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A one-pot method for universal Dengue virus detection by combining RT-RPA amplification and CRISPR/Cas12a assay

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Abstract

Dengue Virus (DENV) is a life-threatening pathogen leading to dengue fever, which brings about huge public health challenges globally. However, traditional detection methods currently fail to meet the increasing demands of clinic practice in terms of speed, simplicity, and accuracy. To address these limitations, we developed a novel, rapid, and highly sensitive diagnostic method for universal DENV detection by integrating recombinase polymerase amplification (RPA) assay and the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and associated (Cas) protein 12a (CRISPR/Cas12a) system into one-pot. This approach achieves exceptional sensitivity and specificity for DENV detection, with the entire process completed within 40 min, without the need for sophisticated equipment. The limit of detection (LOD) was determined to be 91.7 copies/test. Using this one-pot RT-RPA CRISPR/Cas12a detection system, all four serotypes of DENV (1 to 4) were successfully identified. In terms of specificity, the assay accurately detected DENV-infected positive samples without cross-reactivity with four other interfering viruses-infected samples (VSV, SeV, HSV-1 and IAV). Furthermore, we established a universal DENV RT-RPA-CRISPR/Cas12a-lateral flow dipstick (LFD) platform, which successfully identified all four serotypes of DENV with a sensitivity of approximately 250 copies/test. Collectively, our method not only provides a robust alternative for universal DENV detection but also offers valuable insights for the identification of other viruses.

Keywords DENV, RPA, CRISPR/Cas12a, One-pot, LFD, Universal detection

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Introduction

As a prominent mosquito-borne virus, Dengue virus (DENV) is endemic in tropical and subtropical regions worldwide [1]. The DENV is a single-stranded positive-sense RNA virus with a genome approximately 11 kb in length, classified within the genus *Flavivirus* of the *Flaviviridae* family. Infection with DENV can lead to a spectrum of clinical manifestations, ranging from mild dengue fever (DF) to severe and potentially fatal conditions such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2]. There are four genetically distinct serotypes of DENV: DENV-1, DENV-2, DENV-3, and DENV-4 [3]. Each serotype further comprises multiple genotypes, highlighting the extensive genetic diversity of DENV [4]. Among the serotypes, DENV-1 is the most widespread serotype and highly prevalent in Southeast Asian countries, East Asian countries, as well as regions in Africa, the Americas, Malaysia, Australia, Indonesia, and the Philippines. DENV-2, which includes six genotypes, is endemic in Southeast Asia, the Americas, and the Philippines. DENV-3, with five genotypes, is distributed across Southeast Asia, the Indian subcontinent, the South Pacific, East Africa, and the Americas. DENV-4, comprising three genotypes, is commonly found in Malaysia, South Asia and Southeast Asia [5, 6].

DENV is responsible for over 390 million infections and approximately 25.000 deaths annually worldwide [7], with DENV-2 being the primary contributor, followed by DENV-3, DENV-4, and DENV-1 [6]. Despite its significant global impact, effective vaccines or specific therapeutics remain unavailable, hindering efforts to curb its rapid emergence and widespread transmission [8, 9]. Currently, traditional methods for DENV detection include virus isolation, serological assays, enzyme-linked immunosorbent assay, reverse-transcription polymerase chain reaction (RT-PCR), and real-time quantitative RT-PCR (qRT-PCR), all of which have been widely used for diagnosis of dengue infections [10–12]. However, these methods often rely on specific DENV antibodies, sophisticated equipment, and highly trained personnel, limiting their applicability in resource-limited regions. Consequently, there is an urgent need for an early, simple, and accurate universal detection method for DENV to facilitate timely preventive measures against infection.

The Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and CRISPR-associated (Cas) proteins (CRISPR/Cas) technology has been adapted for nucleic acid detection, with several innovative platforms developed to date [13–16]. The Cas protein, guided by a specific CRISPR RNA (crRNA), can recognize and cleave the target viral nucleic acid sequence, followed by collateral cleavage of non-target single-stranded nucleic acids in the system [14]. By employing a fluorescent dye and quencher-labeled single-stranded DNA (ssDNA) reporter, a strong fluorescence signal is generated in the presence of the target nucleic acid sequence, while no signal is detected in its absence [13, 16, 17]. Due to the exceptional sensitivity of the Cas protein, which cannot tolerate even a single base mismatch in the target sequence, the CRISPR/Cas system demonstrates remarkable specificity for nucleic acid detection. To further enhance sensitivity, a pre-isothermal amplification assay is frequently integrated into the CRISPR/Cas system. Recently, Vikrant Nain and colleagues developed a CRISPR/Cas13-based method using specific gRNAs to distinguish the four DENV serotypes, achieving universal detection of DENV [18]. However, most CRISPR-based systems involve two separated steps: pre-amplification and CRISPR/Cas detection, requiring the transfer of amplified products into the CRISPR/Cas system through uncapping operations. This process increases the risk of cross-contamination, posing a fatal limitation to these platforms.

In this study, we developed a novel one-pot system for universal DENV detection by integrating RT-RPA assay with CRISPR/Cas12a system. This was achieved by preparing the RT-RPA reaction at the bottom of the tube and placing the CRISPR/Cas12a reaction mixture in the tube cap. Following amplification, the CRISPR/Cas12a detection system was seamlessly mixed into the RT-RPA system through a brief centrifugation step, thereby entirely eliminating the risk of cross-contamination. Additionally, we established an RT-RPA-CRISPR/Cas12a-based lateral flow dipstick (LFD) platform for the detection of all four DENV serotypes. Through this study, we optimized the reaction conditions of the RT-RPA-CRISPR/Cas12a system and evaluated its performance in universal DENV detection, demonstrating its potential value as a robust diagnostic tool. The findings are expected to provide an alternative approach for other virus detection.

Materials and methods

Cells and virus

Huh7 and A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS) and a penicillin–streptomycin mix (100U/mL penicillin and 0.1 mg streptomycin/mL; Gibco, Invitrogen, USA). Cells were kept at 37 ℃ in a humidified incubator with 5% CO_2. *Aedes albopictus* mosquito (C6/36) cells (kindly provided by Prof. Jing An, Capital Medical University, China) were cultured in Roswell Park Memorial Institute (RPMI)−1640 medium (Gibco, Invitrogen, USA) supplemented with 10% FBS and antibiotics, but maintained at 28 ℃.

The DENV-2 (strain Tr1751) was kindly donated by Prof. Jing An(Capital Medical University, China) and was propagated in C6/36 cells. Four other viruses were selected as the negative control, including Sendai virus (SeV, strain Ohita), Herpes simplex virus-1 (HSV-1, strain 8F), Parainfluenza A virus (IAV, strain A/PR/8/34 H1N1), and Vesicular stomatitis virus (VSV, Indiana strain 98COE). They were kindly provided by Prof. Bing Sun (the Center for Excellence in Molecular Cell Science, Shanghai, China), Prof. QiHan Li (Chinese Academy of Medical Science, Beijing, China), Dr. Yujia Wang (National Key Laboratory of Immunity & Inflammation, Shanghai, China) and Prof. Wei Pan (Naval Medical University, Shanghai, China), respectively. These viruses were cultured as described previously[19, 20]. Viral stocks were titrated via standard plaque assays, with infectivity quantified as plaque-forming units (PFU) per mL.

Design of the DENV-specific RT-RPA assay primer and Cas12a crRNA

Based on the highly conserved regions in the genomes of all four DENV serotypes, a set of DENV-specific primers were designed, comprising 2 forward and 7 reverse primers for RT-RPA assay. All the RT-RPA primers were synthesized by Sangon Biotech (Shanghai, China). The amplification efficiency and specificity of these primers were validated in the viral RNA samples extracted from various reference DENV strains. The Cas12a crRNA was designed to target the amplified sequence of the DENV genome, and then was purified using the Cas12a High Yield crRNA synthesis and purification kit (#31,903–01, ToloBio, China), following the manufacturer's instructions. The sequences of the primers and crRNAs are detailed in Tables 1 and 2, respectively.

Table 1 The sequence of RPA primers that used in the present study

Primer	Sequence (5′–3′)
DENV_3′UTR-F1	GACTAGYGGTTAGAGGAGACCCCTCCC
DENV_3′UTR-F2	AAGGACTAGAGGTTAGAGGAGACCCCCC
DENV_3′UTR-R1a	AGAACCTGTTGATTCAACAGCACCATTCCAT
DENV_3′UTR-R1b	AGAACCTGTTGGATCAACAACACCAATCCAT
DENV_3′UTR-R2	CTGGTCTYTCCCAGCGTCAATATGCTGTTT
DENV_3′UTR-R3	TCTCTGGTCTYTCCCAGCGTCAATATGCTG
DENV_3′UTR-R4	CAGGATCTCTGGTCTYTCCCAGCGTCAATATGC
DENV_3′UTR-R5	CAGCAGGATCTCTGGTCTYTCCCAGCGTCA
DENV_3′UTR-R6	AGACAGCAGGATCTCTGGTCTYTCCCAGCGTC

Table 2 The Cas12a crRNAs for DENV detection in the present study

crRNA	Sequence (5′–3′)
crRNA1	UAAUUUCUACUAAGUGUAGAUGAGGUUAGAGGAGACCCCCC
crRNA2	UAAUUUCUACUAAGUGUAGAUCCAGCGUCAAUAUGCUGUUU
crRNA3	UAAUUUCUACUAAGUGUAGAUAGCGUCAAUAUGCUGUUU
crRNA4	UAAUUUCUACUAAGUGUAGAUCAGCGUCAAUAUGCUGUU

The target sequences were underlined

The one-pot RT-RPA-CRISPR/Cas12a DENV universal detection system

The RT-RPA reaction was performed using the commercial RNA Isothermal Rapid Amplification Kit (Basic)-II (WLRB8207KIT, AMP Future, China). The reaction was carried out in a final volume of 10 μL, prepared at the bottom of the tube, with the following components: 5.88 μL of A Buffer, 0.4 μL each of F1 (10 μM) and R1 (10 μM) primers, 0.5μL of B Buffer, 1.82 μL of nuclease-free water, and 1 μL of template. The reaction was incubated at 38℃ for 30 min, and the amplification products were verified by gel electrophoresis.

For the CRISPR/Cas12a detection, a 10 μL mixture was prepared in tube cap, containing 2 μL of HOLMES Buffer(10×), 1 μL of LbCas12a (#32,108, ToloBio, China), 2 μL of HOLMES ssDNA reporter (FAM, #31,101, ToloBio, China), 2 μL of crRNA, and 3 μL of nuclease-free water. Following amplification, the CRISPR/Cas12a reaction system was centrifuged into the RT-RPA system and incubated at 48 ℃ for 10 min. The dynamic FAM fluorescence signals were monitored every 30 s using the Applied Biosystems QuanStudio 5 real-time PCR system (QuantStudio 5, ThermoFisher, USA).

Specificity and sensitivity of RT-RPA-CRISPR/Cas12a for DENV

To evaluate the specificity, 22 cell lysates from DENV-infected samples and 4 interference samples (from VSV or IAV-infected A549 and Huh7 cells) were prepared as previously described [19, 21]. RNA extraction was performed using TRIZOL regent (Invitrogen, USA) following the established protocol described previously [22], and its concentration (od260 ssRNA) was analyzed by nano-drop (Pono-550, ThermoFisher, USA). A high-concentration synthetic RNA transcript of DENV served as the positive control (PC). Additionally, four other viruses were tested to assess the specificity of the RT-RPA-CRISPR/Cas12a platform, including SeV (RNA virus), HSV-1 (DNA virus), IAV (RNA virus), and VSV (RNA virus). The total RNAs were extracted from the cell lysis from cells infected with these viruses and was further synthesized as cDNA followed by RT-PCR assays, which was performed using a HiScript Q RT SuperMix

for qPCR (+gDNA wiper) reverse transcription kit (R123-01, Vazyme), according to the manufacturer's directions. Nuclease-free water was applied as the negative control (NC). To evaluate the effectiveness of the current platform for universal DENV detection, recombinant plasmids containing the corresponding nucleic acid sequences of DENV-1, DENV-2, DENV-3, and DENV-4 were synthesized and used as positive controls (PCs). The information on their reference strains and nucleic acid sequences is listed below. DENV-1 (NC_001477 Dengue virus 1): TGCAAACCATGGAAGCTGTACGCATGG GGTAGCAGACTAGTGGTTAGAGGAGACCCCTCC CAAGACACAACGCAGCAGCGGGGCCCAACACCA GGGGAAGCTGTACCCTGGTGGTAAGGACTAGAG GTTAGAGGAGACCCCCCGCACAACAACAAACAG CATATTGACGCTGGGAGAGACCAGAGATCCTGC TGTCTCTACAGCATCATTCCAGGCACAGAACGC CAAAAAATGGAATGGTGCTGTTGAATCAACAGG TTC. DENV-2 (NC_001474 Dengue virus 2): ACAAAC CATGGAAGCTGTACGCATGGCGTAGTGGACTAG CGGTTAGAGGAGACCCCTCCCTTACAAATCGCA GCAACAATGGGGGCCCAAGGCGAGATGAAGCTG TAGTCTCGCTGGAAGGACTAGAGGTTAGAGGAG ACCCCCCCGAAACAAAAAACAGCATATTGACGC TGGGAAAGACCAGAGATCCTGCTGTCTCCTCAG CATCATTCCAGGCACAGAACGCCAGAAAATGGA ATGGTGCTGTTGAATCAACAGGTTCT. DENV-3 (NC_001475 Dengue virus 3): GAAGCTGTACGCATG GCGTAGCAGACTAGCGGTTAGAGGAGACCCCTC CCATGACAAAACGCAGCAAGCGGGGCCCGAACA CGAGAGGAAGCTGTACCCTCGTGGGAAGGACTA GAGGTTAGAGGAGACCCCCCCGCAAAACAAAAAC AGCATATTGACGCTGGGAGAGACCAGAGATCCT GCTGTCTCTTCAGCATCATTCCAGGCACAGAAC GCCAGAAAATGGAATGGTGCTGTTGAATCAACA GGTTCT. DENV-4 (OL314747 Dengue virus 4 isolate JBB-055): ATGCGCCACGGAAGCTGTACGCGTGGG ACTAGCGGTTAGAGGAGACCCCTCCCATCATTG ACAAAACGCAGCAAAAAAGGGGGCCCGAAGCCA GGAGGAAGCTGTACTTCTGGTGGAAGGACTAGA GGTTAGAGGAGACCCCCCCAACACAAAAACAGC ATATTGACGCTGGGAAAGACCAGAGATCCTGCT GTCTCTGCAACATCAATCCAGGCACAGAGCGCC GCAAGATGGATTGGTGTTGTTGATCCAACAGGT TCT.

To analyze the sensitivity of the DENV RT-RPA-CRISPR/Cas12a assay, the recombinant plasmids were subjected to a two-fold serial dilution, with concentrations ranging from 3.125 copies/test to 100 copies/test respectively. The copy number of the template was calculated based on the molar weight and concentration of the recombinant plasmids (1ng≈7.6E9 copies). Eight replications were performed at each concentration. The limit of detection (LOD) was determined using a Sigmoid function for statistical analysis.

Detection with test strip using RT-RPA-CRISPR/Cas12a lateral flow dipstick (LFD) platform for DENV universal detection

Following the RT-RPA and CRISPR/Cas12a detection steps, 5 µl of the reaction mixture was diluted in 95 µl of nuclease-free water, and a colloidal gold test strip (#31,203, TOLO biotech, China) was inserted for visual detection. After incubation at room temperature for 2–5 min, the results were interpreted based on the color development on the test strip. In the absence of the target nucleic acid sequence, only the control line (C line) is visible, while the test line (T line) remains invisible. In the presence of the target nucleic acid, the ssDNA reporter molecule was trans-cleaved by the activated Cas12a protein, separating the FITC and biotin moieties. The FITC–anti-FITC complex was subsequently captured by the goat anti-rabbit IgG antibody immobilized on the T line, yielding a positive result. Due to the incomplete trans-cleavage of the ssDNA reporter molecule, both cleaved and intact ssDNA reporters were present in a positive assay, leading to the appearance of red bands at both the T line and the C line.

Statistical analysis

Each experiment was conducted in triplicate, and the data were expressed as mean ± standard deviation (SD). For comparison between two groups, a Student's t-test was employed for statistical analysis. For comparative analysis involving three or more groups, Analysis of Variance (ANOVA) was applied. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using Graphpad Prism software (version 9.0).

Results

The workflow of one-pot RPA CRISPR/Cas12a method for DENV detection

The workflow of the one-pot RPA CRISPR/Cas12a method for DENV detection is illustrated in Fig. 1. Initially, nucleic acid was extracted from DENV-infected samples and used as the template. The target nucleic acid sequence was then amplified using the RT-RPA assay with universal primers designed based on the conserved regions of the four genetically distinct DENV serotypes. The RT-RPA amplification reaction was prepared at the bottom of the tube. Simultaneously, the CRISPR/Cas12a detection reaction was prepared in tube cap and subsequently centrifuged into the RT-RPA amplification for detection, utilizing a FAM-labeled single-stranded DNA reporter. Upon recognition of the target nucleic acid

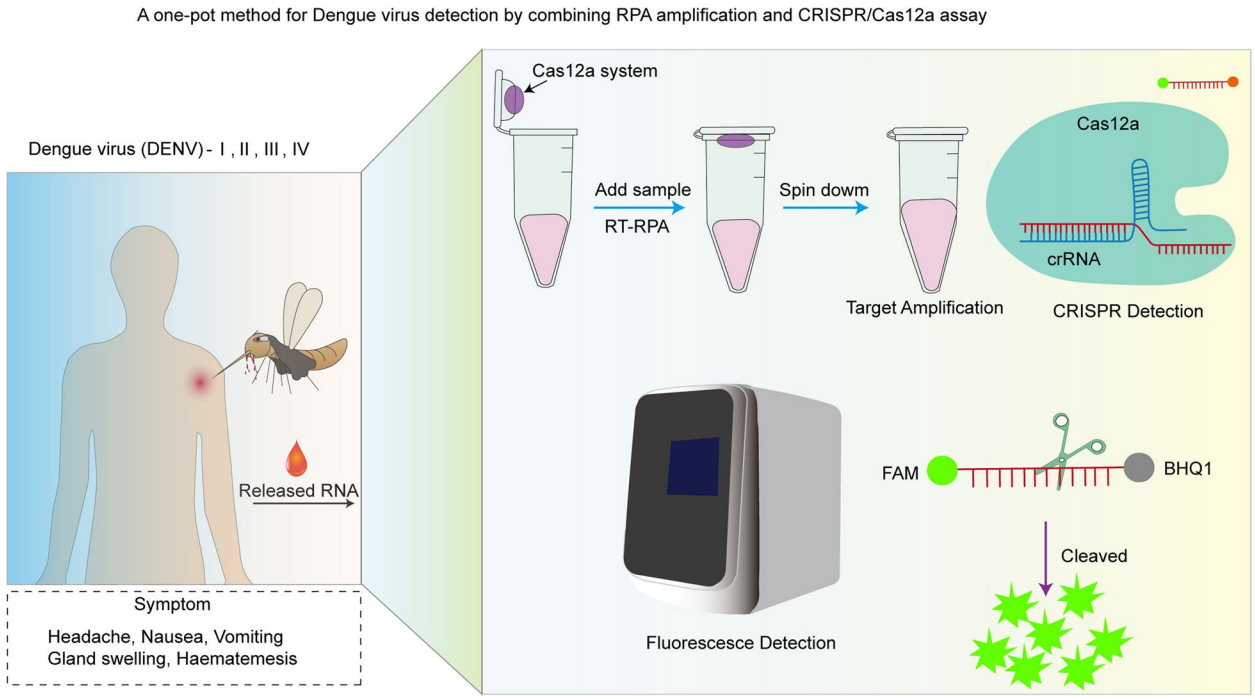

Fig. 1 Schematic illustration of one-pot RPA CRISPR/Cas12a workflow for DENV detection

sequence by the specific crRNA, the CRISPR/Cas12a system was activated, cleaving the FAM-labelled reporter and generating a fluorometric signal. This integrated approach enabled contamination-free, universal DENV detection without the need for additional uncapping steps. The entire detection process was completed within 40 min, consisting of 30 min for RT-RPA amplification and 10 min for CRISPR/Cas12a detection. Furthermore, a corresponding LFD platform was successfully established and demonstrated effective performance for universal DENV detection.

The RPA primer and crRNA selection

Based on the conserved nucleic acid sequence of DENV, a total of 2 forward and 7 reverse RPA primers were designed and synthesized. As illustrated in Fig. 2, the amplification product generated using the primer pair F1/R1 exhibited superior quality compared to those produced by other primer pairs. Consequently, the F1/R1 primer pair was selected for subsequent RPA amplification. Following this, four crRNAs were designed based on the amplified fragment. After evaluating the threshold time and fluorescence intensity, crRNA2 exhibited the optimal performance (Fig. 3). As a result, the crRNA2 was chosen for the detection assays.

Optimized the one-pot RT-RPA CRISPR/Cas12a detection reaction

To enhance the efficiency of the detection system, the concentrations of Cas12a and crRNA were systematically optimized. Cas12a was tested at concentrations of 125 nM, 250 nM, 500 nM, while crRNA was evaluated at concentrations of 125 nM, 250 nM, 500 nM, and 1000 nM. As shown in Fig. 4A, the fluorescence intensity

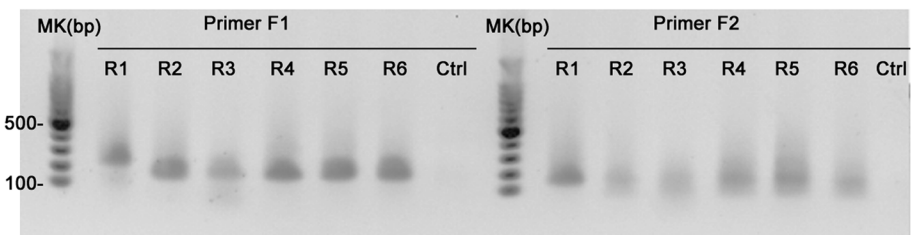

Fig. 2 Selection of RPA primers for the one-pot RPA CRISPR/Cas12a system. Two forward primers (F1 and F2) and six reverse primers were individually combined, and the resulting amplification products were analyzed using 2% agarose gel electrophoresis

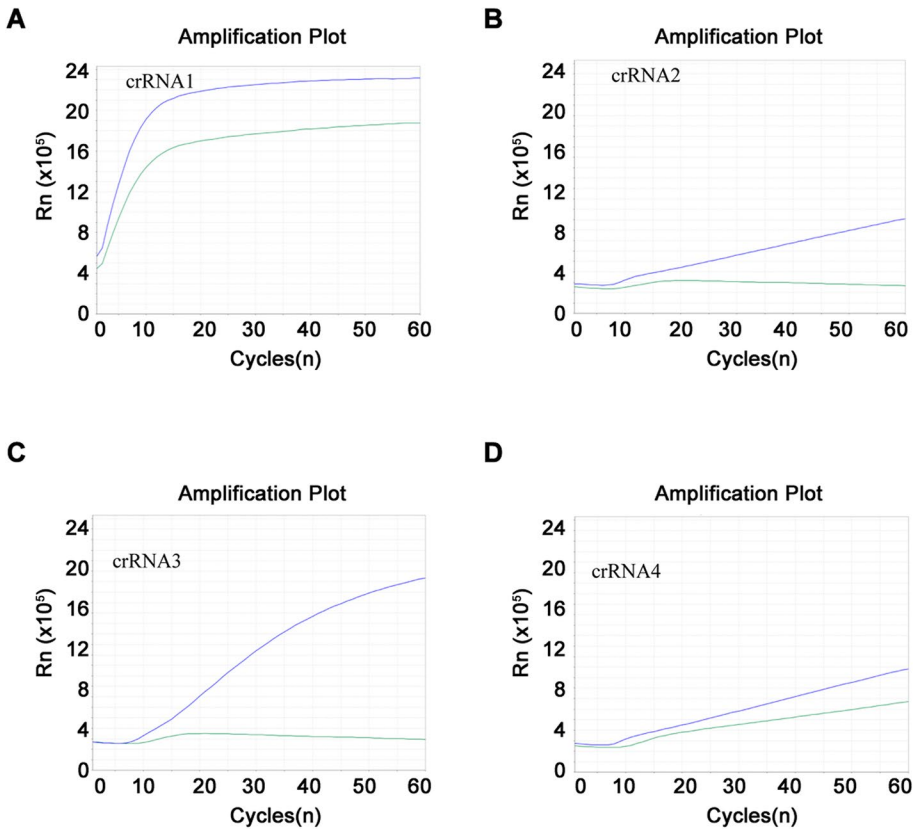

Fig. 3 Selection of the Cas12a crRNA for the one-pot RPA CRISPR/Cas12a system. The detection performance of four crRNAs (crRNA1, crRNA2, CrRNA3, and crRNA4) is shown in panels (**A**), (**B**), (**C**), and (**D**), respectively. The purple curve represents the signal from the crRNA, while the green curve represents the negative control

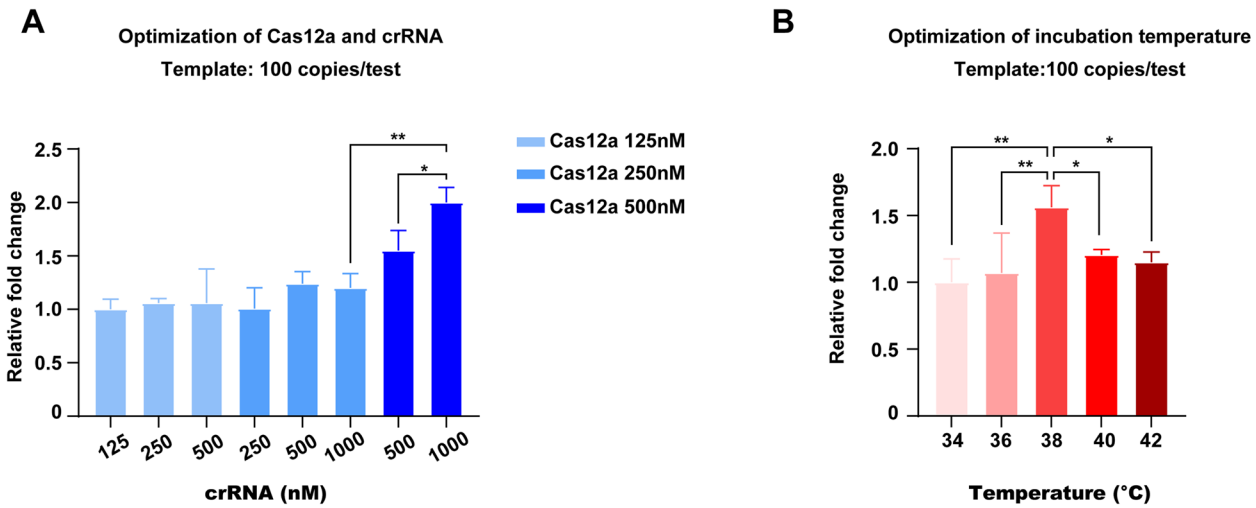

Fig. 4 Optimization of the one-pot RPA CRISPR/Cas12a system for DENV detection. **A** Optimization of Cas12a and crRNA concentrations. Cas12a was tested at 125 nM, 250 nM, and 500 nM, while crRNA was tested at 125 nM,250 nM, 500 nM, and 1000 nM. **B** Optimization of the incubation temperature for the one-pot RPA CRISPR/Cas12a system. The reaction temperature was evaluated at 34 ℃, 36 ℃, 38 ℃, 40 ℃ and 42 ℃. Each condition was tested in four repetitions. *$p < 0.05$; **$p < 0.01$

of the detection reaction peaked when using 500 nM Cas12a and 1000 nM crRNA.

Temperature is a critical factor influencing the detection reaction. To determine the optimal temperature, the reaction was tested at 34 ℃, 36 ℃, 38 ℃, 40 ℃ and 42 ℃. The highest fluorescence intensity was observed at 38 ℃, which was subsequently selected as the optimal temperature for the assay (Fig. 4B).

Sensitivity and specificity analysis of one-pot RT-RPA CRISPR/Cas12a detection reaction

To assess the sensitivity of the detection system, the LOD was determined by performing a two-fold serial dilution of the recombinant plasmid, ranging from 3.125 copies/test to 100 copies/test respectively. As illustrated in Fig. 5, the LOD of the system was determined to be 91.7 copies/test at a 95% probability.

To evaluate specificity, a total of 26 virus-infected cell samples were tested. The A549 or Huh7 cells were infected with DENV virus or four non-DENV virus, including Sendai virus (SeV), Herpes simplex virus-1 (HSV-1), Parainfluenza A virus (IAV), and vesicular stomatitis virus (VSV). And each sample was tested in triple repetitions. The recombinant DENV plasmid was served as the positive control (PC) and the nuclease-free water was used as the negative control (NC). All DENV-positive samples were accurately identified, and no cross-reactivity was observed, suggesting 100% specificity of the detection system for DENV-infected cells with different infection time and multiplicity of infection (MOI) (Fig. 6A).

Additionally, this one-pot RT-RPA CRISPR/Cas12a detection reaction was performed in A549 cells infected with 4 non-DENV virus, including SeV, HSV, IAV, and VSV. The recombinant DENV plasmid served as the PC, while nuclease-free water was used as the NC. We obtained both RNA and cDNA from the A549 cells treated as described previously and performed one-pot RT-RPA CRISPR/Cas12a detection reaction. As shown in Fig. 6B, only the PC sample produced a significant fluorescence signal, with no notable signals detected from the other interfere samples. These results confirmed that the system is highly specific for DENV detection, with no cross-reactivity observed.

Validation of the one-pot RT-RPA CRISPR/Cas12a DENV detection system

To assess the effectiveness of the platform for universal DENV detection, recombinant plasmids containing the nucleic acid sequences of DENV-1, DENV-2, DENV-3, and DENV-4 were synthesized and tested. As shown in Fig. 7A, all four DENV serotypes were successfully identified using our CRISPR-based detection system.

Establishment and validation of the RT-RPA CRISPR/Cas12a-LFD DENV detection system

Additionally, we developed the DENV RT-RPA-CRISPR/Cas12a-LFD platform and used it to detect the four recombinant DENV plasmids. As illustrated in Fig. 7B, all four DENV serotypes were accurately identified using the RT-RPA-CRISPR/Cas12a-colloidal gold test strip platform (Fig. 7B).

To optimize the FAM-T7-Biotin reporter used in the reaction, its concentration was serially adjusted in two-fold increments from 200 to 25 nM. As shown in Fig. 7C, the efficiency of the test strip showed no significant

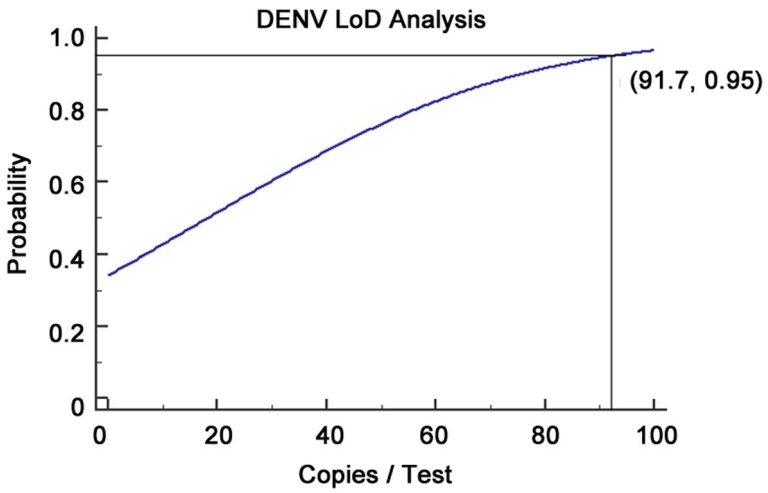

Fig. 5 Analysis of the LOD for the DENV one-pot RPA CRISPR/Cas12a detection system. The LOD of the system was determined to be 91.7 copies/test at a 95% probability

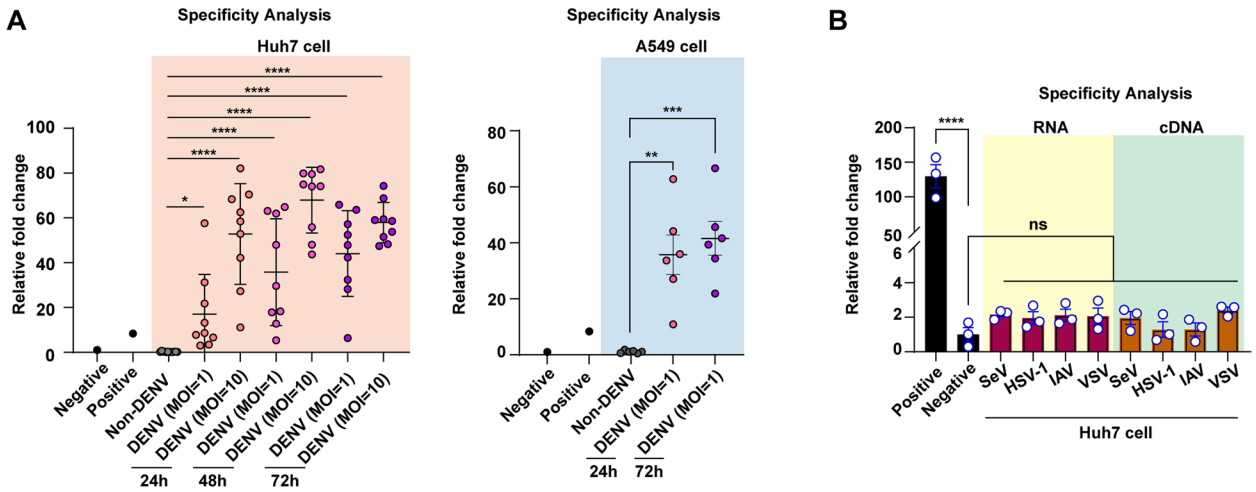

Fig. 6 Specificity analysis of the DENV one-pot RPA CRISPR/Cas12a detection system. **A** Nucleic acid samples were derived from Huh7 or A549 cells infected with DENV or non-DENV virus. **B** The RNA and cDNA samples from A549 cells infected with SeV, HSV, IAV or VSV were used to evaluate the specificity of the system. At least three technical replicates were performed for each biological sample. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$

difference across the tested concentrations. To minimize costs, ssDNA reporter concentration was set at 25 nM for subsequent test strip assays.

To validate the sensitivity of the test strip assay, cultured DENV-2 virus was used for detection. The DENV-2 nucleic acid template was diluted to 2000, 1000, 500, 250, and 125 copies/test, with four test replications tested for each concentration. As shown in Fig. 7D, all four replicates were positive at the concentration of 2000, 1000, and 500 copies/test, while three replicates were positive at 250 copies/test and two at 125 copies/test. All these results demonstrate that the RT-RPA CRISPR/Cas12a-LFD platform is highly effective for universal DENV detection.

Discussion

DENV is a deadly virus that deeply threaten the human health and life quantity, with over 4 billion people currently at a risk of dengue infection [23]. Despite its global impact, no approved specific therapies or vaccine are available to date. Therefore, the development of early detection methods for DENV is essential to prevent its pandemic and optimize public health responses.

With advance in molecular detection technologies, PCR-based methods have become widely adopted as routine assay for DENV diagnosis due to their high sensitivity [24–26]. Compared with PCR-based method, RT-RPA-assist methods have emerged as promising alternatives for DENV detection [27–29]. However, non-specific amplification products cannot be entirely eliminated, leading to a high rate of false-positive results [30, 31]. Xia et.al and colleagues have developed a bio-enzyme method for DENV detection based on Cas13a and Cas12a. [32]. In this study, we developed a novel universal DENV detection system by combining RT-RPA with the CRISPR/Cas12a assay in one-pot, achieving remarkable sensitivity (LOD = 91.7 copies /test) and specificity for DENV detection. This system offers several advantages over traditional methods. First, it eliminates the need for sophisticated equipment. Second, the entire detection process can be completed within 40 min. Third, it mitigates the risk of cross-contamination caused by aerosol generation. Last but not the least, high specificity RT-RPA CRISPR/Cas12a platform minimizes the risk of misdiagnosis. Collectively, this system represents a significant advancement in the rapid, simple, and accurate diagnosis of universal DENV infection.

In China, the incidence of DENV infection is increasingly serious in recent years, with all the four serotypes of DENV implicated in [33, 34]. Due to the genetic diversity of the DENV, including their multiple serotypes and genotypes, most existing detection platform fails to identify dominant serotypes in specific regions. A universal DENV detection platform has remained elusive due to technical limitations. Huangxian Ju and colleagues developed an electrochemical method based on CRISPR/Cas13a-assisted catalytic hairpin assembly (CHA), achieving a LOD of 0.78 fM (femtomolar, 1 fM = 6.02×10^5/ml) for DENV-1 detection [35]. Despite its high sensitivity, this method requires costly electrochemical biosensor, specialized technicians, and cannot identify the other DENV serotypes. By performing multiple sequence alignment of the four DENV serotypes, we designed a pair of primers capable of amplifying a target sequence encompassing the conserved regions of all four DENV serotypes. A Cas12a crRNA was then designed to

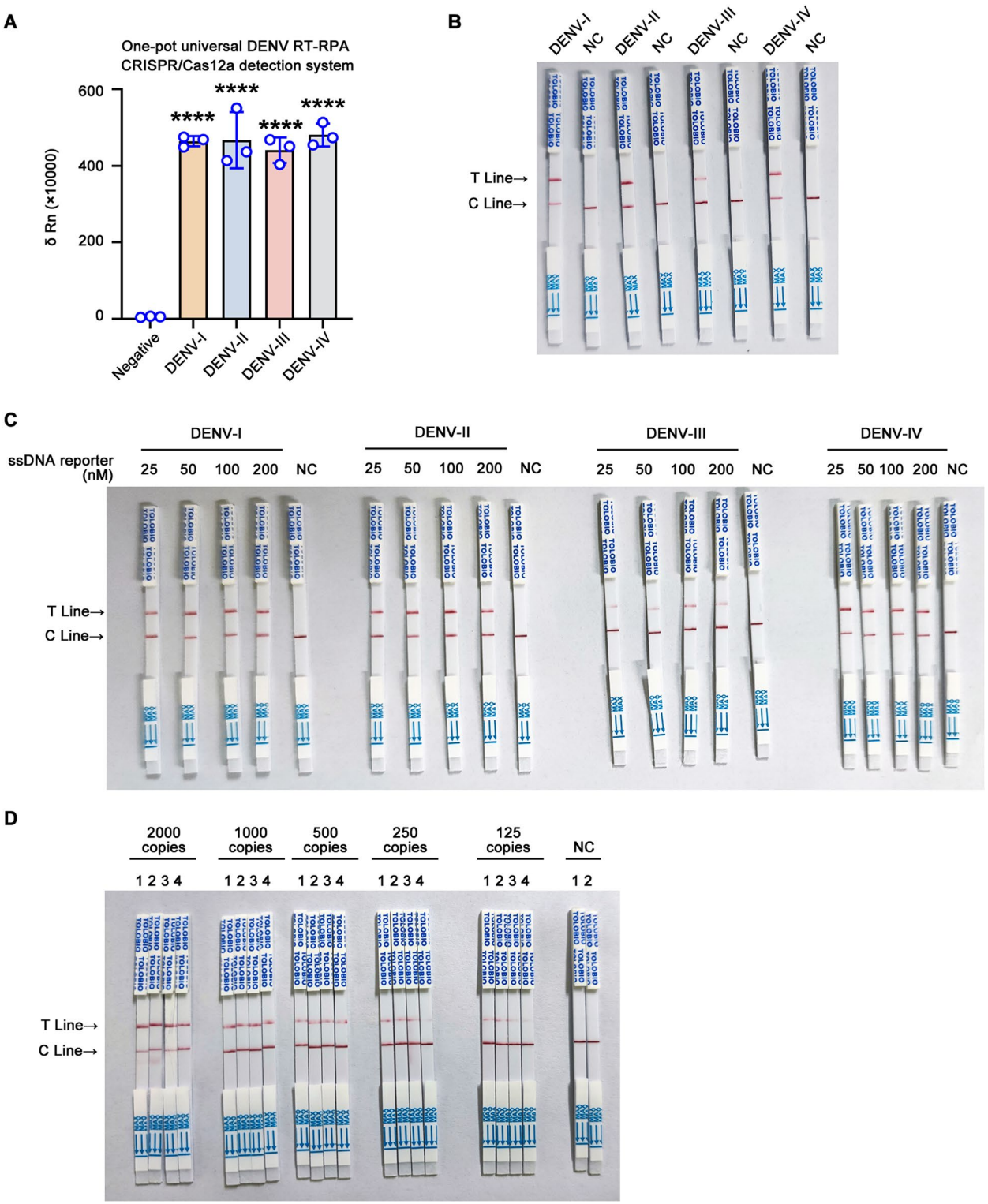

Fig. 7 Establishment of the RT-RPA CRISPR/Cas12a-LFD system. **A** The recombinant plasmids of DENV-1, DENV-2, DENV-3, and DENV-4 were successfully identified using the one-pot universal DENV RT-RPA CRISPR/Cas12a detection system. **B** The recombinant plasmids of DENV-1, DENV-2, DENV-3, and DENV-4 were detected using the DENV RT-RPA CRISPR/Cas12a-LFD detection system. **C**. Optimization of the ssDNA reporter concentration for the DENV RT-RPA CRISPR/Cas12a-LFD detection system. The ssDNA reporter was serially diluted by two-fold, ranging from 200 to 25 nM. **D** Sensitivity analysis of the DENV RT-RPA CRISPR/Cas12a-LFD detection system. The template was diluted to 2000, 1000, 500, 250, and 125 copies/test, with $n = 4$ replicates for each concentration

recognize this target sequence. Consequently, the one-pot DENV universal detection system successfully identified recombinant plasmids containing the conserved region of DENV-1 to DENV-4. This indicates that our RT-RPA-CRISPR/Cas12a system is capable of detecting all four DENV serotypes, making it a promising diagnostic tool for clinical applications in dengue fever detection.

Furthermore, the corresponding RT-RPA CRISPR/Cas12a-LFD platform was successfully established and displayed excellent performance in detecting recombinant plasmid. Given the affordability and convenience of LFD, our DENV detection system holds great potential for application in resources-limited regions, particularly in developing countries, which contributes to the development of Point-of-care Testing (POCT) and in vitro diagnostic (IVD) products for DENV. Moreover, all components of the assay can be pre-prepared as lyophilized powders, and the entire process can be performed using a simple thermal heater or even at room temperature. This makes the RPA-CRISPR/Cas12a platform highly suitable for deployment in resource-poor settings. In brief, our findings not only provide a promising and practical approach for universal DENV detection but also offer valuable insight for developing diagnostic methods for other human viruses. However, the LoD of the LFD test is not the same as the fluorescence result, in which the LOD was calculated based on the fluorescence data collected by the qRT-PCR machine. In LFD experiment, 500 copies might have a high probability of being detected, while 250 copies might have a false negative result.

Nevertheless, the off-target effects of the CRISPR/Cas12a system could not be overlooked, which refers to the unintended cleavage or binding of crRNA with the non-target nucleic acid sequences [36]. Off-target activity particularly occurs when the crRNA shares partial homology with the non-target sequences, resulting in false-positive results that compromise the accuracy and reliability of the system. So far, several strategies have been proposed to minimize off-target effects in CRISPR/Cas12a-based systems, including crRNA design optimization, utilization of Cas12a variants with high-fidelity and suitable concentration, protospacer-adjacent motif (PAM) recognition modification, and dual crRNA validation [37].

Conclusion

In this study, we developed a one-pot RT-RPA-CRISPR/Cas12a system for universal DENV detection, and established a corresponding RT-RPA CRISPR/Cas12a-LFD system. The RT-RPA CRISPR/Cas12a method exhibited high specificity and sensitivity for DENV, with no observed false-positive results. This system offers a rapid, accurate, simple, and contamination-free approach, making it a highly effective diagnostic tool for DENV infection.

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Authors' contributions
Yunkai Zhang, Xingguang Liu. and Xiaomeng Ren supervised and secured funding for the project; Yunkai Zhang, Xingguang Liu. and Xiaomeng Ren conceived the study, designed experiments, analyzed data, and revised the manuscript; Yunkai Zhang, Yan Xiang and Dengyong Hou designed experiments, performed experiments, analyzed data, interpreted data and drafted the manuscript. Liben Fang, Shuqi Cai, Jianping Zhang, Yujia Wang, Yuyu Jiang, Bin Liu, Jie Bai, Yue Ding, Jingjing Fang, Shuanghong Chen performed experiments and collected data.

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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