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Development of a Lateral Flow Strip Membrane Assay for Rapid and Sensitive Detection of the SARS-CoV-2

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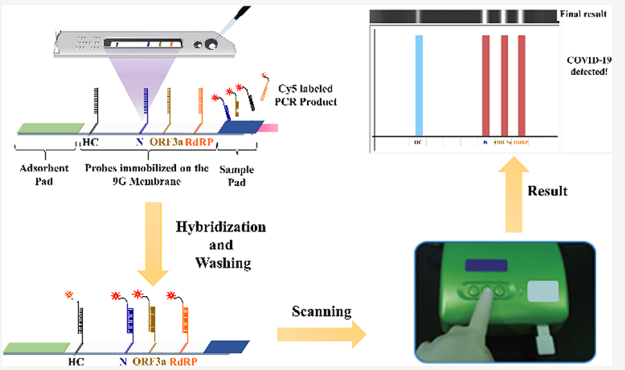
ABSTRACT: The infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that causes the coronavirus disease 2019 (COVID-19) has threatened public health worldwide. The easy human-to-human transmission of this virus has rapidly evolved into a global pandemic. Therefore, to control the community spread of the virus, it is crucial to identify the infected individuals, including asymptomatic people. Hence, a specific and rapid assay is crucial for the early diagnosis and active monitoring of individuals potentially exposed to SARS-CoV-2 for controlling the COVID-19 outbreak. In this study, we have developed the novel lateral flow strip membrane (LFSM) assay that allows the simultaneous detection of RdRp, ORF3a, and N genes using the PCR product obtained by using the single-tube reverse transcription polymerase chain reaction (RT-PCR). The LFSM assay allows detection of SARS-CoV-2 in 30 min at 25 °C after the RT-PCR with the detection limit of 10 copies/test for each gene. The clinical performance of the LFSM assay for the detection of SARS-Cov-2 was evaluated using 162 clinical samples previously detected by using the commercial assay. The percent positive agreement, percent negative agreement, and overall percent agreement of the LFSM assay with the commercial assay were 100% (94.2−100%), 99.0% (94.6−100%), and 99.4% (96.6−100%), respectively. Therefore, the results of the LFSM assay showed significantly high concordance with the commercial assay for the detection of SARS-CoV-2 in clinical specimens. Therefore, we conclude that the developed LFSM assay can be used alone or complementary to the RT-PCR or other methods for the diagnosis and monitoring of the patients to curb community transmission and the pandemic.



The novel coronavirus (SARS-CoV-2) that causes coronavirus disease 2019 (COVID-19) was originated in the province of Wuhan, in December 2019.[1] The easy transmission of SARS-CoV-2 from human-to-human to close contact has rapidly evolved into a worldwide pandemic.[2] Despite several emergency measures taken by many countries worldwide, the current situation to contain the spread of the virus is grim. However, the trace, treat, and track strategy has helped countries like South Korea to control the spread of the virus and to reduce community transmission.[3] The extensive and focused research on the clinical features of COVID-19 and route of transmission of SARS-CoV-2 established the incubation period of 5−12 days.[4] Furthermore, the relapse of COVID-19 in patients who had been discharged after a negative PCR has increased the risk of community spread.[5] Therefore, the early diagnosis and active monitoring of people potentially exposed to SARS-CoV-2 is critical for controlling the COVID-19 outbreak.

A rapid and accurate test that can detect the SARS-CoV-2 as earliest as possible during the incubation period and monitoring the treated patients until two weeks after recovery is desired.[6] However, the vast number of samples and complex situations in hospitals pose significant challenges in an early diagnosis and active monitoring.[7] Furthermore, the low sensitivity of reported methods results in the false-negative cases among infected individuals, and incomplete treatment in patients can lead to the widespread relapse of COVID-19.[8,9]

The chest computed tomography (CT) is used as a confirmatory diagnostic method for patients with COVID-19 symptoms.[10] Though facilities for CT are available in major cities across the globe, this method fails to diagnose the patients in the early stage of the incubation period, and it is not applicable for the active monitoring of treated patients.[11] The immunodiagnostic methods are highly applicable for the diagnosis of the COVID-19.[12] However, the available tests can only determine if an individual has, at some point, been infected with COVID-19.[13,14] Hence, the immunoassay methods though good for mass screening, cannot detect the

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infected people during the incubation period and are not suitable for the monitoring of the patients to avoid relapse.

Currently, the reverse transcriptase real-time quantitative PCR (qRT-PCR) that detects the nucleic acid of SARS-CoV-2 is used as a gold standard for the diagnosis of COVID-19.[15] Many RT-PCR kits have been developed for the detection of SARS-CoV-2 since the start of the COVID-19 outbreak. However, the false-negative results as high as 20 to 40% are reported in cases where the clinical symptoms and imaging results raised strong suspicions of the disease.[16] Several factors could contribute to the false-negative results of the tests based solely on the RT-PCR for both amplification and detection.[17] To date, the majority of RT-PCR-based tests have targeted different SARS-CoV-2 genomic regions, including the ORF1b, nucleocapsid (N), spike (S) protein, RNA-dependent RNA polymerase (RdRp), or envelope (E) gene regions individually or in combination. The possibility of recombination and mutations increase the risk of false-negative results of RT-PCR assays that use a single region. Hence, a test that can detect the nucleic acid of SARS-CoV-2 with high sensitivity and specificity is required for the diagnosis of infection early during the incubation period and active monitoring of patients after treatment to control the COVID-19 outbreak.

The lateral flow strip membrane (LFSM) assay that allows simultaneous detection of the multiple regions of nucleic acids in a single test would be a useful adjunct or a more practical alternative to the CT, RT-PCR, or immunoassays. In addition, the LFSM-based assays have the advantages of being rapid and straightforward. Though the RT-PCR assays are readily available for the diagnosis of COVID-19, their false-negative rate poses challenges in controlling community transmission. Therefore, a novel method that can be used alone or complementary to the RT-PCR for the diagnosis and monitoring of suspected COVID-19 patients can help to curb the community transmission and control the pandemic.

With our ongoing research on the LFSM assay based on the 9G membranes, we propose a test that can simultaneously detect the RdRp gene, ORF3a gene, and N gene of SARS-CoV-2 within 30 min after a PCR at room temperature. The simultaneous detection of RdRp, ORF3a, and N genes allows avoiding potential cross-reaction with other endemic coronaviruses as well as possible false-negative results due to the mutations in the nucleic acid of SARS-CoV-2. The limit of detection of 10 copies/test enables the proposed method to be applicable for the early diagnosis of the infected people during the incubation period. Furthermore, the proposed method has high potential for the active monitoring of the treated patients, which is essential to avoid the relapse and contain the epidemic.

EXPERIMENTAL SECTION

Reagents, Samples, and Instrumentation. The one-step RT-PCR premix (SuPrimeScript RT-PCR Premix 2X) and RNA extraction kits were obtained from GeNet Bio, Korea, and Qiagen, Germany, respectively. Glass slides (2.5 × 7.5 cm) were purchased from Paul Marienfeld GmbH & Co. KG, Germany. All washing solvents for the substrates are of HPLC grade from SK Chemicals, Korea. All standard template RNA, primers, and probes designed for this study were obtained from Bioneer Corp., Daejeon, Korea. Oligonucleotides were spotted using a Qarray2 microarrayer, Genetix Technologies, Inc., to produce DNA chips used for the probe and primer selections. Hybridization of the Cy5-labeled PCR products with probes immobilized on DNA Chips was performed at 25 °C for 30 min in the commercial incubator, and then the slides were dried using the commercial centrifuge (1000 rpm). The fluorescence signal of the microarray was measured on a ScanArray Lite, GSI Lumonics, Bedford, U.S.A. The images were analyzed by Quant Array software, Packard Bioscience. The glass fiber membrane (2.5 × 7.5 cm) to produce the LFSM was purchased from Whatman, Springfield, U.K. Ultrapure water (18 MΩ cm) was obtained from a Milli-Q purification system, Millipore, Burlington, U.S.A. Oligonucleotides were lined using the FrontLine microliter contact dispenser, BioDot Technologies Inc., Irvine, U.S.A. The fluorescence intensities for the LFSM assays were recorded on the BMT reader (Biometrix Technology Inc., Chuncheon, South Korea).

Nucleic Acid Extraction from the Clinical Specimens. Total RNA clinical specimens were extracted from 140 μL of each clinical sample using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany), according to the manufacturer instructions. The RNA samples were diluted in 60 μL of nuclease-free water and stored at −80 °C until use. Five microliters of this solution was used for the RT-PCR.

Primer and Probe Design. The genome similarity analysis of SARS-CoV-2-related viruses reveals that the E gene and most of the regions of the ORF1 gene show considerable similarity with the human SARS-CoV-2 genome (Figure S1).[18] ORF3a is the most variable gene except for the fast-evolving regions. Genomes of SARS-CoV-2 are closely related to other beta-coronaviruses, including SARS-CoV-1 and bat-derived SARS-like coronaviruses. Therefore, the discrimination of SARS-CoV-2 from the other beta-coronaviruses is crucial to enhance the specificity and accuracy of the diagnostic assay. Therefore, even though the ORF3a is the most variable gene between SARS-CoV-2 and the other beta-coronaviruses except for the fast-evolving regions, we chose to target this region of ORF3a. Furthermore, it is reported that two or more regions in the SARS-CoV-2 genome should be targeted to avoid false negatives.[19] Therefore, in this study, the RdRp gene, ORF3a gene, and N gene were chosen to develop the LFSM assay for the accurate detection of SARS-CoV-2. The primer and probes were designed according to the previously published generalized probe selection method.[20] To optimize the primer set targeting the RdRp gene, forward primers RdRp-F1−RdRp-F8 and reverse primers RdRp-R1 and RdRp-R2 were designed (Figure S2 and Table S1). To optimize the primer set targeting the ORF3a gene, forward primers ORF3a-F1−ORF3a-F5 and reverse primers ORF3a-R1−ORF3a-R3 were designed. To optimize the primer set targeting the N gene, forward primers N-F1−N-F3 and reverse primers N-R1−N-R6 were designed. The probes (Table S2) for the detection of SARS-CoV-2 were also designed, and finally, three probes, one each corresponding to the RdRp gene, ORF3a gene, and N gene were selected to produce the LFSM.

Reverse Transcription Polymerase Chain Reaction (RT-PCR). The reaction components included 5 μL of the template solution (or a solution containing extracted RNA); 5 μL of solution containing primers (4X) designed in this study; and 10 μL of the SuPrimeScript RT-PCR premix that includes SuPrimeScript RTase, HS Prime Taq DNA Polymersae, RNase inhibitor, reaction buffer, enzyme stabilizer, a dNTP mixture, and loading dye. After vortexing and centrifugation, the reaction tube was then transferred to the thermocycler SimpliAmp (Thermo Fisher Scientific, U.S.A.). The one-step

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RT-PCR amplification contained the following steps: 50 °C for 30 min and 95 °C for 5 min, followed by 50 cycles at 95 °C for 10 s, 60 °C for 20 s, 72 °C for 15 s, and then 72 °C for 1 min. Twenty microliters of the Cy5-labeled PCR products were used for the detection of SARS-CoV-2 on the developed LFSM.

Preparation of DNA Chips, Hybridization, and Washing. The DNA chips were prepared according to our previous reports on the 9G DNA chips.[21] In brief, the DNA chips were prepared by spotting the immobilization solution containing oligonucleotide probes with the microarray, and the spots were arranged to make six by three pixels on the 9G slides.[22] The microarrayed 9G slides were then kept in the incubator (25 °C, 50% humidity) for 4 h to immobilize the oligonucleotides. The slides were then suspended in the blocking buffer solution at 25 °C for 30 min, in order to remove the excess oligonucleotides and to deactivate the nonspotted area. Then the slides were rinsed with washing buffer solutions A and B for 5 min each, and then dried with a commercial centrifuge to obtain ready-to-use DNA chips. Before hybridization, the DNA chips were covered with Secure-Seal hybridization chambers. Hybridizations were done by using Cy5-labeled PCR products at 25 °C for 30 min in the commercial incubator. Then DNA chip was rinsed with washing buffer solutions A and B (Table S3) successively for 2 min each, in order to remove the excess target DNA, and dried with a commercial centrifuge (1000 rpm). The fluorescence signal of the microarray was measured on the ScanArray Lite, and the images were analyzed by Quant Array software (Packard Bioscience).

Preparation of Lateral Flow Strip Membranes (LFSM), Hybridization, and Washing. The LFSMs were obtained by a slight modification of the 9G DNA chip technology used for the production of the 9G DNA chips.[23] A glass membrane was used to produce the 9G membrane owing to their similar physicochemical properties with glass slides. In brief, by lining the 18 pmol mL^{-1} solution containing selected probes appended with 9 consecutive guanines were immobilized on the AMCA (aminocalix[4]arene) membrane in a 4 h immobilization step. After immobilization, the membranes were washed with the blocking solution and then dried to produce the 9G membranes. The dried 9G membranes were assembled into strips to obtain the LFSM. For the detection of SARS-CoV-2 in the clinical samples, 5 μL of the Cy5-labeled PCR products were first mixed with 110 μL of the hybridization buffer (pH = 7.3). Then, 110 μL of this mixture was loaded at 25 °C on the LFSM and allowed to incubate for 20 min. Then, 200 μL of the washing buffer solution was loaded and allowed to stand for 12 min to wash the excess of the Cy5-labeled PCR product. Final results were obtained by scanning the LFSMs in the BMT membrane reader (Biometrix Technology Inc., Chuncheon, South Korea), which is a lightweight, portable fluorescence scanner.

Detection of SARS-CoV-2 Virus from Clinical Samples. The clinical samples (n = 162) used in this study (Table 1) were collected from subjects as part of the registered protocols approved by the Institutional Review Board (IRB) of Yeungnam University Medical Center, Daegu, South Korea. All patients provided a written, informed consent (IRB registration number: YUMC 2020-04-023). Nasopharyngeal swabs and sputum from COVID-19 patients and healthy subjects were stored in a universal transport medium (UTM) (Noble Biosciences, South Korea). Clinical samples were inactivated by heating at 100 °C for 10 min and were stored at −80 °C for further use. All samples were tested by using the commercial assay (Allplex 2019-nCoV Assay; Seegeen, Korea) according to the manufacturer protocol. The RT-PCR products obtained by following the procedure mentioned above were loaded on the LFSM for the detection of SARS-CoV-2 in clinical samples. The results of the clinical study are presented in Table 1.

Table 1. Performance of the LFSM Assay for the Detection of SARS-CoV-2 in Clinical Samples

SARS-CoV-2 detection		LFSM assay positive	LFSM assay negative	total
commercial assay	positive	62	0	62
	negative	1	99	100
total		63	99	162
percent positive agreement (PPA)		100% (95% CI: 94.2−100%)		
percent negative agreement (PNA)		99.0% (95% CI: 94.6−100%)		
overall percent agreement (OPA)		99.4% (95% CI: 96.6−100%)		

Statistical Analysis. The trials were performed in triplicate. The kappa and p values of the LFSM assay were calculated. The statistical analysis was carried out with SPSS 21.0 (IBM, Armonk, NY).

RESULTS AND DISCUSSION

There are more than 200 genome sequences that were uploaded in the GISAID, GWH, and NMDC databases after the first SARS-CoV-2 genome sequence was published on January 10, 2020 (GeneBank accession number MN908947). These genome sequences are identical to each other. Therefore, an 80 mer, 117 mer, and 75 mer sequences in the RdRp gene, ORF3a gene, and N gene, respectively, were selected as targets, and primers were meticulously designed for the PCR amplification of these three genes.

For the optimization of the primer sets and PCR conditions targeting the cDNA of RdRp, ORF3a, and N genes obtained by the RT-PCR using 100 copies/test of RNA templates (20 copies/μL; Figure S3), various combinations of forward and reverse primers were tested. For the RdRp gene, eight forward primers (RdRp-F1−RdRp-F8) with melting temperatures (T_M) in the range of 64.3−71.1 °C and two reverse primers (RdRp-R1 and RdRp-R2) with T_M in the range of 60.3−63.2 °C were studied. The details of the primer concentration optimization data are not presented here. The gel data obtained after the RT-PCR using optimized concentrations of the forward primer (3 pmol/test) and reverse primer (9 pmol/test) indicated that the RdRp-F6 and RdRp-R2 produce a high PCR yield with low impurities (Figure 1A). Similarly, the gel data indicated that the ORF3a-F2 (2 pmol/test) and ORF3a-R2 (6 pmol/test) show good PCR yields (Figure 1B). The primers N-F1 (1.5 pmol/test) and N-R1 (4.5 pmol/test) showed a high yield (Figure 1C). Therefore, the primer sets RdRp-F6 and RdRp-R2, ORF3a-F2 and ORF3a-R2, and N-F1 and N-R1 were selected for the simultaneous RT-PCR in a single reaction targeting the amplification of the genomic sequences in the RdRp gene, ORF3a gene, and N gene, respectively.

About 11 probes (Table S2) were designed using a generalized probe selection method for DNA chips[21] to finally select four optimized probes for the highly efficient detection of SARS-CoV-2. The designed probes appended with nine consecutive guanines were immobilized on the AMCA slides to obtain the DNA chips using a reported method (Figure 2A).[22]

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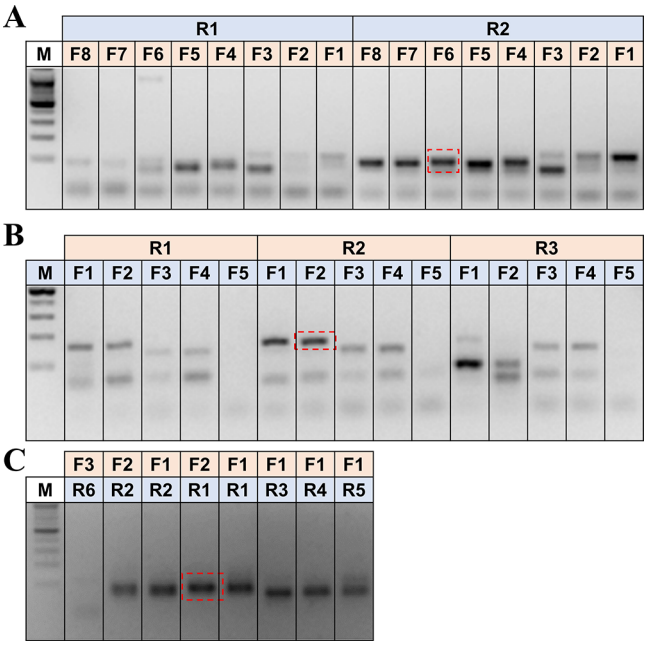

Figure 1. Optimization of primer sets for the RT-PCR amplification of (A) RdRp gene, (B) ORF3a gene, and (C) N gene of SARS-CoV-2 (Optimized primer sets are marked with dotted red boxes).

The Cy5-labeled PCR products were obtained individually by using RNA templates of RdRp gene, ORF3a gene, and N gene in the presence of the mixture of three optimized primer sets corresponding to these genes. The PCR products were allowed to hybridize with the immobilized probes on the DNA chip to obtain the results (Figure 2).

As depicted in Figure 2**B-F**, the probes P1, P6, and P10 showed high fluorescence intensities upon hybridization of the PCR products obtained from the RNA templates RdRp gene, ORF3a gene, and N gene, respectively. Further, these probes did not show any cross-reactivity with the non-target PCR products. The probe P11 showed significant fluorescence intensity upon hybridization with PCR products obtained in the absence (Negative control) and in the presence of the RNA templates of RdRp, ORF3a, and N genes.

Hence, as shown in Scheme 1, the probes P1, P6, P10, and P11 were chosen to produce the LFSM for the detection of SARS-CoV-2. Scheme 1 depicts the working procedure of the developed LFSM. As mentioned earlier, the detection of SARS-CoV-2 by targeting any single genome leads to the false-negative results. Therefore, we immobilized three probes specific to the RdRp gene, ORF3a gene, and N gene on the LFSM along with the probe for hybridization control. The sample is considered as COVID-19 negative if the LFSM assay shows the fluorescence signals only for the HC probe. Whereas, the sample is identified as COVID-19 positive if any one of the probes, two probes, or all three probes show fluorescence signals (Figure S4). Therefore, the detection method used in the LFSM presented here enables highly specific detection of human SARS-CoV-2 for the diagnosis of COVID-19.

To confirm the specificity of the immobilized probes for the detection of the RdRp gene, ORF3a gene, and N gene, the PCR products targeting these genes were loaded on the LFSM. The obtained results are presented in Figure 3 (Figure S5). Each experiment was repeated eight times.

As shown in Figure 3, the probes P1, P6, and P10 highly specific hybridization with the respective PCR products were obtained from the RNA template of the RdRp, ORF3a, and N genes, respectively. The ratio for specific to nonspecific hybridizations for each probe was over 130 times. Therefore, these probes assure the highly specific detection of SARS-CoV-2 for the diagnosis of COVID-19.

The LFSM assay was evaluated for its sensitivity in the detection of the RdRp, ORF3a, and N genes. A single RT-PCR was performed by using the mixture of the RdRp gene, ORF3a gene, N gene RNA templates (1000, 100, 10, and 0 copies/test) and three primer sets corresponding to each template. The PCR products were loaded on the LFSM, and the fluorescence intensities for respective probes were recorded (Figure 4, Figure S6).

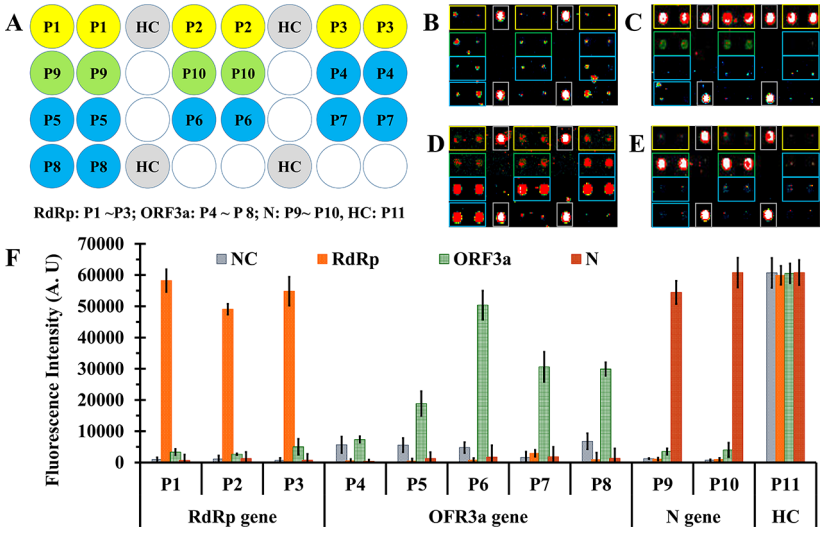

Figure 2. Selection of probes for the detection of SARS-CoV-2. (A) Chip map indicating positions of immobilized probes, hybridized immobilized probes with the Cy5-labeled RT-PCR products obtained by using (B) no RNA templates (Negative Control; NC), (C) RNA template of RdRp gene, (D) RNA template of ORF3a gene, and (E) RNA template of N gene, and (F) summarized fluorescence intensity of each probe upon hybridization with the PCR products.

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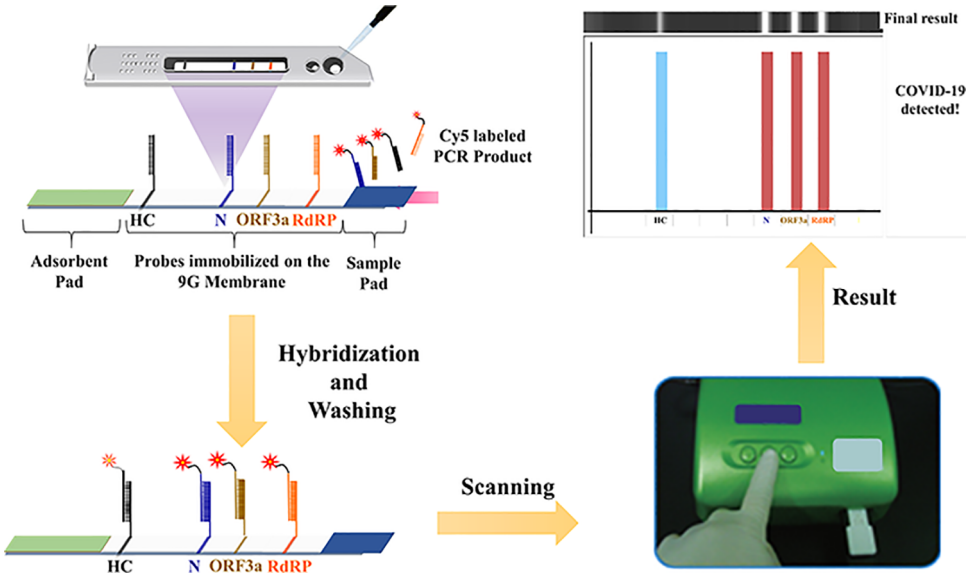
Scheme 1. LFSM Assay for the Highly Specific and Sensitive Detection of SARS-CoV-2 to diagnose COVID-19



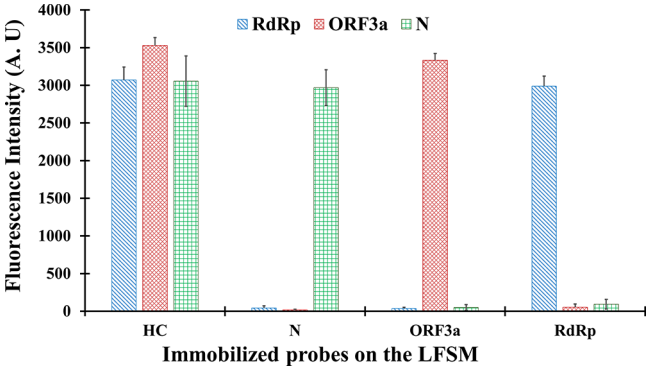


Figure 3. Specificity of the LFSM assay (100 copies/test of respective RNA templates) for the detection of SARS-CoV-2.

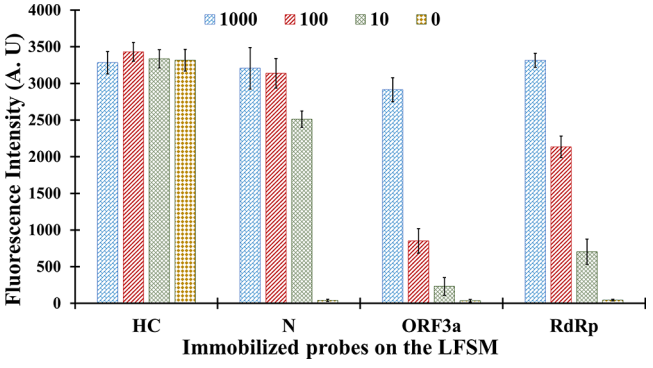


Figure 4. Sensitivity of the LFSM assay for detection of SARS-CoV-2: (A) 1000 copies/test, (B) 100 copies/test, (C) 10 copies/test, and (D) 0 copies/test.

As shown in Figure 4, the LFSM assay can simultaneously detect the RdRp gene, ORF3a gene, and N gene in a single PCR. The limit of detection for the simultaneous detection of these three genes was found to be 10 copies/test. As mentioned earlier, the simultaneous detection of more than two genes of SARS-CoV-2 ensures the high accuracy of the assay for the diagnosis of COVID-19. The assays that focus on the detection of a single gene suffer from the high rate of false-negative results among the infected individuals leading to the spread of COVID-19 with large clusters. The LFSM presented in this article detects three genes in SARS-CoV-2 and ensures accurate detection of the virus in the symptomatic as well as COVID-19 suspected individuals.

We evaluated the diagnostic performance of the newly developed LFSM assay for the detection of SARS-CoV-2 in comparison with commercial assay (Allplex 2019-nCoV Assay; SeeGeen, Korea) using 162 clinical samples. Of these 162 samples, the commercial assay detected 62 samples as positive and 100 samples as negative. The LFSM assay detected 62/62 positive samples as positive and 99/100 negative samples as negative. The LFSM assay showed one false-positive result in comparison with the commercial assay. The results of the LFSM assay and commercial assay are presented in Table 1.

As shown in Table 1, the PPA, PNA, and OPA of the LFSM assay with the commercial assay were 100% (94.2−100%), 99.0% (94.6−100%), and 99.4% (96.6−100%). Hence, the results of the LFSM assay showed significantly high concordance with the commercial assay for the detection of SARS-CoV-2 in clinical specimens. Therefore, we conclude that the newly developed LFSM assay is highly sensitive in the detection of SARS-CoV-2. The high sensitivity of the LFSM assay is justified by the fact that it can simultaneously detect RdRp, ORF3a, and N genes using a single PCR with the detection limit of 10 copies/test.

The main advantage of the LFSM assay is its multiplexed detection. However, the current tests based on the qRT-PCR are indeed designed to target at least two regions of the SARS-CoV-2 genome. Even though the RT-PCR is used most widely for the detection of SARS-CoV-2, its major disadvantages include the requirement of expensive laboratory instruments, highly trained personnel, and the high cost per test.[24] On the contrary, the LFSM assay presented in this article requires portable instrumentation, uses a simple experimental protocol, and can be modified into an automatic testing system. The presented method allows detection of SARS-CoV-2 in 12 min after a PCR at room temperature. However, the total time for the detection of SARS-CoV-2 is about 2 h (100 min PCR, 12 min LFSM assay) after extraction. The commercial qRT-PCR kits require at least 2−3 h to obtain final results after

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extraction. Usually, LFSM assays have a high potential for point-of-care testing (POCT) settings. However, the assay presented here requires a PCR machine to amplify the genomic copies and hence limits its ability to be developed into a POCT assay.

In summary, the LFSM assay has advantages of high sensitivity and specificity for the detection of RdRp, ORF3a, and N genes. It is a first-ever report on the detection of SARS-CoV-2 by targeting more than one gene. The simultaneous detection of RdRp, ORF3a, and N genes allows accurate detection of the SARS-CoV-2 and overcomes the disadvantage of false-negative results shown by many RT-PCR-based assays. Furthermore, the limit-of-detection (LoD) of 10 copies/test for all three genes enables the LFSM assay to be applicable for the early diagnosis in the infected people during the incubation period. We firmly believe that the proposed method has a high potential for active monitoring of the patients under treatment until a complete cure, which is essential to avoid the relapse and contain the epidemic.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c03202.

The information on genomic alignment, primer regions, probe regions, results of LFSM in clinical samples, and the sensitivity in terms of copy numbers per test (Figures S1−S6). The information on primer sequences; probe sequences; and composition of various solutions used for immobilization, blocking, hybridization, and washing (Table S1−S3) (PDF).

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Author Contributions

§S.Y. and S.B.N. contributed equally. Hence, both should be considered as first authors. S.Y., S.B.N., K.S., and T.K. designed the study; S.Y., J.K., and S.B.N. performed the experiments; S.Y., S.B.N., J.K., and T.K. analyzed and interpreted the data; S.B.N. and T.K. wrote the paper. All authors provided a critical review and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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