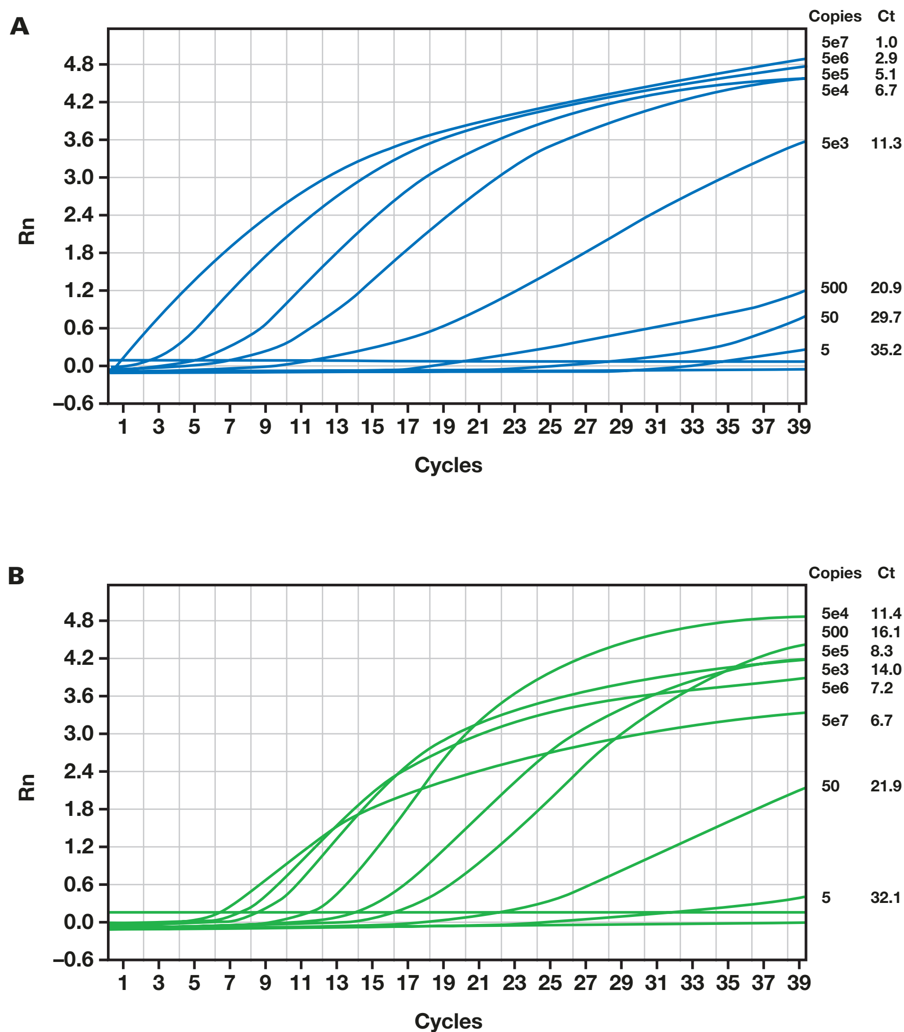
FIGURE 2. The limit of detection of the auto-RPA–fluorescence detection platform in detecting SARS-CoV-2 gene fragments. A, The limit of detection with FAM-labeled N256For/N689Rev/ProbeN645 in detecting SARS-CoV-2 N gene. Fluorescent curves of reactions with 10-fold gradually diluted the N gene template from 50 million copies/μL to 5 copies/μL. B, The limit of detection with HEX-labeled S2531For/S2850Rev/ProbeS2560 in detecting SARS-CoV-2 S gene. Fluorescent curves of reactions with 10-fold gradually diluted the S gene fragments from 50 million copies/μL to 5 copies/μL.



5 copies/μL prior to the 38 Ct cutoff, which suggested positive results in detecting SARS-CoV-2 gene fragments. Additionally, both primer/probe sets were able to detect as few as 5 copies/μL S gene or N gene templates. (FIGURE 2).

The Auto-RPA–Fluorescence Detection Platform Is Specific in Detecting SARS-CoV-2

To assess its detection specificity, we tested the auto-RPA-fluorescence platform on the nucleotide sequences of influenza, HCoV-229E, measles virus, mumps, HIV, and coxsackie viruses. The FAM-labeled N256For/N689Rev/ProbeN645 and HEX-labeled S2531For/S2850Rev/ProbeS2560 were applied in the reaction. Only the RNA from SARS-CoV-2 generated positive signals (FIGURE 3), demonstrating that the primers and probes were highly specific for SARS-CoV-2 detection.

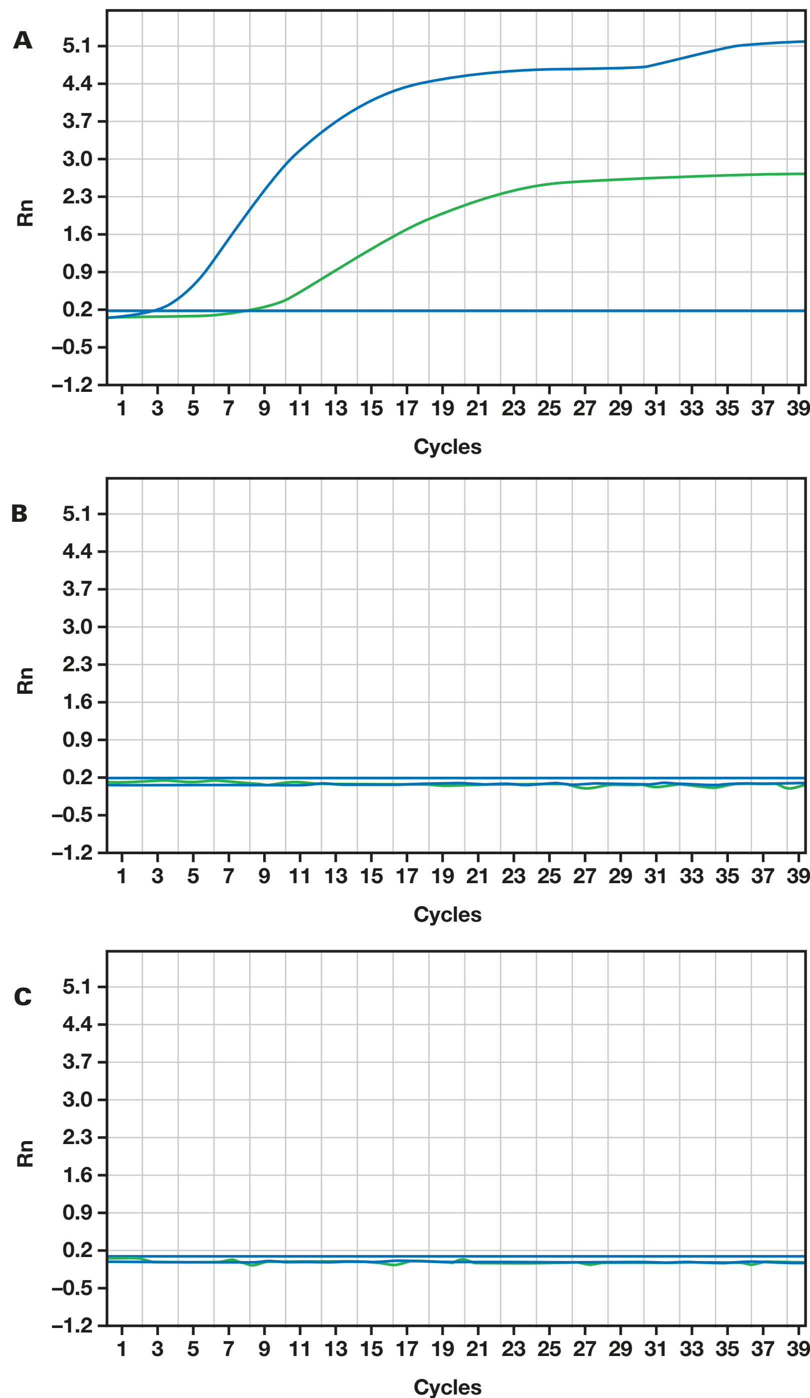
The Auto-RPA–Fluorescence Platform Was Accurate and Reliable for the Detection of SARS-CoV-2 in Clinical Samples

To investigate the diagnostic accuracy and reliability of the auto-RPA–fluorescence detection method, we applied it to oropharyngeal swab samples obtained during the 2020 epidemic. A total of 16 samples from SARS-CoV-2–infected patients and 2 SARS-CoV-2–negative samples—which were initially detected using qPCR—were tested using the auto-RPA–fluorescence assay. The N256For/N689Rev/ProbeN645 and HEX-labeled S2531For/S2850Rev/ProbeS2560 were applied in the PRA reaction. The results showed that all the samples validated by our assay were consistent with those detected using the qRT-PCR method. Moreover, the results were reported in less than 15 min with Ct values less than 10 (FIGURE 3). The SARS-CoV-2–negative samples did not show fluorescence signals (FIGURE 3). The Ct values of 16 positive samples detected by auto-RPA–fluorescence are shown in TABLE 2. The qRT-PCR results of samples that were used in the auto-RPA–fluorescence detection platform are shown in **Supplementary Figure S1**.

Discussion

Although the most popular approach for SARS-CoV-2 detection is based on qRT-PCR, it is unsuitable for large-scale diagnostics since it is time-consuming and requires special equipment and well-trained

FIGURE 3. **The specificity of the auto-RPA–fluorescence detection platform in detecting SARS-CoV-2. Figures represent the fluorescent curves generated by the platform in detecting SARS-CoV-2–positive samples (A), SARS-CoV-2–negative samples (B), and an influenza sample (C) as representative of the control virus. FAM-labeled N256For/N689Rev/ProbeN645 and HEX-labeled S2531For/S2850Rev/ProbeS2560 were applied in the reaction.**



personnel. The emerging isothermal amplification-based technologies have mostly been reported for SARS-CoV-2 detection. For rapid and convenient testing, colorimetric methods coupled with RT-LAMP amplification have been proposed.[18,19] However, the colorimetric methods developed for LAMP lack specificity because their detection objects are based on factors such as H^+-induced pH change and the level of magnesium pyrophosphate.[7,19] To improve specificity and limits of detection, researchers have developed LAMP- or RPA-based methods using CRISPR-Cas9/Cas12. Such methods can achieve nearly single-copy detection of CRISPR-RNA and can recognize specific target sequences.[6,13,20,21] However, such isothermal reactions are followed by the mixing of amplicons with Cas9/Cas12 reagents for probe cleavage, which complicates SARS-CoV-2 detection. Recently, 1-step CRISPR-based methods coupled with RPA have been developed.[22,23] However, these assays present a high

TABLE 2. The Ct Values of 16 Positive Samples Detected by the Auto-RPA–Fluorescence Platform

Sample	Ct Value (N Gene)	Ct Value (S Gene)
1	3.1	7.8
2	5.0	13.2
3	2.8	7.9
4	4.2	14.5
5	5.3	14.3
6	5.1	14.7
7	4.3	12.4
8	4.0	11.9
9	2.1	9.0
10	4.0	8.8
11	2.3	7.3
12	5.1	15.3
13	2.3	1
14	7.0	18.1
15	5.1	15.7;
16	3.1	9.9

Ct, cycle threshold, N, nucleocapsid; S, spiked.

background in visual detection, which decreases the analytical sensitivity and specificity of CRISPR detection. Therefore, there is still an urgent need for a more rapid, accurate, and simple methods for SARS-CoV-2 detection.

Herein, we combined the multienzyme RPA principle with a fluorescence detection system to create a new auto-RPA–fluorescence platform that simplifies laboratory operation and can be implemented for point-of-care testing. The probe modification strategy deployed in the new auto-RPA–fluorescence assay generates reaction yields that are suitable for real-time fluorescent monitoring with high analytical sensitivity. We propose an operation protocol that requires minimal equipment—that is, pipettes, heat blocks, and reagent tubes—and takes place at the relatively low temperature of approximately 42°C.[16] Thus, it has potential for next-generation point-of-care molecular diagnosis. We expect this assay could be widely applied to detect various pathogenic microbes, including infectious bacteria, fungi, and viruses.

In this study, the single auto-RPA–fluorescence assay for SARS-CoV-2 demonstrated several comparative advantages over its counterparts. First, it was much faster than qRT-PCR. The entire amplification process of single auto-RPA–fluorescence can be completed within 15 min, whereas qRT-PCR usually takes approximately 2 h. To our knowledge, single auto-RPA-fluorescence requires the least amount of time to diagnose SARS-CoV-2 infection of any system. Second, the single auto-RPA–fluorescence assay can be performed at a constant temperature of approximately 39 to 42°C, which can be achieved in a water bath. It is very convenient for use where an energy supply is unavailable. The auto-RPA–fluorescence platform provided 100% accuracy in testing SARS-CoV-2–positive and –negative samples. Third, the detection limit of the auto-RPA–fluorescence platform was as low as 5 copies/μL, which is comparable with other CRISPR/isothermal-based methods. Isothermal methods such as LAMP are relatively inaccurate because they involve a robust reaction.[10–12] Further, the limit of detection makes this platform adaptable for analyzing the mixed nucleic acids of samples from suspected COVID-19 patients and thus improves the throughput of detection.

In conclusion, we establish a promising single auto-colorimetric assay that can detect SARS-CoV-2 infection with high analytical sensitivity using a more rapid and simple detection process. The assay also has potential for the detection of other major pathogenic microorganisms. However, the present assay still requires an RNA preparation module. Thus, further efforts are required to combine RNA extraction with the single auto-RPA method to achieve simplified SARS-CoV-2 detection.

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