

Rapid, Ultrasensitive, and Highly Specific Diagnosis of COVID-19 by CRISPR-Based Detection

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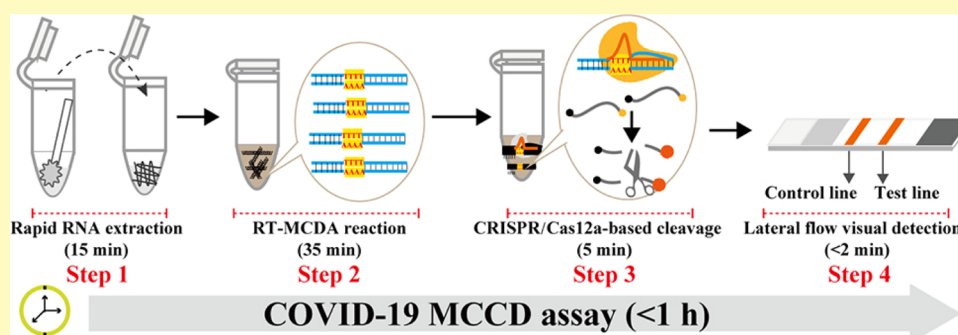
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ABSTRACT: Coronavirus Disease 2019 (COVID-19), which is caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), has rapidly spread leading to a global pandemic. Here, we combined multiple cross displacement amplification (MCDA) with CRISPR-Cas12a-based detection to develop a novel diagnostic test (MCCD) and applied for the diagnosis of COVID-19, called COVID-19 MCCD. The MCCD protocol conducts reverse transcription MCDA (RT-MCDA) reaction for RNA templates followed by CRISPR-Cas12a/CrRNA complex detection of predefined target sequences after which degradation of a single-strand DNA (ssDNA) molecule confirms detection of the target sequence. Two MCDA primer sets and two CrRNAs were designed targeting the opening reading frame 1a/b (ORF1ab) and nucleoprotein (N) of SARS-CoV-2. The optimal conditions include two RT-MCDA reactions at 63 °C for 35 min and a CRISPR-Cas12a/CrRNA detection reaction at 37 °C for 5 min. The COVID-19 MCCD assay can be visualized on a lateral flow biosensor (LFB) and completed within 1 h including RNA extraction (15 min), RT-MCDA reaction (35 min), CRISPR-Cas12a/CrRNA detection reaction (5 min), and reporting of result (within 2 min). The COVID-19 MCCD assay is very sensitive and detects the target gene with as low as seven copies per test and does not cross-react with non-SARS-CoV-2 templates. SARS-CoV-2 was detected in 37 of 37 COVID-19 patient samples, and nonpositive results were detected from 77 non-COVID-19 patients. Therefore, the COVID-19 MCCD assay is a useful tool for the reliable and quick diagnosis of SARS-CoV-2 infection.

KEYWORDS: SARS-CoV-2, COVID-19, CRISPR, MCCD, multiple cross displacement amplification, lateral flow biosensor

SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), as a new coronavirus, was first detected in Wuhan, China, and has caused an unexpected outbreak.¹ This virus has led an infectious respiratory illness-Coronavirus Disease 2019 (COVID-19).² COVID-19 has become a pandemic worldwide, affecting more than 200 countries/regions with 10,922,324 confirmed cases including 523,011 death cases (World Health Organization, COVID-19 Situation Report-166).¹ The increasing new cases, along with the possible high fatal rate, challenges current public health and social security systems globally.³ Therefore, reliable and fast diagnostics of SARS-CoV-2 infection is needed.

The early diagnosis of COVID-19 is extremely difficult as SARS-CoV-2 infection displays a range of clinical manifestations.⁴ While genomic sequencing has been used for the

diagnosis of COVID-19, it is not practical for rapid and large-scale diagnosis because of the time-consuming process and expensive experimental equipment requirement.⁵ Reverse transcription-real time quantitative PCR (RT-qPCR) was another option as it is a sensitive and reliable diagnostic assay for detecting pathogens, which has already been employed in SARS-CoV-2 infection in various laboratories.⁶ However, RT-qPCR diagnostic services strongly rely on

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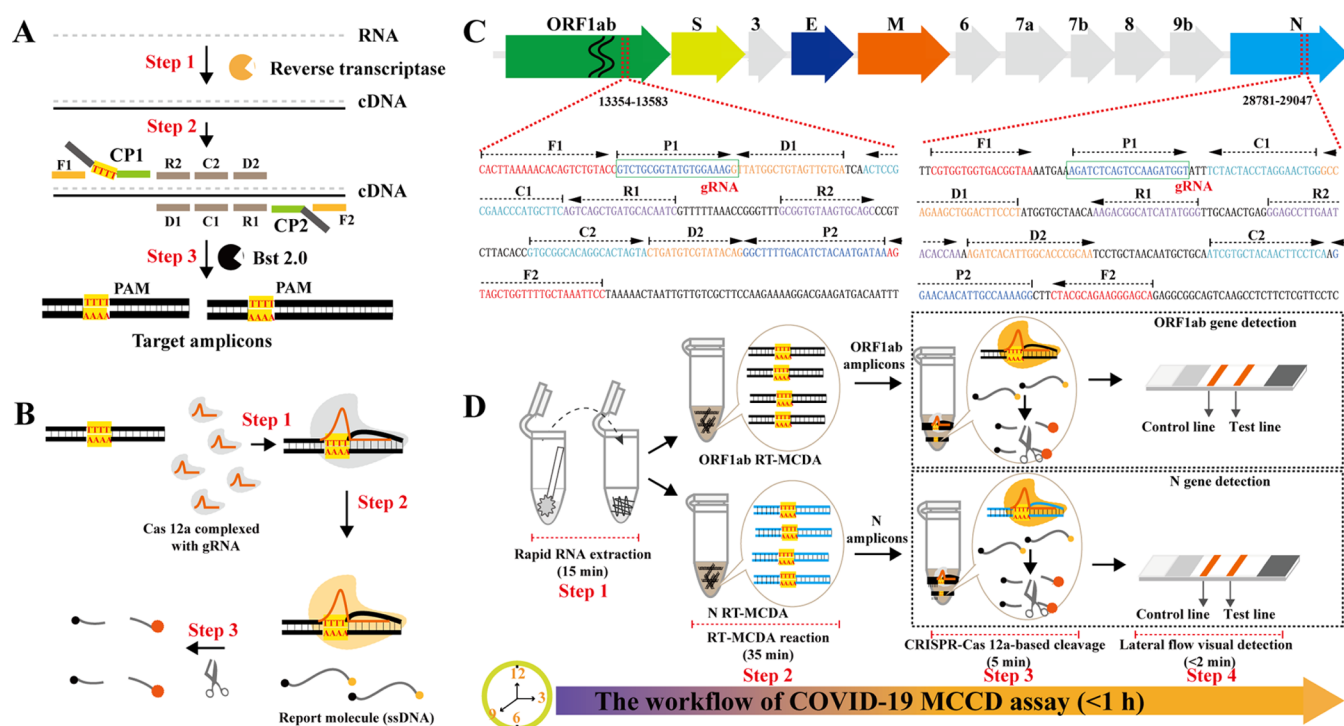


Figure 1. Outline of COVID-19 MCCD assay. (A) Schematic illustration of the principle of MCDA with the modified CP1 primer. The linker region of the regular CP1 primer was replaced with a PAM site (TTTT). After MCDA amplification, a CRISPR-Cas12a recognition site was constructed derived from the modified CP1 primer. (B) Schematic illustration of the principle of CRISPR-Cas12a/CrRNA detection system. Amplicons produced from step A can induce the CRISPR-Cas12a/CrRNA complex to nonspecifically cleave a single-strand reporter molecule modified with biotin and fluorescein isothiocyanate (FITC) at each terminus. (C) Primer and CrRNA design of COVID-19 MCCD assay. Top row, COVID-19 genome organization (GenBank: MN908947, Wuhan-Hu-1), and the length of all genes are not drawn in scale. ORF1ab (open reading frame 1a/b); S (spike protein); E (envelope protein); M (membrane protein); N (nucleoprotein); and accessory proteins (3, 6, 7a, 7b, and 9b). Bottom row, nucleotide sequence and location of the ORF1ab and N gene used to design the primers and CrRNAs. Part of nucleotide sequences of ORF1ab (left) and N (right) are shown. The sites of the primer sequence were underlined and CrRNAs are boxed. Right arrows and left arrows showed the sense and complementary sequence that are used. (D) Schematic of the COVID-19 MCCD workflow. COVID-19 MCCD employs four closely linked steps: RNA preparation, isothermal amplification, CRISPR-Cas12a cleavage, and LFB detection.

complex apparatus, skilled personnel, and a stable power supply.⁷ It is also time-consuming.⁸ Herein, further development of simpler, easy-to-use, and more rapid methods to diagnose COVID-19 are still needed.

The CRISPR-Cas system (clustered regularly interspaced short palindromic repeat and CRISPR-associated protein) has become an attractive tool for nucleic acid detection and displayed huge potential for the development next-generation molecular diagnostics methodology.⁹ On account of the reliability, high specificity, and sensitivity, a variety of CRISPR effectors (e.g., Cas9, Cas12a, Cas12b, Cas13a, and Cas13b) have been explored and the prospect of RNA-guide CRISPR-Cas nuclease-based diagnostic tests are encouraging.¹⁰ In particular, several Cas nucleases including Cas12a, Cas12b, Cas13a, and Cas14 show strong single-strand nucleic acid shredding activity (collateral cleavage activities) in which a crRNA-target-binding-activated Cas nuclease is able to non-specifically and indiscriminately cleave surrounding nontarget ssDNA and ssRNA.^{10b,c,11} By coupling with isothermal amplification technique RPA (recombinase polymerase amplification), the Cas12a and Cas13, respectively, have been employed for devising DETECTR (DNA Endonuclease-Targeted CRISPR Trans Reporter) and SHERLOCK (Specific High-sensitivity Enzymatic Reporter UNLOCKing) assays for rapid and sensitive nucleic acid analysis.^{10a,c,12}

To expand the COVID-19 testing capacity, the novel COVID-19 diagnostic techniques that eliminate the use of

complex apparatus or technical expertise are needed. Isothermal amplification-based assays, e.g., loop-mediated isothermal amplification (LAMP), RPA, and multiple cross displacement amplification (MCDA), which allow nucleic acid amplification under isothermal conditions using simple instrument, are being developing for COVID-19 diagnosis.^{7,13} In particular, some CRISPR-based diagnostic tests, which incorporated isothermal amplification techniques with CRISPR-Cas/gRNA complexes to allow rapid detection of nucleic acids including SARS-CoV-2 RNA, have recently been successfully developed.¹⁴ Importantly, these CRISPR-based diagnostic tests developed can offer analytical sensitivities better than or similar to PCR-based techniques without complex apparatus and are suitable for field, clinic, and point-of-care (POC) diagnostic application.

In this work, we combined a powerful innovative isothermal amplification technique (multiple cross displacement amplification; MCDA) with CRISPR-Cas12a-based detection to develop a novel diagnostic test (MCCD) and applied for detection of SARS-CoV-2 RNA, called COVID-19 MCCD (Figure 1). In particular, we first reported a strategy that engineered the amplification primer with a PAM site for CRISPR-based detection assay; thus, our design was able to detect any sequences (even as these targets do not contain any PAM sites) as long as they meet the requirement of primer design. Here, we expounded the basic mechanism of MCCD

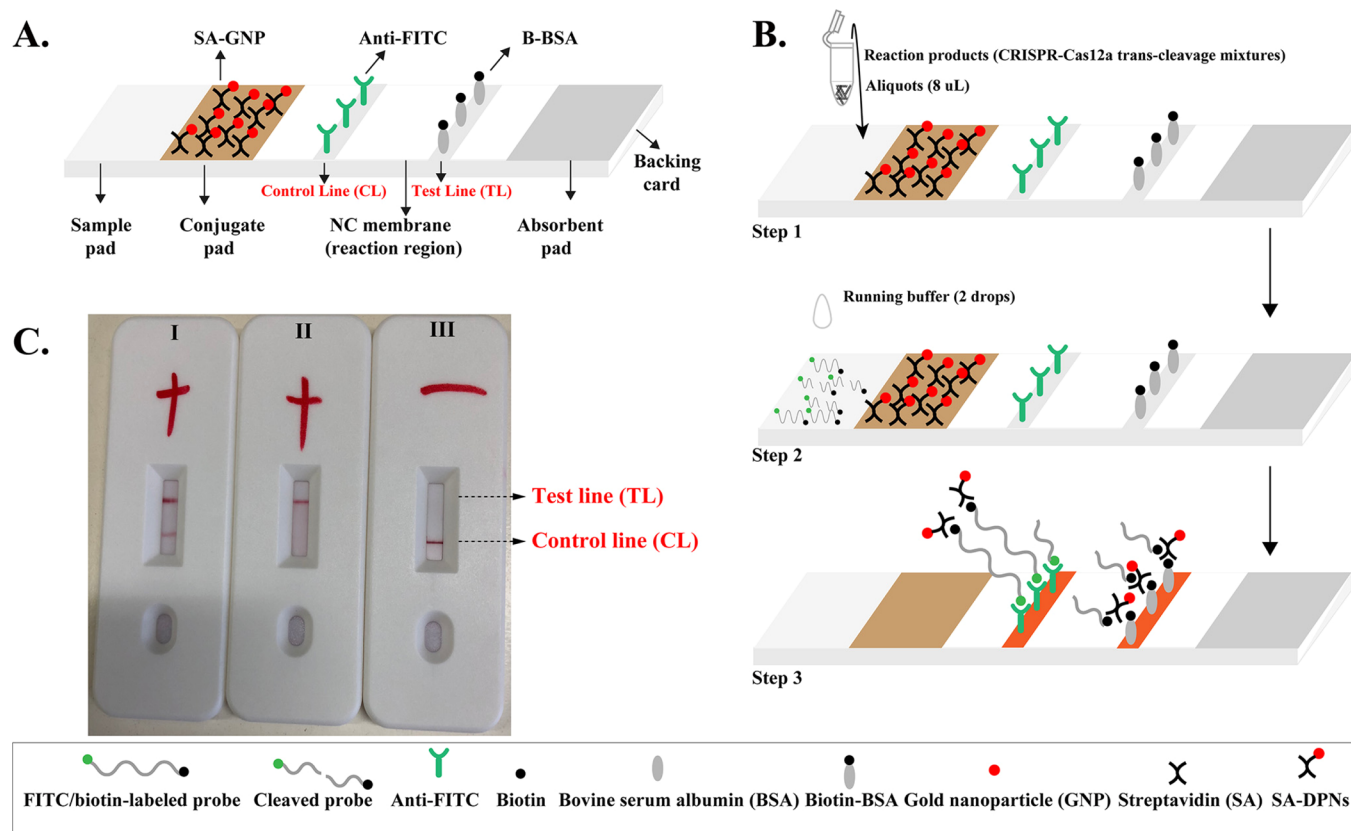


Figure 2. Principle of the LFB for visualization of COVID-19 MCCD products. (A) Details of the LFB. (B) Principle of the LFB for COVID-19 MCCD products. (C) Interpretation of the COVID-19 MCCD results. I and II, positive results for the COVID-19 MCCD test (CL and TL appear on the LFB, or only the TL appears on the LFB); III, negative (only the CL appears on the LFB).

assay and initially validated its application in SARS-CoV-2 detection using RNA templates extracted from clinical patients.

MATERIALS AND METHODS

Primer and CrRNA Design. Two MCDA primer sets (ORF1ab-MCDA and N-MCDA), which targeted ORF1ab and N genes of SARS-CoV-2 (GenBank MN908947, Wuhan-Hu-1), were designed using Primer3 online software version 0.4.0 (<http://bioinfo.ut.ee/primer3-0.4.0/>) based on the principle of MCDA. A total of ten primers for each target gene (ORF1ab and N) were designed, and the primer sets with better efficiency, specificity, and rapidity were employed for developing COVID-19 MCCD assay (Figures 1, and Figures S1, and S2). Each MCDA primer set recognizes ten different regions to amplify each gene marker and consists of two displacement primers (F1 and F2), six amplification primers (C1, D1, R1, C2, D2, and R2), and two cross primers (CP1 and CP2).¹⁵ Then, OligoAnalyzer online software version 3.1 (Integrated DNA Technologies, Coralville, IA) was employed for secondary structure and primer dimer investigation. The specificity of the MCDA primers was also analyzed using National Center for Biotechnology Information BLAST. Moreover, two CrRNAs were designed according to the COVID-19 MCCD principle. More details of MCDA primer design, sequences, locations, and CrRNA are given in Table S1, Figure 1C, and Figures S1, and S2. All of the oligomers were synthesized and purified by TianYi-HuiYuan Biotech. Co., Ltd. (Beijing, China) with an HPLC purification grade.

Reverse Transcription MCDA Reaction (RT-MCDA). The RT-MCDA was performed using a commercial reverse transcription isothermal amplification kit (HuiDeXing Biotech. Co., Ltd. Tianjing, China) and conducted in a heat-blocker (ThermoCell, Bioer Biotech. Co., Ltd. Hangzhou, China). In brief, the RT-MCDA was carried out in a 25 μ L mixture containing 12.5 μ L 2 $\times$ isothermal reaction buffer, 1.5 μ L enzyme mix (8 U of Bst 2.0 DNA polymerase and 5 U of

reverse transcriptase), 0.3 μ L Bst 3.0 polymerase, 1.6 μ M each of CP1 and CP2, 0.8 μ M each of C1, C2, D1, D2, R1, and R2, 0.4 μ M each of F1 and F2, and template (1 μ L for each of the standard plasmid, 5 μ L for samples). The real-time turbidity analysis (LA-320C) was employed for optimizing the isothermal reaction temperature.

CRISPR-Cas12a-Based Detection (CRISPR-Cas12a Trans-cleavage Assays). The CRISPR-Cas12a-based detection was conducted using CRISPR-Cas12a for the trans-cleavage assay, which was carried out similarly to the one previously described.^{10a} First, a total of 100 nM CrRNA was preincubated with 75 nM CRISPR-Cas12a (NEB, #M0653) in 1 $\times$ NEBuffer 2.1 at 37 $^{\circ}$ C for 10 min using a heat-blocker (ThermoCell, Bioer Biotech. Co., Ltd. Hangzhou, China); thus, the CRISPR-Cas12a/CrRNA complex was successfully formed. The CRISPR-Cas12a/CrRNA complexes should be used immediately or stored at 4 $^{\circ}$ C for up to 12 h before use.

The CRISPR-Cas12a trans-cleavage assay was performed in a 100 μ L mixture containing 50 μ L of 2 $\times$ NEBuffer 2.1, 2.5 μ L single-strand DNA reporter molecule (5'-FITC-TTATTATTATT-biotin-3', 10 μ M), 2 μ L of the RT-MCDA product, 18 μ L of the CRISPR-Cas12a/CrRNA complex, and 27.5 μ L of distilled water. The CRISPR-Cas12a trans-cleavage assay was carried out at 37 $^{\circ}$ C for 5 min. For real-time monitoring the CRISPR-Cas12a-based detection, the single stranded DNA (ss-DNA) reporter molecule (5'-FITC-TTATTATTATT-biotin-3', 10 μ M) used in lateral flow assay should be replaced using a F-Q probe (5'-FAM-TTATTATTATT-BHQ1-3', 10 μ M).

Lateral Flow Biosensor (LFB) Assay. The LFB (4 mm $\times$ 60 mm), shown in Figure 2A, incorporated an absorbent pad, a nitrocellulose membrane (NC), a conjugate pad, and a sample pad assembled on a plastic adhesive backing card. Anti-FITC (rabbit anti fluorescein antibody) and biotin-BSA (biotinylated bovine serum albumin), as the capture reagents, were dispensed onto the NC membrane. On the reaction region (NC membrane), there are areas

marked as the CL (control line, conjugated with anti-FITC) and TL (test line, conjugated with biotin-BSA), with each band separated by 5 mm. Streptavidin-immobilized gold nanoparticles (SA-GNPs) were deposited on the conjugate region of the LFB. In this report, we entrusted the HuiDeXing Biotech. Co., Ltd. (Tianjing, China) to produce the LFB according to our design.

A volume of 8 μL of the CRISPR-Cas12a trans-cleavage mixture was added to the sample region of the LFB; then, two drops of running buffer (100 mM PBS, pH 7.4 with 1% Tween 20) also was dropped to this region. The strip was allowed to absorb the whole buffer, and the lateral flow readout was visualized in the form of red bands on the NC region within 2 min.

Sensitivity and Specificity of the COVID-19 MCCD Assay.

The ORF1ab-plasmid and N-plasmid, which contain the ORF1ab and N sequences, respectively, were commercially constructed by TianYi-HuiYuan Biotech. Co., Ltd. (Beijing, China). Tenfold serial dilutions of the two standard plasmids, arranging from 7×10^4 to 7×10^{-2} copies, were employed for determining the sensitivity of the COVID-19 MCCD assay. Then, the assay's specificity was evaluated using synthesized sequences and templates extracted from various pathogens (Table S2).

Feasibility of COVID-19 MCCD Using Clinical Samples.

Thirty-seven respiratory samples, which were diagnosed as SARS-CoV-2 infection using RT-qPCR assay (Table S3), were collected from SanYa People's Hospital, Hainan. After clinical and laboratory diagnosis, these RNA templates, which were extracted from respiratory samples, were employed for validating our protocol. Aliquots of 5 μL of templates were used for examining the COVID-19 MCCD testing. Particularly, collection and analysis of these RNA templates were approved by SanYa People's Hospital.

■ RESULTS

COVID-19 MCCD Design. As shown in Figure 1, the RNA molecules were first converted to cDNA with the assistance of reverse transcriptase (Figure 1A, Step 1), and the cDNA served as the templates for subsequent MCDA amplification. Then, a total of ten primers initiated and completed the exponential amplification at a constant temperature within 35 min (Figure 1A, Step 2, and Figure S3). In the MCCD design, we engineered the MCDA core primer CP1 or CP2 with a PAM (protospacer adjacent motif) site (TTTT) for the Cas12a effector at its linker region (Figures 1A and S3). By the use of the modified CP1 primer, the MCDA amplicons obtain a new TTTT PAM site (protospacer adjacent motif) (Figure 1A, Step 3, and Figure S3). In particular, the MCDA core primer CP1 or CP2 includes three regions: the 5'-terminal region (20–25 bp), which is target-dependent, is reverse complementary to the target sequence for exponential amplification; the 3'-terminal region (18–22 bp), which also is target-dependent, is complementary to the target sequence for primer amplification; and a linker region (0–4 bp), which is in the middle of the two regions, is target sequence-independent. Thus, the linker region of CP1 or CP2 is an ideal candidate region, which could be used for constructing the engineered primer for CRISPR-Cas12a-based detection. As a result, our design is able to detect any sequence (even as these targets do not contain any PAM sites) as long as they meet the MCDA design. For COVID-19 diagnosis, the ORF-1ab- and N-CP1 primers were engineered at their linker regions with a PAM site (TTTT).

At the CRISPR-Cas12a-based detection stage (Figure 1B), the TTTT PAM site derived from the modified CP1 primer can guide the corresponding CRISPR-cas12a/CrRNA complex to its location (Figure 1B, Step 1), which activates Cas12a proteins (Figure 1B, Step 2). As a result, the binding of the CRISPR-Cas12a-CrRNA complex to a guide-complementary

MCDA product enables ultrafast cleavage of single-strand DNA reporter molecules (Figure 1B, Step 3). Thus, our assay conducts simultaneous reverse transcription and MCDA reaction for the RNA template followed by CRISPR-Cas12a-based detection of predefined target genomes after which degradation of a ssDNA molecule validates detection of the target pathogen.

Then, we designed two sets of MCDA primers targeting the opening reading frame 1a/b (ORF1ab) and nucleoprotein (N) genes of SARS-CoV-2 (Figures 1C, S1, and S2). The CP1 of the ORF1ab- and N-MCDA primer sets was modified with a PAM site (TTTT) at the linker region (Table S1), and the ORF1ab- and N-CrRNA were designed to specially detect ORF1ab and N sequence of SARS-CoV-2 according to the COVID-19 MCCD design (Figure 1B, and Figures S1, S2, and Table S1). The whole diagnostic test of COVID-19 MCCD assay, including rapid preparation of SARS-CoV-2 RNA (15 min), isothermal reaction (35 min), and CRISPR-Cas12a-based detection (within 10 min), can be finished within 60 min (Figure 1D). Particularly, the detection results of COVID-19 MCCD assay can be reported using an LFB (Figures 1D and 2) and real-time fluorescence analysis (Figure S4).

Visualization of COVID-19 MCCD Results by an LFB.

The details of the LFB are shown in Figure 2A. After CRISPR-Cas12a cleavage, a volume of 8 μL of reaction mixtures were deposited on the sample region of the LFB (Figure 2B, Step 1), then two drops of running buffer were also added to the same region (Figure 2B, Step 2). The running buffer moved along the LFB through capillary action, which rehydrated the immobilized indicator (SA-GNPs) in the conjugate sample. The FITC labeled at the ssDNA molecule was captured by an anti-FITC antibody at the CL, and the biotins of the ssDNA reporter molecule bind SA-GNPs for visualization (Figure 2B, Step 3). Once the ssDNA reporter molecule was cleaved by activated the CRISPR-Cas12a protein, FITC and biotin were separated. Thus, the biotin/SA-GNP complex was captured by biotin-BSA at the test line, which indicated a positive readout (Figure 2B, Step 3). The interpretation of the COVID-19 MCCD assay using an LFB is shown in Figure 2C and Figure S5.

Optimal Conditions for COVID-19 MCCD Assay. To confirm the optimal amplification temperature of COVID-19 MCCD assay at the isothermal stage, ORF1ab- and N-RT-MCDA were performed using ORF1ab and N plasmids, respectively. As shown in Figures S6 and S7, a temperature of 63 $^{\circ}\text{C}$ was employed for performing the COVID-19 MCCD assay at the isothermal amplification stage. Then, we optimized the reaction time of CRISPR-Cas12a detection and also compared the signal yielded by CRISPR-Cas12a when using an LFB and real-time fluorescence. COVID-19 RT-MCDA reactions were performed using ORF1ab (700 copies) and N (700 copies) plasmid templates and analyzed the results of the CRISPR-Cas12a readout using a real-time fluorescence instrument and an LFB at 0, 2.5, 5, 7.5, and 10 min. As shown in Figure S8, a visual signal by the LFB was detectable within 5 min (Figure S8A, B), and the CRISPR-Cas12a fluorescence signal was obtained within 1 min (Figure S8C, D). Hence, a reaction time of 5 min was recommended for COVID-19 MCCD assay at the CRISPR-Cas12a cleavage stage (Figure 1D, Step 3, and Figure S8).

Sensitivity of COVID-19 MCCD Assay. Using the ORF1ab- and N-plasmid templates at various dilutions, the sensitivity of COVID-19 MCCD lateral flow assay was seven

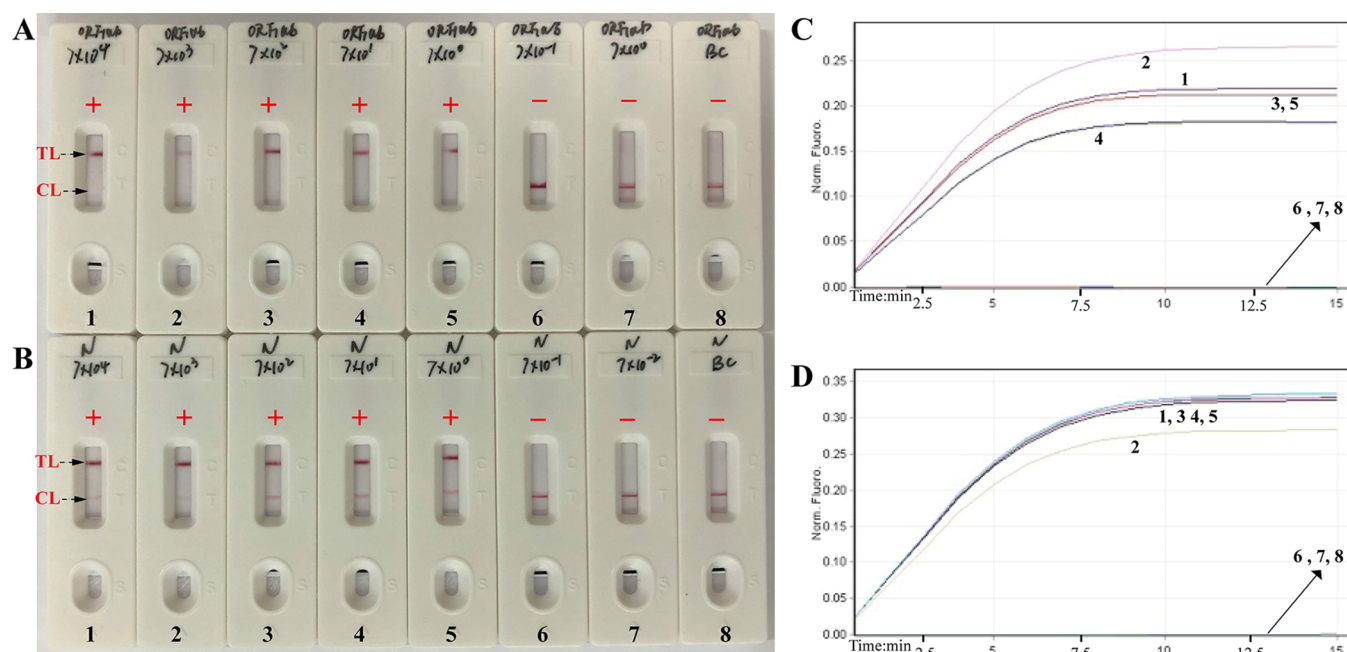


Figure 3. Sensitivity of COVID-19 MCCD assay. (A) LFB applied for reporting the results; (B) real-time fluorescence applied for further confirming the results. LFB (A)/Signals (C) 1–8 represented the plasmid levels (ORF1ab-plasmid) of 7×10^4 , 7×10^3 , 7×10^2 , 7×10^1 , 7×10^0 , 7×10^{-1} , and 7×10^{-2} copies per reaction and blank control (DW). LFB (B)/Signals (D) 1–8 represented the plasmid levels (N-plasmid) of 7×10^4 , 7×10^3 , 7×10^2 , 7×10^1 , 7×10^0 , 7×10^{-1} , and 7×10^{-2} copies per reaction and blank control (DW).

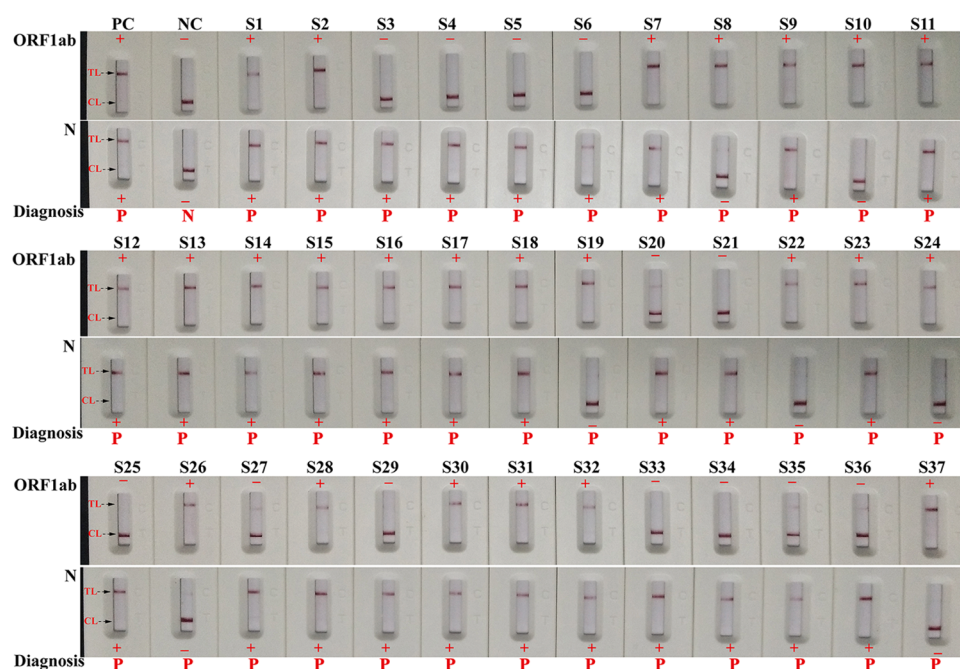


Figure 4. COVID-19 MCCD results on 37 COVID-19-infected patient samples. S1 to S37 represents clinical samples 1 to 37; PC, positive control; NC, negative control; “+”, positive; “−” negative; P, samples were diagnosed as SARS-CoV-2 infection using our protocol.

copies (for each of the target template) per test (Figure 3). An easy-to-interpret qualitative readout, which was a visual signal for the presence or absence of the target sequence, was produced by the LFB at the limit of detection (LoD) (Figure 3A,B). Using the COVID-19 MCCD test by self-trail, the LoD of COVID-19 MCCD lateral flow assay was further confirmed by COVID-19 MCCD fluorescence assay. By the fluorescence analysis, the LoD of COVID-19 MCCD assay was also seven copies (for each of the target template) per reaction (Figure

3C,D), which was in agreement with the LFB detection (Figure 3).

Specificity of COVID-19 MCCD Assay. A specificity evaluation was manipulated by using synthesized templates and various templates extracted from virus, bacteria, and fungi. All positive results were obtained from ORF1ab- and N-plasmid templates (positive controls), whereas non-ORF1ab- and N-plasmid templates produced negative results (Table S2). No cross-reactions were generated from COVID-19 MCCD assay;

thus, the diagnostic test designed in this report was highly selective to target sequences.

Validation of the COVID-19 MCCD Assay to Clinical Samples. To examine the feasibility of COVID-19 MCCD assay as a SARS-CoV-2 diagnostic tool, we determined these templates extracted from 37 respiratory swab samples obtained from 37 RT-qPCR-positive COVID-19 patients and 77 non-COVID-19 respiratory swab samples (Tables S3, S4, and S5). SARS-CoV-2 RNA was detected in 37 of 37 patient samples (Figure 4, Tables S3 and S4); thus, the analytical sensitivity of COVID-19 MCCD was 100%. Moreover, the specificity of COVID-19 MCCD assay was also 100% because nonpositive signals were observed from non-SARS-CoV-2 respiratory samples using COVID-19 MCCD tests (Figure 4, and Tables S3 and S5).

DISCUSSION

In this report, a novel MCCD test, which integrated isothermal amplification (MCDA) with CRISPR-Cas12a-based detection, was established and applied for detection of SARS-CoV-2 RNA in clinical samples. To date, several CRISPR-Cas12a-based diagnostic assays (Table S6), including diagnostic techniques for the SARS-CoV-2 detection, have been devised and exhibited the advantages of sensitivity, specificity, and versatility.^{14b,16} In such techniques, isothermal amplification assays, including LAMP and RPA, were employed for nucleic acid amplification, and the CrRNA-guided Cas12a effector is programmed to detect amplicons from SARS-CoV-2 with high specificity (single base pair specificity) and sensitivity (arranging from 2 copies to 10 copies). In this report, MCDA assay was first used as an attractive alternative for conventional isothermal amplification assays due to its rapidity, low cost, and high efficiency. Furthermore, our CRISPR-Cas12a-based diagnostic assay eliminated the protospacer adjacent motif (PAM) site limitation because the engineered core primer contained the PAM site at the linker region and simultaneously met design requirements for the MCDA technique. Thus, the MCCD can detect any sequences even as they lack suitable PAM sites for Cas12a CrRNA.

Only simple instruments (e.g., heating block) were required for conducting COVID-19 MCCD assay, including the MCDA reaction at 63 °C for 35 min and CRISPR-Cas12a cleavage at 37 °C for 5 min, which eliminated the use of thermocycling. Thus, the MCCD diagnostic test may be more suitable than RT-qPCR assays for detection of SARS-CoV-2 infection in resource-poor setting and field laboratories (Figure 1). Apart from the modest equipment requirement, the MCCD assay also showed a key advantage over COVID-19 RT-PCR methods, namely, rapid turnaround time. The whole test process, including SARS-CoV-2 RNA preparation (15 min), RT-MCDA reaction (35 min), Cas12a cleavage (5 min), and reporting of results (within 5 min), can be finished within 1 h (Figure 1D).

COVID-19 MCCD assay targeting ORF1ab and N genes were developed. At the isothermal amplification stage, two sets of MCDA primers (ORF1ab-MCDA and N-MCDA primer sets), which recognized ten regions of ORF1ab and N genes, respectively, ensured the high specificity for SARS-CoV-2 RNA detection. After RT-MCDA amplification (Figure 1A), each amplicon was decoded by CRISPR-Cas12a-based detection (Figure 1B,D), which is a CRISPR-based diagnosis technique for rapid and precise detection of the target sequence.^{10a} Most importantly, the MCCD assay showed extremely high

specificity (single nucleotide target specificity) for target sequence analysis by a PAM site and a target-dependent CrRNA. Attributing to the characteristic of specificity for MCDA and MCCD assays, our COVID-19 MCCD assay enabled accurate detection of SARS-CoV-2 RNA. Moreover, these data also demonstrated that no positive signals were obtained from non-SARS-CoV-2 templates (Table S2). Hence, COVID-19 MCCD assay did not cross-react with other pathogens and the synthetic nucleic acid templates (Table S2).

In regular MCDA, there is a linker region (0–4 bp) in the CP1 primer (Figure S3). For the MCCD detection, we replaced this region with a PAM site (TTTT). The CP1 primer was modified to meet the requirements for CRISPR-Cas12a-based MCCD. Thus, the MCCD can detect any sequences (including these sequences that lack suitable PAM sites for the Cas12a/CrRNA) as long as they meet the design requirement of the MCDA method. In the COVID-19 MCCD system, instead of the regular CP1 primers, the modified CP1 primers specific to two different targets, which were labeled with PAM sites at the linker region, were added into RT-MCDA mixtures for amplifying the possible targets. After RT-MCDA, the amplification products were subject to CRISPR-Cas12a-based analysis. A key point of our assay is to successfully devise the CrRNA enabling each target corresponding to a unique CrRNA sequence in the CRISPR-based detection process (Figure 1B, and Figures S1, and S2). Although RT-MCDA products are complicated, the PAM site will assist the CRISPR-Cas12a/CrRNA complex to accurately recognize all the specific amplicons, which were derived from the modified CP1 primers. Thus, the possible target genes existing in the SARS-CoV-2 genome are specially detected and accurately differentiated by the CRISPR-Cas12a-based analysis (Figure 1D and Figure S4).

The RT-MCDA assay was employed for amplifying the marker genes ORF1ab and N at the isothermal amplification stage due to its high sensitivity. Previous reports have demonstrated that MCDA-based methods were 10-fold to 1000-fold more sensitive than PCR assays, even at least 10-fold more sensitive than the most popular LAMP assay.¹⁷ The data of analytical sensitivity suggested that COVID-19 MCCD was able to detect down to seven copies (of each target gene) per test (Figure 3). The results obtained using an LFB were in complete agreement with the fluorescence readout (Figure 3). Apart from the advantage on sensitivity, MCDA assay also showed another trait of high amplification efficiency, only a 30 min isothermal time was recommended as the cutoff value for DNA amplification, and 35 min for RNA detection.^{17a} Herein, for the COVID-19 MCCD protocol, a 35 min incubation time was employed for setting the cutoff value for SARS-CoV-2 RNA detection during the isothermal signal amplification stage.

The COVID-19 MCCD protocol was initially validated using the extracted RNA from clinical samples. We first examined these RNA templates from 37 respiratory swab samples collected from 37 RT-PCR-positive COVID-19 patients. SARS-CoV-2 RNA was detected in 37 of 37 patient swabs (Figure 4 and Table S3). Given the concordance between COVID-19 MCCD and RT-PCR assays (37 of 37 tests), the COVID-19 MCCD assay showed high reliability for SARS-CoV-2 detection. Then, we also determined these RNA templates from 77 respiratory swab samples collected from 77 non-COVID-19 patients, and no samples were diagnosed as the SARS-CoV-2 infection. These data initially confirmed that

the COVID-19 MCCD assay is a potential detection tool for the diagnosis of SARS-CoV-2 infection. For the clinical application, we recommended LFB-based readout over fluorescence-based readout, because the LFB was disposable, instrument-free, easy to use, visually indicated the result within 2 min, did not require any equipment, and was suitable for large-scale screening. Hence, the COVID-19 MCCD using LFB-based readouts offered a measure of practicality for POC (point-of-care), on-site, and field laboratories in resource-poor settings.

CONCLUSIONS

In this study, we report the development of a CRISPR-Cas12a-based detection technology for rapid detection of SARS-CoV-2, named COVID-19 MCCD assay. Assay's result can be visualized on an LFB, and the whole test was completed within 1 h. The data of analytical sensitivity, specificity, and clinical evaluation initially validated that our protocol is able to accurately differentiate SARS-CoV-2 from non-SARS-CoV-2 and reliably diagnose SARS-CoV-2 infection in clinical samples. Thus, these traits of our COVID-19 MCCD assay provide the need for quick diagnosis of the current global pandemic of COVID-19 in a variety of settings including field, clinical, and resource-limited environments.

FUNDING

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ETHICAL APPROVAL

This study was approved by the Ethics Committee of Sanya People's Hospital (SYPH-2019(41)-2020-03-06).

TRANSPARENCY DECLARATION

The lead author and guarantor affirms that the manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned and registered have been explained.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssensors.0c01984>.

The supporting information includes primer and CrRNA sequences (Table S1, Figures S1, and S2), pathogen information (Table S2), clinical samples and detection results (Table S3, S4, S5, and S6), details of MCDA amplification with the PAM site (Figure S3), fluorescence COVID-19 MCCD assay (Figure S4), interpretation of lateral flow MCCD results (Figure S5), optimal MCDA reaction temperature (Figures S6 and S7) and optimal CRISPR test time (Figure S8) (PDF)

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Notes

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