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Research Article

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A dual-probe one-pot RPA-CRISPR/Cas12a: a highly sensitive and rapid method for detection of *Phytophthora vignae*

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Abstract

BACKGROUND: *Phytophthora vignae* is the primary pathogen affecting cowpea plants. Early on-site and accurate detection of the causal pathogen is critical for successful management. Therefore, it is both essential and urgent to create a rapid, sensitive, and accurate detection for *P. vignae* in cowpeas.

RESULTS: In this study, the one-pot RPA-CRISPR/Cas12a assay demonstrated the ability to effectively differentiate closely related oomycete species, exhibiting excellent specificity. The entire detection process can be completed within 15–20 min, with results being visually determined using a lateral flow strip (LFS) or by fluorescence detection under blue or ultraviolet (UV) light. Additionally, we have added dual probes to the one-pot RPA-CRISPR/Cas12a assay detection system, this modification improved reaction efficiency and significantly enhanced sensitivity (200 fg μL^{-1}).

CONCLUSION: This study developed a dual-probe one-pot RPA-CRISPR/Cas12a detection platform that demonstrates rapid and highly sensitive identification of *P. vignae*. The proposed technology enables field-deployable detection without requiring specialized technical expertise, representing a promising approach with substantial potential for practical applications.
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Supporting information may be found in the online version of this article.

Keywords: cowpea; dual-probes; one-pot RPA-CRISPR/Cas12a; visual detection

1 INTRODUCTION

Phytophthora stem rot caused by *Phytophthora vignae* was first reported in 1957 and has become a significant threat to cowpea production.[1] It primarily affects stems, leaves, and pods, with an incidence rate typically ranging from 25% to 35%, and in severe cases, reaching as high as 75%. Under conditions of high temperature and heavy rainfall, the disease progresses rapidly, exhibiting strong epidemic potential and devastating effects, leading to substantial losses in cowpea yield.[2] To effectively manage cowpea Phytophthora stem rot, early detection technologies are critical for disease diagnosis.

The traditional detection methods are often cumbersome, time-consuming, and require detailed taxonomic information about the pathogen, failing to meet the demands for rapid, sensitive, and reliable detection in disease management. Due to the limitations and inconveniences associated with traditional morphological identification, many pathogen detection methods have shifted to conventional molecular techniques, such as conventional polymerase chain reaction (PCR) and quantitative PCR (qPCR).[2–4] Several reports have been published on the development of DNA-based methods for the detection and identification of *Phytophthora* spp. which provides strategies for effective Phytophthora control, however little is known about detection methods for *P. vignae* in cowpea.[5,6] However, even in well-equipped laboratories with skilled personnel, the application of these molecular techniques is hindered in complex field settings and environments with limited resources. In contrast, the widespread use of assorted isothermal nucleic acid amplification techniques, for instance, loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA), has gained prominence due to their ability to amplify nucleic acids at specific temperatures.[4,7–9] This feature enables on-site detection in a resource-limited environment. Nevertheless, these methods carry potential risks, primarily due to aerosol contamination, which can lead to non-specific amplification and false-positive results.[10,11]

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Currently, CRISPR-Cas systems are closely integrated with molecular detection technologies, demonstrating significant practical value.[12–16] Methods such as DETECTR for CRISPR-based detection are highly rapid and efficient for pathogen identification.[12,13,16,17] Due to the fast detection time, high specificity, and sensitivity of CRISPR-based molecular detection, it not only matches but often surpasses traditional methods like PCR and qPCR.[18,19] Moreover, these detection methods are user-friendly for non-specialists and can be adapted to most resource-limited environments, including on-site detection.[16–18] CRISPR-based detection technologies (utilizing Cas9, Cas12, and Cas13) are being employed across various platforms,[14–20] However, different Cas proteins exhibit distinct enzymatic characteristics. For instance, Cas9 is a powerful tool for gene editing but has limited trans-cleavage activity, while Cas13 is an RNA-target-dependent endonuclease that specifically recognizes and cleaves RNA targets.[21] Although CRISPR/Cas12a has also demonstrated an outstanding performance in detecting plant pathogens.[16,22–26] Since these detection technologies separate nucleic acid amplification from the CRISPR-Cas system, they do not perform reactions in a one-pot, which significantly increases the risk of contamination during step transfers, as opening the tube during pre-amplification can lead to non-specific amplification and cross-contamination.[27,28] Therefore, to address these challenges, it is essential to integrate all ingredients of the RPA-CRISPR/Cas experiment into a one-pot reaction.[10,27] This approach not only decreases the hazard of cross-contamination but also enhances detection specificity.[20,22,29–31]

In this study, we developed a dual-probe one-pot RPA-CRISPR/Cas12a technology. Compared to the conventional single-probe one-pot CRISPR/Cas12a assay, simply adding a second probe to the existing system enhances the sensitivity of both the two-step and one-step methods. In the two-step one-pot RPA-CRISPR/Cas12a method, after RPA, the pre-prepared Cas12a solution is rapidly centrifuged from the tube cap to the bottom, where it combines with the RPA solution to initiate the reaction. Upon completion, the results are visualized under blue or ultraviolet (UV) light. In the one-step one-pot RPA-CRISPR/Cas12a technology, the RPA and optimized Cas12a solutions are directly mixed to start the cleavage reaction, with results similarly detected under blue or UV light. To facilitate on-site experiments, we have also integrated the one-pot RPA-CRISPR/Cas12a technology with lateral flow strip (LFS). In summary, we have developed a versatile, user-friendly, ultrasensitive, and visually intuitive one-pot detection method for *P. vignae*, offering broad applications powerful in the detection of plant pathogens.

2 MATERIALS AND METHODS

2.1 Materials and reagents

All the primers, probes, CRISPR RNA (crRNA), and DNA fragments were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). Reagents were purchased from Tiangen Biotech (Beijing, China) including High-Affinity HotStart Taq (ET108-1), 2× PCR SuperMix, and the genomic DNA extraction kit. REnGen® LbaCas12a and NEBuffer™ were obtained from New England BioLabs Ltd (Beijing, China). The RPA kit was purchased from AMP-Future Biotech Co., Ltd (Changzhou, China).

2.2 Isolates and DNA extraction

The isolates pathogens used in this study were isolated from infected cowpea plants gathered in the field or obtained from Hainan University (Supporting Information, Table S1). All isolates were routinely maintained in liquid nitrogen at the School of Breeding and Multiplication, Hainan University, China. Genomic DNA was extracted from mycelial pellets using the 2% CTAB (cetyltrimethylammonium bromide) extraction method.[32] The DNA was diluted to a concentration of 200 ng μL^{-1} using nuclease-free water and stored at 4 °C.

2.3 Design of primers and crRNAs

To find a conserved and specific detection target for *P. vignae*, a BLAST (Basic Local Alignment Search Tool) search was conducted using all available gene sequences of *P. vignae* and genome sequences of related taxa. The gene sequence (ABJUN010000202.1 Up-Downstream 5 kb JABJXB010000007.1: 772555-783124, *P. vignae* strain PVMG4, Contig 7) was ultimately selected as the specific target for the detection used in this study. Primers for RPA (Pv-RPA-F/Pv-RPA-R), PCR (Pv-F/Pv-R), and qPCR (Pvq-F/Pvq-R) were designed to be consistent and analyzed using Primer5. The crRNA consists of a structural sequence (5′-UAAUUUCUACUAAGUGUAGAU-3′) and 20 bases following the PAM site. It was devised by operating EuPaGDT (http://www.grna.ctegd.uga.edu). The sequences of all primers are listed in Table 1.

2.4 Conventional PCR detection of *P. vignae*

In this study, the conventional PCR detection was set up in a 10 μL reaction volume. The experiment mixture comprised

Table 1. List of primers, CRISPR RNA (crRNA), and probes used in this study

Primers or probes	Sequences (5′ → 3′)	Purpose
Pv-F	GCAAGATTGCTTGGTTGAGTT	For PCR
Pv-R	GCGGTTGGAGAACTCGCCAT	
Pvq-F	GCAAGATTGCTTGGTTGAGTT	For real-time PCR
Pvq-R	GCGGTTGGAGAACTCGCCAT	
PvFQ-reporter 1	FAM-TCTCGGCTGTTTCGTACCGTCCTCTA-BHQ1	
Pv-RPA-F	GCAAGATTGCTTGGTTGAGTTCTACAGCAG	For RPA
Pv-RPA-R	GCGGTTGGAGAACTCGCCATGGTACTTCTC	
crRNA	UAAUUUCUACUAAGUGUAGAUCCUUAGAGGACGGUACGAAA	For Cas12a cleavage
FQ-reporter 1	FAM-CCACCC-BHQ1	
FQ-reporter 2	FAM-CCCCCC-BHQ1	
LFS-FQ-reporter 1	FAM-CCACCC-Biotin	For LFS

Note: LFS, lateral flow strip; PCR, polymerase chain reaction; RPA, recombinase polymerase amplification.

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5 μL of 2× Taq PCR Master Mix, 0.25 μL (Pv-F/Pv-R) (10 μM), 1 μL target DNA (200 ng μL^{-1}), the remaining volume was filled with nuclease-free water to achieve a total of 10 μL. After preparing the experiment mixture, it was briefly centrifuged and placed in a PTC-200 thermal cycler (MJ Research, Inc., Watertown, MA, USA). The steps are as follows: initial denaturation at 94 °C for 30 s, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 65 °C for 35 s, extension at 68 °C for 10 s, with a final extension at 68 °C for 15 min, and then held at a low temperature for storage. Each experiment was performed in triplicate.

2.5 Detection of *P. vignae* by real-time fluorescence qPCR

Real-time PCR detection was carried on in a 20 μL reaction volume. Each reaction included 0.5 μL primers (Pvq-F/Pvq-R) (10 μM), 0.5 μL Pv-FQ-reporter 1 (10 μM), 1.6 μL of dNTPs (2.5 mM), 0.2 μL of High-Affinity HotStart Taq, 4 μL of 5× Probe qPCR Buffer, 2 μL of 10× HA Buffer, and 1 μL of target DNA (200 ng μL^{-1}). Finally, 9.7 μL of nuclease-free water was added. Real-time fluorescence qPCR was carried out using the AriaMx qPCR system (Agilent Technologies, Inc., Santa Clara, CA, USA). The reaction process is composed of an initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 30 s. Each experiment was performed in triplicate.

2.6 Optimization of the one-pot RPA-CRISPR/Cas12a detection

The concentration and ratio of Cas12a/crRNA significantly influence the entire velocity of the CRISPR/Cas12a experiment. To improve the one-pot RPA-CRISPR/Cas12a assay for better reaction efficiency, the following optimization steps were performed. Initially, the concentration scope of Cas12a and crRNA (50–800 nM) was tested. Next, the different ratios of Cas12a/crRNA from 1:10 to 2:1 were optimized under the optimal concentration of Cas12a and crRNA. Additionally, the RPA reaction time (5–30 min) and the RPA-CRISPR/Cas12a reaction temperature (37–42 °C) were evaluated, with a negative control included in each experiment. Finally, fluorescence curves and intensity values were analyzed, and the fluorescence tubes were visually inspected under blue or UV light to determine the optimal reaction conditions. All experiments were repeated three times.

2.7 Detection of two probes

The fluorescent reporter probes consist of 6-carboxyfluorescein (FAM) at the 5′ end and a quencher at the 3′ end, connected by a sequence of bases (5′ FAM-CCACCC-BHQ1 3′) or (5′ FAM-CCCCCC-BHQ1 3′).[33,34] In their intact state, these probes do not emit fluorescence. However, when cleaved by the ternary complex, they gradually release green fluorescent signals until complete cleavage is achieved.

2.8 The one-pot RPA-CRISPR/Cas12a assay

The two-step one-pot RPA-CRISPR/Cas12a system consists of 10 μL of RPA mixture and 10 μL of CRISPR/Cas12a mixture. Specifically, 10 μL of RPA mixture was taken from a pre-prepared 50 μL RPA system according to the RPA kit instructions and combined with 10 μL of CRISPR/Cas12a mixture. The 10 μL CRISPR/Cas12a mixture contained 3 μL of Cas12a (final 1.2 μM), 3 μL of crRNA (final 600 nM), 2 μL of FQ-reporter 1 (final 10 μM), 1.5 μL of 10× NEB Buffer 2.1, and was supplemented with nuclease-free water to reach 10 μL. The one-step one-pot RPA-CRISPR/Cas12a experiment consists of 30 μL of RPA mixture and 10 μL of CRISPR/Cas12a mixture. Similarly, 30 μL of RPA mixture was taken from a pre-prepared 50 μL RPA system and combined with 10 μL of CRISPR/Cas12a mixture, which contained 3 μL of Cas12a (1.2 μM), 3 μL of crRNA (600 nM), 2 μL of FQ-reporter 1 (10 μM), 1.5 μL of 10× NEB Buffer 2.1, and was supplemented with nuclease-free water to reach 10 μL. In the two-step method, the CRISPR/Cas12a mixture was initially entered into the tube cap, while in the one-step method, the CRISPR/Cas12a mixture was directly added to the bottom of the tube to mix with the RPA solution. For the two-step method, after a 10 min RPA reaction at 39 °C, the CRISPR/Cas12a mixture was centrifuged to the bottom of the tube and incubated at 39 °C for 5–10 min. Positive reaction tubes were visualized under blue or UV light. For the one-step method, the experiment only requires 5–15 min at 40 °C, the positive reaction tubes similarly showed green fluorescence under blue or UV light after completion.

2.9 LFS visualization of RPA-CRISPR/Cas12a assays

Initially, when there is no target gene in the LFS detection system, the control line appears red, while the test line shows no color change. However, when the target gene shows up, the CRISPR/Cas12a system cleaves the fluorescent reporter gene, resulting in both the control line and the test line displaying a red stripe. So, the process involves first reacting 10 μL of RPA mixture for 10 min, followed by combining it with 10 μL of CRISPR/Cas12a mixture and reacting for an additional 5–10 min. Then, 40 μL of nuclease-free water is entered to dilute it, and LFS results are read out after 5 min. After completion, positive results are visually observed as a red stripe on both the control and test lines of the LFS or as a red stripe only on the test line, while negative results show a red stripe solely on the control line. All lateral flow assays were performed in triplicate.

2.10 Specificity and sensitivity of one-pot RPA-CRISPR/Cas12a assays

To evaluate the specificity of the one-pot RPA-CRISPR/Cas12a assay, a total of 15 different oomycete species and four species of fungi isolated from cowpea were used (Table S1). To assess the sensitivity of the one-pot RPA-CRISPR/Cas12a assay, the DNA template of *P. vignae* was serially diluted in ten-fold concentration gradients. Nuclease-free water was used as a negative control included in each experiment. All experiments were repeated three times.

2.11 Practicability of using RPA-CRISPR/Cas12a for detecting field samples

To assess the practical application of the RPA-CRISPR/Cas12a detection for *P. vignae*, eight samples of cowpea plants with symptomatic and asymptomatic (Supporting Information, Fig. S1) were collected from different regions, including Sanya and Ledong County in Hainan Province. The DNA was extracted from cowpea plants as described previously.[35] These DNA samples were then used as templates for detection using the one-pot RPA-CRISPR/Cas12a assay developed in this study. DNA from *P. vignae* served as the positive control, while DNA from healthy cowpea plants was used as the negative control. The *P. vignae* infection was confirmed using the traditional isolation and cultivation methods employed to identify the pathogen based on its morphological characteristics. All experiments were repeated three times.

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3 RESULTS

3.1 Create a one-pot RPA-CRISPR/Cas12a test

To facilitate better on-site detection of *P. vignae*, we created a one-pot RPA-CRISPR/Cas12a detection assay, which includes both a two-step and a one-step approach. A schematic diagram of the experiment and the time required for each step are shown in (Fig. 1). The two-step one-pot RPA-CRISPR/Cas12a assay (Fig. 1(A)) consists of four steps: (1) preparation of the test sample, (2) targeted DNA amplification via RPA using an isothermal device, (3) Cas12a-mediated reaction, (4) visual readout. In contrast, the one-step one-pot RPA-CRISPR/Cas12a detection technology (Fig. 1(B)) involves three steps: (1) preparation of the test sample, (2) direct incubation of RPA with CRISPR/Cas12a in an isothermal device, where fluorescence signals are generated through crRNA-mediated cleavage, and (3) visual readout. DNA templates were extracted from cowpea plants infected with *P. vignae*. In the two-step method, the prepared RPA mixture was added directly to the bottom of the fluorescence quantification tube, while the CRISPR/Cas12a mixture was entered into the tube cap. The RPA experiment was completed within 10–15 min. Subsequently, the CRISPR/Cas12a reagent in the cap was centrifuged to the bottom to mix with the RPA solution. Guided by crRNA, the Cas protein specifically recognizes the target amplification fragment, activating its collateral activity to cleave the FQ reporter, resulting in visual fluorescence. The fluorescence signal emitted by the reaction solution was visually determined under blue or UV light (Fig. 1(A)). In the one-step method (Fig. 1(B)), RPA was entered into the bottom of the reaction tube, and CRISPR/Cas12a was directly blended with the RPA solution. Since the limited targets cannot initiate the cleavage activity of CRISPR/Cas12a, the RPA reaction activated rapidly and promoted the accumulation of initial templates, then those amplicons were recognized by CRISPR/Cas12a, resulting in the emission of fluorescence signals from the reporter. In summary, our study demonstrates the fruitful establishment of a one-pot RPA-CRISPR/Cas12a experiment for detecting *P. vignae*.

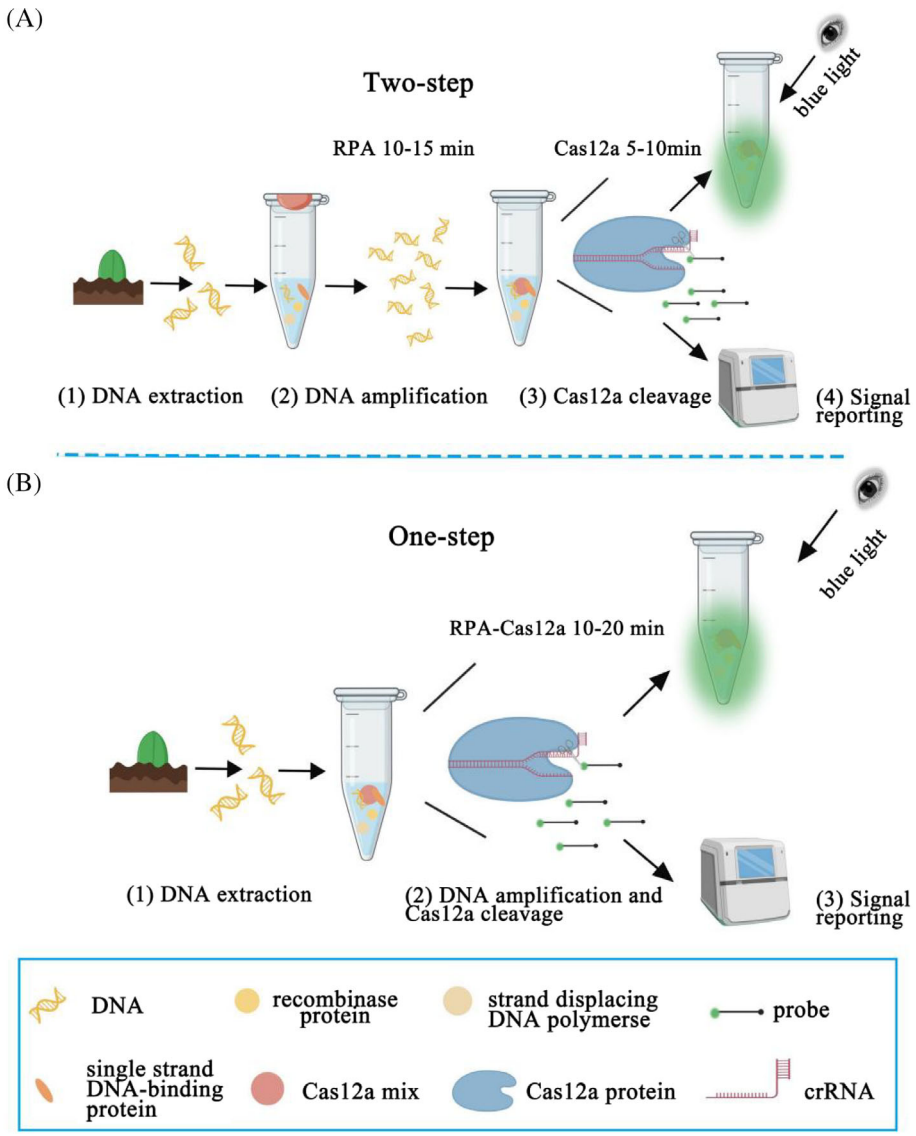


Figure 1. Diagrammatic sketch of the workflow for one-pot RPA-CRISPR/Cas12a with two-step and one-step detection methods. (A) One-pot RPA-CRISPR/Cas12a with two-step method: (1) DNA extraction from field samples; (2) recombinase polymerase amplification (RPA); (3) combination of RPA amplicons with CRISPR/Cas12a; (4) visualization of fluorescence signals. (B) One-pot RPA-CRISPR/Cas12a with one-step method: (1) DNA extraction from field samples; (2) simultaneous RPA of the target gene and Cas12a reaction; (3) visualization of fluorescence signals.

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3.2 Establish and optimization of a one-pot RPA-CRISPR/Cas12a assay

To systematically evaluate the one-pot RPA-CRISPR/Cas12a assay, seven reaction systems containing different components were subjected to testing. As depicted in Fig. 2(A), after incubating the RPA mixture at 39 °C for 10 min and performing CRISPR/Cas detection for 5–10 min, only reaction #1, containing Cas12a, crRNA, fluorescent reporter, target DNA, and RPA reaction mix, allows for visual readout of results under blue or UV light. This is to confirm the fluorescence signal intensity, we analyzed the one-pot RPA-CRISPR/Cas12a experiment using the AriaMx qPCR instrument. The fluorescence values indicated that the one-pot RPA-CRISPR/Cas12a experiment provided better fluorescence intensity (> 28 000 AU) within 10–15 min (Fig. 2(B)). Analysis of the fluorescence curves demonstrated that the one-pot RPA-CRISPR/Cas12a assay offers a specific, simple, rapid detection approach for *P. vignae*.

To ensure a simpler and more efficient one-pot RPA-CRISPR/Cas12a reaction, we initially optimized the concentration ratio and proportion of Cas12a/crRNA in a one-pot RPA-CRISPR/Cas12a reaction. The results showed that the best final concentration of Cas12a/crRNA was determined to be 600 nM (Figs 2(C) and S2). Under this concentration, different ratios of Cas12a and crRNA from 1:10 to 2:1 for both the one-step and two-step in the one-pot RPA-CRISPR assay were performed. The results show that a ratio of Cas12a/crRNA at 1:2 or 2:1 was found to be the most effective in both the one-step or two-step one-pot RPA-CRISPR/Cas12a assay (Figs 2(D) and S3). Subsequently, using the optimized Cas12a/crRNA conditions, we further optimized the temperature (from 37 °C to 42 °C) for the one-pot RPA-CRISPR/Cas12a assay. The results showed that the best temperature of one-step and one-pot RPA-CRISPR/Cas12a was 40 °C (Figs 2(E) and S4) which is higher than the two-step 39 °C (Fig. S5), this temperature ensured efficient RPA reaction activity and facilitated accumulation initial

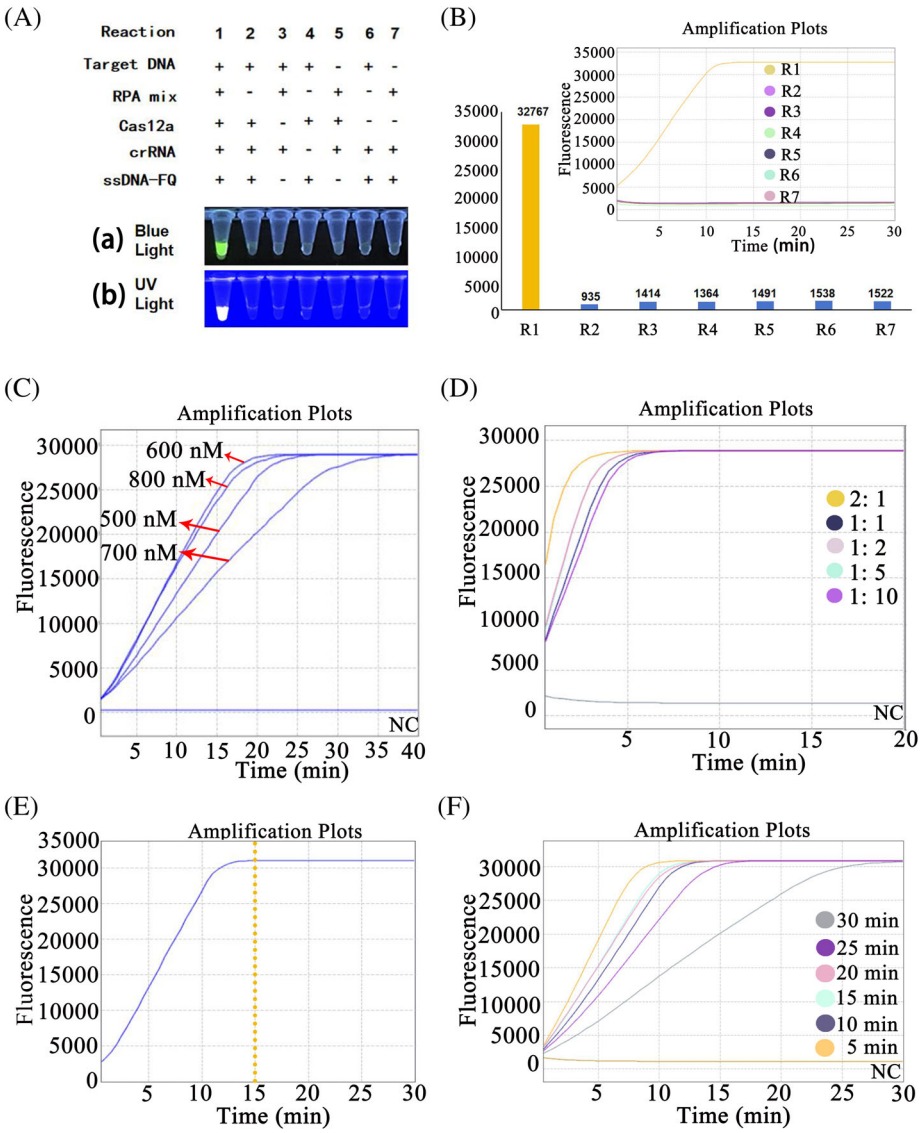


Figure 2. Evaluation and optimization of one-pot RPA-CRISPR/Cas12a assay with various components. (A) Detection of one-pot RPA-CRISPR/Cas12a experiments with five reaction ingredients: (a) visualization under blue light after 15 min of reaction; (b) visualization under UV light after 15 min of reaction. (B) Fluorescence intensity readings using a quantitative PCR instrument. (C) One-step one-pot RPA-CRISPR/Cas12a with varying concentrations of Cas12a and crRNA. (D) One-step one-pot RPA-CRISPR/Cas12a with various ratios of Cas12a to crRNA. (E) One-step one-pot RPA-CRISPR/Cas12a experiment at various temperatures. (F) RPA reactions of one-pot RPA-CRISPR/Cas12a experiment at various time points; NC: negative control.

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templates. Additionally, we also tested the RPA reaction time in one-pot RPA-CRISPR/Cas12a assay, and the best RPA reaction time was determined to be 10–15 min (Fig. 2(F)). The optimization of these reaction conditions provides the basis for an excellent one-pot RPA-CRISPR/Cas12a assay.

3.3 Specificity and sensitivity of one-pot RPA-CRISPR/Cas12a detection

To test the specificity of the detection method, target DNA was extracted from the target pathogen *P. vignae* and 18 non-target pathogens. Leveraging the requirement of a PAM sequence near the crRNA binding site, crRNAs for the CRISPR/Cas12a were designed and screened within conserved regions. Specific RPA primers were then devised based on the crRNA binding sites to achieve targeted amplification of the specific gene. This approach ensures precise and efficient testing of *P. vignae*. In Fig. 3, positive amplification was observed only for *P. vignae* using PCR (Fig. 3(A)), qPCR (Fig. 3(B)), one-step and one-pot RPA-CRISPR/Cas12a fluorescence detection (Fig. 3(C)), and visual readout by blue light and UV light (Fig. 3(D)). No detection was observed for other control strains, including *Fusarium oxysporum* (9097 and 9098). These conclusions testify that the one-pot RPA-CRISPR/Cas12a detection method created in this article exhibits better specificity for *P. vignae*, making it a reliable tool for accurate pathogen identification.

Combining RPA with CRISPR/Cas12a not only reduces non-specific amplification in the RPA reaction but also enhances the overall sensitivity of the detection. For the sake of better appraising the sensitivity of the experiment, this study performed a ten-fold serial dilution of the target DNA, with the detection range spanning from 200 ng μL^{-1} to 20 fg μL^{-1}. The diluted samples were analyzed using conventional PCR (Fig. 4(A)), qPCR (Fig. 4(B)), and one-pot RPA-CRISPR/Cas12a experiment. The results show that under the single-probe assay, the two-step one-pot RPA-CRISPR/Cas12a experiment could test target DNA at concentrations as low as 2 pg μL^{-1} (Fig. 4(C),(D)), however, the test limit of the one-step one-pot RPA-CRISPR/Cas12a is 20 pg μL^{-1}(Fig. 4(E),(F)). These results showed that under single-probe conditions the limit of detection (LOD) of the two-step one-pot RPA-CRISPR/Cas12a assay was 2 pg μL^{-1}, and the one-step and one-pot was 20 pg μL^{-1}.

3.4 The incorporation of dual probes enhances the performance of the detection

Since the single-probe system in the one-pot RPA-CRISPR/Cas12a experiment, whether in the two-step or one-step approach, demonstrated non-optimal sensitivity, we explored the possibility of enhancing sensitivity by adding a second probe without altering the existing reaction. According to previous reports, the concentration and type of

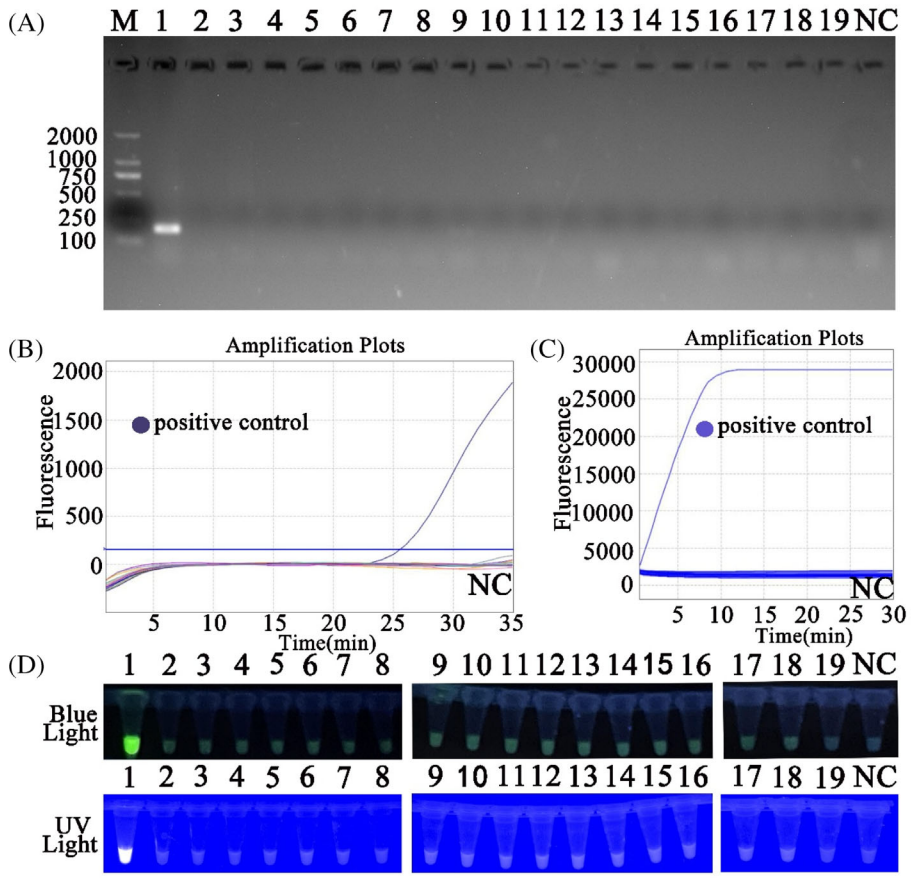


Figure 3. Specificity detection of *Phytophthora vignae* using PCR, qPCR, and one-pot RPA-CRISPR/Cas12a. (A) PCR; (B) qPCR; (C) specificity detection using CRISPR-Cas12a-based RPA assay; (D) specificity visualization under blue and UV light. M: DL2000 DNA marker; PC: positive control; NC: negative control; 1: *Phytophthora vignae*; 2: *Phytophthora palmivora*; 3: *Phytophthora sojae*; 4: *Phytophthora cinnamomi*; 5: *Phytophthora drechsleri*; 6: *Phytophthora infestans*; 7: *Phytophthora cryptogea*; 8: *Phytophthora parasitica*; 9: *Phytophthora cactorum*-1; 10: *Phytophthora citrophthora*; 11: *Phytophthora colocasiae*; 12: *Phytophthora cactorum*-2; 13: *Phytophthora nicotiana*; 14: *Phytophthora capsici*; 15: *Peronophythora litchii*; 16: *Diaporthe phaseolorum* var. *caulivora*; 17: *Fusarium oxysporum* 9097; 18: *Fusarium* spp.; 19: *Fusarium oxysporum* 9098.

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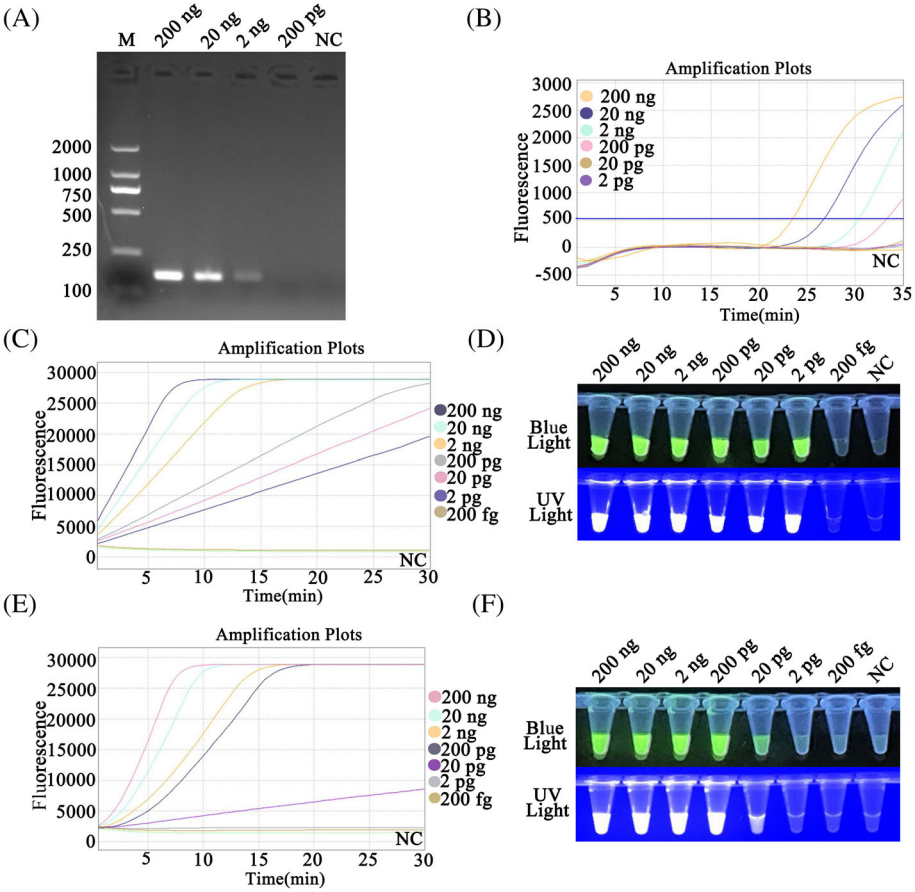


Figure 4. Sensitivity of one-pot RPA-CRISPR/Cas12a for the test of *Phytophthora vignae*. The sensitivity assessment was conducted through (A) PCR; (B) qPCR; (C, D) two-step one-pot RPA-CRISPR/Cas12a probe sensitivity evaluation; (E, F) one-step one-pot RPA-CRISPR/Cas12a probe sensitivity evaluation; M: DL2000 DNA marker; NC: negative control.

FAM-BHQ1 (FQ) probe also play a crucial role in results readout based on Cas-mediated target recognition, cleavage, and fluorescent release.[36,37] In fluorescence readout of detection, reaching the plateau phase earlier means time–cost efficiency and more sensitivity. Therefore, we first tested the reaction time of using a single FQ probe to reach saturation fluorescence at different concentrations, the results showed the final concentrations from 2.5 to 10 μM were most effective, and it took 15–20 min to reach the platform, while the FQ concentration is 500 nM, the total fluorescence yield decreases significantly and the detection requires a longer time (Fig. S6). Therefore, the final concentration of 10 μM was used in subsequent analyses. In Fig. 5(A),(B) the dual-probe (each probe was 5 μM) one-pot RPA-CRISPR/Cas12a experiment, in both the two-step and one-step formats, reached the plateau phase faster than the single-probe system, indicating that dual-probes can significantly improve reaction efficiency and thus shorten reaction time. In addition, we also tested the detection limits of dual probes for two-step and one-step in one-pot RPA-CRISPR/Cas12a assay. The results show that the LOD of one-pot RPA-CRISPR/Cas12a experiments could test target DNA at concentrations as low as 0.2 pg μL^{-1}, both in the two-step (Fig. 5(C),(D)) and one-step methods (Fig. 5(E),(F)). Compared to our previous Cas-OPRAD study (10 pg μL^{-1}), the dual-probe provided a 20-fold increase in sensitivity. These results validate that the dual-probe method can improve the detection limit of the one-step one-pot RPA-CRISPR/Cas12a assay.

3.5 Comparison of PCR, qPCR, and one-pot RPA-CRISPR/Cas12a

To intuitively demonstrate the detection performance of the one-pot RPA-CRISPR/Cas12a, we compared it with traditional PCR and qPCR. In Table 2, traditional PCR exhibited a sensitivity of only 200 ng μL^{-1}, while qPCR demonstrated a sensitivity of 200 pg μL^{-1}. In contrast, the one-pot RPA-CRISPR/Cas12a experiment achieved significantly higher sensitivity: the one-probe two-step method tested target DNA at concentrations as low as 2 pg μL^{-1}, and the one-probe one-step method reached 20 pg μL^{-1}. Furthermore, the dual-probe two-step and one-step methods both achieved an impressive sensitivity of 200 fg μL^{-1}. These results highlight the superior sensitivity and exactitude of the one-pot RPA-CRISPR/Cas12a experiment, underscoring its strong potential for rapid and accurate pathogen detection.

3.6 Preparation of LFS detection platform for *P. vignae*

One-pot RPA-CRISPR/Cas12a experiment operates under isothermal conditions, making it highly appropriate for on-site field experiments. We make use of LFS for visual one-pot RPA-CRISPR/Cas12a detection, providing a more convenient approach for field applications. The on-site testing steps include: (1) sample collection and

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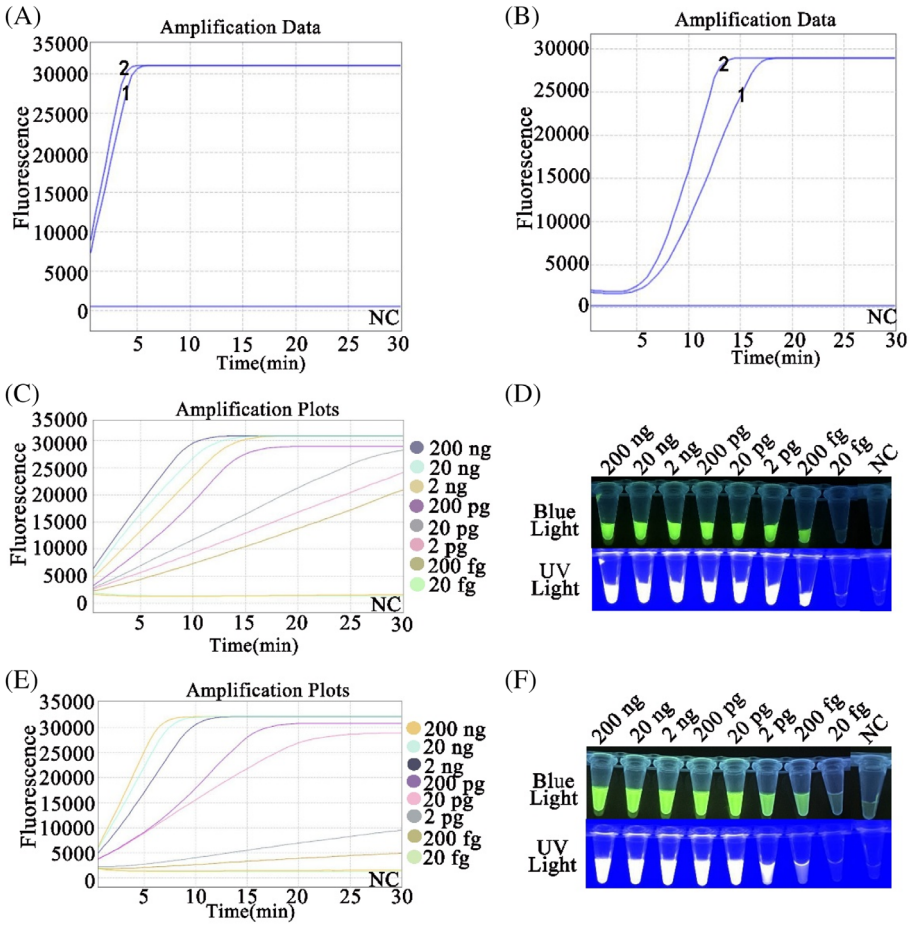


Figure 5. Enhanced sensitivity of one-pot RPA-CRISPR/Cas12a experiment for detecting *Phytophthora vignae* using dual-probes. (A) Comparison of single-probe and dual-probe two-step RPA-CRISPR/Cas12a methods. (B) Comparison of single-probe and dual-probe one-step RPA-CRISPR/Cas12a methods. (1) Single-probe (2) Dual-probe. (C, D) Sensitivity evaluation of the dual-probe one-pot RPA-CRISPR/Cas12a two-step experiment. (E, F) Sensitivity evaluation of the dual-probe one-pot RPA-CRISPR/Cas12a one-step experiment. NC: negative control.

Table 2. Comparison of the sensitivity of the two-step method operating one probe and two probes in conventional polymerase chain reaction (PCR), quantitative PCR (qPCR), and one-pot RPA-CRISPR/Cas12a, as well as the one-step method using one probe and two probes in RPA-CRISPR/Cas12a

	Different concentration gradients						
Methods	200 ng μL^{-1}	20 ng μL^{-1}	2 ng μL^{-1}	200 pg μL^{-1}	20 pg μL^{-1}	2 pg μL^{-1}	200 fg μL^{-1}
Conventional PCR	+	+	+	–	–	–	–
qPCR	+	+	+	+	–	–	–
Two-step RPA-CRISPR/Cas12a-one probe	+	+	+	+	+	+	–
Two-step RPA-CRISPR/Cas12a-two probes	+	+	+	+	+	+	+
One-step RPA-CRISPR/Cas12a-one probe	+	+	+	+	+	–	–
One-step RPA-CRISPR/Cas12a-two probes	+	+	+	+	+	+	+

Positive (+) or negative (–) are based on the presence of a PCR, qPCR, or RPA-CRISPR/Cas12a result of amplification.

nucleic acid extraction; (2) one-step and one-pot RPA-CRISPR/Cas12a assay; and (3) visual readout. The whole testing time is approximately 15–30 min (Fig. 6(A)). The principle of LFS is the same as the work we previously reported. Specifically, the RPA-amplified nucleic acid product binds to the conjugate pad, and the presence of the target triggers CRISPR/Cas12a to Cleavage the fluorescent reporter, separating FAM from biotin. In this case, both the control line (C line) and the test line (T line) on the LFS display a red stripe, or only the T line shows a red stripe while the C line remains colorless. Both scenarios indicate effective cleavage by Cas12a and activation of the reporter group, resulting in a positive outcome. If only the C line shows a red stripe and the T line remains colorless, the result is negative, indicating that Cas12a failed to cleave the reporter and activate the reporter group.

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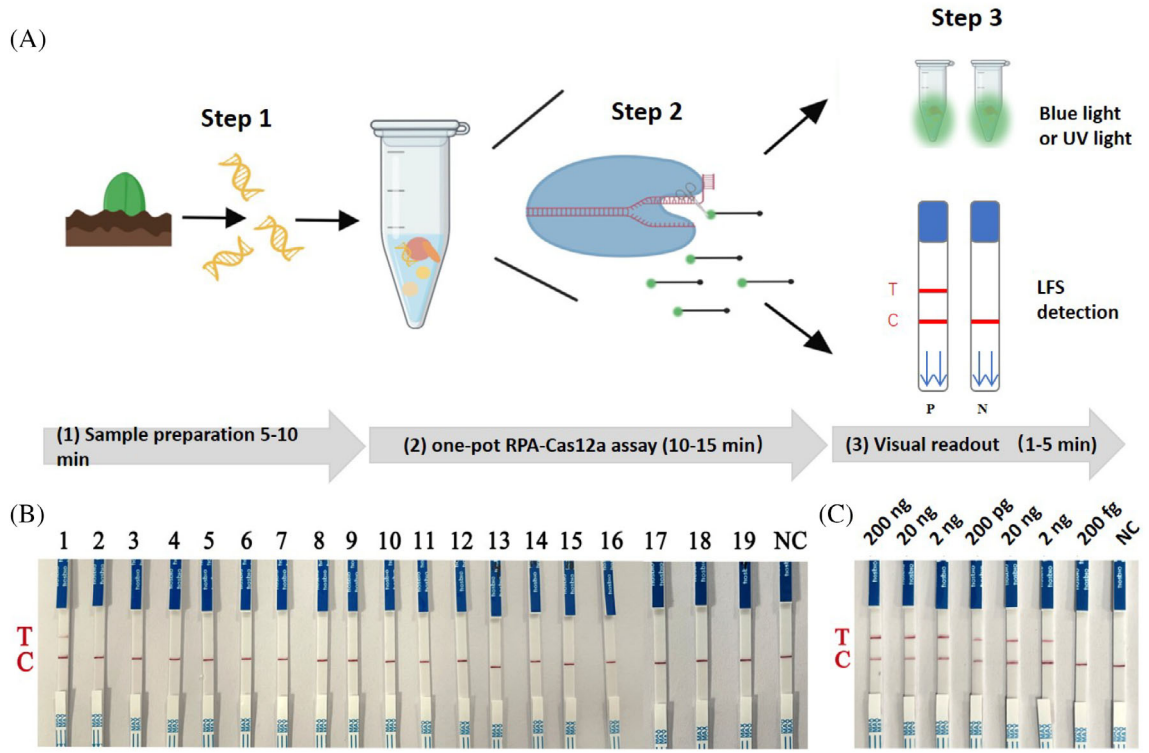


Figure 6. On-site lateral flow strip (LFS) visualization of one-step one-pot RPA-CRISPR/Cas12a assay. (A) The workflow of on-site LFS detection based on one-pot RPA-CRISPR/Cas12a assay. (B) LFS readout specific to *Phytophthora vignae*. NC: negative control; 1: *Phytophthora vignae*; 2: *Phytophthora palmivora*; 3: *Phytophthora sojae*; 4: *Phytophthora cinnamomi*; 5: *Phytophthora drechsleri*; 6: *Phytophthora infestans*; 7: *Phytophthora cryptogea*; 8: *Phytophthora parasitica*; 9: *Phytophthora cactorum*-1; 10: *Phytophthora citrophthora*; 11: *Phytophthora colocasiae*; 12: *Phytophthora cactorum*-2; 13: *Phytophthora nicotiana*; 14: *Phytophthora capsici*; 15: *Peronophythora litchii*; 16: *Diaporthe phaseolorum* var. *caulivora*; 17: *Fusarium oxysporum* 9097; 18: *Fusarium* spp.; 19: *Fusarium oxysporum* 9098. (C) Sensitivity analysis of LFSs for *Phytophthora vignae*. 1: 200 ng μL^{-1}; 2: 20 ng μL^{-1}; 3: 2 ng μL^{-1}; 4: 200 pg μL^{-1}; 5: 20 pg μL^{-1}; 6: 2 pg μL^{-1}; 7: 200 fg μL^{-1}; NC: negative control.

We also analyzed the specificity and sensitivity of the LFS for *P. vignae*. Specificity testing results were visualized using LFS. During specificity testing, only the LFS for *P. vignae* DNA samples displayed a red stripe on the T line, while no red stripes were observed on the T lines for other pathogens (Fig. 6(B)). These conclusions demonstrate that the LFS-RPA-CRISPR/Cas12a test method exhibits better specificity for *P. vignae*. In sensitivity testing, the LFS results were comparable to those of the two-step one-pot RPA-CRISPR/Cas12a method, with a test limit of 2 pg μL^{-1}, as evidenced by the appearance of a red stripe on the T line (Fig. 6(C)). This detection platform enables the identification of *P. vignae* without the need for specialized equipment.

3.7 Validation of one-pot RPA-CRISPR/Cas12a assay for on-site sample

This study collected ten field samples from Hainan Province, China, and analyzed them using PCR (Fig. 7(A)), qPCR (Fig. 7(B)), fluorescence-based one-pot RPA-CRISPR/Cas12a experiment (Fig. 7(C)), visual detection under blue or UV light (Fig. 7(D)), and LFS methods (Fig. 7(E)). The results showed that *P. vignae* was detected in six out of eight diseased samples, while the remaining two samples tested negative, likely due to other pathogens causing cowpea root rot. Healthy cowpea seedlings also tested negative, indicating that the cowpea plant genomic DNA did not interfere with the detection results. Furthermore, the qPCR results and visual detection under blue or UV light were fully consistent with the LFS readings. These findings testify to the excellent detection capability of one-pot RPA-CRISPR/Cas12a compared with the traditional methods of PCR, and qPCR for *P. vignae*.

4 DISCUSSION

The outbreak of cowpea *Phytophthora* blight poses a persistent threat to cowpea production, necessitating the urgent rapid and effective on-site detection method to prevent and mitigate losses. There are molecular diagnostic methods available for detecting *Phytophthora* spp., including PCR-based techniques and LAMP.[6,9] However, these methods often rely on thermal equipment or lack specificity for *Phytophthora* strains assay in cowpeas, leading to potential issues with sensitivity and specificity. Additionally, limited genomic data on regional pathogen variants and a lack of field-deployable diagnostic kits hinder effective disease management. Therefore, the detection of cowpea *Phytophthora* blight remains constrained by the limited sensitivity of PCR and other techniques that are less suitable for convenient field application.[1–3]

In this study, through bioinformatics analysis, we compared the genomic sequences of *P. vignae* with those of other pathogens. Extensive experimental results identified a highly specific target gene. The optimized one-pot RPA-CRISPR/Cas12a technology demonstrates significant capability compared to conventional methods such as PCR and qPCR (Table 2).[38,39] When analyzing infected samples, the sensitivity of one-pot RPA-CRISPR/Cas12a technology is significantly higher than that of traditional PCR.[23,40] Additionally, this method operates under isothermal conditions, reducing reliance on specialized equipment. Compared to other

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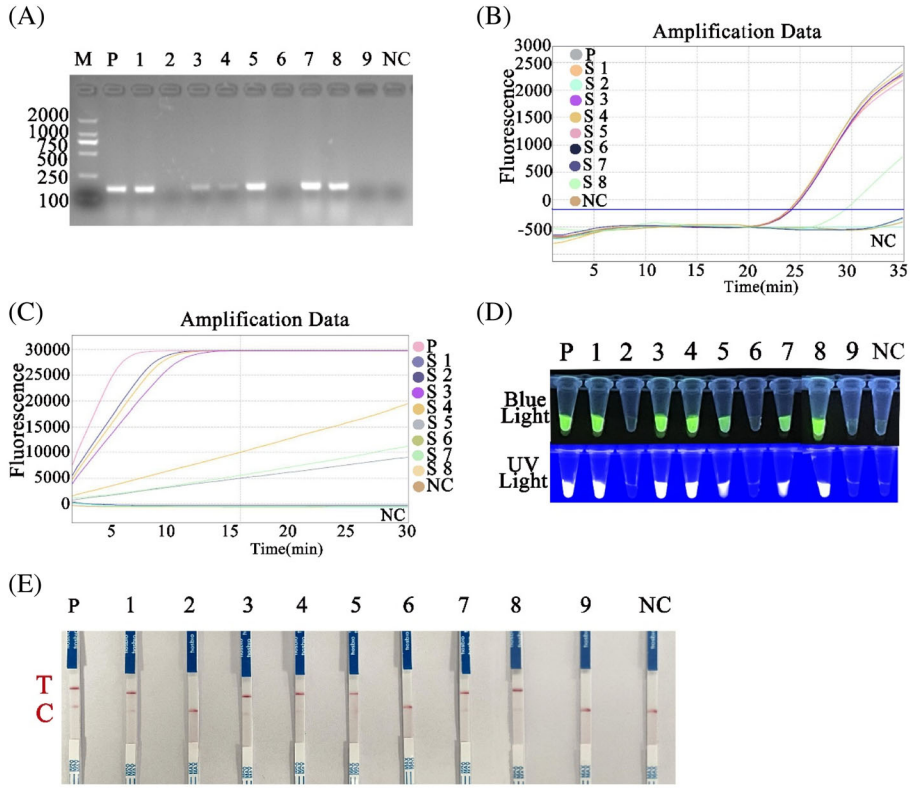


Figure 7. Detection of *Phytophthora vignae* in field samples using PCR, qPCR, RPA-CRISPR/Cas12a, and LFS. (A) PCR detection experiment for field cowpea samples; (B) qPCR detection experiment for field cowpea samples; (C) one-pot RPA-CRISPR/Cas12a detection experiment for field cowpea samples; (D) one-pot RPA-CRISPR/Cas12a experiments under blue and UV light for field cowpea samples; (E) RPA-CRISPR/Cas12a LFS detection for field cowpea samples. +, Positive control; −, negative control; 1–8, naturally infected cowpea samples; 9, healthy cowpea sample.

isothermal amplification techniques based on the CRISPR/Cas assay, the RPA offers shorter reaction times and higher efficiency.[30] Moreover, the reaction temperature of RPA aligns with the optimal activity of the CRISPR/Cas12a nuclease, enabling seamless integration into a single reaction tube (Table 3). Therefore, one-pot RPA-CRISPR/Cas12 can be more easily integrated into CRISPR/Cas detection analysis compared with other isothermal amplification techniques such as LAMP or rolling circle amplification (RCA).[41–43] Additionally, the dual-probe one-pot RPA-CRISPR/Cas12a system developed in this study demonstrates greater rapidity, higher sensitivity, and enhanced field-deploy ability for non-expert users compared to previously reported two-step RPA-CRISPR/Cas12a assays and other one-step RPA-CRISPR/Cas12a platforms.[44,45] Combined with RPA portable dry powder formulations, it provides more stable, convenient, and reliable on-site analysis for field testing.

The combination of CRISPR/Cas with Isothermal Amplification Technology (IAT) necessitates multiple steps, inevitably increasing the risk of contamination during reagent transfer and potentially leading to non-specific amplification and cross-contamination. To address this issue, numerous studies are investigating one-pot strategies for integrating IAT with CRISPR/Cas assays.[42,45,46] The primary advantage of this work include, the lower temperature requirement, as reactions can proceed at normal physiological temperatures (37–40 °C). Secondary, the two-step one-pot RPA-CRISPR/Cas12a experiment can be completed within 15–30 min, while the one-step method achieves detection in 10–20 min. Both approaches allow all reaction steps to be completed in a one-pot without reopening, minimizing contamination risks. Finally, this study further optimized the system by introducing a dual-probe one-pot RPA-CRISPR/Cas12a detection method. Incorporating dual probes into both the two-step and one-step reactions accelerates the reaction time, enabling faster plateau attainment and improved sensitivity compared with our previous research (10 pg μL^{-1}),[45] thereby creating a more efficient and rapid detection method. However, the complex ingredients synergistic mechanisms of one step one-pot RPA-CRISPR/Cas12a are worth further study to produce a more efficient response. Although the one-step method is faster and simpler than the two-step approach, the lack of pre-amplification in RPA results in insufficient RPA product accumulation before Cas12a binding, leading to reaction delays and instability. In contrast, the two-step method ensures stable reactions due to adequate RPA product generation before Cas12a interaction. To address this issue, we found that increasing the volume of the RPA mixture before combining it with Cas12a significantly enhances stability (data not published).

The integration of LFS with one-pot RPA and CRISPR/Cas12a represents a cutting-edge approach for rapid, visual, and portable nucleic acid detection.[47,48] Furthermore, RPA is a highly sensitive and selective isothermal amplification technique, that amplifies target DNA at 37–42 °C within 15–30 min, eliminating the need for thermal cyclers.[38] As an engineered programmable biosensor, CRISPR/Cas systems have extremely high specificity recognition and signal amplification ability. Upon recognizing the target DNA, Cas12a exhibits collateral cleavage activity, the cleavage of a reporter molecule. This activity can generate a visible signal on the LFS by FAM-biotin-labeled ssDNA probe design. The cleaved reporter produces a test line (T-line) signal, while an unmodified control line (C-line) confirms assay validity.[30] These provide a

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Table 3. Comparison of performance between recent detection techniques and dual-probe one-pot RPA-CRISPR/Cas12a

Methods	Operation steps	Specificity	Sensitivity	Duration	One-pot	One-step	References
SHINE	(1) RT-RPA	100%	10 copies/μL	~1.3 h	No	No	21
	(2) CRISPR/Cas fluorescence						
POC-CRISPR	(2) RT-RPA	High	1 genome/100 μL	~30 min	No	No	46
	(2) CRISPR/Cas LFS assay						
ECS-CRISPR	One-pot RT-RPA CRISPR/Cas fluorescence/LFS assay	100%	3 copies/μL	25–30 min	Yes	No	42
DAMR	One-pot RT-RPA CRISPR/Cas fluorescence/LFS assay	100%	10–100 copies	1 h	Yes	No	41
OPERATOR	One-pot RCA-CRISPR/Cas fluorescence/LFS assay	100%	1.625 copies per reaction	~30 min	Yes	Yes	43
sPAMC	One-pot RPA-CRISPR/Cas fluorescence /LFS assay	100%	94.2%	~20 min	Yes	No	44
Cas-OPRAD	(1) RPA	100%	10 pg μL^{-1}	~30 min	Yes	No	45
	(2) CRISPR/Cas12 fluorescence/LFS assay						
Cas-PfLAMP	(1) LAMP or RT-LAMP	100%	3–9 copies per reaction	~50 min	No	No	30
	(2) CRISPR/Cas fluorescence/LFS assay						
Dual-probe one-pot RPA-CRISPR/Cas12a.	One-step one-pot RPA-CRISPR/Cas12a fluorescence /LFS assay	100%	200 fg μL^{-1} (~18.5 copies per reaction)	~15–20 min	Yes	Yes	This work

foundation for rapid and on-site analysis. In addition, we also can leverage smartphone technology to potentially record, analyze, and monitor results.[49,50] These conclusions can be quickly imported into designated software on smartphones, offering convenience for farmers and researchers.

5 CONCLUSION

In comparison of PCR, qPCR, LFS, and one-pot RPA-CRISPR/Cas12a detection methods, the sensitivity of RPA-CRISPR/Cas12a was found to be comparable to that of LFS, followed by qPCR and PCR. The isothermal RPA-Cas12a detection method was employed to analyze cowpea samples. The one-pot format not only helps prevent contamination but also provides intuitive visual readouts. Finally, this study established a dual-probe one-pot RPA-CRISPR/Cas12a technology, which is not only exceptionally rapid but also highly efficient in detecting target pathogens. Particularly, it enables on-site detection and can be operated without the need for specialized personnel, making it a promising technology with significant application potential.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

DATA AVAILABILITY STATEMENT

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

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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