EUROPEAN RESPIRATORY *journal*

FLAGSHIP SCIENTIFIC JOURNAL OF ERS

Early View

Original article

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Please cite this article as: Li S, Jiang W, Huang J, *et al*. Highly sensitive and specific diagnosis of coronavirus disease 19 (COVID-19) by reverse transcription multiple cross displacement amplification-labelled nanoparticles biosensor. *Eur Respir J* 2020; in press (https://doi.org/10.1183/13993003.02060-2020).

Title Page

Highly sensitive and specific diagnosis of coronavirus disease 19 (COVID-19) by reverse transcription multiple cross displacement amplification-labeled nanoparticles biosensor

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Abstract

Background: The ongoing outbreak of the novel human coronavirus SARS-CoV-2 (also known as 2019-nCoV) has become a global health concern. Rapid and ease-to-use diagnostic techniques are urgently needed to diagnose SARS-CoV-2 infection.

Methods: We devised a reverse transcription multiple cross displacement amplification (RT-MCDA) coupled with nanoparticles-based biosensor (BS) assay (RT-MCDA-BS) for rapid, sensitive and specific diagnosis of COVID-19. Two primer sets were designed to target the open reading frame 1a/b (F1ab) and nucleoprotein gene (N) of SARS-CoV-2. A total of 183 clinical samples, including 65 patients with COVID-19 infections and 118 patients with other pathogen infections, were used to testify the assay's feasibility. The diagnosis results were visually reported using BS.

Findings: The designed assay was performed using a simple instrument which could maintain the reaction in a constant temperature at 64^{o}C for only 35 min. The total procedure of COVID-19 RT-MCDA-BS test could be finished within 1 h. The COVID-19 RT-MCDA-BS could detect down to 5 copies of target sequences. Among 65 clinical samples from the COVID-19 patients, 22 (33.8%) positive results were obtained from face, nasal, pharyngeal and anal swabs via COVID-19 RT-MCDA-BS assay, while rRT-PCR assay only detected 20 (30.7%) positive results in these samples. No positive results were obtained from clinical samples with non-COVID-19 infections.

Interpretation: COVID-19 RT-MCDA-BS was a rapid, reliable, low cost and easy-to-use assay, which could provide an attractive laboratory tool to diagnose COVID-19 in multiple clinical specimens, especially for field, clinic laboratories and primary care facilities in resource-poor settings.

Keywords: SARS-CoV-2; COVID-19; Coronavirus; Rapid diagnosis; Multiple cross displacement amplification; Biosensor.

Introduction

In late December 2019, an outbreak of pneumonia caused by a novel human coronavirus SARS-CoV-2 (also referred to 2019-nCov) emerged in Wuhan, Hubei province in China [1, 2]. The novel emerging virus has rapidly spread across China, and to more than 200 countries/regions outside of China (World Health Organization, COVID-19 Situation Report-129) [3]. Data as of 28 May 2020, SARS-CoV-2 has affected more than 5,593,631 patients (353,334 deaths) worldwide (WHO, COVID-19 Situation Report-129). Due to its rapid spreading speed (R_0 3.28), possible fatal progression and strong infectivity, COVID-19 has stirred a grave global concern [4]. Hence, a rapid, easy-to-use and reliable test tool was urgently required for diagnosing infections, treating the patients and controlling the spreading of COVID-19.

Early diagnosis of COVID-19 is extremely difficult only according to manifestations, which are highly variable [5]. The clinical manifestations of COVID-19 patients range from asymptomatic infection, non-specific flu-like clinical presentations (such as cough and fever) to respiratory failure [3]. Obvious clinical manifestations may occur in two days, even up to two weeks after exposure. Thus, designing a rapid and early diagnostic method, is vital for treatment and disease control. Currently, the definitive diagnosis of COVID-19 infections strongly relies on polymerase chain reaction (PCR)-based methods, such as reverse transcription PCR (RT-PCR) and real-time reverse transcription PCR (rRT-PCR) [5]. However, they require an expensive thermal cycle apparatus and very skillful laboratory workers. In addition, only 62% positive rate obtained by PCR-based technologies because of their sensitivity limitation among clinical diagnosed COVID-19 patients is not effective for control of infection [5, 6]. Thus, an easy-to-use and cost-effective test with higher sensitivity is needed as a first-line technique to tackle the COVID-19 in field and clinical settings.

Recently, several loop-mediated isothermal amplification (LAMP) assays have been devised for rapid detection of COVID-19 in clinical and stimulated patient samples [7-9]. However, the developed COVID-19 LAMP assays only detect one

target gene (Open reading frame 1a/b; F1ab). False-positive results could be produced when they were applied to detect clinical samples containing highly homologous sequences, such as gene from bat severe acute respiratory syndrome-like coronavirus (GenBank KY417152.1). In addition, LAMP results were reported via agarose gel electrophoresis, SYBR dyes or PH indicator. Particularly, the electrophoresis is a tedious and time-consuming process, and the assessment of color change with the unaided eye is potentially subjective in an ambiguous test caused by the low concentration of templates. Herein, novel techniques, which could overcome these technical difficulties, are continuously demanded.

Multiple cross displacement amplification (MCDA) technique is an easy-to-perform and reliable diagnostic assay, which amplifies the target nucleic acid under conditions (usually between 58^{o} C and 69^{o} C) within a short time (20 min-40 min, usually 30 min) [10]. Only a simple equipment (such as a water bath, a heating block, even a thermo cup) is sufficient for MCDA test. The MCDA technique has been used for diagnosing various infectious diseases producing positive results from down to 3 copies of target sequence, which has shown its rapidity, simplicity, high specificity characteristics. [11, 12]. To achieve a simultaneous detection of multiple targets and rapid analysis of MCDA products, nanoparticles-based biosensor has been coupled with MCDA to achieve an objective detection results. Biosensor is simple, easy-to-conduct and low-cost, and does not need complex instrument and skilled personnel [13].

In this report, we devised a one-step, single-tube reverse transcription MCDA (RT-MCDA) coupled with nanoparticles-based biosensor (BS) assay (RT-MCDA-BS) (**Figure 1** and **2**), which could diagnose COVID-19 by simultaneously detecting F1ab (open reading frame 1a/b) and N (nucleoprotein) genes. The whole detection process could be completed within 1 hour with high accuracy. Thus, the RT-MCDA-BS method may enable the diagnosis of COVID-19 easier, faster and more reliable, even in resource-limited settings.

Methods

Preparation of nanoparticles-based biosensor

The nanoparticles-based biosensor (BS, 4 mm×60 mm), depicted in **Figure 2A**, consisted of a sample pad, a conjugate pad, a nitrocellulose membrane (NC membrane) with an absorbent pad assembled on a plastic adhesive backing card (Jie-Yi Biotechnology. Co., Ltd. Shanghai, China). The capture reagents, including anti-Dig (sheep anti-digoxigenin antibody, 0.25 mg/mL, Abcam. Co., Ltd.), anti-FITC (rabbit anti-fluorescein antibody, 0.2 mg/ml, Abcam. Co., Ltd.) and biotin-BSA (biotinylated bovine serum albumin, 4 mg/mL, Abcam. Co., Ltd.), were dispensed onto the NC membrane as the test line 1 (TL1), test line 2 (TL2) and control line (CL) , respectively, with each line separated by 5 mm.. The detector reagents (Dye streptavidin coated polymer nanoparticles, 129 nm, 10mg mL-1, 100mM borate, pH 8.5 with 0.1% BSA, 0.05% Tween 20 and 10mM EDTA) were sprayed onto the conjugate region of the biosensor. Thus, the biosensor can detect three targets, including two target products (F1ab MCDA amplicons and N MCDA amplicons) and a chromatography control. Finally, the assembled biosensors were cut into 4-mm dipsticks, and dryly stored at the room temperature for use.

RT-MCDA Primer design

Two sets of RT-MCDA primers (F1ab-MCDA and N-MCDA primer sets), which targeted F1ab gene and N gene of COVID-19 (GenBank MN908947, Wuhan-Hu-1), were designed according the MCDA principle (**Figure 3**). Each MCDA primer set consists of two displacement primers (F1 and F2), two cross primers (CP1 and CP2), and six amplification primers (C1, D1, R1, C2, D2 and R2). The specificity of F1ab- and N-MCDA primer sets were analyzed by National Center for Biotechnology Information BLAST. Moreover, the OligoAnalyzer online software (V3.1, Integrated DNA Technologies, Coralville, IA) was employed for primer dimer and secondary structure investigation. The details of RT-MCDA primer design, primer location, sequences and modifications were listed in **Figure 3** and **Table S1**. All oligomers were synthesized and purified by Tianyi-Huiyuan Biotech. Co., Ltd. (Beijing, China)

at HPLC purification grade.

Reverse transcription MCDA (RT-MCDA) reaction

The standard RT-MCDA (F1ab-MCDA/N-MCDA) was conducted in a one-step reaction in a 25-μl mixture containing 12.5 μl 2×isothermal reaction buffer [40 mM Tris-HCl (pH 8.8), 40 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH4)_2SO_4$, 2 M betaine and 0.2 % Tween-20], 8 U of Bst 2.0 DNA polymerase (New England Biolabs), 10 U of the avian myeloblastosis virus reverse transcriptase (Invitrogen), 1.4 mM dATP, 1.4 mM dCTP, 1.4 mM dGTP, 1.4 mM dTTP, 0.1 mM biotin-14-dCTP, 0.1 mM biotin-14-dATP, 0.4 μM each of displacement primers F1 and F2, 0.8 μM each of amplification primers C1, C2, D1, D1, R1 and R2, 0.8 μM each of cross primers CP1* and CP1, 1.6 μM cross primers CP2 and template (1μl for the standard plasmid).

The COVID-19 RT-MCDA was also performed in a one-step reaction in a 25-μl mixture containing 12.5 μl 2×isothermal reaction buffer [40 mM Tris-HCl (pH 8.8), 40 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH4)_2SO_4$, 2 M betaine and 0.2 % Tween-20], 8 U of Bst 2.0 DNA polymerase (New England Biolabs), 10 U of the avian myeloblastosis virus reverse transcriptase (Invitrogen), 1.4 mM dATP, 1.4 mM dCTP, 1.4 mM dGTP, 1.4 mM dTTP, 0.1 mM biotin-14-dCTP, 0.1 mM biotin-14-dATP, 0.275 μM of F1ab-F1 and F1ab-F2, 0.55 μM of F1ab-C1, F1ab-C2, F1ab-D1, F1ab-D2, F1ab-R1 and F1ab-R2, 0.55 μM of F1ab-CP1* and CP1, 1.1 μM of F1ab-CP2, 0.125 μM of N-F1 and N-F2, 0.25 μM of N-C1, N-C2, N-D1, N-D2, N-R1 and N-R2, 0.25 μM of N-CP1* and CP1, 0.5 μM of N-CP2, and template (1μl for the each standard plasmid, 5μl for samples).

Real-time turbidity (LA-320C), visual detection reagents (VDR) and BS were applied to demonstrating the MCDA reactions and confirming the optimal amplification temperature.

Sensitivity of the RT-MCDA-BS assay

Two plasmids (F1ab-plasmid and N-plasmid), which contain the F1ab and N genes,

respectively, were commercially constructed by Tianyi-Huiyuan Biotech. Co., Ltd. (Beijing, China). According to manual, the initial concentrations of F1ab- and N-plasmids were 5×10^{8} copies/µl. Then, ten-fold serial dilutions (5×10^{4} to 5×10^{-2} copies) of F1ab-plasmid and N-plasmid were used to assess the limit of detection (LoD) of the COVID-19 RT-MCDA, F1ab-RT-MCDA and N-RT-MCDA assays. The examinations were conducted in triplicate independently. Simultaneously, the concentration of plasmid at the LoD level was used for confirming the optimal isothermal time of COVID-19 RT-MCDA assay.

Specificity of the COVID-19 RT-MCDA-BS assay

The analytical specificity of the COVID-19 RT-MCDA-BS assay was evaluated by comparing COVID-19 templates with the templates extracted from various viruses, bacteria and fungi (**Table S2**).

Validating the feasibility of COVID-19 RT-MCDA-BS using clinical samples

A total of 54 clinical samples (including face, anal, nasal, pharyngeal swabs) were obtained from acute phase and convalescent of COVID-19 sent to the Guizhou province CDC (**Table S3**). Analysis of these RNA templates using COVID-19 RT-MCDA-BS assay was approved by Guizhou province CDC. The pharyngeal, nasal and anal swabs samples were collected using a Flocked sterile plastic swab applicator, which was placed in a Universal Viral Transport Medium (UVIM) for viruses (HiDNA biotech. Co., Ltd.). For faecal sample, aliquots (approximately 1 g) of the stools were placed into a tube, which contained 1.5 ML of UVIM. Then, the faecal sample was centrifuged at 5000 g for 10 min, and the supernatant was placed into a new tube. Particularly, aliquots (200 µl) of UVIM (pharyngeal, nasal and anal swabs samples) and supernatant of faecal samples were subjected to extract the RNA templates, and the procedure only required 15 min using a rapid RNA Extraction Kit (TianLong Biotech. Co., LtD.). Aliquots of 5 µl of templates were used for conducting the RT-PCR and COVID-19 RT-MCDA-BS methods. The RNA templates extracted from different types of clinical samples were collected after rRT-PCR performance in

Guizhou province CDC, which was conducted by officially approved clinical RT-PCR kit. The LoD (limit of detection) of RT-PCR diagnosis kit was 500 copies/mL according its manual. A Ct value is < 37, which is defined as a positive result for COVID-19 infection. A Ct value is >40, or no Ct value is obtained, which is defined as a negative result for COVID-19 infection. A Ct value between 37-40 is indeterminate, and the test should be examined again. In addition, 118 pharyngeal swabs samples collected from non-COVID-19 patients also were tested for validating the specificity of the COVID-19 RT-MCDA-BS method.

Results

The schematic mechanism of COVID-19 RT-MCDA-BS assay

In the COVID-19 RT-MCDA system, two core primers, including F1ab-CP1 and N-CP1 primer, are labeled at the 5' end with digoxigenin (Dig) and fluorescein (FITC), respectively. The new F1ab-CP1 and N-CP1 primers were termed as F1ab-CP1* and N-CP1*. Simultaneously, two components (biotin-14-dCTP and biotin-14-dATP) are added into the COVID-19 RT-MCDA reaction mixtures (**Figure 1A**). As shown in **Figure 1B**, the SARS-CoV-2 templates (RNA) are firstly converted to cDNA with the supplement of avian myeloblastosis virus reverse transcriptase (AMV) at the amplification temperature (64^{o} C). Then, the cDNA serves as the template for MCDA amplification. The F1ab-CP1* and N-CP1* primers anneal to the target regions, and is extended by the displacement enzyme (*Bst* 2.0), thus biotin-14-dATP and biotin-14-dCTP are incorporated into the newly synthesized products. As a result, a plenty of detectable double-labeled products are formed with F1ab-MCDA amplicons simultaneously labeled with Dig and biotin, and N-MCDA amplicons for FITC and biotin (**Figure 1C**).

The principle of BS visualization of COVID-19 RT-MCDA results

The details of nanoparticles-based biosensor (BS) were shown in **Figure 2A**. After amplification, aliquots (1 μl) of COVID-19 RT-MCDA products were deposited on the sample pad of BS (**Figure 2B**, step 1), followed by the addition of an aliquot (100

μl) of running buffer to the same region (**Figure 2B**, step 2). The running buffer can move along BS through capillary action, and then rehydrate the immobilized SA-DNPs in conjugate region. F1ab-MCDA amplicons were specifically captured by the anti-Dig fixed on the TL1, and the N-MCDA amplicons captured by the anti-FITC immobilized in the TL2. The biotins of COVID-19 MCDA products can bind SA-DNPS for visualization. The excess SA-DNPs were captured by biotinylated bovine serum albumin immobilized in CL, which evaluated the working condition of BS (**Figure 2B**, step 3). The interpretation of the COVID-19 RT-MCDA results via BS was displayed in **Figure 2C**.

Confirmation and analysis of F1ab-, N- and COVID-19 RT-MCDA products

Using VDR, the positive F1ab-, N- and COVID-19 RT-MCDA vessels were visualized by unaided eye as light green, while the vessels of negative and blank controls remained colorlessness (**Figure S1**, top row). In BS, two red bands (TL1 and CL) were seen in positive F1ab-RT-MCDA amplification (**Figure S1A**, bottom row), TL2 and CL for positive N-RT-MCDA amplification (**Figure S1B**, bottom row). TL1, TL2 and CL simultaneously appeared in the detection region of BS indicate positive result of COVID-19 RT-MCDA amplification (**Figure S1C**, bottom row). Only the CL shown represents negative and blank controls (**Figure S1A**, **S1B**, **S1C**, bottom row). These data demonstrated that F1ab- and N-MCDA primer sets, and COVID-19 RT-MCDA assay were feasible for target sequence detection. Then, the optimal reaction temperature of 64^{o} C of COVID-19 RT-MCDA was also determined (**Figure S2** and **S3**).

Sensitivity of COVID-19 RT-MCDA-BS assay

As shown in **Figure 4**, the COVID-19 RT-MCDA-BS assay can detect down to 5 copies of either F1ab-plasmid or N-plasmid in a vessel. Of note, F1ab and N gene of COVID-19 were simultaneously amplified correctly with their own primers in a one-step, single-tube reaction (**Figure 4A**). The results obtained via BS were consistent with VDR reagent and real-time turbidity, while the VDR and real-time

turbidity methods could not achieve multiple target detection (**Figure 4B** and **4C**). Most importantly, F1ab- and N-RT-MCDA assays also revealed a detection limit of 5 copies of target sequences in a reaction, which was in consistent with COVID-19 RT-MCDA assay (**Figure S4, S5** and **Figure 4**).

The optimal reaction time required for the COVID-19 RT-MCDA-BS assay during the amplification stage was also tested. The lowest template level (5×10^0 copies of either F1ab- or N-plasmids displayed three red bands (TL1, TL2 and CL) when the isothermal reaction was conducted for 25 min at 64°C (**Figure S6**). For the clinical sample analysis, therefore, a reaction time of 35 min was recommended including the reverse transcription process (10 min). Thus, the whole diagnosis procedure of COVID-19 RT-MCDA-BS technique, including sample collection (3 min), rapid template preparation (15 min), RT-MCDA reaction (35 min) and result reporting (< 2 min), can be completed within 1 h (**Figure 5**).

Specificity of COVID-19 RT-MCDA-BS assay

COVID-2019 RT-MCDA-BS assay specifically detected the positive control (containing 5×10^3 copies of each F1ab- and N-plasmids), while other target templates from non-COVID-19 virus, bacteria and fungi were not detected (**Table S2**). This suggested that the COVID-2019 RT-MCDA-BS assay has a very good specificity.

Evaluation of COVID-19 RT-MCDA-BS assay in clinical samples

A total of 65 RNA samples from the acute phase and convalescent patients, which were initially detected using rRT-qPCR in Guizhou province CDC in 2020, were tested to verify the feasibility of our assay. Our COVID-19 RT-MCDA-BS assay detected 22 (33.8%) positive results from different types of clinical specimens (e.g., face, nasal, anal and pharyngeal swabs) while rRT-PCR only found 20 (30.7%) positive results (**Table S3**).. The data suggested our COVID-19 RT-MCDA-BS assay is more powerful in detecting COVID-19 patients, especially for those with very low virus loads. Furthermore, no positive results were obtained from these samples

collected from non-COVID-19 patients (Table S4).

Discussion

With the unexpected occurrence of SARS-CoV-2 infection in Wuhan, the novel human coronavirus has since spread within China and other regions/countries [14]. Hence, it is vital to develop adequate diagnosis techniques, which can provide a simple, rapid, reliable and easy-to-use strategy to detecting SARS-CoV-2 infection. Such diagnosis methods are required not only in countries where COVID-19 are spreading but also in countries threatened by COVID-19. To tackle the pandemic efficiently, we successfully devised a novel method for diagnosis of COVID-19, termed COVID-19 RT-MCDA-BS assay.

COVID-19 RT-MCDA-BS assay merges reverse transcription, cDNA isothermal amplification, multiplex detection with nano-biosensor achieving the rapid diagnosis of COVID-19 in a one-step and single-tube reaction. An extremely simple instrument, such as a heating block, a water bath or even a thermos cup which can maintain a fixed temperature (64^{o} C) for 30 min, is sufficient for COVID-19 RT-MCDA-BS test. Importantly, the biosensor provides an easy-to-use platform, which could objectively and visually indicate the COVID-19 RT-MCDA results, and eliminate the use of special colorimetric indicator such as electrophoresis and optical equipment. The whole diagnosis process of COVID-19 RT-MCDA-BS assay- including clinical sample collection (3 min), rapid template preparation (15 min), isothermal amplification (35 min) and result reporting (within 2 min)-can be completed within 1 h. Herein, COVID-19 RT-MCDA-BS is a economical, rapid and technically simple method, which provides a measurement of practicality for 'on-site', field and clinical settings, especially for economically impoverished regions.

F1ab-MCDA and N-MCDA primer sets were designed targeting ten regions of F1ab and N gene, respectively, ensuring the high selectivity for COVID-19 diagnosis. The specificity analysis demonstrated that COVID-19 RT-MCDA-BS could correctly diagnose the target pathogens with no false positive results observed from non-SARS-CoV-2 templates, including bacterial, fungi and viral genomic templates

(**Table S2**). Furthermore, COVID-19 RT-MCDA-BS assay could simultaneously detects two target genes (F1ab and N) in a on-step isothermal reaction, which ensures the reliability for COVID-19 diagnosis, eliminating the chance getting false-positive or false-negative results compared to other COVID-19 diagnosis methods that only detects a molecular marker (e.g., F1ab biomarker).

A detection limit analysis demonstrated that COVID-19 RT-MCDA-BS is sensitive for the reliable detection of target sequences. The sensitivity of COVID-19 RT-MCDA-BS is very high with pure plasmid template down to 5 copies (each of F1ab-plasimd or N-plasmid) per reaction, which was completely accordance with the detection results obtained from F1ab-RT-MCDA-BS and N-RT-MCDA-BS assays (**Figure 4**, **S4** and **S5**). Compared to the singlex F1ab-RT-MCDA-BS and N-RT-MCDA-BS assays, COVID-19 RT-MCDA-BS did not show decreased or improved analytical sensitivity.

A total 65 RNA samples isolated form face, nasal, anal and pharyngeal swabs of the COVID-19 patients were examined to demonstrate the assay's feasibility in clinic. The data suggested that COVID-19 RT-MCDA-BS assay was able to analyze different types of clinical samples (**Table S3**). There were 22 RNA samples tested positive by COVID-19 RT-MCDA-BS assay, whereas only 20 RNA samples shown positive by rRT-PCR in Guizhou province CDC. The findings suggested our COVID-19 RT-MCDA-BS assay is more powerful in detecting COVID-19 patients, especially for those with very low virus loads (Table S3). The lower diagnosis rate of rRT-PCR might be reasoned by the presence of inhibitors which specifically affect to the rRT-PCR assay or the low copy numbers of the SARS-CoV-2 RNA templates which is out of rRT-PCR assay's detection limit. Moreover, isothermal amplification-based assays, including MCDA-based methods (e.g., RT-MCDA-BS test), was less sensitive to various inhibitors, or was less affected by the presence of various salts from sample buffer, or could tolerate the inhibitory effect of the large amounts of nucleic acids [15]. Particularly, no positive results were obtained from non-COVID-19 samples, which further validated the analytical specificity of COVID-19 RT-MCDA-BS assay.

Conclusion

We devised a method (COVID-19 RT-MCDA-BS) to diagnose COVID-19 using reverse transcription, multiplex detection and isothermal amplification coupled with a nanoparticles-based biosensor. Our data demonstrated that COVID-19 RT-MCDA-BS was a highly specific and sensitive diagnostic assay, and could be used as an attractive laboratory tool for diagnosis of COVID-19 in different types of clinical specimens. The diagnosis test of COVID-19 RT-MCDA-BS could be finished within 1h, and did not rely on expensive reagents and apparatus. Collectively, its rapidity, low cost and ease-to-use characteristics make the COVID-19 RT-MCDA-BS method an ideal tool for use in field, primary and clinical laboratories, especially for resource-poor settings.

Contributors

Yi Wang and Shijun Li conceived and designed this study. Shijun Li, Weijia Jiang, Junfei Huang, Ying Liu, Lijun Ren, Li ZHuang, Qinni Zheng, Ming Wang, Rui Yang, Yi Zeng and Yi Wang performed the experiments. Shijun Li, Weijia Jiang, Junfei Huang and Yi Wang analyze the data. Shijun Li, Weijia Jiang, Junfei Huang, Ying Liu, Lijun Ren, Li ZHuang, Qinni Zheng, Ming Wang, Rui Yang and Yi Zeng contributed the reagents and analysis tools. Shijun Li, Weijia Jiang, Junfei Huang, Lijun Ren, Li ZHuang, Qinni Zheng, Ming Wang, Rui Yang and Yi Zeng contributed the materials. Yi Wang conducted the software. Shijung Li and Yi Wang drafted the manuscript. Yi Wang revised the manuscript.

Disclosure

The authors report no conflicts of interest in this work.

Acknowledgments

This study was funded by Qian Ke He Support Plan [2019]-2822, by Qian Ke He Platform talent [2018]-5606, by Qian Ke He [2016]-4021 and by Qian Ke He Platform [2018]-5767 from Science and Technology Department of Guizhou Province. We would like to thank Chao Yang (HSS research Institute, Hospital for Special Surgery, Weill Cornell Medicine, New York) for linguistic assistance during the revision of this manuscript.

Abbreviations:

RT: reverse transcription

MCDA: multiple cross displacement

BS: nanoparticles-based biosensor

CDC: Center for Disease Control and Prevention

CoV: coronaviruses

PCR: polymerase china reaction

WHO: World Health Organization

Dig: digoxigenin

FITC: fluorescein

BSA: bovine serum albumin

CL: control line

TL: test line

SA: streptavidin

DNPs: eye streptavidin coated polymer nanoparticles

F1ab: open reading frame 1a/b

N: nucleoprotein gene

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Figure legend

Figure 1. **Mechanistic description of the COVID-19 RT-MCDA-BS assay**

(A), Preparing the COVID-19 RT-MCDA reaction mixtures. **(B)**, One-step, single-tube reverse transcription MCDA reaction. **(C)**, The detectable COVID-19 RT-MCDA products were formed.

Figure 2. **Schematic illustration of the principle of BS for visualization of COVID-19 RT-MCDA products**

(**A**), The details of BS. (**B**), The test principle and steps of BS for COVID-19 RT-MCDA amplicons. (**C**), Interpretation of the detection results. I, negative (only the control line appears on the BS); II, a positive result for F1ab (TL1 and CL appear on the detection region); III, a positive result for N (TL2 and CL appear on the detection region); IV, a positive result for F1ab and N (TL1, TL2 and CL appear on the BS).

Figure 3. **Primer design of COVID-19 RT-MCDA-BS assay**

Up row, COVID-19 genome organization (GenBank: MN908947, Wuhan-Hu-1), and the length of all genes is not drawn in scale. F1ab (Open reading frame 1a/b); S (Spike protein); E (Envelope protein); M (Membrane protein); N (Nucleoprotein); Accessory proteins (3, 6, 7a, 7b, and 9b). **Bottom row**, nucleotide sequence and location of F1ab and N gene used to design COVID-19 RT-MCDA primers. Part of nucleotide sequences of F1ab (**Left**) and N (**Right**) are shown. The sites of primer sequence were underline. Right arrows and Left arrows showed the sense and complementary sequence that are used.

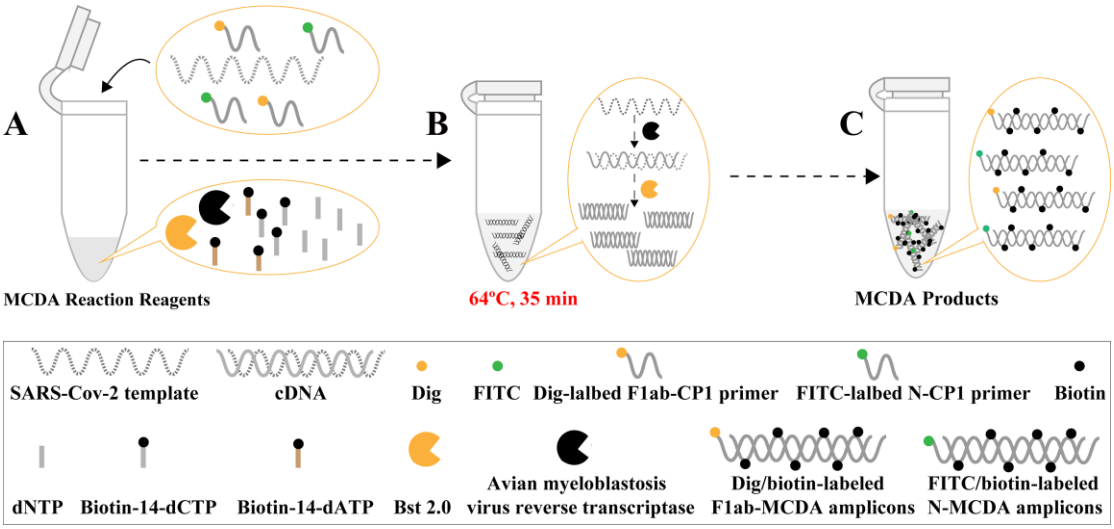
Figure 4. **Sensitivity of COVID-19 RT-MCDA-BS assay using serially diluted plasmid templates**

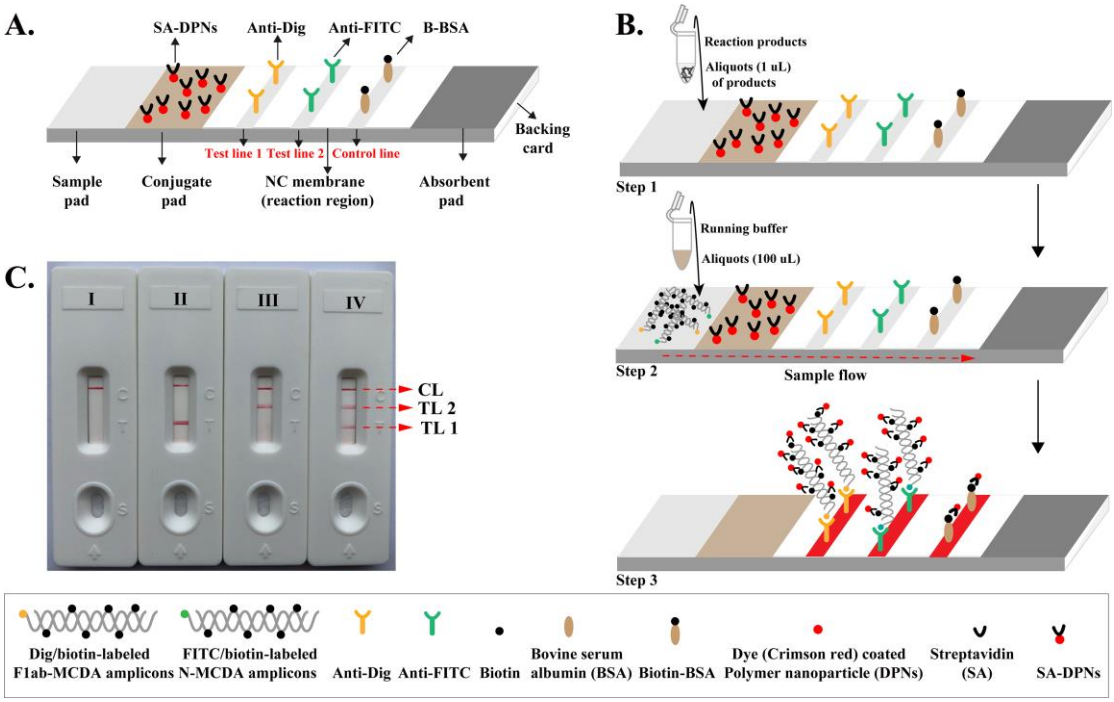
A, BS applied for reporting the results; **B**, Real-time turbidity applied for reporting the results; **C**, VDR applied for reporting the results. BS (A)/Signals (B)/Tubes (C) 1-8 represented the plasmid levels (each of F1ab- and N-plasmids) of $5x10^4$, $5x10^3$,

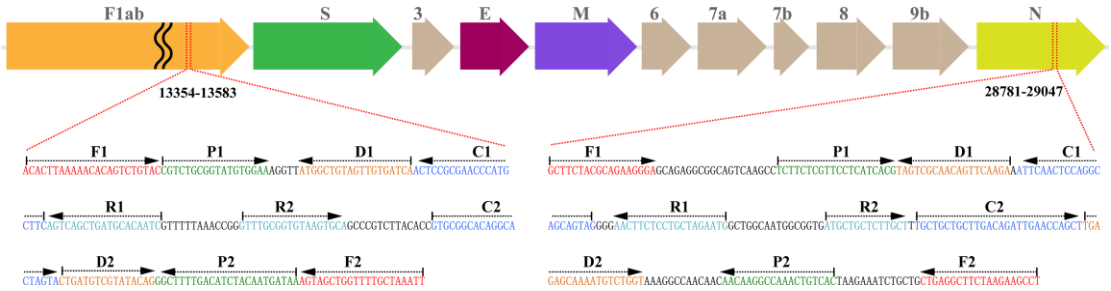
$5x10^2$, $5x10^1$, $5x10^0$, $5x10^{-1}$, $5x10^{-2}$ copies per reaction and blank control (DW). The plasmid levels of $5x10^4$ to $5x10^0$ copies per reaction produced the positive reactions.

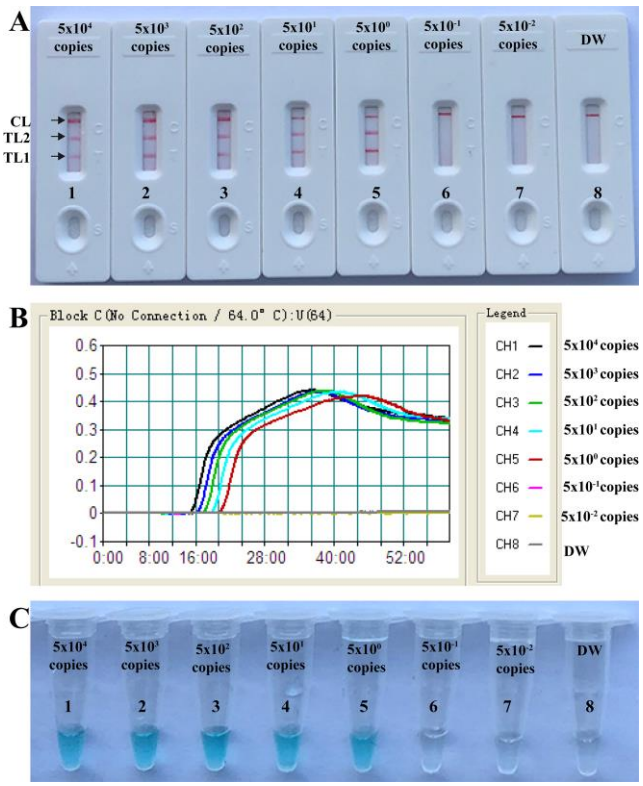
Figure 5. **The workflow of COVID-19 RT-MCDA-BS assay**

A total of four steps, including sample collection (3 min), rapid template preparation (15 min), RT-MCDA reaction (35 min) and result reporting (within 2 min), were required for the COVID-19 RT-MCDA-BS assay, and the total procedure could be completed within 60 min.


A
B
C
MCDA Reaction Reagents
64°C, 35 min
MCDA Products
SARS-Cov-2 template
cDNA
Dig
FITC
Dig-lalbed F1ab-CP1 primer
FITC-lalbed N-CP1 primer
Biotin
dNTP
Biotin-14-dCTP
Biotin-14-dATP
Bst 2.0
Avian myeloblastosis virus reverse transcriptase
Dig/biotin-labeled F1ab-MCDA amplicons
FITC/biotin-labeled N-MCDA amplicons


A.
SA-DPNs
Anti-Dig
Anti-FITC
B-BSA
Backing card
Test line 1
Test line 2
Control line
Sample pad
Conjugate pad
NC membrane (reaction region)
Absorbent pad
B.
Reaction products
Aliquots (1 uL) of products
Step 1
Running buffer
Aliquots (100 uL)
Step 2
Sample flow
Step 3
C.
I
II
III
IV
CL
TL 2
TL 1
Dig/biotin-labeled F1ab-MCDA amplicons
FITC/biotin-labeled N-MCDA amplicons
Anti-Dig
Anti-FITC
Biotin
Bovine serum albumin (BSA)
Biotin-BSA
Dye (Crimson red) coated Polymer nanoparticle (DPNs)
Streptavidin (SA)
SA-DPNs


F1ab
S
3
E
M
6
7a
7b
8
9b
N
13354-13583
28781-29047
F1
P1
D1
C1
ACACTTAAAAACACAGTCTGTACCGTCTGCGGTATGTGGAAAGGTTATGGCTGTAGTTGTGATCAACTCCGCGAACCCATG
R1
R2
C2
CTTCAGTCAGCTGATGCACAATCGTTTTTAAACCGGGTTTGCGGTGTAAGTGCAGCCCGTCTTACACCGTGCGGCACAGGCA
D2
P2
F2
CTAGTACTGATGTCGTATACAGGGCTTTTGACATCTACAATGATAAAGTAGCTGGTTTGCTAAATT
F1
P1
D1
C1
GCTTCTACGCAGAAGGGAGCAGAGGCGGCAGTCAAGCCTCTTCTCGTTCCTCATCACGTAGTCGCAACAGTTCAAGAAATTCAACTCCAGGC
R1
R2
C2
AGCAGTAGGGGAACTTCTCCTGCTAGAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAGATTGAACCAGCTTGA
D2
P2
F2
GAGCAAAATGTCTGGTAAAGGCCAACAACAACAAGGCCAAACTGTCACTAAGAAATCTGCTGCTGAGGCTTCTAAGAAGCCT


A
5x10⁴ copies
5x10³ copies
5x10² copies
5x10¹ copies
5x10⁰ copies
5x10⁻¹ copies
5x10⁻² copies
DW
CL
TL2
TL1
1
2
3
4
5
6
7
8
B
Block C(No Connection / 64.0° C):U(64)
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
0:00
8:00
16:00
28:00
40:00
52:00
Legend
CH1
CH2
CH3
CH4
CH5
CH6
CH7
CH8
5x10⁴ copies
5x10³ copies
5x10² copies
5x10¹ copies
5x10⁰ copies
5x10⁻¹ copies
5x10⁻² copies
DW
C
5x10⁴ copies
5x10³ copies
5x10² copies
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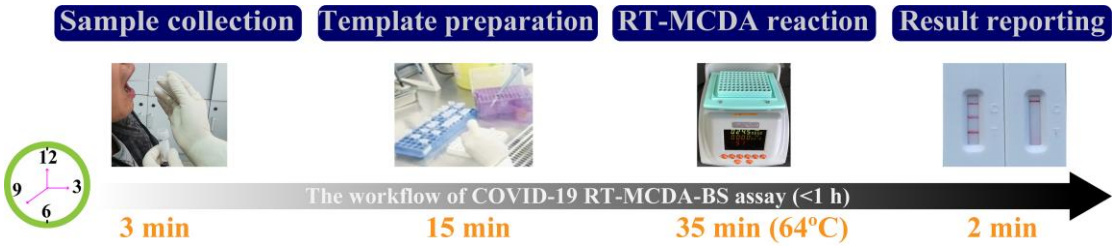
5x10⁻¹ copies
5x10⁻² copies
DW
1
2
3
4
5
6
7
8


Sample collection
Template preparation
RT-MCDA reaction
Result reporting
The workflow of COVID-19 RT-MCDA-BS assay (<1 h)
3 min
15 min
35 min (64°C)
2 min

Supplementary Materials

Highly sensitive and specific diagnosis of coronavirus disease 19 (COVID-19) by reverse transcription multiple cross displacement amplification-labeled nanoparticles biosensor

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Table S

Table S1. The primers used in this study

Primers name [a]	Sequences and modifications [b]	Length [c]	Genes
F1ab-F1	5'-ACACTTAAAAACACAGTCTGTAC-3'	23 nt	
F1ab-F2	5'-AATTTAGCAAAACCAGCTACT-3'	21 nt	
F1ab -CP1	5'-GAAGCATGGGTTCGCGGAGTCGTCTGCGGTATGTGGAA-3'	38 mer	
F1ab -CP1*	5'-Dig-GAAGCATGGGTTCGCGGAGTCGTCTGCGGTATGTGGAA -3'	38 mer	
F1ab -CP2	5'-GTGCGGCACAGGCACTAGTATTATCATTGTAGATGTCAAAAGCC -3'	44 mer	
F1ab -C1	5'-GAAGCATGGGTTCGCGGAGT-3'	20 nt	F1ab
F1ab -C2	5'-GTGCGGCACAGGCACTAGTA-3'	20 nt	
F1ab -D1	5'-TGATCACAACTACAGCCAT-3'	19 nt	
F1ab -D2	5'-CTGATGTCGTATACAG-3'	16 nt	
F1ab -R1	5'-GATTGTGCATCAGCTGACT-3'	19 nt	
F1ab -R2	5'-GTTTGCGGTGTAAGTGCA-3'	18 nt	
N-F1	5'-GCTTCTACGCAGAAGGGA-3'	18 nt	
N-F2	5'-AGGCTTCTTAGAAGCCTCAG-3'	20 nt	
N-CP1	5'-CTACTGCTGCCTGGAGTTGAATTCTTCTCGTTCCTCATCACG-3'	42 mer	
N-CP1*	5'-FITC-CTACTGCTGCCTGGAGTTGAATTCTTCTCGTTCCTCATCACG-3'	42 mer	
N-CP2	5'-TGCTTGACAGATTGAACCAGCTGTGACAGTTTGGCCTTGTT-3'	41 mer	
N-C1	5'-CTACTGCTGCCTGGAGTTGAAT-3'	22 nt	N
N-C2	5'-TGCTTGACAGATTGAACCAGCT-3'	22 nt	
N-D1	5'-TCTTGAACTGTTGCGACTA-3'	19 nt	
N-D2	5'-TGAGAGCAAAATGTCTGGT-3'	19 nt	
N-R1	5'-CATTCTAGCAGGAGAAGTT-3'	19 nt	
N-R2	5'-ATGCTGCTCTTGCTTTGCT-3'	19 nt	

[a] F1ab, open reading frame 1a/b; N, nucleoprotein gene; F1ab-CP1*, 5'-labeled with Dig when used in RT-MCDA-BS assay; N-CP1*, 5'-labeled with FITC when used in RT-MCDA-BS assay.

[b] Dig, digoxigenin; FITC, fluorescein isothiocyanate.

[c] mer, monomeric unit; nt, nucleitide.

Table S2. The pathogens used in this study

Strains	Serotypes (subtypes)	No. of strains	RT-LAMP-NBS
Positive control	Unidentified	1	P
Coronavirus	HKU1	1	N
Infuenza Virus A	H1N1	2	N
Infuenza Virus A	H1N2	1	N
Infuenza Virus A	H5	9	N
Infuenza Virus A	H5N8	1	N
Infuenza Virus A	H4N6	1	N
Infuenza Virus A	H7N9	1	N
Infuenza Virus B	Unidentified	3	N
Syncytial Virus	Unidentified	1	N
Human Adenovirus	Unidentified	2	N
Parainfluenza Virus	Unidentified	1	N
Bocavirus	Unidentified	1	N
Coxsackievirus	CAV16	1	N
Human enterovirus	EV71	1	N
Porcine circovirus	II	1	N
Swine fever virus	Unidentified	2	N
Lelysted Virus	Unidentified	2	N
Mycoplasma	Unidentified	1	N
Chlamydia	Unidentified	1	N
Lpneumophila	Unidentified	1	N
Pseudomonas aeruginosa	Unidentified	1	N
Staphylococcus aureus	Unidentified	1	N
Staphylococcus epidermidis	Unidentified	1	N
Staphylococcus saprophytics	Unidentified	1	N
Klebsiella pneumoniae	Unidentified	1	N
Neisseria meningitidis	Unidentified	1	N
Acinetobacter baumannii	Unidentified	1	N
Enterococcus faecalis	Unidentified	1	N
Enterococcus faecium	Unidentified	1	N
Shigella flexneria	Unidentified	1	N
Listeria monocytogenes	Unidentified	1	N
Staphylococcus suis	Unidentified	1	N
Bacillus cereus	Unidentified	1	N
Enterotoxigenic E. coli	Unidentified	1	N
Salmonella	Unidentified	1	N
Streptococcus pneumona	Unidentified	1	N
Vibrio parahemolyticus	Unidentified	1	N
Candida tropicalis	Unidentified	1	N

Cryptoccus neo formas	Unidentified	1	N
Candida albicans	Unidentified	1	N

Notes: Only positive control ($5x10^3$ copies each of F1ab-plasmid and np-plsmid) could be detected by the COVID-19 RT-MCDA-BS technique, indicating the extremely high selectivity of this method.

Table S3. Detection results of MCDA-BS for different types of samples

Sample number [a]	Sample type	Result of RT-PCR [b]	Ct value of RT-PCR [c]	Judgment result of MCDA-BS	Results of MCDA-BS		Patient status
					F1ab	N	
S1	Face	Positive	34.3	Positive	+	+	Convalescent
S2	Pharyngeal swab	Positive	36.6	Positive	+	+	Convalescent
S3	Nasal swab	Negative	—	Negative	—	—	Convalescent
S4	Face	Positive	35.4	Positive	+	_	Convalescent
S5	Face	Positive	33.7	Positive	+	+	Convalescent
S6	Face	Negative	—	Negative	—	—	Convalescent
S7	Face	Negative	—	Negative	—	—	Convalescent
S8	Face	Positive	36.2	Positive	+	—	Convalescent
S9	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S10	Nasal swab	Negative	—	Negative	—	—	Convalescent
S11	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S12	Anal swab	Negative	—	Negative	—	—	Convalescent
S13	Anal swab	Negative	—	Negative	—	—	Convalescent
S14	Face	Negative	—	Negative	—	—	Convalescent
S15	Face	Negative	—	Negative	—	—	Convalescent
S16	Face	Negative	—	Negative	—	—	Convalescent
S17	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S18	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S19	Face	Negative	—	Negative	—	—	Convalescent
S20	Anal swab	Negative	41.8	Positive	+	+	Convalescent
S21	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S22	Anal swab	Negative	—	Negative	—	—	Convalescent
S23	Anal swab	Negative	—	Negative	—	—	Convalescent
S24	Anal swab	Negative	41.9	Positive	—	+	Convalescent
S25	Nasal swab	Negative	—	Negative	—	—	Convalescent
S26	Nasal swab	Negative	—	Negative	—	—	Convalescent
S27	Pharyngeal swab	Positive	35.2	Positive	+	+	Convalescent
S28	Pharyngeal swab	Positive	34.3	Positive	+	+	Convalescent
S29	Nasal swab	Negative	—	Negative	—	—	Convalescent
S30	Anal swab	Negative	—	Negative	—	—	Convalescent
S31	Nasal swab	Negative	—	Negative	—	—	Convalescent
S32	Anal swab	Negative	—	Negative	—	—	Convalescent
S33	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S34	Anal swab	Negative	—	Negative	—	—	Convalescent
S35	Nasal swab	Negative	—	Negative	—	—	Convalescent
S36	Anal swab	Negative	—	Negative	—	—	Convalescent
S37	Nasal swab	Negative	—	Negative	—	—	Convalescent
S38	Anal swab	Negative	—	Negative	—	—	Convalescent

S39	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S40	Anal swab	Negative	—	Negative	—	—	Convalescent
S41	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S42	Anal swab	Negative	—	Negative	—	—	Convalescent
S43	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S44	Anal swab	Negative	—	Negative	—	—	Convalescent
S45	Nasal swab	Negative	—	Negative	—	—	Convalescent
S46	Anal swab	Negative	—	Negative	—	—	Convalescent
S47	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S48	Anal swab	Positive	33.3	Positive	+	+	Convalescent
S49	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S50	Anal swab	Positive	34.5	Positive	+	+	Convalescent
S51	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S52	Anal swab	Negative	—	Negative	—	—	Convalescent
S53	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S54	Pharyngeal swab	Negative	—	Negative	—	—	Convalescent
S55	Pharyngeal swab	Positive	30.2	Positive	+	+	Acute phase
S56	Pharyngeal swab	Positive	32.4	Positive	+	—	Acute phase
S57	Pharyngeal swab	Positive	29.4	Positive	+	+	Acute phase
S58*	Pharyngeal swab	Positive	35.9	Positive	+	+	Acute phase
S59	Pharyngeal swab	Positive	25.4	Positive	+	+	Acute phase
S60	Pharyngeal swab	Positive	26.6	Positive	+	+	Acute phase
S61	Pharyngeal swab	Positive	29.3	Positive	+	+	Acute phase
S62	Pharyngeal swab	Positive	31.3	Positive	+	—	Acute phase
S63	Pharyngeal swab	Positive	28.4	Positive	+	+	Acute phase
S64	Pharyngeal swab	Positive	29.6	Positive	+	+	Acute phase
S65	Pharyngeal swab	Positive	30	Positive	+	+	Acute phase

[a] S58: this sample was collected from a asymptomatic individual.
[b] Positive: Ct <37; Negative: Ct>40 or no Ct.
[c] '—': no Ct.

Table S4. Detection results of COVID-19 RT-MCDA-BS method for non-COVID-19 samples

Sample number	Sample type	Result of COVID-19 RT-PCR	Result of COVID-19 RT-MCDA-BS	Confirmed pathogens*
S1	Pharyngeal swab	—	—	Influenza A virus
S2	Pharyngeal swab	—	—	Influenza A virus
S3	Pharyngeal swab	—	—	Influenza A virus
S4	Pharyngeal swab	—	—	Influenza A virus
S5	Pharyngeal swab	—	—	Influenza A virus
S6	Pharyngeal swab	—	—	Influenza A virus
S7	Pharyngeal swab	—	—	Influenza A virus
S8	Pharyngeal swab	—	—	Influenza A virus
S9	Pharyngeal swab	—	—	Influenza A virus
S10	Pharyngeal swab	—	—	Influenza A virus
S11	Pharyngeal swab	—	—	Influenza A virus
S12	Pharyngeal swab	—	—	Influenza A virus
S13	Pharyngeal swab	—	—	Influenza A virus
S14	Pharyngeal swab	—	—	Influenza A virus
S15	Pharyngeal swab	—	—	Influenza A virus
S16	Pharyngeal swab	—	—	Influenza A virus
S17	Pharyngeal swab	—	—	Influenza A virus
S18	Pharyngeal swab	—	—	Others
S19	Pharyngeal swab	—	—	Others
S20	Pharyngeal swab	—	—	Others
S21	Pharyngeal swab	—	—	Others
S22	Pharyngeal swab	—	—	Others
S23	Pharyngeal swab	—	—	Others
S24	Pharyngeal swab	—	—	Others
S25	Pharyngeal swab	—	—	Others
S26	Pharyngeal swab	—	—	Others
S27	Pharyngeal swab	—	—	Others
S28	Pharyngeal swab	—	—	Others
S29	Pharyngeal swab	—	—	Others
S30	Pharyngeal swab	—	—	Others
S31	Pharyngeal swab	—	—	Others
S32	Pharyngeal swab	—	—	Others
S33	Pharyngeal swab	—	—	Others
S34	Pharyngeal swab	—	—	Others
S35	Pharyngeal swab	—	—	Others
S36	Pharyngeal swab	—	—	Others
S37	Pharyngeal swab	—	—	Others
S38	Pharyngeal swab	—	—	Others
S39	Pharyngeal swab	—	—	Others
S40	Pharyngeal swab	—	—	Others
S41	Pharyngeal swab	—	—	Others

S42	Pharyngeal swab	—	—	Others
S43	Pharyngeal swab	—	—	Others
S44	Pharyngeal swab	—	—	Others
S45	Pharyngeal swab	—	—	Others
S46	Pharyngeal swab	—	—	Others
S47	Pharyngeal swab	—	—	Others
S48	Pharyngeal swab	—	—	Others
S49	Pharyngeal swab	—	—	Others
S50	Pharyngeal swab	—	—	Others
S51	Pharyngeal swab	—	—	Others
S52	Pharyngeal swab	—	—	Others
S53	Pharyngeal swab	—	—	Others
S54	Pharyngeal swab	—	—	Others
S55	Pharyngeal swab	—	—	Others
S56	Pharyngeal swab	—	—	Others
S57	Pharyngeal swab	—	—	Others
S58	Pharyngeal swab	—	—	Others
S59	Pharyngeal swab	—	—	Others
S60	Pharyngeal swab	—	—	Others
S61	Pharyngeal swab	—	—	Others
S62	Pharyngeal swab	—	—	Others
S63	Pharyngeal swab	—	—	Others
S64	Pharyngeal swab	—	—	Others
S65	Pharyngeal swab	—	—	Others
S66	Pharyngeal swab	—	—	Others
S67	Pharyngeal swab	—	—	Others
S68	Pharyngeal swab	—	—	Others
S69	Pharyngeal swab	—	—	Others
S70	Pharyngeal swab	—	—	Others
S71	Pharyngeal swab	—	—	Others
S72	Pharyngeal swab	—	—	Others
S73	Pharyngeal swab	—	—	Others
S74	Pharyngeal swab	—	—	Others
S75	Pharyngeal swab	—	—	Others
S76	Pharyngeal swab	—	—	Others
S77	Pharyngeal swab	—	—	Others
S78	Pharyngeal swab	—	—	Others
S79	Pharyngeal swab	—	—	Others
S80	Pharyngeal swab	—	—	Others
S81	Pharyngeal swab	—	—	Others
S82	Pharyngeal swab	—	—	Others
S83	Pharyngeal swab	—	—	Others
S84	Pharyngeal swab	—	—	Others
S85	Pharyngeal swab	—	—	Others

S86	Pharyngeal swab	—	—	Others
S87	Pharyngeal swab	—	—	Others
S88	Pharyngeal swab	—	—	Others
S89	Pharyngeal swab	—	—	Others
S90	Pharyngeal swab	—	—	Others
S91	Pharyngeal swab	—	—	Others
S92	Pharyngeal swab	—	—	Others
S93	Pharyngeal swab	—	—	Others
S94	Pharyngeal swab	—	—	Others
S95	Pharyngeal swab	—	—	Others
S96	Pharyngeal swab	—	—	Others
S97	Pharyngeal swab	—	—	Others
S98	Pharyngeal swab	—	—	Others
S99	Pharyngeal swab	—	—	Others
S100	Pharyngeal swab	—	—	Others
S101	Pharyngeal swab	—	—	Others
S102	Pharyngeal swab	—	—	Others
S103	Pharyngeal swab	—	—	Others
S104	Pharyngeal swab	—	—	Others
S105	Pharyngeal swab	—	—	Others
S106	Pharyngeal swab	—	—	Others
S107	Pharyngeal swab	—	—	Others
S108	Pharyngeal swab	—	—	Others
S109	Pharyngeal swab	—	—	Others
S110	Pharyngeal swab	—	—	Others
S111	Pharyngeal swab	—	—	Others
S112	Pharyngeal swab	—	—	Others
S113	Pharyngeal swab	—	—	Others
S114	Pharyngeal swab	—	—	Others
S115	Pharyngeal swab	—	—	Others
S116	Pharyngeal swab	—	—	Others
S117	Pharyngeal swab	—	—	Others
S118	Pharyngeal swab	—	—	Others

*Note： Influenza A virus includes H1N1, H3N2, H5N1,H7 subtype et, al; Others for Parainfluenza Virus, *Mycoplasma*, *Neisseria meningitidis* et, al.

Figure legends

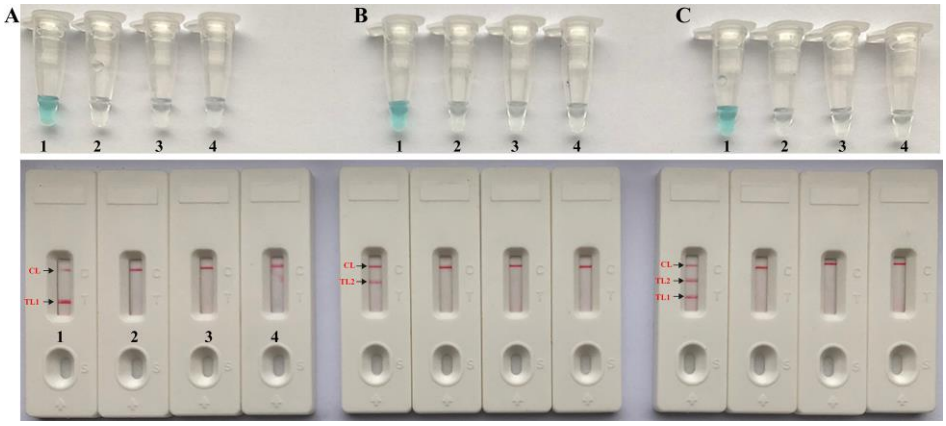

Figure S1. Confirmation and detection of F1ab- and N-RT-MCDA products

Color change of F1ab- (**A**, top row), N- (**B**, top row) and COVID-19 (**C**, top row) RT-MCDA tubes; BS applied for visual detection of F1ab- (**A**, bottom row), N- (**B**, bottom row) and COVID-19 (**C**, bottom row) RT-MCDA products. Tube A1 (BS A1), F1ab-RT-MCDA positive amplification (5×10^3 copies of F1ab-plasmids); Tube A2 (BS A2), F1ab-RT-MCDA negative amplification (H1N1); Tube A3 (BS A3), F1ab-RT-MCDA negative amplification (EV 71); Tube A4 (BS A4), Blank control (DW). Tube B1 (BS B1), N-RT-MCDA positive amplification (5×10^3 copies of N-plasmids); Tube B2 (BS B2), N-RT-MCDA negative amplification (H1N1); Tube B3 (BS B3), N-RT-MCDA negative amplification (EV 71); Tube B4 (BS B4), Blank control (DW). Tube C1 (BS C1), COVID-19 RT-MCDA positive amplification (5×10^3 copies of F1ab-plasmids and N-plasmids); Tube C2 (BS C2), COVID-19 RT-MCDA negative amplification (H1N1); Tube C3 (BS C3), COVID-19 RT-MCDA negative amplification (EV 71); Tube C4 (BS C4), Blank control (DW).

Note*: H1N1, Influenza H1N1 virus; EV 71, Human enterovirus 71; DW, Distilled water.

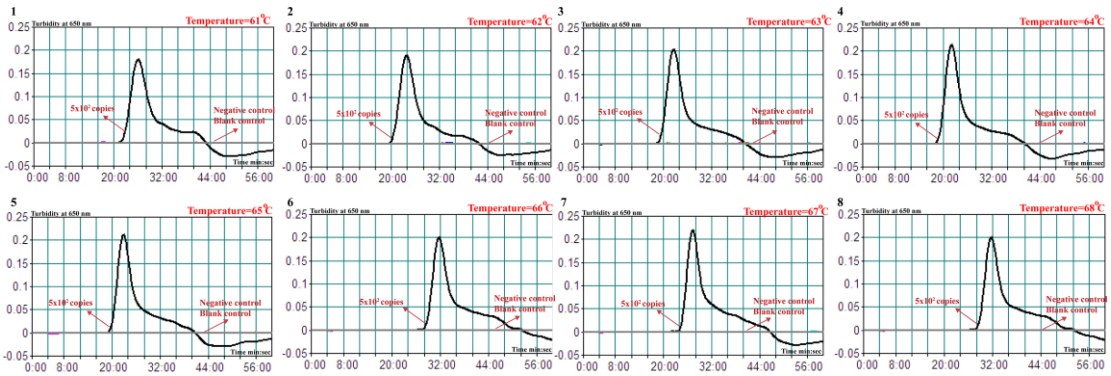

Figure S2. Optimal amplification temperature for F1ab-RT-MCDA primer set

The F1ab-RT-MCDA reactions for detection of F1ab gene of COVID-19 were monitored by real-time measurement of turbidity (LA320c). Turbidity of >0.1 was considered to be positive as the threshold value was 0.1. Eight kinetic graphs (1-8) were yielded at different temperatures (61-68°C, 1^oC intervals) with target template at the level of 5×10^2 copies (F1ab-plamid) per reaction. The graphs from 63°C to 65°C showed faster amplification.

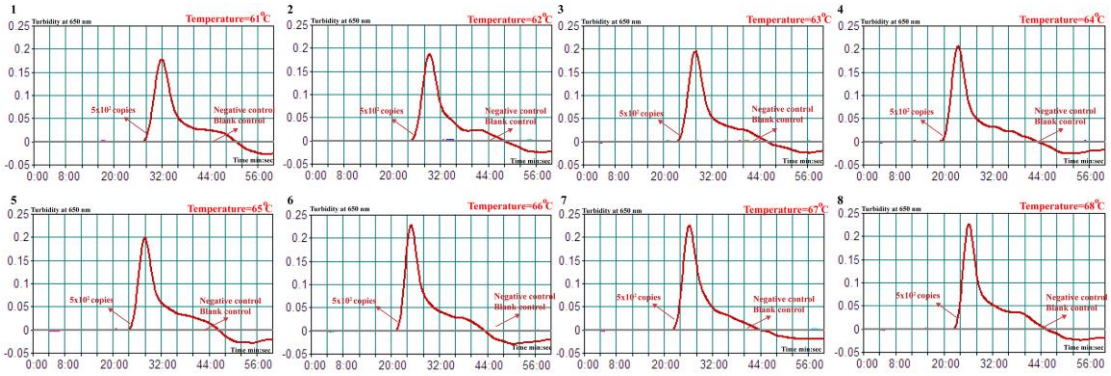

Figure S3. Optimal amplification temperature for N-RT-MCDA primer set

The N-RT-MCDA reactions for detection of N gene of COVID-19 were monitored by real-time measurement of turbidity (LA320c). Turbidity of >0.1 was considered to be positive as the threshold value was 0.1. Eight kinetic graphs (1-8) were yielded at different temperatures (61-68°C, 1°C intervals) with target template at the level of 5×10^2 copies (N-plasmid) per reaction. The graphs from 63°C to 65°C showed faster amplification.

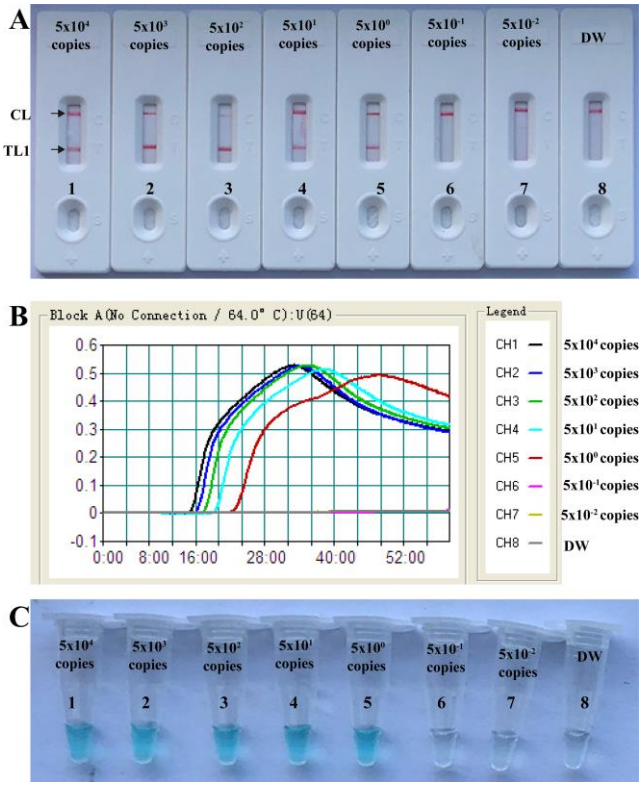

Figure S4. Analytical sensitivity of F1ab-RT-MCDA-BS assay using serially diluted F1ab-plasmid templates

A, BS applied for reporting the results; **B**, Real-time turbidity applied for reporting the results; **C**, VDR applied for reporting the results. BS (A)/Signals (B)/Tubes (C) 1-8 represented the F1ab-plasmid levels of 5×10^4, 5×10^3, 5×10^2, 5×10^1, 5×10^0, 5×10^{-1}, 5×10^{-2} copies per reaction and blank control (DW). The plasmid levels of 5×10^4 to 5×10^0 copies per reaction produced the positive reactions.

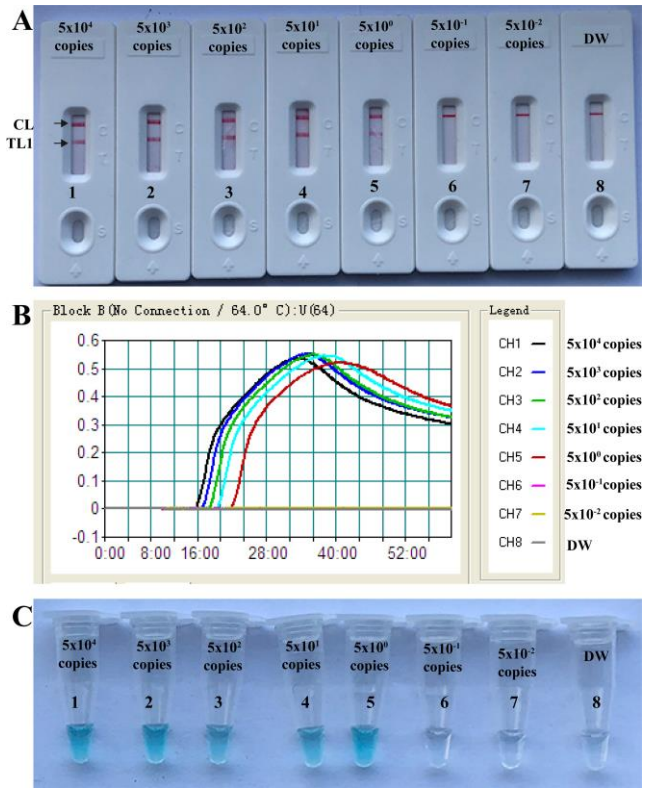

Figure S5. Analytical sensitivity of N-RT-MCDA-BS assay using serially diluted N-plasmid templates

A, BS applied for reporting the results; **B**, Real-time turbidity applied for reporting the results; **C**, VDR applied for reporting the results. BS (A)/Signals (B)/Tubes (C) 1-8 represented the N-plasmid levels of 5×10^4, 5×10^3, 5×10^2, 5×10^1, 5×10^0, 5×10^{-1}, 5×10^{-2} copies per reaction and blank control (DW). The plasmid levels of 5×10^4 to 5×10^0 copies per reaction produced the positive reactions.

Figure S6. The optimal duration of time required for COVID-19 RT-MCDA-BS assay

Four reaction times (**A**, 15 min; **B**, 25 min; **C**, 35 min; and **D**, 45 min) were tested and compared at 64 ^oC. COVID-19 RT-MCDA reactions were conducted using the LoD level of templates ($5x10^0$ copies of each of F1ab-plasmid and N-plasmid), and the templates at the LoD level could be detected when the RT-MCDA reaction only lasted for 25 min (**Biosensor 2**).