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Four thermostatic steps: A novel CRISPR-Cas12-based system for the rapid at-home detection of respiratory pathogens

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Abstract

The outbreak of coronavirus disease 2019 (COVID-19) in 2019 has severely damaged the world's economy and public health and made people pay more attention to respiratory infectious diseases. However, traditional quantitative real-time polymerase chain reaction (qRT-PCR) nucleic acid detection kits require RNA extraction, reverse transcription, and amplification, as well as the support of large-scale equipment to enrich and purify nucleic acids and precise temperature control. Therefore, novel, fast, convenient, sensitive and specific detection methods are urgently being developed and moving to proof of concept test. In this study, we developed a new nucleic acid detection system, referred to as 4 Thermostatic steps (4TS), which innovatively allows all the detection processes to be completed in a constant temperature device, which performs extraction, amplification, cutting of targets, and detection within 40 min. The assay can specifically and sensitively detect five respiratory pathogens, namely SARS-CoV-2, *Mycoplasma felis* (MF), *Chlamydia felis* (CF), *Feline calicivirus* (FCV), and Feline herpes virus (FHV). In addition, a cost-effective and practical small-scale reaction device was designed and developed to maintain stable reaction conditions. The results of the detection of the five viruses show that the sensitivity of the system is greater than 94%, and specificity is 100%. The 4TS system does not require complex equipment, which makes it convenient and fast to operate, and allows immediate testing for suspected infectious agents at home or in small clinics. Therefore, the assay system has diagnostic value and significant potential for further reducing the cost of early screening of infectious diseases and expanding its application.

Key points

- *The 4TS system enables the accurate and specific detection of nucleic acid of pathogens at 37 °C in four simple steps, and the whole process only takes 40 min.*
- *A simple alkali solution can be used to extract nucleic acid.*
- *A small portable device simple to operate is developed for home diagnosis and detection of respiratory pathogens.*

Keywords CRISPR-Cas12 · Point-of-Care Testing · Respiratory pathogens · SARS-CoV-2

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Introduction

Infectious diseases have affected human populations for a long time, and their improper prevention and control can result in major hazards to human health (Talwar et al. 2021). Since the start of the global pandemic of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in December 2019, the virus and its variant strains have been continuously spread and continue to pose enormous challenges to the diagnosis, treatment and management of COVID-19 (Keske et al. 2022; Qiu et al. 2020). The pandemic of COVID-19 has raised concerns about respiratory infectious pathogens (Narasimhan et al. 2022), such as zoonotic MF and CF, as well as FCV and FHV that cause respiratory infections in animals (Michael et al. 2021). Nucleic acids, such as DNA and RNA, are important biomolecules that can be used as biomarkers to diagnose various acute and chronic infectious diseases. Nucleic acid hybridization is a technique based on the principle of complementary base pairing of nucleic acids that can be used to detect specific nucleic acid sequences for the purpose of species identification, genotyping or measuring gene expression levels (Grody et al. 1989). Currently, the most commonly used detection method is qRT-PCR, which is limited by the requirement of expensive instruments, high-standard operation place, highly specialized operators and long detection period. Consequently, it may result in delayed diagnosis and cannot be used to rapidly and conveniently screen early infectious diseases (Qiu et al. 2020). Therefore, more rapid and convenient detection methods are needed for the early diagnosis of infectious pathogens.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated sequences (CRISPR-Cas) is an important RNA-guided adaptive immunity in bacteria and archaea, in which the RNA-guided RNA endonuclease works like a pair of molecular scissors that recognizes and cuts foreign nucleic acids. The CRISPR-Cas system has been used as a gene editing tool (Faure et al. 2019; Koonin and Makarova 2019; Makarova et al. 2020; Sorek et al. 2013). Gene editing based on CRISPR-Cas has been widely used in disease diagnosis and treatment (Hu and Li 2022). In the past decade, the CRISPR-Cas system has been developed into multiple editing tools (Abdelrahman et al. 2021; Singh et al. 2022). Recently, Rahman et al. reviewed the application of CRISPR-Cas-based molecular technologies in the diagnosis of several tropical diseases (Rahman et al. 2022). According to effector modules, the CRISPR-Cas system is divided into type I and type II (Koonin and Makarova 2019). By combining Cas12a ssDNase activation with isothermal amplification, a DNA endonuclease-targeted CRISPR trans reporter (DETECTR) is created to detect single-stranded DNA (ssDNA). In addition, DETECTR combined with isothermal preamplification is capable of rapidly detecting pathogens by measuring fluorescence intensity values or using test strips (Chen et al. 2018a).

Point of care testing (POCT) refers to the testing performed at sampling points, using portable analytical instruments and supporting reagents to quickly obtain test results. With the COVID-19 pandemic, nucleic acid testing and POCT are increasingly accepted by people. As CRISPR-based diagnostic systems do not require precise temperature and complex instruments, they are highly compatible with POCT. In recent years, CRISPR-based diagnostic systems have been extensively explored in the field of POCT. Zhao et al. studied a comprehensive detection method of HIV-1 mediated by CRISPR-Cas12a combined with real-time isothermal reverse transcriptase assisted amplification, which could be directly observed by the naked eye using a blue light imager for fluorescence detection (Zhao et al. 2021). The test results were similar to those of the HIV-1 qRT-PCR assay approved by the Chinese FDA. Li et al. developed a one-tube recombinase polymerase amplification (RPA)-CRISPR-Cas12 detection system to rapidly detect methicillin-resistant *Staphylococcus aureus* (MRSA) using lateral flow test strips (Li et al. 2022). Ma et al. developed a CRISPR-Cas12a-powered dual-model biosensor, which proved to be capable of detecting *Salmonella* in food samples, and broadened the application of the CRISPR-Cas system in biosensor techniques (Ma et al. 2021). Jirawannaporn et al. designed an RPA-CRISPR-Cas12 system targeting lipL32, which is highly sensitive and specifically detects *Leptospira* (Jirawannaporn et al. 2022). Rahman et al. summarized the advantages and disadvantages of CRISPR-based detection methods developed in recent years, and suggested that the promising CRISPR-based methods will promote the application of POCT (Rahman et al. 2021).

In this study, we developed a novel 4 thermostatic step (4TS) system with an adaptable thermostatic step amplification (TSA). By combining the CRISPR-Cas12 system, 4TS enabled the extraction of nucleic acids at a constant temperature (37℃), amplifying DNA fragments, cleaving targets and detecting pathogens within 40 min. Compared with the current detection by qRT-PCR, our newly developed 4TS system is characterized by rapid detection, constant temperature, low risk of aerosol contamination, and high sensitivity and specificity. Therefore, 4TS is suitable for rapid detection and pathogen testing at home. Also, the low cost of the developed small device makes it easy to use even in economically depressed communities as there is no need for complex laboratory equipment. Additionally, it is suitable for rapid detection of infectious diseases at home and in small clinics or pet hospitals, resulting in significant

savings in social resources. In addition, it provides a technical reference for POCT in the future.

Materials and Methods

Preparation of viral plasmids and clinical samples

The respiratory tract infection viral plasmids used in this study were synthesized by GenScript Biotech Corporation (Nanjing, China), including COVID-19 (*SARS-CoV-2 nucleocapsid gene*, NCBI Accession number LC528233.1), FCV (*Orf1* gene, NCBI Accession number KU373057.1), FHV-1 (*TK* gene, NCBI Accession number MH070348.1), MF (*Tuf* gene, NCBI Accession number FJ896388.1) and CF (*OmpA* gene, NCBI Accession number AF269257.1). A SARS-CoV-2 pseudovirus was provided by Yeasen (Shenzhen, China). A total of 27 clinical samples of COVID-19 were obtained from the First Affiliated Hospital of Nanchang University. In addition, 18 MF samples, 20 CF samples, 21 FCV samples and 24 FHV samples were obtained from 13 pet hospitals. Informed consent was obtained from all patients and pet owners.

Extraction of RNA from virus

In this study, three methods of nucleic acid extraction were used for comparison purposes. The first, the Heating Unextracted Diagnostic Samples to Obliterate Nucleases (HUDSON) method, refers to Zhang Feng's reference protocol (Myhrvold et al. 2018). The second, involves heating and inactivating the viral RNA for 5 min at 95 ℃, and the viral RNA extraction is performed using the Virus RNA Isolation Kit obtained from Sangon Biotech Co, Ltd. (Shanghai, China), following the manufacturer's protocol. The third, the alkali extraction method, is performed at room temperature, and involves lysing the virus RNA with 250 mM NaOH for 2 min, immediately followed by the addition of a solution containing equal proportion of HCl to neutralize the RNA solution without inhibiting subsequent amplification or detection.

Design of primers, CRISPR RNAs (crRNAs) and reporter genes

Conserved domains in the SARS-CoV-2 N, FCV *ORF1*, FHV-1 *TK*, MF *Tuf*, and CF *OmpA*, genes (GenBank) were searched using the Primer Premier 5 software (Premier Biosoft International, Palo Alto, CA, USA). Three pairs of specific primers and three crRNAs were designed for each virus by Sangon Biotech Co, Ltd. (Shanghai, China). The specificity of each primer was confirmed using the Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). Long fragments were used for constructing plasmids and those with 30 bp were subjected to reverse transcription-thermostatic step amplification (RT-TSA). The crRNAs with 20 nt were used for fluorescence detection of Cas12. The 5' and 3' ends of the ssDNA-reporter were labeled with FAM-BHQ1 and FAM-Bio, respectively. The sequences of the primers and crRNAs are listed in Table S1 and S2.

Amplification of DNA fragments

A 20 μL PCR system, containing 10 μL of Taq PCR Master Mix, 0.8 μL of forward primer at 10 μM, 0.8 μL of reverse primer at 10 μM, 7.4 μL of nuclease-free water and 1 μL of cDNA, was prepared and used for thermal amplification at 94 °C for 4 min, followed by 28 cycles at 95 °C for 30 s, 72 °C for 1 kb/min and 72 °C for 10 min.

A 50 μL RT-TSA system was prepared by mixing the following reagents: 1 μL M-MuLV reverse transcriptase, 300 ng/μL recombinant enzyme, 600 ng/μL accessory protein, 600 ng/ μL single strand DNA-binding protein, 60 ng/μL DNA polymerase, 50 mM Tris–HCl, 2 mM DTT, 3 mM ATP, 200 μM dNTPs, 20 mM phosphocreatine, 100 ng/μL creatine kinase, 6.5% Carbowax 20 M, 2 μL of primer at 10 μM, 1 μL of template, 17.5 μL of nuclease-free water, and 1 μL RNase inhibitor. Then, the reaction was started by adding 2.5 μL of 280 mM MgCl2, which was used for amplification at 37℃ for 20 min. After purification, the products were subjected to electrophoresis.

Measurement of fluorescence intensity values

The CRISPR-Cas12 system included the Cas12 protein, Cas12 buffer, crRNA and ssDNA-reporter. The reaction buffer contained 1 μL of *Lachnospiraceae bacterium* (Lba) Cas12a at 1 μM, 2 μL of Cas12 buffer (10×), 2 μL of 500 nM crRNA, 2 μL of DNA reporter gene at 2 μM and 11 μL of nuclease-free water. After gentle mixing the mixture, 2 μL of amplified product was mixed with the reaction buffer at 37℃ for 20 min, followed by measurement of fluorescence intensity values.

Lateral flow assay (LFA)

Based on the mechanism of the CRISPR-Cas12 system, FAM-Biotin labeled ssDNA reporter in the control (C) and test (T) bands was prepared for the lateral flow strip test, in which streptavidin was immobilized at the control zone, and protein A in the test zone captured the anti-FAM antibody. A lateral flow strip and buffer were used in the LFA. After Cas12 detection, 100 μL of buffer was sprayed on the lateral flow strip for 2-min observation. The buffer flowed upward

by capillary action. The visualization of one and two bands was considered as positive and negative, respectively.

Kit Reagents

The kit reagents include nucleic acid extraction solution 1 (200 mM NaOH), termination solution 2 (200 mM HCl), RT-TSA reagent A (protein mixture consisting of M-MuLV recombinant enzyme, accessory protein, single strand DNA-binding protein and DNA polymerase), RT-TSA reagent a (buffer consisting of Tris–HCl, dNTP, DTT, ATP, primers, phosphocreatine, creatine kinase, Carbowax 20 M), Cas12a reaction reagent B (consisting of Cas12 and DNA reporter,) and Cas12a reaction reagent b (buffer consisting of Tris–HCl, NaCl, $MgCl_2$, BSA and crRNA). All reagents are stored at—20 ℃.

Statistical analysis

Data from three independent tests were expressed as the mean ± standard deviation ($\bar{x}\pm$SD). Differences between groups were compared by the Student's t test, and those among three or more groups were compared by one-way analysis of variance (ANOVA). Statistical analysis was performed using the GraphPad PRISM 8.01 software (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered as statistically significant.

Results

Establishment of 4TS

4TS consists of four steps, namely extraction of nucleic acids, amplification of sequences, cleavage of targets, and detection of fragments. Samples are incubated in 10 mM EDTA for 2 min to extract nucleic acids. Subsequently, the solution is neutralized by adding a strong acid. Then, the RT-TSA system is prepared by mixing M-MuLV reverse transcriptase, protein powder, buffer, primers, $MgCl_2$, and nuclease-free water, with 5 µL of the template solution containing nucleic acids for 20 min at room temperature. The RT-TSA is a thermostatic nucleic acid amplification method using recombinant enzyme as the core. After reverse transcription, recombinase proteins formed complexes with each primer and scanned DNA for homologous sequences, then the target nucleic acid was exponentially amplified by single-stranded binding proteins (SSB) and DNA polymerase at 37–42℃ for about 20 min. Then, the Cas12a-crRNA complex in the mixture with amplified product is guided by the crRNA and binds to the target nucleic acid. Eventually, the test results can be visualized on fluorescent and lateral flow test strips using ssDNA reporters (FAM-BHQ and FAM-Bio) (Fig. 1).

Optimization of 4TS

Based on the conserved domains in the *SARS-CoV-2*, MF, CF, FCV, and FHV-1 genes, we synthesized 3 pairs of primers and crRNAs for each gene using the Primer Premier 5 software and BLAST program (Fig. 2A, the other four pathogens gene sequences are shown in Table S1). After RT-TSA for amplification and polyacrylamide gel electrophoresis (PAGE), the *SARS-CoV-2* primer 2, *MF* primer 3, *CF* primer 3, *FCV* primer 2 and *FHV* primer 3 were found to be the optimal primers and thus used in subsequent experiments (Fig. 2B). We additionally compared the efficiency of nucleic acid extraction from the SARS-CoV-2 pseudovirus by alkaline lysis at room temperature, using the HUDSON method, and commercial nucleic acid extraction kit. We found that both methods achieved similar outcome (Fig. 2C). However, the HUDSON method requires heating to 95 °C, and the procedures of the commercial nucleic acid extraction kit are time consuming and complicated. Therefore, the alkaline lysis at room temperature was ultimately selected for use in this study. To determine the influence of temperature on amplification, amplification was performed at 17, 22, 27, 32, 37, and 42 °C, and the results revealed that DNA amplification failed at 17 °C, and the optimal amplification was achieved at 37 °C (Fig. 2D).

Most crRNAs were synthesized based on the T-rich protospacer adjacent motif (PAM) TTTN to select the optimal one that was recognized by the target. However, in order to find high-efficiency crRNAs as much as possible, we designed three crRNA on each gene with or without the TTTN PAM site restriction, according to a previous report (Ding et al. 2020). The results showed that *SARS-CoV-2* crRNA1, MF crRNA1, CF crRNA2, FCV crRNA1 and FHV crRNA3 achieved faster fluorescence activation, and were thus selected for subsequent cleavage by the CRISPR-Cas12 system (Fig. 3A). A total of five concentrations (0.25, 0.5, 1, 2, and 4 µM) of ssDNA-reporter were prepared. The results revealed that the highest fluorescence intensity increased in a concentration-dependent manner, with the highest fluorescence intensity value was obtained at 4 µM (Fig. 3B). The design of the lateral flow strip is shown in Fig. 3C. When the nucleic acid was not cleaved by Cas12, streptavidin bound to biotin and the excess solution flowed forward to bind to the anti-rabbit antibody, thus enabling the visualization of two bands, which is indicative of a negative result. Alternatively, the anti-fluorescein isothiocyanate (FITC) conjugated gold nanoparticles bound to the anti-rabbit antibody, enabling the visualization of a single band, which is indicative of a positive result. Consistent with the qRT-PCR results, SARS-CoV-2, MF, CF, FCV and FHV were successfully detected by our lateral flow strips, indicating the acceptable detection efficacy of the 4TS system (Fig. 3D).

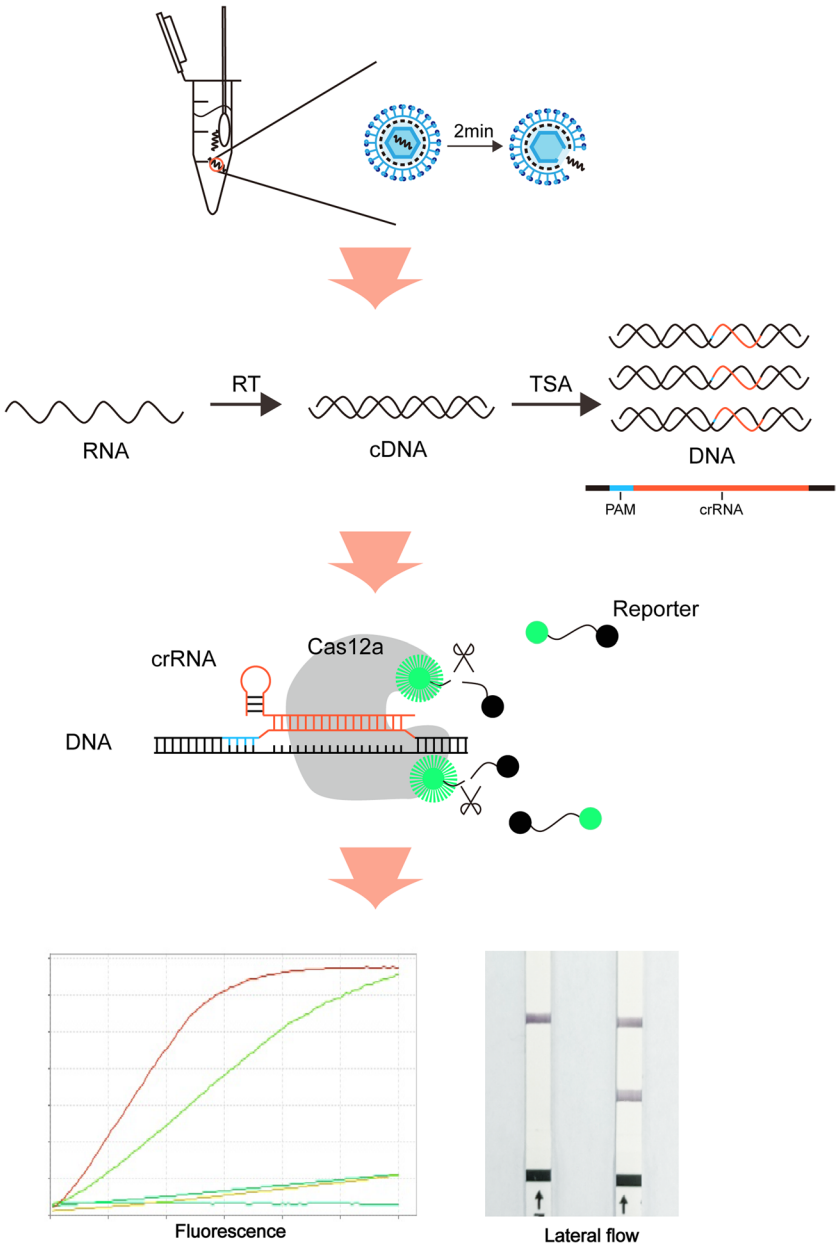

Fig. 1 Schematic diagram of the 4TS system for detecting nucleic acids of infectious pathogens. The nucleic acid sample was collected with swabs and placed in the alkaline extraction solution. Two minutes later, the virus shell was ruptured, and the nucleic acid was released. Reverse transcription and amplification by RT-TSA were then performed, followed by recognition of DNA target by the Cas12-crRNA complex. The activated Cas12 then cleaved the reporter genes, and the result was recorded by a fluorescence instrument and visualized on the lateral flow strip at room temperature within 40 min

Specificity of the 4TS system and its lower limit of detection

The specificity of 4TS was assessed through the detection of MF, CF, FCV and FHV by measuring the fluorescence intensity values and LFA. The four feline respiratory pathogen plasmids were compared with each other (Fig. 4A, B). The results showed that the primers and crRNAs of MF, CF, FCV and FHV could only be activated by themselves to emit fluorescence signals. In addition, they could be accurately detected by the lateral flow strips, indicating the acceptable specificity of the 4TS system (Fig. 4E).

We then determined the lower limit of detection of the 4TS system. To this end, the template concentration was diluted to 10^8, 10^6, 10^4, 10^2, 10^1, 10^0 and 0 copies/μL and performed the detections (Fig. 4C, D). The results showed that fluorescence intensity signals of 10 copies/μL of SARS-CoV-2, MF, CF, FCV, FHV could be detected within 20 min. A positive result was detectable by lateral flow strips above 10 copies/μL (Fig. 4F). Therefore, the lower limit of detection of the 4TS system is 10 copies/μL, demonstrating its high sensitivity.

Clinical validation of the 4TS system

A total of 27 clinical samples of COVID-19, including 22 positive patients and 5 negative throat swab samples, were collected. In addition, 18 MF samples (17 positive and 1

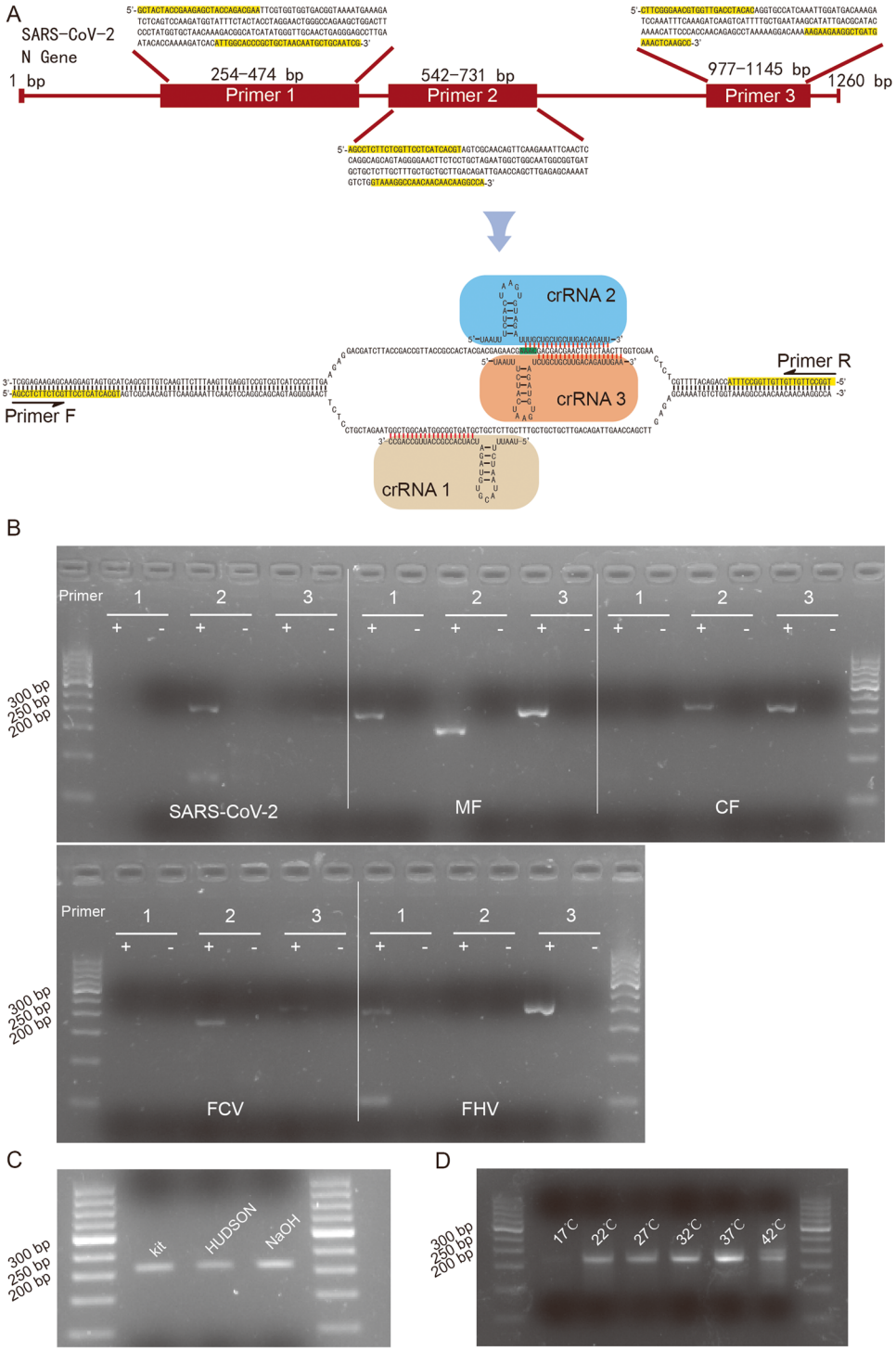

Fig. 2 Primer design for the 4TS platform. (**A**) Screening of 3 pairs of primers and crRNAs for SARS-CoV-2 N by the Primer Premier 5 software and BLAST program; (**B**) RT-TSA for amplification and PAGE showed that SARS-CoV-2 N primer 2, MF primer 3, CF primer 3, FCV primer 2 and FHV primer 3 were the optimal primers and were selected for use in the subsequent experiments; (**C**) The efficiency of nucleic acid extraction of SARS-CoV-2 pseudovirus by alkaline lysis at room temperature, the HUDSON method, and a commercial nucleic acid extraction kit was comparable; (**D**) TSA was performed at 17, 22, 27, 32, 37, and 42 °C, and the best amplification was achieved at 37 °C. SARS-CoV-2, *severe acute respiratory syndrome coronavirus 2*; BLAST, Basic Local Alignment Search Tool; TSA, thermostatic step amplification; MF, *Mycoplasma felis*; CF, *Chlamydia felis*; FCV, *Feline calicivirus*; FHV, *Feline herpes virus*

negative sample), 20 CF samples (18 positive and 2 negative samples), 21 FCV samples (19 positive and 2 negative samples) and 24 FHV samples (20 positive and 4 negative samples) were obtained from 13 pet hospitals. Confusion matrix for the dichotomous model(Kaminski et al. 2020) was adopted to assess the sensitivity and specificity of 4TS in detecting the above samples. Compared with the qRT-PCR data, the sensitivity and specificity of the 4TS system for detecting SARS-CoV-2 and FCV were both 100% (Fig. 5A, B), while for detecting MF, CF and FHV were 94 and 100%, respectively (Fig. 5C, D). Therefore, these results confirm that 4TS is a reliable platform for rapidly and conveniently detecting infectious pathogens with high sensitivity and specificity.

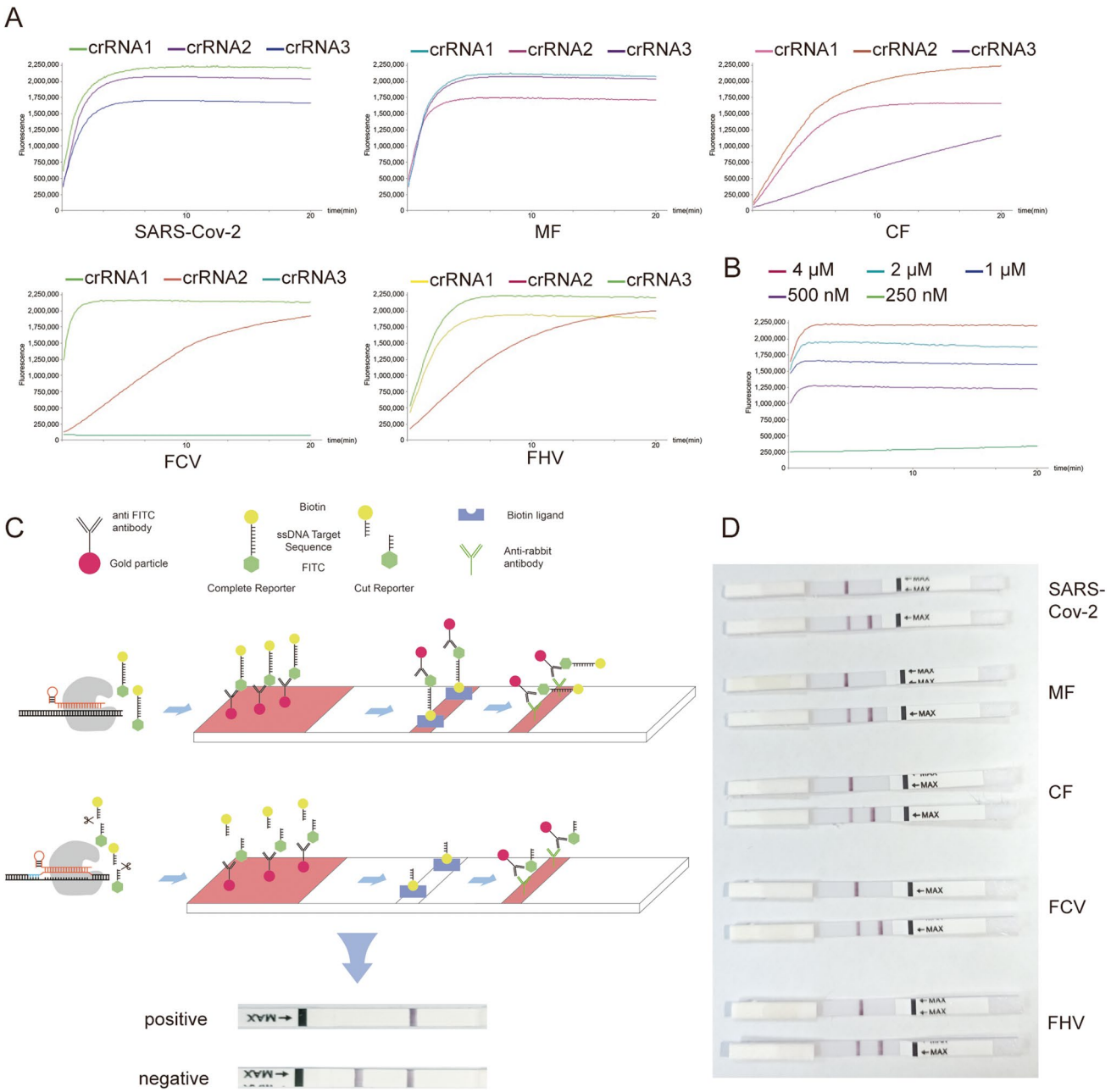

Fig. 3 Optimization of crRNA, ssDNA-reporter and lateral flow strips for the 4TS system. (**A**) Fluorescence detection of 3 pairs of crRNAs for five respiratory infectious pathogens; (**B**) Fluorescence detection by diluting the ssDNA-reporter at 0.25, 0.5, 1, 2, and 4 μM; (**C**) Schematic diagram of the LFA, one and two bands indicated a positive and negative result, respectively; (**D**) Detection of five respiratory infectious pathogens using lateral flow strips. Data are presented as the mean ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$

Design of a 4TS system simulation device

We designed a 4TS system-based at-home testing device for screening infectious pathogens, which consists of a power indicator light, reaction well A (RT-TSA reaction zone), reaction well B (nucleic acid detection zone), sample adding area C, and visual frame window (Fig. 6A). The three-dimensional (3D) disassembly part is shown in Fig. 6B. The material is made of acrylonitrile butadiene styrene (ABS resin), and has a soft touch, good toughness and strong rigidity. The inside of the device contains an indicator light (green light), a small heating device, a precision electronic temperature control (ETC) device, counterweight parts (to make it suitable for outdoor environment), and a

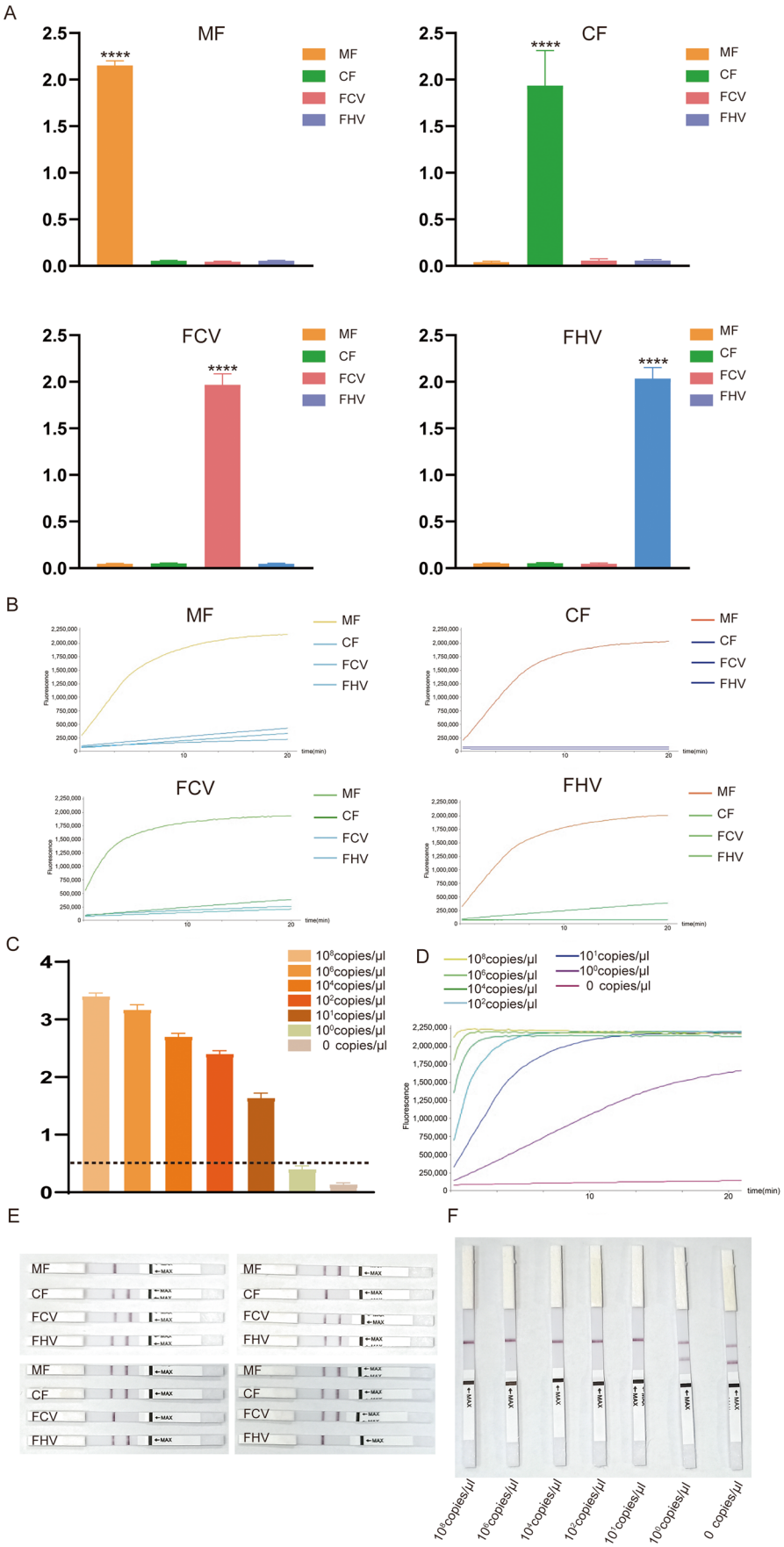

Fig. 4 Specificity of the 4TS system and its lower limit of detection. (**A**, **B**) The specificity of fluorescence detection of SARS-CoV-2, MF, CF, FCV and FHV by the 4TS system; (**C**, **D**) Fluorescence detection of the lower limit of SARS-CoV-2 at 10^8, 10^6, 10^4, 10^2, 10^1, 10^0 and 0 copies/μL; (**E**, **F**) Detection of the specificity and lower limit of SARS-CoV-2, MF, CF, FCV, and FHV by the LFA. Dashed lines represented fluorescence intensity values for activated Cas12. Data are presented as the mean ± SEM from three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$

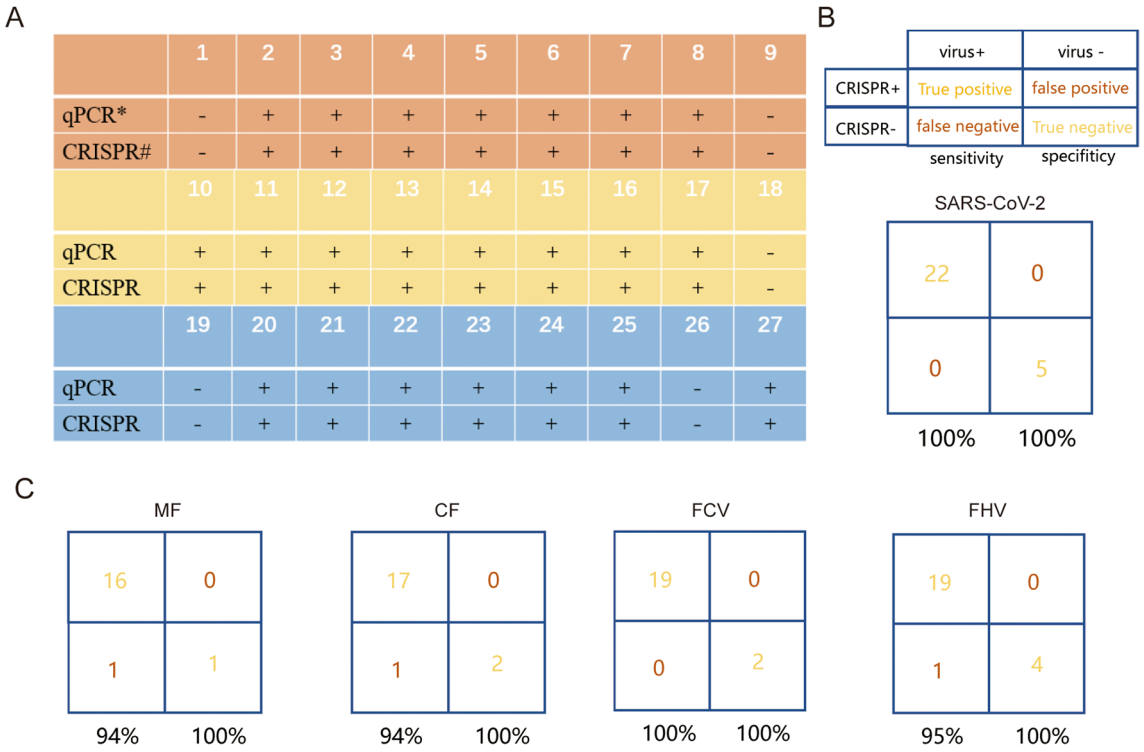

Fig. 5 Clinical validation of the 4TS system. (**A**) Detections of 27 clinical samples of COVID-19 (22 positive patients and 5 negative samples from throat swabs) are consistent with qRT-PCR findings; (**B**) Confusion matrix for the dichotomous model verified the 100% specificity and sensitivity to detect SARS-CoV-2 by 4TS; (**C**) Confusion matrix for the dichotomous model verified the 100% specificity to detect 18 MF samples (17 positive and 1 negative samples), 20 CF samples (18 positive and 2 negative samples), 21 FCV samples (19 positive and 2 negative samples) and 24 FHV samples (20 positive and 4 negative samples), and the sensitivity was 94, 94, 100 and 95%, respectively

battery (Fig. 6C). Reaction reagents include: nucleic acid extraction solution 1, termination solution 2, well A reaction reagent (A/a), well B reaction reagent (B/b), swab, and dropper. The result is negative if the test shows the presence of both C and T bands, and positive if it shows the presence of only the C band (Fig. 6D). The system is simple to operate. After the heating is turned on, nucleic acid samples are collected with a cotton swab, which is then placed in the alkaline extraction solution 1 for 2 min, followed by the addition of an equivalent amount of termination solution 2 for neutralization. Then, A and a are mixed for RT-TSA, and nucleic acid extraction solution is added to well A and incubated for 20 min. Next, B and b are mixed and then a drop from well A is added to well B for Cas12 induced cleavage. After 20 min, a drop of reaction solution is transferred from well B to well C, so that the solution flows to the viewbox window and the result is visible on the transverse flow bar as shown in the operation video S1.

The stability of the 4TS system simulation device

From March to December 2022, we used this device to perform field tests for infectious disease detection in the pet hospital. A total of 20 cases of FCV, FHV, MF and CF were separately tested. qRT-PCR was performed on the samples from the pet hospital animals or small animal community, and the analysis results showed that $Ct \geq 36$ was negative, and $Ct < 36$ was positive. Compared with the results of the qRT-PCR analysis, the accuracy rate of FCV, FHV, MF and CF reached 95, 95, 100 and 100%, respectively (Fig. 7, all data are shown in Table S3). These findings demonstrated the stability of 4TS-based devices through the testing of clinical samples. The results confirmed that the simple operation 4TS-based home detector is effective in home detection of infectious pathogens at low cost.

Discussion

Infectious diseases pose serious threats on public health, causing 17 million deaths every year (Khan et al. 2022). The mortality of infectious diseases account for 15% of global deaths (Li et al. 2021). COVID-19 is a global pandemic that has placed enormous burdens on medical resources and social welfare. Respiratory infectious diseases caused by MF, CF, FCV, and FHV can be widely transmitted in

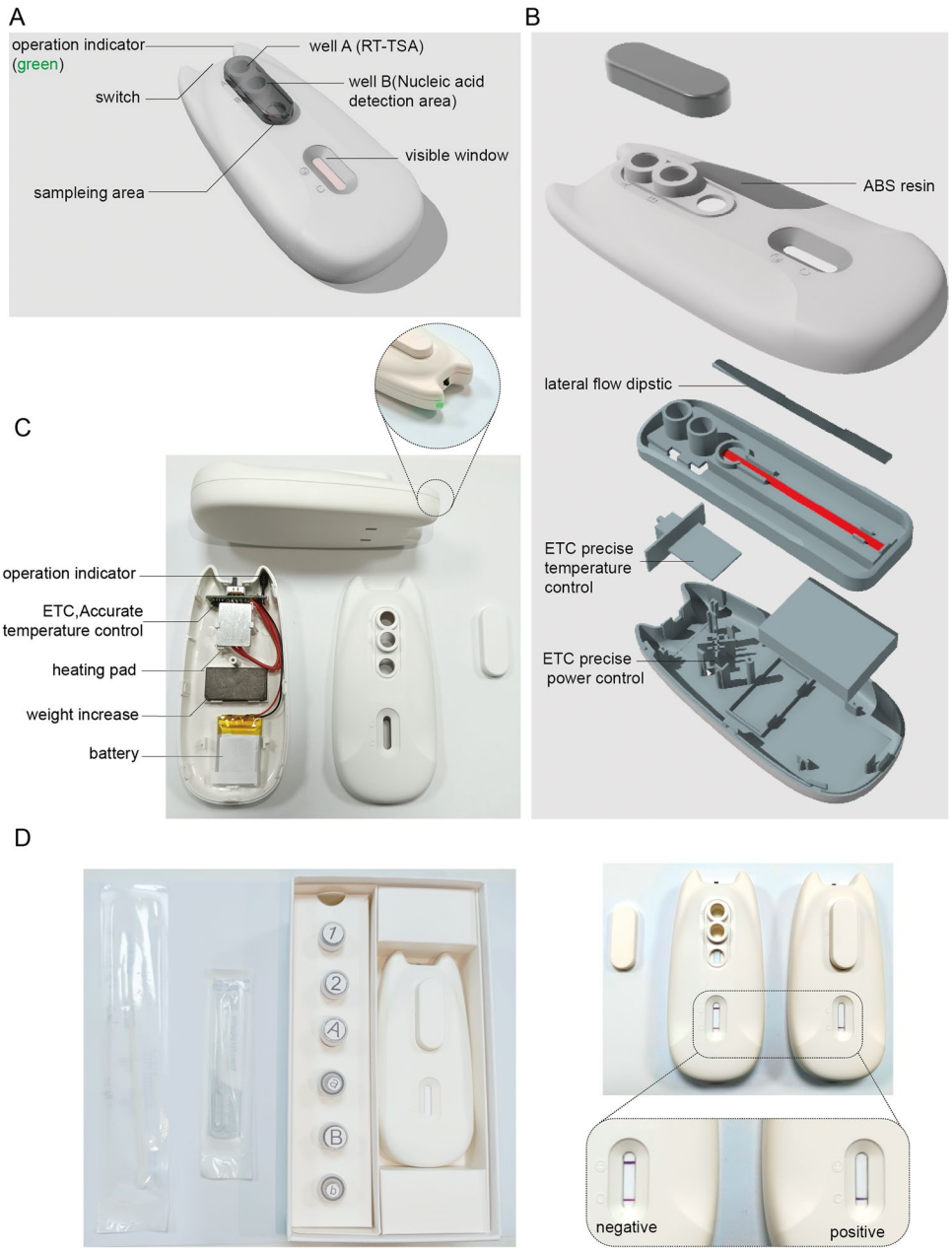

Fig. 6 Design of the 4TS simulation device. (**A**) Schematic diagram of the 4TS system simulation device. The 4TS system consists of a reaction well A (RT-TSA), a reaction well B (nucleic acid detection), a sample adding area and a visual frame window; (**B**) 3D internal structure of the 4TS simulator. The internal structure is divided into three layers, the material is made of ABS resin, the middle is transverse flow measuring strip, the lower layer includes an ETC precision temperature control devices and an ETC precision electricity control device; (**C**) Internal structure of the 4TS physical device, including an indicator light, ETC precision temperature control and electricity control devices, heating device, counterweight and battery; (**D**) 4TS reaction reagent, including swab, dropper, reagent including nucleic acid extraction solution 1 and termination solution 2, reaction reagent a (A and a), reaction reagent B (B and b). Clinical samples were confirmed to be negative and positive using the 4TS physical device, with negative showing the presence of both C and T bands and positive showing the presence of only the C band

humans and animals in a very short period of time. Therefore, the rapid identification of pathogens is of great significance for the subsequent treatment of patients.

Conventional methods for pathogen detection mainly include microscopic observation of bacterial smear, isolation and culture of bacteria, biochemical reactions, serological reactions, and PCR assays. They are greatly limited by complicated procedures, long detection period, and low sensitivity and specificity (Chen et al. 2018b). qRT-PCR is considered the gold standard for detecting pathogens, even though it is complicated and costly. This method requires considerable cost and time to transport samples to specialized laboratories and extract, amplify and test for the virus (Morshed et al. 2007). Moreover, false negative results due to contamination and strict temperature requirements significantly limit the application of qRT-PCR in the rapid detection of pathogens. As a result, insufficient etiological evidence eventually causes delayed diagnosis, emergence of multi-drug resistance and high medical cost (Surkova et al. 2020).

With the global spread of the novel SARS-CoV-2 coronavirus and the number of pet owners increasing every year, the probability of people contracting various pet infectious diseases is increasing rapidly. POCT can provide rapid and immediate diagnosis of infectious diseases. Currently, the rapid and affordable antigen and antibody detection

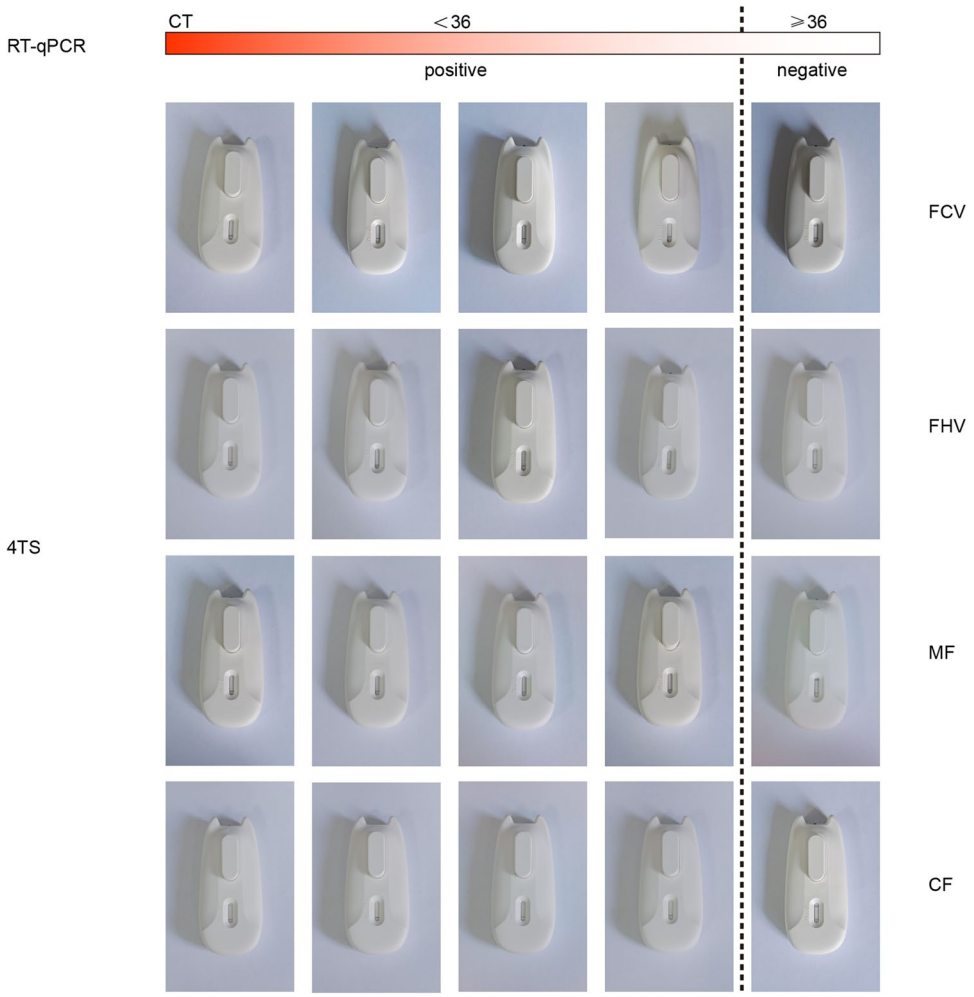

Fig. 7 The stability of the 4TS device test. Samples of 20 cases of FCV, FHV, MF and CF were collected on site in the pet hospital, and tests were performed according to the operation process. Compared with qRT-PCR, the accuracy rates were 95, 95, 100 and 100%, respectively (all data are shown in Table S3)

methods widely used in diagnosis have low sensitivity and specificity (Nafian et al. 2022). Delays in early pathogen screening and disease development may eventually lead to large-scale spread of infectious diseases. Therefore, there is an urgent need to control the spread of diseases through specific, sensitive, low-cost, instrument-free real-time testing that can be rapidly deployed in the field (Yuan et al. 2021). The diagnostic system based on CRISPR-Cas has great potential in the POCT approach due to its rapid and accurate detection and low cost of the raw materials used. In recent years, the CRISPR-Cas system has been extensively studied in the POCT field. Xiong et al. developed a rapid, ultra-sensitive and one-pot DNA detection method combining recombinant-enzyme polymerase amplification (RPA) and CRISPR-Cas12a for African swine fever virus (ASFV) and *Capripoxvirus* (CaPV) (Xiong et al. 2022). The sensitivity was 100 times that of qRT-PCR, which can quickly detect ASFV and CaPV at POCT sites. Lei et al. developed a portable single-tube method for the rapid detection of toxoplasma in heavily contaminated landfill leachate using freeze-dried reagent in a portable suitcase, making it a promising tool for field applications in remote areas (Lei et al. 2022). Zhang et al. developed a gold nanoparticle-based visual assay that, combined with CRISPR-Cas12a-assisted RT-LAMP, was able to detect as few as 4 copies / μL of SARS-CoV-2 with the naked eye within 40 min (Zhang et al. 2021). These methods have great potential applications in screening suspected patients at airports, train stations or in low-resource settings. However, although these studies perform well in terms of detection sensitivity, there is still no good optimization scheme for nucleic acid extraction, and it is difficult for non-professionals to operate. With ASSURE features (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end-users) a gap still exists (Mabey et al. 2004).

In the present study, we developed a novel 4TS platform that is able to detect pathogens with high sensitivity, high specificity and low cost. At constant temperature (37℃), the 4TS system can be used to rapidly extract nucleic acid, amplify sequences, cleave targets and detect viral fragments. Commonly used methods for rapid detection of nucleic acids mainly include the solid-phase silica-based method (Boom

et al. 1990), magnetic bead nucleic acid extraction (Nargessi and Ou 2010), aluminum oxide membrane for nucleic acid extraction (Oblath et al. 2013) and cell lysis for extracting nucleic acid. However, the need to use a centrifuge in the solid-phase silica-based method (Boom et al. 1990), high cost of magnetic beads (Nargessi and Ou 2010) and complicated manufacturing process of aluminum oxide membranes (Oblath et al. 2013) significantly limit their application in the rapid extraction of nucleic acid. Noteworthy, cell lysis at room temperature is simple, low cost and convenient, which make it an ideal method for extracting nucleic acids (Ali et al. 2017). Therefore, we used cell lysis at room temperature in the 4TS system, which allowed the rapid extraction of nucleic acids in minutes. Nucleic acid amplification is a necessary step associated with the accuracy of the subsequent detection, which avoids false negative results due to a too low concentration of sample or a large influence of the background level. In recent years, some isothermal amplification techniques have been commercialized, such as loop-mediated isothermal amplification (LAMP) (Starkie et al. 2022), RPA (Piepenburg et al. 2006), strand displacement amplification (SDA) (Zhang et al. 2014), and nucleic acid sequence-dependent amplification (NASBA) (Sharma et al. 2021). The control of the temperature is crucial during the processes of LAMP and SDA. In this study, we determined amplification efficacy at different temperatures, and ultimately, the optimal temperature was determined to be 37 °C. Our novel 4TS system achieved the demand for nucleic acid amplification at room temperature.

We subsequently optimized the 4TS system and tested its detection efficacy. First, optimal crRNAs with the best efficiency in generating fluorescence signals were screened, showing high specificity and sensitivity at 10 copies/μL. Second, the optimal concentration of ssDNA-reporter was also determined. Third, one band was visualized on the lateral flow strip, which was indicative of a positive result, and was further verified by testing clinical samples of COVID-19 and respiratory infection samples from animals. The sensitivity of the 4TS system to detect these pathogens was 94%, and the specificity was up to 100%. Therefore, our novel 4TS platform was able to detect respiratory infectious pathogens rapidly, effectively and conveniently.

Furthermore, we designed a small temperature-controlled device for real-time on-site detection of infectious pathogens. The 4TS-based field deployable small device system used to analyze samples from animals in pet hospitals or small clinics has high stability. By constructing synthetic raw materials, the cost of raw materials has been greatly reduced. At the moment, with the relaxation of China's prevention and control policy on COVID-19 and the increase in the number of families with pets, home detection may become one of the choices for people to detect infectious diseases in pets. The device based on the 4TS system has several advantages: (1) This device requires no professional staff, and anyone can quickly perform the nucleic acid detection test as long as they simply start to operate it; (2) The device is small and exquisite, portable, suitable for use at home or in clinics without large equipment, and has a good prospect in the field of POCT diagnosis; (3) the 4TS system has the advantages of rapid detection, high sensitivity and specificity, which is suitable for early screening of respiratory infectious diseases, and has great efficacy in preventing further infection and contributes to the timely treatment of infectious diseases. The device is suitable for detecting respiratory infectious diseases in pets at home, which greatly saves social resources. In the future, we will undertake the design and construction of a high-throughput, multiplexed system to detect multiple pathogens.

This study has some limitations that require further research. First, for below 10 copies/μL template, the 4TS with lower copy number may not be detected, which is the sensitivity limit of the 4TS itself. In addition, to avoid false positives caused by enzyme inactivation, we implemented the storage of freeze-dried proteins to ensure the long-term activity of the enzyme. Second, the device is a one-time test, which may result in the waste of batteries and other electrical components in the device. We are currently designing a rechargeable small test device based on the 4TS system that can be recycled. The device can maintain a constant temperature of 37℃ and is equipped with multiple channels. After the reagent reacts in the test tube, a test strip is inserted to directly observe the results, and can simultaneously detect 6–8 samples. The rechargeable design can avoid waste of electrical components. Third, the device developed in this study does not detect several variants of the SARS-CoV-2 virus, which limits the application of the device. In future studies, more research will be conducted on respiratory pathogens including SARS-CoV-2 variants such as Omicron. Fourth, testing tools can quickly and sensitively detect early collected samples for screening, but no monitoring work has been performed on mid- and late-stage samples, and further research is needed to address this issue in the future.

Due to the global COVID-19 pandemic and the continuous emergence of new pathogens, it is urgent to rapidly detect respiratory infectious pathogens. Based on the large potential of CRISPR-Cas systems, our newly developed 4TS system is highly scalable and could be used for home testing and infection surveillance. Our innovation importantly lays the foundation for fast and inexpensive POCT in the future.

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Authors' contribution JF and JL conceived and designed the study. JF, JL, TL conducted experiments. NC, XK, ZH, GW, XX analyzed the

data. ZH, ZG contributed new methods and JC contributed models. JF and JL wrote the manuscript. TX reviewed the manuscript. All authors read and approved the manuscript.

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Data availability All data generated or analysed dur ing this study are included in this article (and its supplementary information files), further inquiries can be directed to the corresponding authors.

Declarations

Ethics approval All samples of people and animal have been reviewed and approved by The First Affiliated Hospital of Nanchang University Medical Research Ethics Committee (approval ID: (2023) CDYFYYLK (03–016) and Shenzhen institute of quality & safety inspection and research Ethics Committee (approval ID: [2022] NO. (1–17).

Conflict of interest The authors declare that they have no conflict of interest.

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