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Aligner-Mediated Cleavage-Based Isothermal Amplification for SARS-CoV-2 RNA Detection

Chao Zhang,[†] Tingting Zheng,[†] Hongliang Fan, Tao Zhang,* and Da Han*

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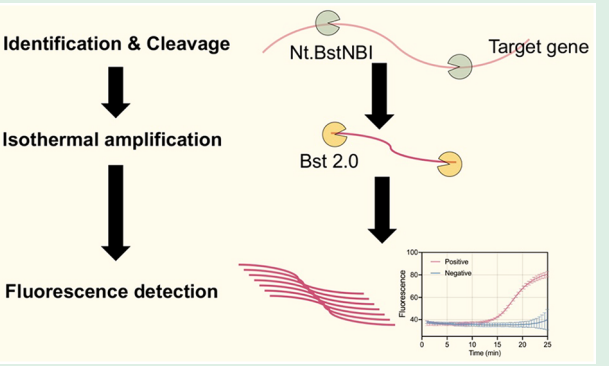
ABSTRACT: Rapid detection of SARS-CoV-2 RNA is critical for reducing the global transmission of COVID-19. Here, we report a simple and versatile assay for detection of SARS-CoV-2 RNA based on aligner-mediated cleavage-based strand displacement amplification (AMC-SDA). The entire amplification procedure takes less than 25 min without professional instruments or requirement of specific target sequences and can reach a limit of detection of attomolar RNA concentration. Using pseudovirus as mimicry of clinical SARS-CoV-2 positive samples, we achieved a diagnostic accuracy of 100% in 10 simulated samples (five positive and five negative). We anticipate that our method will provide a universal platform for rapid and accurate detection of emerging infectious diseases.



KEYWORDS: *aligner-mediated cleavage, strand displacement amplification, isothermal amplification, SARS-CoV-2, RNA detection*

1. INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a global pandemic and caused hundreds of thousands death because of its brilliant capacity for human-to-human transmission and strong pathogenicity,[1−4] which exemplify the urgent need for accurate and rapid diagnostic assays to facilitate clinical and public health interferences. In response, there are several quantitative reverse transcription-PCR (RT-qPCR) methods being applied in clinical and public health laboratories for the diagnosis of SARS-CoV-2.[5−8] However, these tests are dependent on a thermocycler for delicate temperature modulation and therefore are not widely available because of the lack of scalable technology to quickly and inexpensively identify pathogens.[9] Therefore, a rapid diagnostic method with low instrumental dependence, short processing time, and high accuracy is needed to effectively monitor the virus transmission in populations.

As an alternative technique to PCR, isothermal amplification is an important tool in the detection of nucleic acids, particularly in rapid and point-of-care diagnostics.[10−17] Among all kinds of isothermal amplification methods, the strand displacement reaction (SDA) has a simple mechanism and high amplification efficiency. SDA employs a nicking endonuclease to create a nick on one strand of a double-strand DNA, followed by sequence extension from the nicking site catalyzed by strand displacement polymerase.[18] Overall, the SDA method has been demonstrated to be able to detect trace amounts of DNA or RNA targets. However, one obstacle that prevents the universal use of traditional SDA for pathogen detection is the requirement of specific recognition sequences in the target templates for nicking endonuclease, which greatly impedes its universality in detecting different pathogen sequences such as SARS-CoV-2. Therefore, an improved SDA method that allows using conventional nicking endonuclease for versatile detection of pathogen targets with high sensitivity and specificity is still needed.

In this study, we demonstrated a simple and versatile AMC (aligner-mediated cleavage)-SDA-based strategy that enables rapid detection of SARS-CoV-2 with high specificity and sensitivity. Because of the excellent universality of AMC, the cleavage of pathogen DNA followed by primer-dependent amplification to generate recognition sites for endonuclease is no longer dependent on any specific sequence in the target template.[19] In addition, the detection sensitivity can achieve 10 copies/reaction (0.3 aM) for SARS-CoV-2 pseudovirus through a real-time fluorescence detector under isothermal conditions within 25 min, which indicates the applicability of this method for diagnosis of SARS-CoV-2 in clinics. Furthermore, our AMC-SDA platform can be applied to other pathogen detection through a simple change of primers, highlighting the universality of this detection platform.

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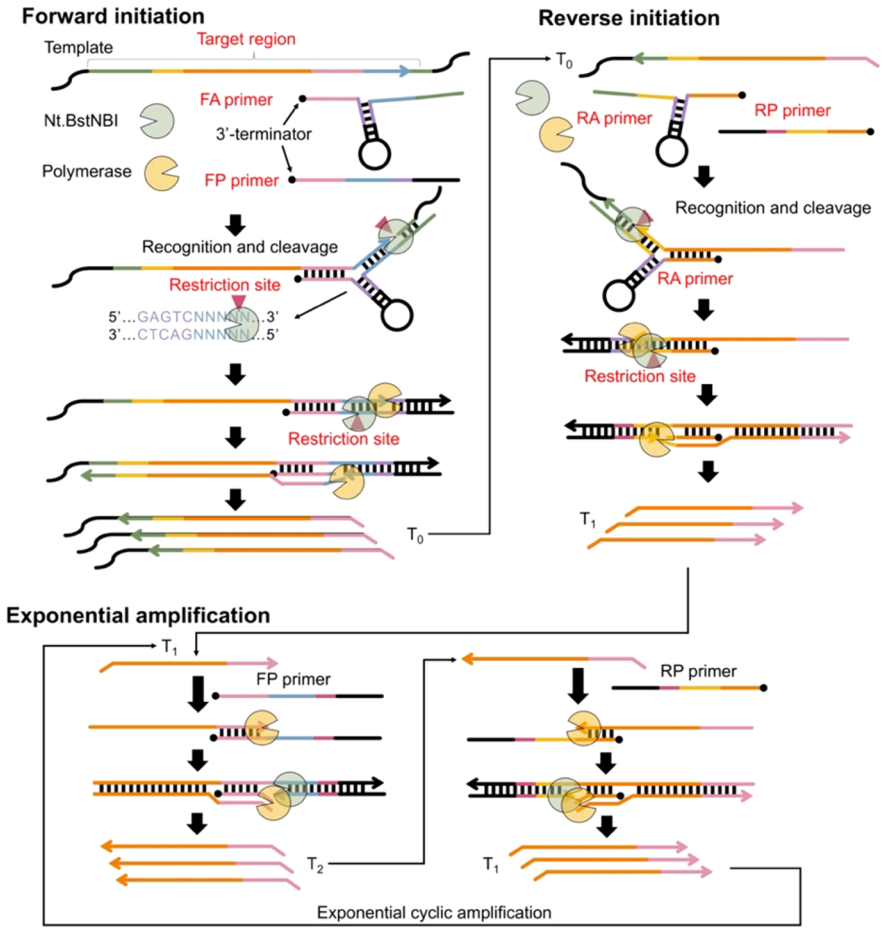

Figure 1. Schematic illustration of AMC-SDA. The forward and reverse initiation step can amplify the target template and generate the T_1 strand continuously. The exponential amplification step can repeatedly generate a large amount of T_1 and T_2 strands as amplification products.

2. EXPERIMENTAL SECTION

2.1. Preparation of Oligonucleotides. All oligonucleotides used in this study were purchased from Beijing Tsingke Biotechnology Co. LTD (Beijing, China), and the sequence details are listed in Table S1. Purified lyophilized oligonucleotides were dissolved in nuclease-free water to make a 10 μM stock solution and stored at 4 °C. The plasmid with in vitro transcribed gene fragments used in this study was resuspended at 1×10^{12} copy/mL in nuclease-free water and stored at −20 °C.

2.2. Verification of AMC. The verification of AMC was carried out by incubation of 100 nM target sequences (synthetic in vitro-transcribed SARS-CoV-2 nucleoprotein gene fragment) and 100 nM of forward aligner and forward linear primers in 1× reaction buffer (100 mM NaCl, 50 mM Tris-HCl, 4 mM $MgCl_2$, 100 μg/mL BSA, and 0.4 mM dNTP) with/without 6 U Nt.BstNBI (New England Biolabs, United States) at 55 °C for 1 h. Afterward, polyacrylamide gel electrophoresis (PAGE) was used to characterize the cleavage fragments. Five microliters of each resultant (mixed with loading buffer) were loaded onto 15% denaturing urea-PAGE gel and subjected to 100 V constant voltage in 0.5× TBE buffer for 60 min at room temperature. Finally, the gel was soaked in 3× Gelred dye solution (Sangon, China) for 15 min and then imaged on the Gel Doc EZ imager with *Image Lab* analysis software (BioRad, United States).

2.3. Isothermal Exponential Amplification Based on AMC. A fresh solution containing 6 U Nt.BstNBI, 0.8 U Bst 2.0 WarmStart DNA polymerase (New England Biolabs, United States), 1× reaction buffer with 0.5× SYBR Green I (Solarbio, China), 100 nM forward aligner (FA) and primer (FP), and 100 nM reverse aligner (RA) and primer (RP) was prepared as the stock solution. The final reaction solutions were obtained by mixing the stock solution with various concentrations of target sequences. Afterward, the reaction solutions were subjected to a Roche Cobas Z480 real-time PCR system (Roche, Switzerland) and incubation at 55 °C for 30 min to real-time monitor the fluorescence of SYBR Green I (50 uL per reaction). Finally, the amplification resultants were loaded onto a 12% denaturing urea-PAGE to verify the occurrence of reactions. After being stained with 3× Gelred dye solution, the PAGE gel was imaged on the Gel Doc EZ imager with *Image Lab* analysis software.

2.4. Analytical Specificity of the AMC-SDA Assay. The specificity of the AMC-SDA for SARS-CoV-2 RNA detection was evaluated using synthetic plasmids (20 copies/μL) from 13 common pathogenic microorganisms, including N gene fragments of five human coronaviruses (SARS-CoV, MERS-CoV, HCoV -NL63, HCoV-HKU1, and HCoV-229E), the nucleocapsid protein (NP) gene of the influenza A virus (H7N9, H5N1, HIN1, and H3N2) and influenza B virus (Victoria), and 16S rRNA of *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Streptococcus pneumoniae*.

2.5. Verification of AMC-SDA Assay for Detecting SARS-CoV-2 RNA Using Pseudovirus Samples. Pseudoviruses of SARS-CoV-2 were provided by Zeesan Biotech (Xiamen, China), product batch number 20020101. The pseudovirus was composed of an adenovirus capsid with RNA fragments containing nucleoprotein gene sequence of SARS-CoV-2 virus. The detection of SARS-CoV-2 RNA using pseudovirus samples was conducted to evaluate the feasibility of this AMC-SDA method for diagnosis of SARS-CoV-2. First, RNA was extracted from pseudovirus using the QIAamp Viral RNA Mini kit (Qiagen, USA) according to the manufacturer's instructions. The extracted RNA was reverse transcription by an Evo M-MLV kit (Accurate biotechnology, China) following the operation manual and the incubation program (37 °C for 15 min, 85 °C for 5 s, and then rapid cooling to 4 °C). Afterward, the reverse transcription DNA

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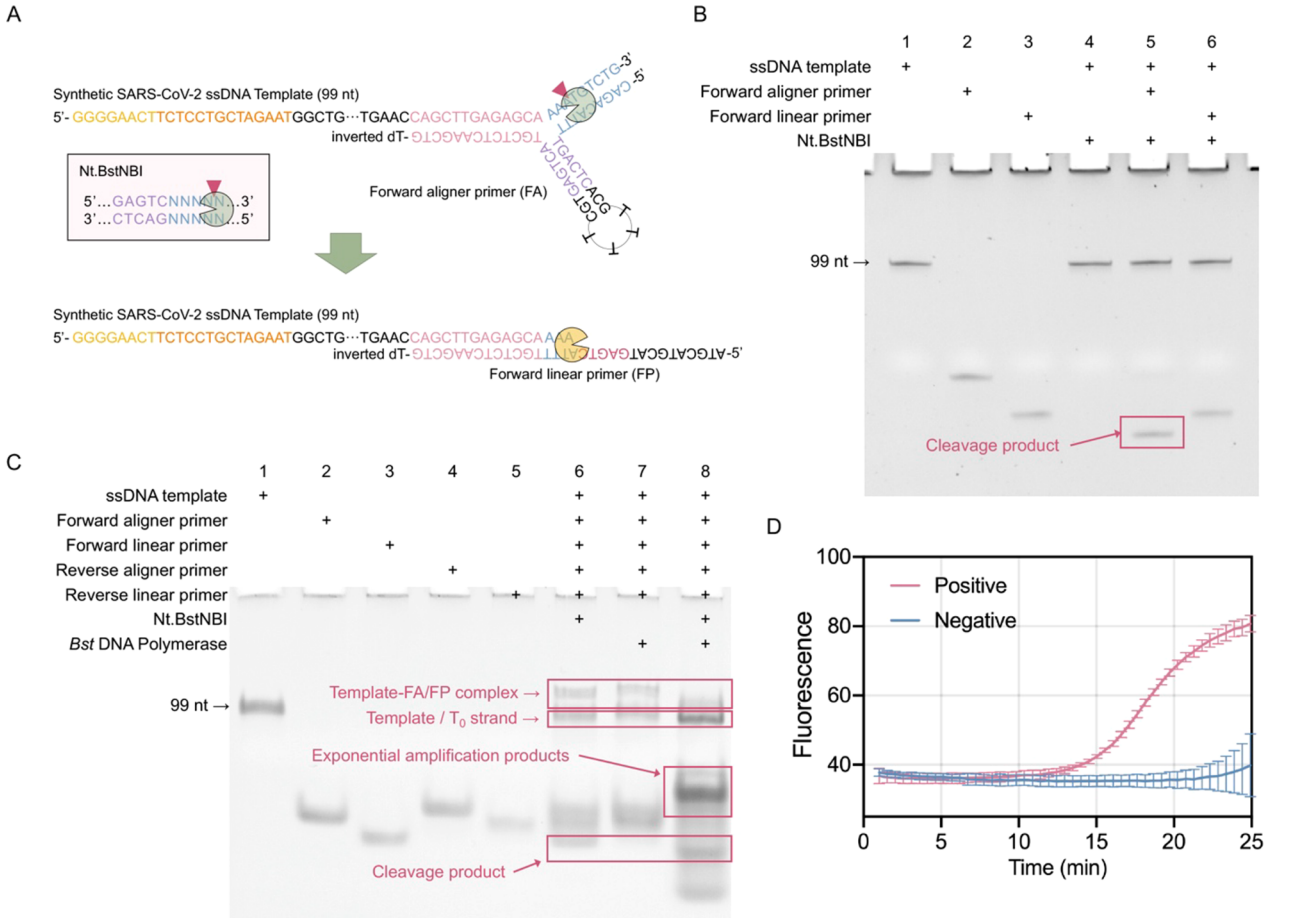

Figure 2. Verification of AMC-SDA. (A) Schematic illustration of AMC and SDA process with the assistance of FA and FP. (B) Denatured gel electrophoresis analysis of the AMC process with different combinations of reactants. (C) Denatured gel electrophoresis of SDA products followed by AMC with different combinations of reactants. (D) Fluorescent kinetics of AMC-SDA process using SYBR Green I as the indicator. The corresponding amplification products (positive and negative) are shown in the electrophoresis images.

products were subjected to AMC-SDA detection procedure described in section 2.3.

2.6. Preparation of the Healthy Clinical Samples. All healthy clinical RNA samples used in this research were isolated from swab samples from the anonymized volunteers. All samples had been analyzed using a standard RT-qPCR to confirm the absence of the SARS-CoV-2. The primers and experimental protocols for RT-qPCR were performed according to the recommended protocols published by the Chinese CDC, and the primers were synthesized by Sangon (Shanghai, China). The study was approved by the Ethics Committee at the Renji Hospital, School of Medicine, Shanghai Jiao Tong University (Shanghai, China). All methods were performed in accordance with the approved guidelines.

3. RESULTS

3.1. AMC-SDA Platform. The scheme of AMC-SDA for SARS-CoV-2 RNA detection involves three steps of forward initiation, reverse initiation, and exponential amplification, as shown in Figure 1. First, the target DNA template hybridizes with a forward aligner (FA) that contains the recognition sites for nicking endonuclease of Nt.BstNBI and is subsequently cleaved four bases downstream of the recognition site. After cleavage, the fragment dehybridizes with FA because the cleaved sequence is not adequate to preserve a stable Y-shaped structure under the reaction conditions. Afterward, a linear primer (FP) containing the recognition sequence of Nt.BstNBI can initiate polymerase-based elongation to generate dsDNA with a new recognition site for Nt.BstNBI. In this way, a new round of cleavage can free the FP binding site again and initiate another round of polymerase-based elongation for the synthesis of the antisense chain (T_0) of target template. Similarly, a reverse initiation step uses T_0 as the template and reverse aligner/linear primer (RA/RP) as primers to generate the amplification product strand (T_1) with the help of Nt.BstNBI and DNA polymerase. It should be noted that we artificially applied a 3-terminator (e.g., 3′ inverted-dT) to FP and RP to eliminate the unexpected extensions of both primers along the target sequence. The goal of the first two steps is not only to amplify the target sequence in a linearly fashion but also to identify and cut the initiation strand with high specificity. Finally, the combination of T_1 and FP resulted in the formation of T_2 strand under the joint action of Nt.BstNBI and polymerase, followed by an identical process with T_2 and RP for the regeneration of T_1. In this way, an exponential amplification can be achieved with accumulation of a large amount of T_1 and T_2 as the final amplification products.

3.2. AMC-SDA for SARS-CoV-2 RNA Detection. To demonstrate the AMC-SDA platform can be used for detecting SARS-CoV-2 RNA, we first designed primers that target the nucleoprotein (N) gene section of SARS-CoV-2 suggested by the Chinese Center for Disease Control and Prevention.[20] The primers for AMC-SDA were subsequently designed and listed in Table S1. We first examined the performance of AMC by

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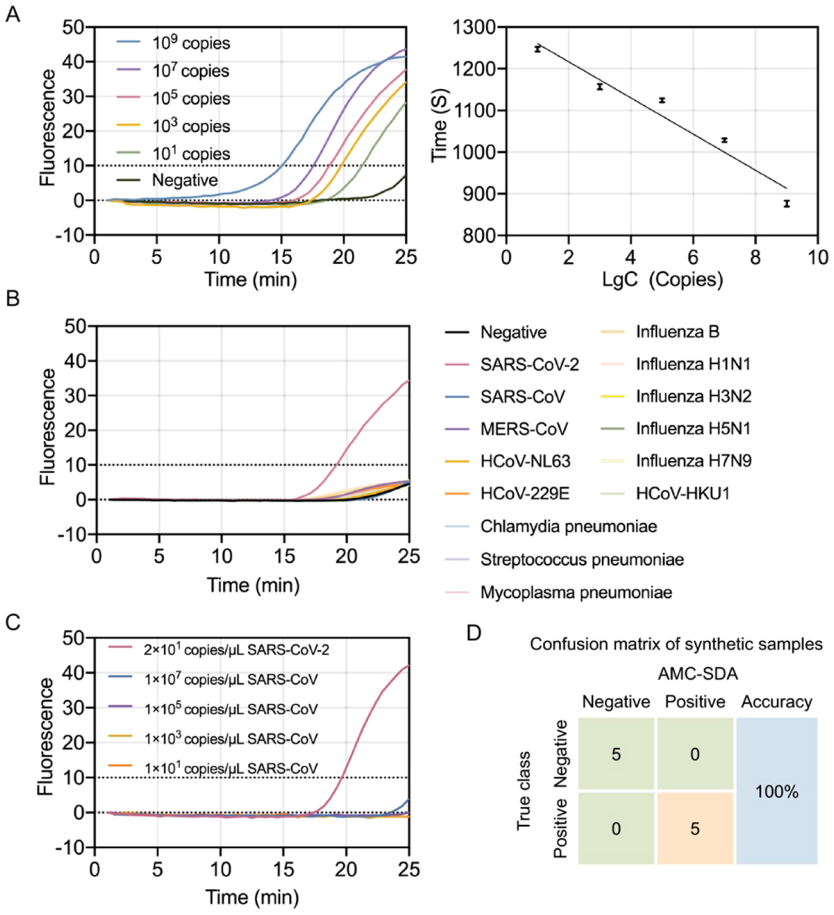

Figure 3. Performance of AMC-SDA platform and diagnostic results using synthetic samples. (A) Fluorescence kinetics (left) and standard curve (right) for the detection of different concentrations of pseudovirus templates of SARS-CoV-2 N gene (reverse transcript before use). R^2 = 0.95. Data of the standard curve are presented as mean ± s.d. (B) Diagnostic results of genes from different pathogenic microorganisms using SARS-CoV-2 primer set. The template concentrations were 20 copies/μL (33 aM) for SARS-CoV-2 pseudovirus (reverse transcript before use) and 20 copies/μL (33 aM) for synthetic plasmids of other pathogenic microorganisms. (C) Fluorescence kinetics for different concentrations of SARS plasmids and 20 copies/μL SARS-CoV-2 pseudovirus (reverse transcript before use). (D) Diagnostic results of five positive (synthetic samples with pseudovirus) and five negative samples (healthy individuals). All reactions were performed under optimal conditions.

using Nt.BstNBI that cleaved only one strand of double-stranded DNA four bases downstream of the recognition site (Figure 2A). Herein, we used a synthetic ssDNA fragment that is identical to the N gene of SARS-CoV-2 as the template. As shown in Figure 2B, a newly formed shorter band can only be found in the case of ssDNA + FA + Nt.BstNBI along with the disappearance of FA band in the electrophoresis image (Figure 2B), which indicated cleavage of the ssDNA template with Nt.BstNBI specifically and efficiently.

We next assessed the capability of AMC-SDA assay for amplification of the synthetic ssDNA template of N gene of SARS-CoV-2. As shown in Figure 2C, a clear and bright band of amplification products can only be found in the group of Template + FA + FP + RA + RP + Nt.BstNBI + Bst DNA Polymerase, which indicated that the target DNA was tailored by Nt.BstNBI exclusively and amplified by Bst DNA polymerase. We further used fluorescence to monitor the real-time reaction kinetics with SYBR Green I as the indicator. A distinct fluorescence increase was seen with the presence of 1000 copies/reaction template (~33 aM, 50 μL per reaction), whereas no obvious signal was seen without template in 25 min of the reaction window. These data confirmed that the AMC-SDA can be used for detecting target DNA with the predesigned mechanism.

3.3. Performance of AMC-SDA Platform for SARS-CoV-2 Diagnostics. Once the AMC-SDA platform have been established for target DNA detection, we further investigated the detection limit of the AMC-SDA system for SARS-CoV-2 RNA. One hundred-fold serial dilutions of the SARS-CoV-2 pseudovirus with concentrations ranged from 1×10^1 to 1×10^9 copies/reaction were first reverse transcripted to DNA and then studied using the AMC-SDA platform (Figure 3A). Negative and positive results can be determined by calculating the threshold time (TT) value, which is defined as the time point when the normalized fluorescence intensity reaches the threshold (set as normalized fluorescence intensity of 10 in this study). Herein, a TT value less than 25 will be considered as a positive result, whereas a TT value more than 25 will be considered negative. We confirmed that the assay can generate obvious fluorescence signals with the concentration of SARS-CoV-2 pseudovirus down to 10^1 copies/reaction (0.33 aM, 50 μL per reaction) within 25 min. Our method showed a similar sensitivity to PCR-based assay, but a much shorter time for amplification and no need for delicate temperature modulation with thermocyclers.[5,21,22] Next, we further tested the specificity of our primer pairs for SARS-CoV-2 diagnostics. Reverse transcripted cDNA from SARS-CoV-2 pseudovirus and 13 types of synthetic plasmids from other common pathogenic

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microorganisms, including synthetic SARS-CoV, MERS-CoV, HCoV-NL63, HCoV-229E, HCoV-HKU1, *Chlamydia pneumoniae*, *Streptococcus pneumoniae*, *Mycoplasma pneumoniae*, influenza B virus, influenza H1N1, influenza H3N2, influenza H5N1, and influenza H7N9, were tested with the AMC-SDA platform under the same conditions, and no positive signals were observed for other pathogens except for SARS-CoV-2 within 25 min (Figure 3B). Finally, we further validated the specificity of our method with different concentrations of SARS plasmids as controls, and the results (Figure 3C) showed that high concentrations of SARS-CoV plasmids could not produce false positive signals. These results demonstrated that our platform can be a reliable system for diagnostics of SARS-CoV-2 with good specificity.

Finally, to evaluate if this AMC-SDA platform can be used for diagnostics of SARS-CoV-2, we further tested its performance using simulated positive samples (five positive samples created by adding pseudovirus that replicated the concentrations appearing in the real medical record.[23]). Briefly, we prepared solutions with different concentrations of pseudovirus followed by RNA extraction. The extracted RNA was then reverse transcripted to DNA and used as template for AMC-SDA. For negative samples, five swab samples collected from healthy people were also subjected to RNA extraction and reverse transcription. Of the five samples with detectable pseudovirus (Table S3), we obtained positive results for all of them using the AMC-SDA platform (Figure S1), whereas negative results were seen with all five swaps from healthy individuals (Figure 3D). The total accuracy of this method for SARS-CoV-2 diagnosis in simulated samples was 100%. This indicates that our method can achieve rapid and accurate detection of SARS-CoV-2.

4. DISCUSSION & CONCLUSIONS

Rapid and accurate testing of pathogenic microorganisms is essential for early discovery, early reporting, early quarantine, early treatment, and efficient control of the epidemic transmission.[24,25] Although many in vitro diagnostic companies are trying to develop new diagnostic kits for rapid and inexpensive detection of pathogen genes, our AMC-SDA approach provides an alternative route for rapid diagnosis while maintaining the advantages of aligner-mediated cleavage and isothermal amplification in terms of simplicity, convenience, and affordability.

However, our study also has limitations to consider. For instance, we used pseudovirus as a mimicry of the real virus for pathogen detection because of the difficulties in acquiring real clinical samples. Therefore, we cannot completely understand the performance of this AMC-SDA platform for detecting infectious individuals who are transmitting SARS-CoV-2 virus.[26,27] Moreover, our method still needs to use standard operating procedures for reverse transcription of virus RNA, which requires extra time and specialized kits, although the AMC-SDA reaction itself only takes 25 min.[28,29]

In summary, we have developed a simple and universal platform of using AMC-SDA to detect the SARS-CoV-2 virus rapidly and accurately. Given the simple probe design and rapid detection processes, AMC-SDA will be a suitable diagnostic method for emerging infectious diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsabm.0c01674.

Table S1, detailed primer sequences for the current study; Table S2, detailed sequences of synthetic pathogenic microorganism gene fragments; Table S3, details of synthetic samples; Figure S1, fluorescence diagnostic results of simulated samples using AMC-SDA (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Da Han − *Institute of Molecular Medicine (IMM), Shanghai Key Laboratory for Nucleic Acid Chemistry and Nanomedicine, State Key Laboratory of Oncogenes and Related Genes, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China;* orcid.org/0000-0002-0804-2964; Email: dahan@sjtu.edu.cn

Tao Zhang − *Research Center for Analytical Instrumentation, Institute of Cyber-Systems and Control, State Key Laboratory of Industrial Control Technology, Zhejiang University, Hangzhou 310058, China;* orcid.org/0000-0003-3782-0478; Email: zhtao@zju.edu.cn

Authors

Chao Zhang − *Institute of Molecular Medicine (IMM), Shanghai Key Laboratory for Nucleic Acid Chemistry and Nanomedicine, State Key Laboratory of Oncogenes and Related Genes, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China;* orcid.org/0000-0003-0399-1201

Tingting Zheng − *Institute of Molecular Medicine (IMM), Shanghai Key Laboratory for Nucleic Acid Chemistry and Nanomedicine, State Key Laboratory of Oncogenes and Related Genes, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200127, China*

Hongliang Fan − *Research Center for Analytical Instrumentation, Institute of Cyber-Systems and Control, State Key Laboratory of Industrial Control Technology, Zhejiang University, Hangzhou 310058, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsabm.0c01674

Author Contributions

†C.Z. and T.Z. contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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