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RESEARCH ARTICLE

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Highly sensitive and rapid detection of SARS-CoV-2 via a portable CRISPR-Cas13a-based lateral flow assay

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Abstract

To rapidly identify individuals infected with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and control the spread of coronavirus disease (COVID-19), there is an urgent need for highly sensitive on-site virus detection methods. A clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas)-based molecular diagnostic method was developed for this purpose. Here, a CRISPR system-mediated lateral flow assay (LFA) for SARS-CoV-2 was established based on multienzyme isothermal rapid amplification, CRISPR-Cas13a nuclease, and LFA. To improve the limit of detection (LoD), the crispr RNA, amplification primer, and probe were screened, in addition to concentrations of various components in the reaction system. The LoD of CRISPR detection was improved to 0.25 copy/μl in both fluorescence- and immunochromatography-based assays. To enhance the quality control of the CRISPR-based LFA method, glyceraldehyde-3-phosphate dehydrogenase was detected as a reference using a triple-line strip design in a lateral flow strip. In total, 52 COVID-19-positive and 101 COVID-19-negative clinical samples examined by reverse transcription polymerase chain reaction (RT-PCR) were tested using the CRISPR immunochromatographic detection technique. Results revealed 100% consistency, indicating the comparable effectiveness of our method to that of RT-PCR. In conclusion, this approach significantly improves the sensitivity and reliability of CRISPR-mediated LFA and provides a crucial tool for on-site detection of SARS-CoV-2.

KEYWORDS

CRISPR, lateral flow assay, SARS-CoV-2, sensitive detection

Hongbo Liu, Shuailei Chang, Sijia Chen, and Yue Du contributed equally to this study.

1 | INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection has caused the coronavirus disease (COVID-19) pandemic, leading to a huge loss of life.[1–3] As the impact of the pandemic continues, efficient and accurate diagnosis of the pathogen is crucial for the rapid identification of infected patients and control of COVID-19 transmission.[4,5] Common methods for SARS-CoV-2 detection mainly include lateral flow assay (LFA)-based antigen detection and reverse transcription polymerase chain reaction (RT-PCR)-based nucleic acid detection.[6–9] The SARS-CoV-2 antigen LFA detection method is simple and rapid, which has emerged as a tool widely used for SARS-CoV-2 field test or self-test.[10] However, its performance is unsatisfactory in terms of sensitivity and accuracy, especially for samples with low pathogen concentrations. In contrast, RT-PCR is considered the gold standard for SARS-CoV-2 detection and provides sufficient sensitivity and reliability. However, it is time consuming and relies on sophisticated experimental conditions, including complicated equipment and skilled operators. Furthermore, owing to the lack of portability, it is difficult to arrange RT-PCR experiments in remote areas or as a self-test method. Thus, it is necessary to develop a sensitive SARS-CoV-2 detection method with high portability and accuracy.[4]

Clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein (Cas) technology has been recognized as a potent gene-editing tool and was further developed as a promising molecular diagnostic method in recent years.[11,12] Since the beginning of the COVID-19 pandemic, various CRISPR-based assays have been developed to rapidly detect the virus.[13–16] Among these, the SHERLOCK platform has attracted extensive attention; this approach utilizes Cas13a nuclease to magnify the test signal and a lateral flow strip to detect the presence of SARS-CoV-2 RNA.[14] It involves simple equipment and training for operation, thus making it a convenient approach for on-site virus detection. However, previous studies have shown that the sensitivity of the lateral flow strip-based CRISPR detection technique is not equal to that of fluorescence-based detection. The limit of detection (LoD) was always found to be lower than that of the gold-standard RT-PCR method.[14–17] Importantly, the CRISPR-based lateral flow immunochromatography employed in former studies did not include a human gene target as an internal reference control, whereas the RT-PCR method commonly did.[14,18] In most cases, a human housekeeping gene was set as an internal reference control in RT-PCR methods to guarantee the quality of sample collection, storage, transfer, and nucleic acid extraction.[17] The lack of reference gene controls reduced the reliability of CRISPR-based lateral flow immunochromatography assays. Thus, the LoD and quality control should be improved to make CRISPR-based immunochromatography a reliable field detection or self-testing method.

In this study, we developed a CRISPR-based lateral flow strip and developed a CRISPR-Cas13a-based SARS-CoV-2 detection method with enhanced sensitivity and quality control. Through multiple optimizations of the reaction system, our method demonstrated a LoD level comparable to those of RT-PCR assays. A triple-line CRISPR lateral flow strip was designed to display a human-gene reference (HR) line. Our CRISPR-based lateral flow technique revealed high sensitivity in the detection of clinical samples and hence could be applied as an ideal on-site detection or self-test method in the fight against the COVID-19 pandemic.

2 | METHODS

2.1 | Reagents

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a housekeeping gene with stable transcription, and primers amplifying conserved sequence of GAPDH gene were designed as internal quality control,[19] and the 5′ end of the upstream and downstream primers were labeled with digoxin and tetramethylrhodamine (TAMRA), respectively. Primers for the SARS-CoV-2 N gene (SARS-CoV-2 reference genome: NC_045512.2) were designed in conserved region by Oligo7. crispr RNA (crRNA) was selected from SARS-CoV-2 N gene-specific conserved sequences. The 3′ and 5′ ends of the probe were labeled with fluorescein isothiocyanate (FITC) and biotin, respectively. All oligonucleotides (Supporting Information: Table S1) were synthesized by Tianyi Huiyuan Biotechnology Co. Ltd. Cas13a protein and the reaction buffer were purchased from Kexin Biotechnology Co., Ltd. Original species of the Cas13a system used in this study was *Leptotrichia wadeimade*. The recombinant Cas13a nuclease was acquired via *Escherichia coli* prokaryotic expression. The purity of these proteins is greater than 95% according to the manufactory's instruction. T7 RNA polymerase, ribonucleotide solution mix, and RNase inhibitor were purchased from Kangrun Chengye Biotechnology Co. Ltd. $MgCl_2$, taurine, and diethylpyrocarbonate (DEPC) water were purchased from Beyotime Biotechnology. All reagents to be diluted were diluted with DEPC water. An immunochromatographic strip was developed by Wondfo Biotech Co., Ltd. The national reference material (GBW(E)091099) produced by the Chinese National Institute of Metrology was used as the SARS-CoV-2 RNA standard. The nucleic acid quality control products of six human coronaviruses and two influenza viruses were produced by BDS Technology Co., Ltd.

2.2 | Apparatus and conditions

A metal bath or water bath was used to provide the appropriate temperature for the amplification and CRISPR reaction steps during CRISPR immunochromatography. CRISPR fluorescence detection was performed using a 7500 Fast System Fluorescence quantitative PCR instrument (Applied Biosystems). The C_t values of clinical specimens were measured using a CFX96 fluorescence quantitative PCR instrument (Bio-Rad Laboratories).

2.3 | Multienzyme isothermal rapid amplification (MIRA)

Ct value analysis of SARS-CoV-2 RT-PCR detection in the clinical samples showed that the N-gene is one of the favorable targets for amplification.[20] Thus, N gene was selected as MIRA target. The MIRA reactions were performed to enrich the target site in the SARS-CoV-2 N gene with products from AMP-Future Biotech Co. Ltd.[21] The principle for the MIRA primer design is the same as that of recombinase polymerase amplification, recommending 30−35nt length. For the amplification, 29.4 µlA buffer, 0.2 µl 100 µM quality control probes, 2 µl 5 µM primer mixture of the SARS-CoV-2 N gene, 5.9 µl DEPC water, 10 µl sample RNA, and 2.5 µl B buffer were mixed and heated at 42°C for 30 min.

2.4 | CRISPR fluorescence detection

In the 25 µl CRISPR reaction system, 0.2 µl 5 µM Cas13a, 0.1 µl 10 µM crRNA, 2 µl ribonucleotide solution mix, 0.1 µl 100 µM fluorescence probe, 2.5 µl x10 reaction buffer, 2.5 µl 0.1 M taurine, 0.75 µl 50000 U/ml T7 RNA polymerase, 0.5 µl 40000 U/ml RNase inhibitor, 0.5 µl 1 M $MgCl_2$, 10 µl MIRA products, and 5.85 µl DEPC water were mixed, incubated at 37°C for 30 min, and detected using a fluorescence spectrometer or RT-PCR instrument.

2.5 | CRISPR immunochromatography detection

In the 50 µl CRISPR reaction system, 0.4 µl 5 µM Cas13a, 0.2 µl 10 µM crRNA, 4 µl ribonucleotide solution mix, 0.2 µl 100 µM strip probe, 5 µl 10x reaction buffer, 5 µl 0.1 M taurine, 1.5 µl 50000 U/ml T7 RNA polymerase, 1 µl 40000 U/ml RNase inhibitor, 1 µl 1 M $MgCl_2$, 10 µl MIRA products, and 21.7 µl DEPC water were mixed and incubated at 37°C for 30 min. Next, 5 µl of CRISPR reaction solution and 80 µl of propulsion buffer were mixed and dripped onto the loading area of the immunochromatographic strip. Concentration of each component in LFA were adjusted to achieve better color effect of the strip. Twenty µg/ml antibody associate with strip buffer containing 1% BSA, 0.3% Tween-20, 0.3% PEG20000, and 0.05% NaN_3 can reduce the HOOK effect and prevent false-negative result. The optimal loading volume of LFA contain 5 µl CRISPR reaction product and 80 µl strip buffer. After loading, the detection results were interpreted within 2–10 min to ensure the reliability of the results.

2.6 | RT-PCR assay

RT-PCR assays were performed using a commercial detection kit from Biogerm Technology Co., Ltd. (Shanghai, China) following the manufacturer's guidelines. In the 25 µl reaction system, 12 µl nucleic acid amplification reaction fluid, 4 µl enzyme mixture, 4 µl ORF1ab/N reaction liquid, and 5 µl sample nucleic acid were mixed and immediately centrifuged. Next, C_t values of clinical specimens were determined using a Bio-Rad CFX96 fluorescence quantitative PCR instrument. The interpretation criteria of RT-PCR based on the threshold of C_t values. Samples were considered as positive when the C_t value of the N and OPR1ab genes were not more than 35, and negative when there is no detectable C_t value. Samples with the C_t value between 35 and 40 should be retested. Repeated test shows C_t value under 40 represents a positive result.

3 | RESULTS

3.1 | CRISPR detection of SARS-CoV-2

Ten detection targets were selected from the specific conserved sequence of the SARS-CoV-2 N gene (Figure 1A) and the corresponding crRNA was designed accordingly. The SARS-CoV-2 RNA reference material was used as a template for MIRA, followed by CRISPR detection. There are two CRISPR detection methods, namely fluorescence and immunochromatography (Figure 1B). In the crRNA screening process, amplified N gene DNA was used in crRNA screening. The crRNAs were screened by CRISPR fluorescence detection using the MIRA product of N gene. The template of MIRA was synthesized N gene DNA, whose sequence derived from reference genome (NC_045512.2). The experiment was repeated three times in each group. The crRNA revealed highest fluorescence intensity by the end of 30 min CRISPR reaction was considered the optimal one. The detection results are shown in Figures 1C,D, and the fluorescence values of nine tested crRNAs were statistically significant compared with those of the control ($p < 0.05$). High fluorescence value appeared at the start of detection especially for crRNA N8 and 10, mainly due to violent nuclease reaction by these two crRNAs. The fluorescence values of crRNA N8 was the highest and reached the cap value within 30 min; hence, it was selected as the crRNA for detection of the N gene. N8 was aligned with sequence of SARS-CoV-2 variants including Omicron, and none of mutant appeared (Supporting Information: Figure S1).

3.2 | Optimization of CRISPR fluorescence detection

Oligo7 software was used to design the MIRA-specific amplification primers for crRNA N-8 detection targets, and the detailed sequences are shown in Supporting Information: Table S2. The screening results of n-8 target MIRA primers are shown in Supporting Information: Figure S2. Finally, F1/R2 was selected as the specific primer pairs for MIRA. Reaction component and condition of CRISPR detection assay was optimized (Figure 2). SARS-CoV-2 RNA reference material at a low concentration (1 copy/µl) was used as positive template. Improved detection performance was obtained under the following conditions: final concentration of $MgCl_2$, 20 mM; final concentration

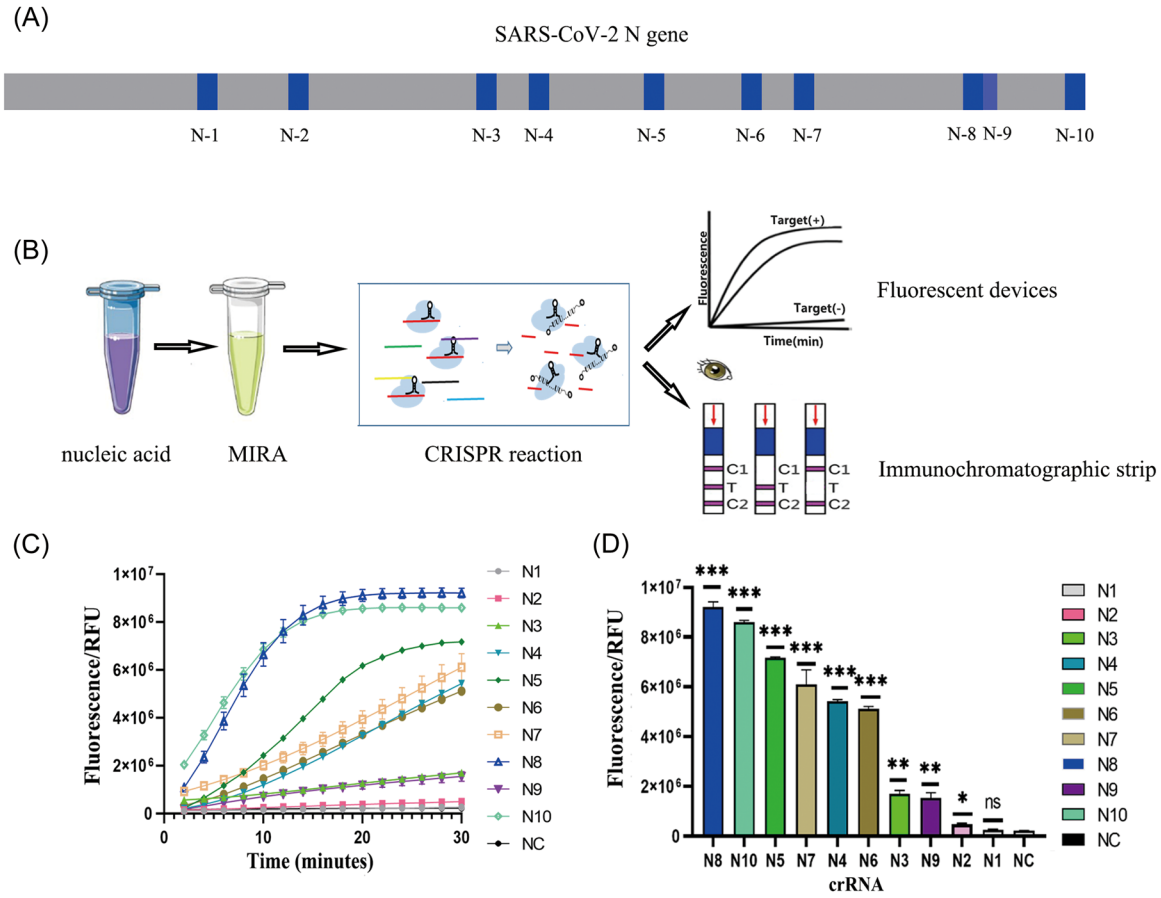

FIGURE 1 (A) Location of each detection target in the SARS-CoV-2 N gene. (B) Flowchart of the two CRISPR methods (fluorescence and immunochromatography) for detecting SARS-CoV-2. (C) Fluorescence curves of the different detection targets detected via the CRISPR fluorescence method within 30 min. (D) The end-point fluorescence values of the different detection targets were determined via the CRISPR fluorescence method within 30 min. CRISPR, Clustered regularly interspaced short palindromic repeat; SARS-CoV-2, Severe acute respiratory syndrome coronavirus-2.

of T7 RNA polymerase, 1.5 U/μl; final concentration of RNase inhibitor, 0.8 U/μl; final concentration of taurine, 10 mM; final concentration of MIRA primers of the N gene, 5 μM (Figure 2).

3.3 | CRISPR immunochromatography detection

The structure of the immunochromatographic strip is shown in Figure 3a; the strip contained a sample area, gold pad, control 1 line (C1 line), control 2 line (C2 line), and test line (T line). An FITC-biotin probe was used for CRISPR immunochromatographic detection. In negative samples, colloidal gold particles were bound to FITC-biotin probes, and the conjugate was intercepted by streptavidin at the C1 line. For positive samples, the FITC-biotin probes were cleaved, colloidal gold particles were bound to FITC, and the conjugate was intercepted by the FITC secondary antibody at the T line. The upstream and downstream primers of GAPDH (a commonly used human quality control gene) were added for RNA MIRA, and the primers were labeled with TAMRA and digoxin, respectively. Human genes were present in the samples, and the amplification products of colloidal gold combined with human genes were intercepted by the TAMRA antibody in the C2 line. No human genes were present in the samples, no color was displayed on the C2 line, and the concentration and sequence of the probe had an important influence on the experimental results. As shown in Figure 3B, an optimized detection performance was obtained using a 400 nM strip probe. To investigate the quality control ability of CRISPR immunochromatography, clinical specimens and SARS-CoV-2 RNA reference material were analyzed. As shown in Figure 3C, both positive and negative clinical samples showed a color on the quality control line 2 of the immunochromatographic strip, whereas SARS-CoV-2 RNA reference material with a Ct value of approximately 32.5 and DEPC water were not observed.

3.4 | LoD and specificity of the method

DEPC water (negative control, NC) and gradient diluted SARS-CoV-2 RNA reference material (10^2 copies/μl, 10 copies/μl, 5 copies/μl, 1 copy/μl, 0.5 copy/μl, 0.25 copy/μl, and 0.125 copy/μl) were used as samples for the CRISPR fluorescence and immunochromatography

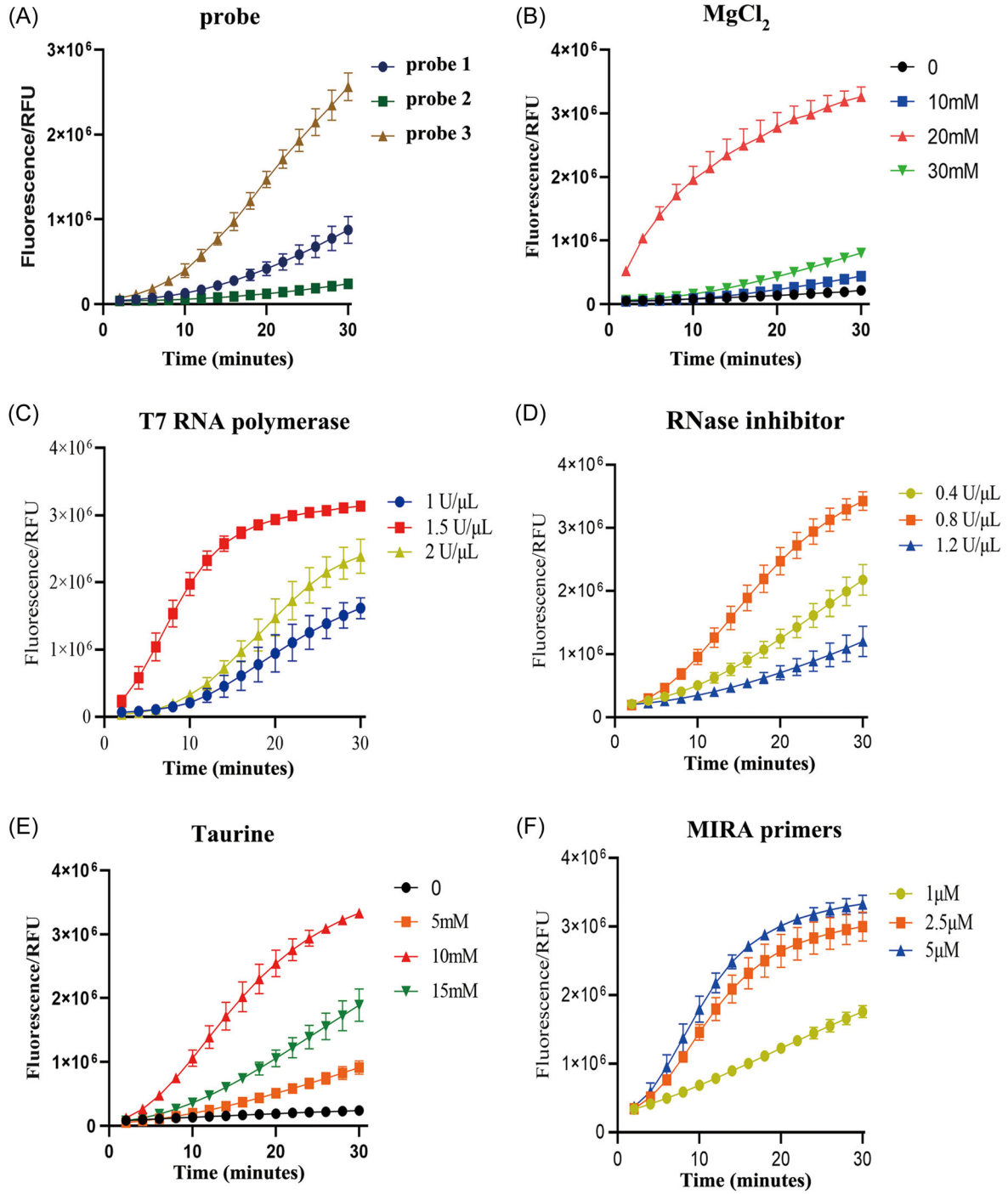

FIGURE 2 (A) Results of CRISPR fluorescence detection with different probes. (B) Results of the CRISPR fluorescence assay using different final concentrations of $MgCl_2$. (C) Results of the CRISPR fluorescence assay using different final concentrations of T7 RNA polymerase. (D) Results of the CRISPR fluorescence assay using different final concentrations of RNase inhibitor. (E) Results of the CRISPR fluorescence assay using different final concentrations of taurine. (F) Results of the CRISPR fluorescence assay using different final concentrations of primers of SARS-CoV-2 N gene in MIRA. CRISPR, Clustered regularly interspaced short palindromic repeat; MIRA, multienzyme isothermal rapid amplification; SARS-CoV-2, Severe acute respiratory syndrome coronavirus-2.

system; three replicates were used for each sample. The results are shown in Figure 4. In the CRISPR fluorescence assay, when the concentration of the SARS-CoV-2 RNA reference material was 0.25 copy/μl or higher, the fluorescence signal intensity was higher than that of the NC, and the difference was statistically significant ($p < 0.05$). In the CRISPR immunochromatography assay, when the concentration of the SARS-CoV-2 standard RNA material was 1 copy/μl, 5 copies/μl, 10 copies/μl, or 10^2 copies/μl. When the T line

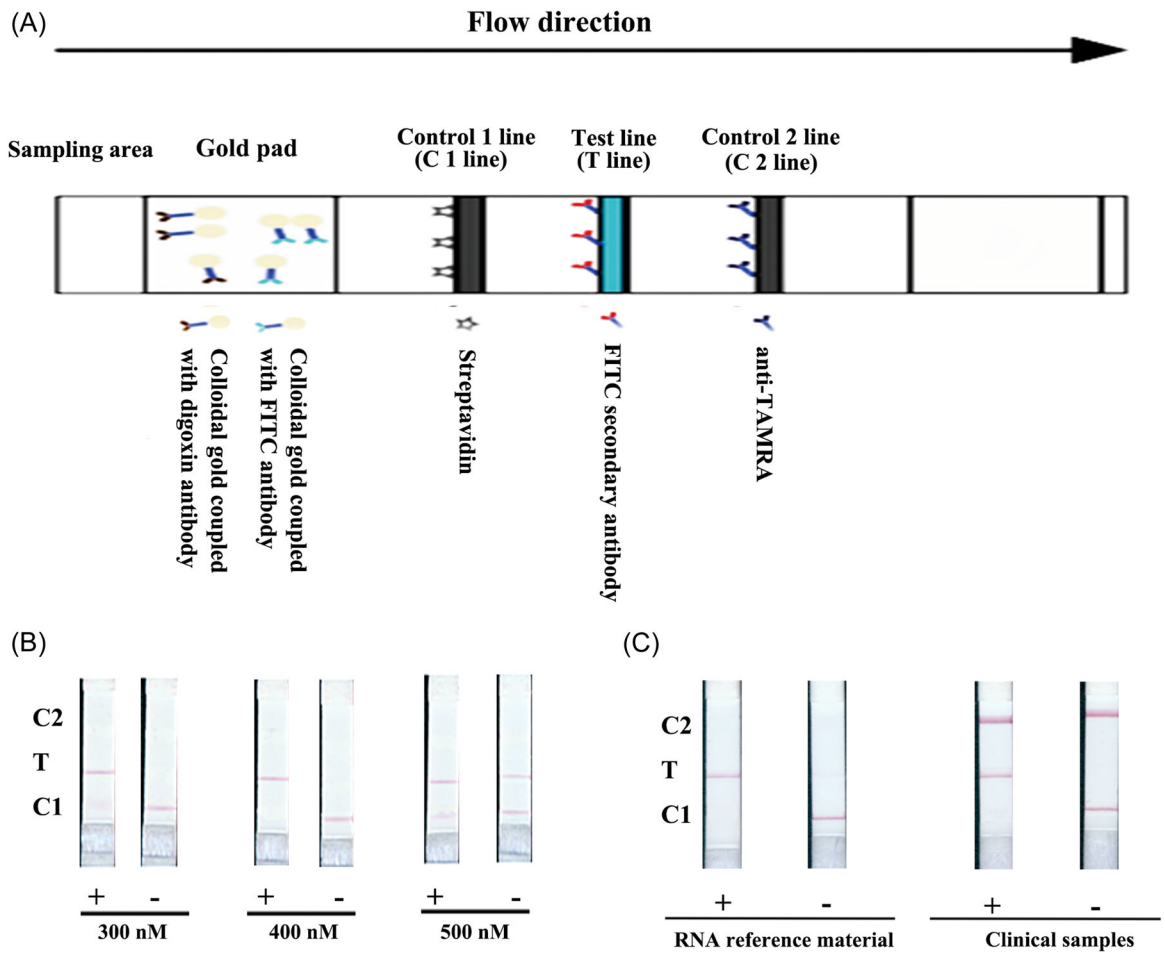

FIGURE 3 (A) Structure of the immunochromatographic strip. (B) Results of CRISPR immunochromatography using different concentrations of the strip probe. (C) Results of CRISPR immunochromatography detection of nucleic acids from different sources. CRISPR, Clustered regularly interspaced short palindromic repeat.

and C1 line were colored, the results were interpreted as positive. The SARS-CoV-2 nucleic acid standard material did not contain human nucleic acid; hence, the internal control line (C2 line) did not show a colored band. At a concentration of 0.25 or 0.5 copy/μl, both T and C1 lines showed a colored band, and the results were also positive. When the concentration was 0.125 copy/μl, the result was the same as that of the NC; the C1 line was colored, the T line was not colored, and the result was negative. These results indicated that the LoD of both CRISPR fluorescence and immunochromatography detection for SARS-CoV-2 was 0.25 copy/μl. The merit of sensitivity and quality control effect of this study compared with former research was concluded in Supporting Information: Table S3.

CRISPR fluorescence and immunochromatography were used to detect the nucleic acids of six human coronaviruses (SARS-COV, MERS, HKU1, OC43, NL63, and 229E), influenza A (H3N2), influenza B (Victoria), and SARS-CoV-2 RNA reference material. Tests were repeated three times for each sample. As shown in Figures 4B,D, only the results of the SARS-CoV-2 RNA standard were positive; all other pathogens tested negative. These results indicate the eligibility specificity of CRISPR fluorescence and immunochromatography methods.

3.5 | Detection of clinical samples

Clinical samples were collected during pathogen surveillance from January 2021 to March 2022 in Beijing. All samples are oropharyngeal swabs. The CRISPR immunochromatography method established in this study was used to detect 52 positive and 101 negative clinical samples, which were verified using the gold-standard RT-PCR method. The C_t value of the positive samples ranged from 17.9 to 38.2. The test results are presented in Table 1 and Figure 5. All 52 RT-PCR-positive clinical samples tested positive following our CRISPR immunochromatography method. Similarly, all 101 RT-PCR-negative samples tested negative following our CRISPR immunochromatography method. The positive and negative coincidence rates with the gold standard of CRISPR immunochromatography were 100%.

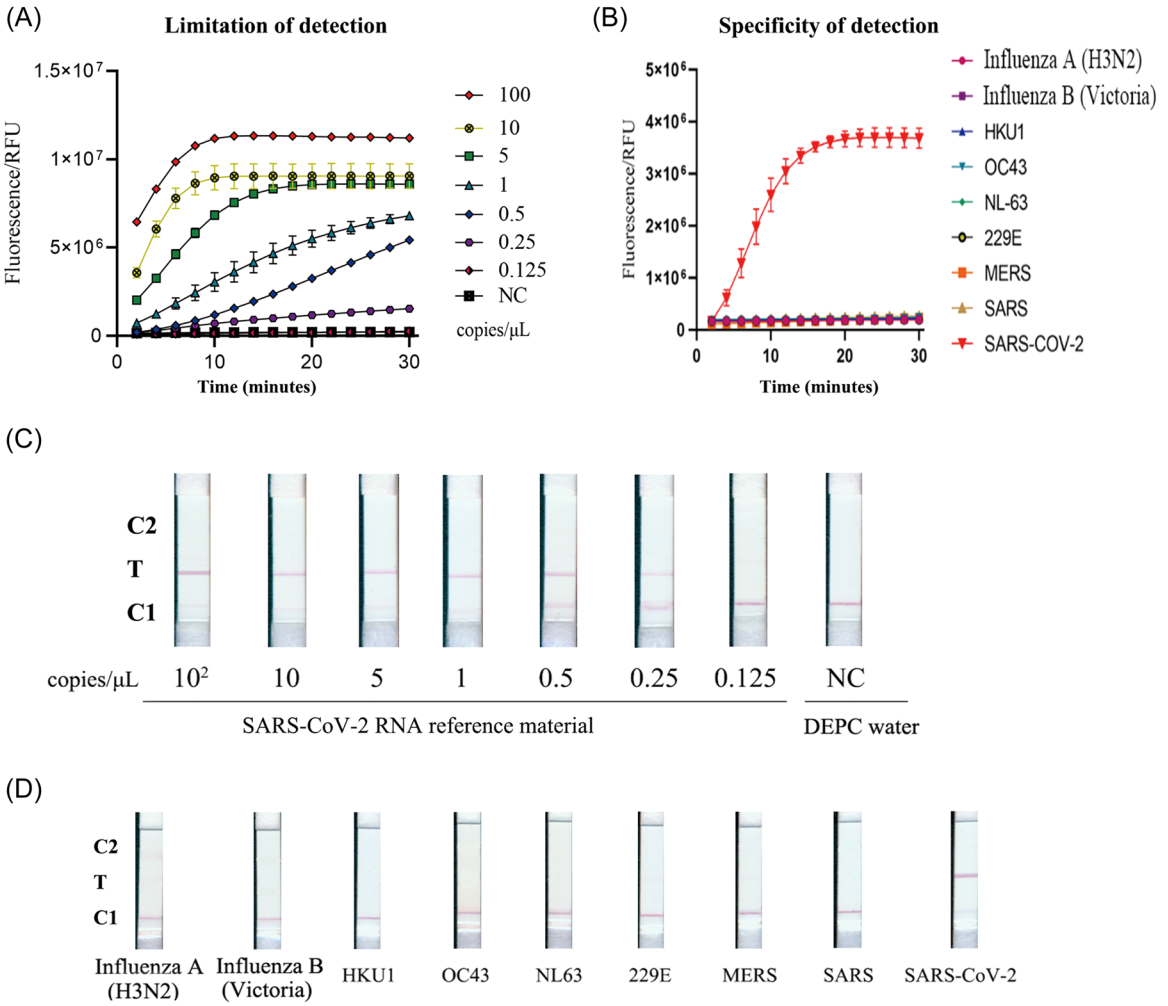

FIGURE 4 (A) Results of CRISPR fluorescence for the SARS-CoV-2 RNA reference material at different concentrations. (B) Results of CRISPR fluorescence for SARS-CoV-2 and eight RNA viruses. (C) CRISPR immunochromatography results of the SARS-CoV-2 RNA reference material at different concentrations. (D) Results of CRISPR immunochromatography for SARS-CoV-2 and the eight RNA viruses. CRISPR, Clustered regularly interspaced short palindromic repeat; SARS-CoV-2, Severe acute respiratory syndrome coronavirus-2.

TABLE 1 Comparison of CRISPR immunochromatography and RT-PCR

CRISPR immunochromatography	RT-PCR		
	Positive	Negative	Total
Positive	52	0	52
Negative	0	101	101
Total	52	101	153

Abbreviation: RT-PCR, reverse transcription polymerase chain reaction.

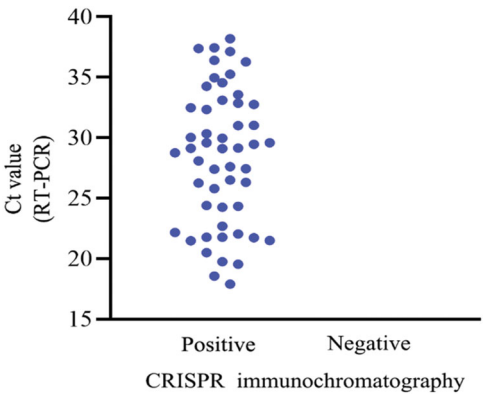

FIGURE 5 Results of CRISPR immunochromatography and RT-PCR of clinical specimens. CRISPR, Clustered regularly interspaced short palindromic repeat; RT-PCR, reverse transcription polymerase chain reaction.

4 | DISCUSSION

Detection of SARS-CoV-2 using an accurate and reliable method is essential to prevent the spread of COVID-19. In this study, a convenient, sensitive, and reliable CRISPR-based LFA method was

established for the sensitive detection of the virus in clinical samples. The LoD restriction for the commercial pathogenic nucleic acid detection kit in China is no more than 1 copy/μl; our CRISPR-LFA method demonstrated an LoD of 0.25 copy/μl and was found to be ultrasensitive. Importantly, this LoD, namely the analytical sensitivity, was comparable to that of the RT-PCR method or even higher than that of many commercial RT-PCR detection kits.[17,22,23] The improved sensitivity of our method is mainly owing to various optimizations of the whole reaction system. The sequences of the MIRA primer, crRNA, and probe were screened in detail. The concentrations of $MgCl_2$, T7 RNA polymerase, RNase inhibitor, taurine, primers, and probes were carefully evaluated using a single variable method. As the Omicron variant has resulted in a significant increase in transmissibility and produces more asymptomatic infections,[24] the molecular diagnosis of pathogens has proved to be a particularly useful method for identifying infected individuals.[5] Owing to its high sensitivity, our method would be helpful for early screening of infected individuals, even in samples with a low viral load.

To avoid false-negatives during the testing of suspected samples, introducing an internal reference is a common method.[17] The lateral flow strip reported in this study was designed to establish internal controls by targeting the human housekeeping gene. This additional control line was formed by capturing the labeled MIRA product in the strip. If an improper operation occurred during sample collection, transportation, or nucleic acid extraction, the HR control line would not be colored. In this way, the operation quality of the entire process of pathogenic nucleic acid detection could be controlled.[25] This design provides a powerful visualization tool for CRISPR detection and could significantly improve the reliability of on-site detection of SARS-CoV-2.

To validate our method, 153 clinical samples were collected and tested using the CRISPR-based lateral flow and RT-PCR methods. All RT-PCR-positive samples, with C_t values ranging from 17.9 to 38.2, tested positive following our CRISPR immunochromatography method. Similarly, all RT-PCR-negative samples tested negative following our CRISPR immunochromatography method. Although the sample size was inadequate to evaluate the overall validity of our method, the available data indicate that the effectiveness of this CRISPR immunochromatography method was consistent with that of the gold-standard RT-PCR method.[22] Notably, the CRISPR immunochromatography method can report testing results within 1 h, thus saving 30 min to 1 h when compared with RT-PCR. For sample nucleic acid preparation, magnetic beads based quick extraction method was suitable for CRISPR detection assays, as it can provide high quality of purified nucleic acid in a relative short time.[14] In addition, our CRISPR immunochromatography method requires minimal instrumentation, which can provide a constant temperature of 37–42°C, thus saving costs related to expensive instruments. Aerosol pollution has been a risk to cause false-positive results for amplification-related nucleic acid detection methods. To address this problem, we take several measures such as frequent ventilation of work area, using nucleic acid cleaner reagent after each experiment, and performing amplification and CRISPR reaction in different room. In addition, we are also developing a one-pot device to prevent release of aerosol pollution by integrating the amplification tube, the Cas13a reaction tube and LFA strip.

Our study greatly advanced the CRISPR-LFA method through the improvement of sensitivity and quality control effect. In comparison with exist research relevant with CRISPR-LFA, LoD of our study were much superior, which achieved comparable level with RT-PCR method.[15,16,26–33] The rational design and optimization of LFA strip ensured the detection sensitivity of SARS-CoV-2 and innovatively illustrated the results of human gene control in one test. In conclusion, our CRISPR immunochromatography method is a sensitive, portable, and reliable approach for SARS-CoV-2 nucleic acid detection. As new SARS-CoV-2 variants continue to evolve, the virus has become increasingly infectious. The ability to rapidly identify infected individuals is critical for the timely control of virus transmission. The approach reported in this study significantly advances the CRISPR-Cas13a-based molecular diagnostic technique in terms of reliability and effectiveness and will be of great value for application as a point-of-care test, particularly in low-resource conditions.

AUTHOR CONTRIBUTIONS

Hongbin Song, Shaofu Qiu, Shengqi Wang, and Zhenjun Li designed this study; Hongbo Liu, Yue Du, and Shuailei Chang performed the experiments; Sijia Chen, and Hui Wang participated in sample collection; Chao Wang, Ying Xiang, and Qi Wang analyzed the data. Hongbo Liu, Shuailei Chang, Shaofu Qiu, and Hongbin Song drafted the manuscript. All authors have read and approved the manuscript.

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CONFLICT OF INTEREST

The authors declare that there are no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All patients provided consent to participate in the study. All study procedures related to patient samples were approved by the Ethics Committee of the Chinese PLA Center for Disease Control and Prevention.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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