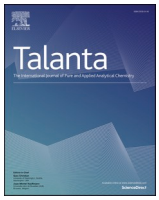
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A rapid visual detection method for *Sugarcane streak mosaic virus* based on one-tube RPA-CRISPR/Cas12a

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ABSTRACT

Sugarcane is the most important crop for sugar production. *Sugarcane streak mosaic virus* (SCSMV) triggered sugarcane mosaic disease can lead to substantial reductions in both yield and sucrose content. In the process of disease prevention and control, target pathogen detection technology is indispensable. However, traditional detection methods are time-consuming and require expensive equipment, making them less efficient for timely disease control and unfavorable to disease resistance breeding. Here, we introduce a novel detection technology that combines recombinase polymerase amplification (RPA) with CRISPR-Cas12a. The method utilizes crude extracts from sugarcane leaves as the reaction template, significantly simplifying and expediting the preparation process. By combining RPA and CRISPR-Cas12a in a single reaction tube, the risk of aerosol contamination has decreased markedly. The entire process, from sample preparation to result interpretation, only takes 50 min, and the reaction equipment only a water bath pot, and results can be blue light spectrometer or UV flashlight assessed visually. Importantly, the method demonstrates high sensitivity, detecting a minimum of 50 copies of the plasmid, which surpasses the sensitivity of reverse transcription polymerase chain reaction (RT-PCR) and is comparable to quantitative RT-PCR (RT-qPCR). The method exhibits excellent specificity, showing no cross-reactivity with other common sugarcane viruses, including *Sugarcane mosaic virus*, *Sugarcane yellow leaf virus*, and *Sorghum mosaic virus*. The practicality of this technique was validated through the detection of leaf crude extracts from 40 field samples. The detection results were consistent with those obtained from RT-PCR and RT-qPCR using leaf RNA as the template, indicating its suitability for laboratory detection and field applications.

1. Introduction

Sugarcane (*Saccharum* spp. hybrids) ~80 % [1,2] of the global and more than 85 % of Chinese [3] sugar production, respectively. Sugarcane mosaic disease (SMD) represents a major challenge in sugarcane cultivation, leading to reduced cane yield and sucrose content [4]. This disease is primarily caused by three pathogens belonging to Potyviridae, namely *Sugarcane mosaic virus* (SCMV), *Sugarcane streak mosaic virus* (SCSMV), and *Sorghum mosaic virus* (SrMV) [5]. The virion of SCSMV is linear, enveloped, and possesses a single-stranded positive-sense RNA genome [6,7].

SCSMV had been classified as the F strain of SCMV since its initial identification in Louisiana in 1932. It was renamed SCSMV in 1998 following partial genomic sequencing and phylogenetic analysis [8]. In China, SCSMV was first reported in Yunnan in 2012. The virus spreads easily and is primarily transmitted through infected seedlings, vectors, and agricultural practices such as cutting [9,10]. It thus rapidly spread to sugarcane-growing areas across the country, becoming one of the predominant pathogens associated with SMD [11]. Upon infection, the virus induces the appearance of greenish stripes or spots on the leaves, resulting in characteristic mosaic symptoms, as well as stunted growth and reduced tillering [12,13]. SCSMV can lead to a 10–50 % reduction [14]. Importantly, the virus-free rates for SrMV, SCMV, and SCSMV reached 100 % [15], 90.62 % and only 10.12 % [16] respectively if application of virus-free sugarcane stem tip technology. Therefore, it is more difficult to remove SCSMV by sugarcane detoxification technology. Consequently, it is essential to quickly identify which plants are truly SCSMV-free seedlings during the production of virus-free seedlings. At the same time, the requirements for testing equipment and technical difficulty should also be as simple as possible. In addition, such

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detection techniques are also needed for monitoring of disease epidemics and disease resistance breeding.

Current detection methods primarily rely on serological and molecular biology technologies, including enzyme-linked immunosorbent assay (ELISA), reverse transcription polymerase chain reaction (RT-PCR), quantitative RT-PCR (RT-qPCR), and reverse transcription loop-mediated isothermal amplification (RT-LAMP) [14,17–19]. Additionally, serological techniques such as, double antibody sandwich-ELISA (DAS-ELISA) and direct antigen coating-ELISA (DAC-ELISA) were also employed to detect SCSMV, and found DAS-ELISA exhibited greater sensitivity than serological technique [20]. Not only that, Reddy et al. [21] integrated serological methods with molecular biology techniques to create the method of Duplex immunocapture-RT-PCR (D–IC–RT-PCR), which can concurrently detect and distinguish SCSMV and SCMV, demonstrating higher sensitivity than DAC-ELISA. Although these techniques are effective for SCSMV detection, they have limitations, i.e. ELISA involves a complex and time-consuming procedure and relies highly on the quality and specificity of antibodies [22]. Using molecular detection, Xie et al. [23] developed an one-step quadruple RT-PCR method through innovative primer design and optimization of conditions, enabling simultaneous detection and differentiation of four viruses: SCSMV, SCMV, SrMV, and *Sugarcane yellow leaf virus* (SCYLV). RT-PCR, while offering high sensitivity and specificity, necessitates the use of RNA as a template. The process of RNA extraction, PCR amplification, and product detection necessitates a high level of skill from the operator, along with extended time commitments, and costly equipment. Moreover, LAMP is prone to aerosol contamination during the reaction, and designing primers is challenging, along with necessitating the extraction of DNA or RNA [24–28]. Consequently, there is a pressing need for an accurate, rapid, and straightforward detection method.

Recombinase polymerase amplification (RPA) is a novel nucleic acid amplification technology used for the diagnosis of pathogens with DNA or RNA [29], first reported by Piepenburg et al. in 2006 [30]. It can rapidly generate billions of DNA copies within 30 min at 37 °C, exhibiting excellent specificity and sensitivity. Additionally, the operational requirements for personnel conducting these experiments are comparatively low [31–33]. Though its reaction products can be detected by agarose gel electrophoresis [30], these products have to purified before loading, because the proteins present in the RPA reaction mixture can hinder the visualization of amplicons forming massive smear migrating products [34,35]. Other detection method, such as the side-flow chromatography strips or biochips [36,37], can also be used for detecting the reaction products, while all of them are complex. Currently, some plant viruses, such as *Yam mosaic virus* [38], *Plum pox virus* [39], and *Banana bunchy top virus* [40] have been detected using RPA technology.

The CRISPR/Cas system represents a sophisticated adaptive immune mechanism that has evolved over a long period in archaea and bacteria [41,42]. This system is capable of recognizing and remembering previously encountered nucleic acid sequences, enabling it to defend against and eliminate the same genetic material upon subsequent invasions [43–45]. Recent studies have demonstrated that the Cas12a protein can detect double-stranded DNA [46]. When the target sequence is present, Cas12a specifically recognizes and cleaves double-stranded DNA under the guidance of crRNA, while also making non-specific cuts to single-stranded DNA [47–49]. To exploit the characteristics of this non-specific cleavage, DNA single-strand fluorescence reporter molecules were designed. Upon detection of the target sequence, the fluorophores and quenched groups of the fluorescence reporter molecule are separated, resulting in fluorescence emission [50–52]. Utilizing this feature, researchers have developed a CRISPR/Cas system for the detection of the *Monkeypox virus* [53], *Nipah virus* [54], and *Largemouth bass ranavirus* [55]. Currently, this system has also been utilized to detect SrMV in sugarcane [56]. However, studies indicate that the side cleavage activity of Cas12a is insufficient to generate detection signals when the concentration of target DNA is too low [42,57,58]. Therefore, rapid and efficient nucleic acid amplification is critical for enhancing its sensitivity [59].

Combining the systems of RPA and CRISPR/Cas can significantly enhance detection sensitivity [60], because this system increases the target sequence content through constant temperature amplification, and thus resulting in significantly improved detection sensitivity and doubled the specificity of target sequence recognition [61,62]. This indicates its considerable potential for enhancing both the sensitivity and specificity of detection methods. This system has been successfully applied to the detection of *Seneca valley virus* [63] and *Neisseria gonorrhoeae* [64], while it has not yet been reported for the detection of SCSMV.

Here, we integrate the systems of RPA and CRISPR/Cas12a, together with a simple method for preparing template RNA, to develop a rapid and straightforward technology for detecting SCSMV in sugarcane (Fig. 1), which is characterized by a high sensitivity, strong specificity and robust stability. This method utilizes leaf crude extract as the template, demonstrating detection sensitivity superior to that of nucleic acid-based RT-PCR and comparable to RT-qPCR. Furthermore, the results can be evaluated visually, enhancing the practicality of this approach.

2. Materials and methods

2.1. Construction of plasmid pUC57-SCSMV

A total of 15 SCSMV genome sequences were identified in the NCBI database. The conserved sequences were determined using DNAMAN software for comparison, and synthesized by Sangong Bioengineering (Shanghai) Co., LTD. Subsequently, the pUC57 plasmid possessing the conserved sequence was constructed. The copy number of plasmid (copies/μL) is calculated using the formula: $(6.02 \times 10^{23}$ copies/mol) $\times$ $(10^{-9} \times$ N)/(660 $\times$ M g/mol/dp), where N (ng/μL) represents the concentration of the plasmid, and M indicates the length of the plasmid in base pairs.

2.2. RPA primer and crRNA design

According to the principles of RPA Primer design, Primer Premier 5.0 software (Premier Biosoft International, USA) was utilized to design RPA-specific amplification primers based on the conserved sequences. RPA-F:5 ′-ACAAGTGGGTTCAGTTCTCGGTTCGTAG-3′, RPA-R: 5 ′-TCTACGCAGGTCCGTGTCCTCATCTCCC-3′, These primers were synthesized by Beijing Qingke Biotechnology Co., LTD. The RPA reaction system comprised a total volume of 50 μL. It was A Buffer 29.4 μL (Keenow, Suzhou, China), 1.0 μL each of the upper and downstream primer (10 pmol/μL), SCSMV cDNA template 1.0 μL, B Buffer 2.5 μL (Keenow, Suzhou, China), sterile water (A129-100, Conrun Bio, Beijing, China) to reach a final volume of 50 μL. RPA reaction procedure was 37 °C for 30 min. Following the reaction, 10 μL of 6 $\times$ Loading buffer was added to the mixture, which was then incubated at 56 °C for 5 min. Finally, 5 μL of the resulting mixture was taken for detection via agarose gel electrophoresis.

The traditional classic protospacer adjacent motif (PAM) recognized by Cas12a is 5 ′-TTTN-3’. However, according to the previous study that Cas12a can also recognize other types of PAM, including suboptimal PAMs such as VTTV, TCTV and TTVV [65]. In this study, crRNA was designed within the amplified fragment of RPA primer. The BLAST tool from the NCBI database was utilized for a specific analysis of all crRNA sequences that conform to the designed sequence in the amplified fragment. This approach can reduce the reaction time of the one-tube method while enhancing sensitivity and specificity [65]. The Scaffold sequence of Cas12a (UAAUUUCUACUAAGUGUAGAU) is then combined with the scaffold sequence of Cas12a. crRNA:5 ′- UAAUUUCUACUAAGUGUAGAUCCCCGGAUGGGAAAGCCUUUAUUC -3′ was synthesized by sent to Genscript biotechnology co., LTD.

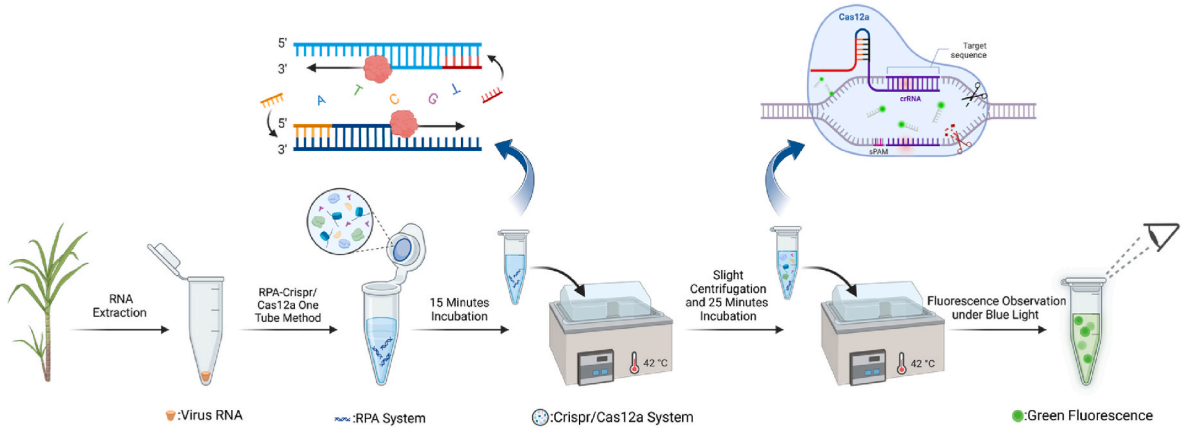


Fig. 1. Flow chart of *Sugarcane streak mosaic virus* detection using RPA-CRISPR/Cas12a visualization method.

2.3. Establishment of RT-RPA-CRISPR/Cas12a reaction system

The RT-RPA-CRISPR/Cas12a reaction system was prepared with a total volume of 35 μL. It contained two reaction systems. CRISPR/Cas12a system, i.e. Lb Cas12a (Cpf1) nuclease 10 pmol/μL 0.5 μL (32108-01, Tolo Port Biology, Shanghai, China), crRNA 100 ng/μL 2.0 μL, Cas12a-ssDNA-reporter (5′-6-FAM-TTATTAT-BHQ2-3′) 10 pmol/μL 0.5 μL, and 10 × HOLMES Buffer 1.0 μL. RPA system (A Buffer 14.7 μL, 1.0 μL each of upper and downstream primers (10 pmol/μL), 1.0 μL of SCSMV template, and B Buffer 1.25 μL), and the volume was adjusted with sterile water to a final total of 35 μL (Table 1).

According to the above system, the RPA system was added to the 200 μL centrifuge tube, while the CRISPR/Cas12a system was added to the cap of the tube, due to the fact that RPA and CRISPR/Cas12a are in competition with each other. Meanwhile, we optimized the reaction time and temperature for the RPA and CRISPR systems, respectively, and finally chose to incubate the centrifuge tubes in a water bath pot at 42 °C for 15 min. Following this incubation, the liquid from the lid was fleetingly centrifuged into the tube then it was incubated at 42 °C for an additional 25 min. Fluorescence signals were collected every 30 s using a quantitative PCR instrument (Thermo Fisher Scientific Waltham, MA, USA), and both a negative control group and a blank control group lacking target cDNA were established. After the reaction, the products were analyzed using 470 nm blue light analyzer (B2042, UElandy, Suzhou, China) or 365 nm UV flashlight (V2-s, Yunfeng Electronics, Ningbo, China) to interpret the results.

2.4. Specificity and sensitivity tests of RT-RPA-CRISPR/Cas12a system

In the RT-RPA-CRISPR/Cas12a system, RNA extracted from sugarcane leaves infected with SCSMV, SCMV, SrMV, and SCYLV, using Trozol kit (Magen Biotechnology, Guangzhou China), was utilized as templates to assess the specificity of the RT-RPA-CRISPR/Cas12a method. Concurrently, RNA from sugarcane leaves free of these viruses served as a negative control, while water was used as a blank control.

Table 1
RT-RPA-CRISPR/Cas12a reaction system.

System composition	Usage amount(μL)
A Buffer	14.7
RPA-F	1.0
RPA-R	1.0
Template	1.0
B Buffer	1.25
Lb Cas12a(Cpf1)nuclease	0.5
crRNA	2.0
Cas12a-ssDNA-reporter	0.5
10 × HOLMES Buffer	1.0
Sterile water	12.05
Total volume	35

The SCSMV plasmid was diluted to concentrations of 1×10^3 copies/μL, 1×10^2 copies/μL, 75 copies/μL, 50 copies/μL, 25 copies/μL, 10 copies/μL, and 1 copy/μL. These diluted plasmids were employed as templates in the RPA-CRISPR/Cas12a reaction system, with cDNA lacking SCSMV serving as the negative control and water as the blank control, to determine the minimum detection limit of the system. The reaction system followed the parameters outlined in Table 1, and the methodology and results were documented as described in 2.3. The PCR primers used were PCR-F: 5′-TCATMTCTTCATCRGCCGC-3′ and PCR-R: 5′-ATCTTCYCTACGCAGGTCCG-3′ based on the previous report [66]. Reaction system: positive cDNA 1.0 μL (900 ng/μL), 2 × Taq PCR StarMix (GenStar, Beijing, China) 20.0 μL, 1.0 μL of each forward and reverse primer, and sterile water to a final volume of 35 μL. The PCR reaction procedure included an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, concluding with a final extension at 72 °C for 10 min.

The qPCR primers were QPCR-F: 5′-TAGAAACGCCAAACCTGG-3′ and QPCR-R: 5′-GTGAGAGGATATTCACCATCA-3′ according to the report [19]. The qPCR reaction system consisted of 1.0 μL of each upper and lower primer, 20.0 μL of 2 × PerfectStart®Green qPCR SuperMix, 1.0 μL of template, and sterile water to a total volume of 35 μL. The reaction conditions adhered to the kit instructions (AQ602, TransGen Biotech, Beijing, China), with an annealing temperature set at 60 °C.

2.5. Preparation of template RNA crude extract from sugarcane

A total of 40 sugarcane varieties were used for verification, including Badila, ROC22, DZ03-83, FN14-15, FN14-28, FN14-30, FN14-38, FN14–39, GT02–467, GT29, GT30, GT31, GT42, GT49, LC03-182, LC07-150, LC05 -136, YG34, YG40, YT00-236, YT00-318, YT93-159, YZ01-1413, YZ03-103, YZ03-194, YZ03-258, YZ05-49, YZ05-51, YZ08-1609, YZ99-91, as well as 10 lines named as FN14-61, FN14-62, FN14-88, FN14-98, FN14-103, FN14-130, FN14-134, FN14-138, FN14-159, and FN14-171, from the F_1 progeny of the cross YT93-159 × ROC22. The leaf samples were weighed 15 mg and ground in liquid nitrogen. A leaf crude extract containing RNA was prepared by adding 130 μL 6 % PEG 200 (A601780-0500, Sangong Biology, Shanghai, China) and 170 μL 20.0 mM NaOH (S817971, McLean, Shanghai, China) to the ground leaf sample. After standing at room temperature for 10 min, an RNA crude extract was obtained. Since the high concentration of crude extracts at this point would appear red under blue light, which interferes with the judgment of fluorescence by the naked eye [67], therefore, the extracts were diluted tenfold to be used in subsequent

assays. We kept the crude extract at 4 °C for up to 7 days for repeated use.

3. Results

3.1. RPA-CRISPR/Cas12a one tube method

Initially, the base sequence of the generated pUC57-SCSMV plasmid perfectly matched the anticipated sequence of the coat protein (CP) gene fragment (572 bp) (Fig. S1). Its total length is 3282 bp determined by sequencing, and a plasmid concentration measured by spectrophotometry was 131 ng/μL, corresponding to a plasmid copy number of 3.64×10^{10} copies/μL. Subsequently, the designed primers underwent amplification via RPA, successfully yielding bands that matched the expected target length of 245 bp (Fig. 2a). In addition, during the amplification process, the fluorescence values of the sample tubes containing SCSMV exhibited a rapid increase (Fig. 2c), while no fluorescence was observed in the negative and blank controls (Fig. 2 b_1, b_2). Furthermore, the fluorescence values for positive samples were significantly higher than those of the negative and blank controls (Fig. 2d). These results indicate that the RPA-CRISPR/Cas12a system developed here is effective for the detection of SCSMV. After that, we optimized the reaction time and temperature for RPA and CRISPR systems respectively to screen the most suitable reaction conditions. First, we performed RPA amplification and CRISPR cutting at 37 °C, 39 °C, and 42 °C, respectively, and each generated a bright electrophoretic band with the size ~250 bp (Fig. S2a) and the strongest fluorescence was emitted at 42 °C in terms of fluorescence values (Fig. S2c). In addition, considering the consistency of the reaction temperature in the process of amplification and cleavage, we chose 42 °C for the reactions of RPA and CRISPR. Meanwhile, in order to evaluate the effect of reaction time on RPA amplification, 10, 15, and 20 min were tested. The electrophoretic bands showed that both 15 and 20 min were more stronger than that of 10 min, and thus a shorter time (15 min) is regarded as the optimal time (Fig. S2b). Finally, we optimized the reaction time for CRISPR by performing CRISPR cuts at 15, 20, 25, and 30 min. Obviously the fluorescence value of the CRISPR system increased gradually with the increase of time (Fig. S2d), but bright fluorescence could be observed by the naked eye at 25 min and 30 min (Fig. S2e), so a shorter time (25 min) was chosen as the subsequent CRISPR reaction time. Finally, to demonstrate the competitive relationship between RPA and CRISPR-Cas12a, we conducted a comparative test between mixed reaction (MR) and separate reaction (SR) in the one-tube method (Fig. S3). When the two reagents were mixed, the reaction only emitted a very weak fluorescence due to the competition between RPA and CRISPR reagents, and thus the results were not clearly recognized by the naked eye (Fig. S3a), resulting in false negatives. This conclusion can also be supported by the fluorescence values when compared SR with MR (Fig. S3b). This defect can be avoided if the CRISPR reagent is added to the cap of the tube to keep apart during RPA reaction.

3.2. Sensitivity evaluation

The fluorescence detection results of the SCSMV RPA-CRISPR/Cas12a system ranged from 1×10^3 copies/μL to 1 copy/μL, as illustrated in the figure. Following amplification via RPA, fluorescence was clearly observed in the range of 1×10^3 copies/μL to 50 copies/μL (Fig. 3a_1, 3a_2). No fluorescence was detected in either the negative control or the blank control. Furthermore, the fluorescence curve indicates that the fluorescence value of the RPA-CRISPR/Cas12a system increased rapidly with reaction time, and the positive plasmid remaining detectable at 50 copies/μL (Fig. 3b). At the endpoint, this value was considerably greater than those of the negative and blank controls (Fig. 3c). These results demonstrate that the detection sensitivity of the RPA-CRISPR/Cas12a system can reach as low as 50 copies/μL.

The sensitivity of PCR detection for the SCSMV plasmid revealed that concentrations of 1×10^3 copies/μL and 1×10^2 copies/μL produced clear bands. However, at a concentration of 75 copies/μL, only a very faint band was observed, and no amplification of the target band occurred at 50 copies/μL (Fig. 3d). This indicates that while the PCR technique can detect down to 75 copies/μL, its sensitivity is lower than that of the RPA-CRISPR/Cas12a method.

Subsequently, the quantitative sensitivity of SCSMV was assessed using qPCR. To effectively illustrate the changes in CT values, the plasmid was diluted from 1×10^7 copies/μL, resulting in a gradient of CT values ranging from 1×10^7 copies/μL to 10 copies/μL, which confirmed the efficacy of the dilution. Notably, CT values were not detected at 1 copy/μL and in the blank control, while the negative control did not show a significant difference at 10 copies/μL, making accurate judgment difficult (Fig. 3e). Consequently, the qPCR reaction system was established with a range from 1000 copies/μL to 1 copy/μL for agarose gel electrophoresis. The results indicated that neither the 1 copy/μL sample, nor the negative and blank controls exhibited target bands (Fig. 3f). Therefore, we conclude that the lowest detection limit of qPCR is 10 copies/μL.

3.3. Specificity of RT-RPA-CRISPR/Cas12a

The RT-RPA-CRISPR/Cas12a system successfully detected SCSMV in sugarcane leaf crude extract. The test results indicated that the RPA-CRISPR/Cas12a detection system generated fluorescence signals exclusively in leaf crude extract samples containing SCSMV, while no fluorescence signal was observed in samples containing other viruses, as well as in negative and blank controls (Fig. 4a_1, 4a_2). Analysis of the

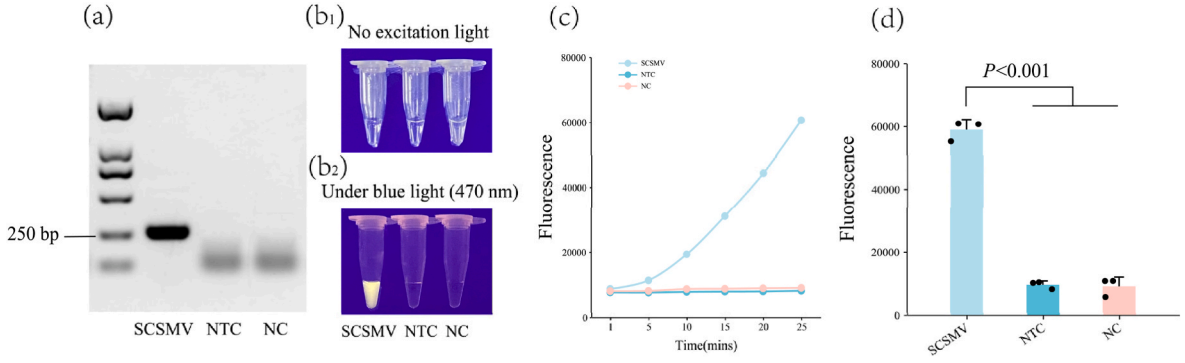


Fig. 2. One-tube detection of the *Sugarcane streak mosaic virus* (SCSMV) based on RPA-CRISPR/Cas12a. (a) Recombinase polymerase amplification (RPA) reaction result. The marker length was established at 2000 bp. SCSMV: positive plasmid template. NTC: negative control. NC: blank control. (b_1, b_2) Results were visualized following the reaction. (c) Real-time fluorescence profiles of the CRISPR cutting probes were recorded 15 min post-RPA amplification. (d) The endpoint fluorescence values were measured. The experiment was conducted in triplicate, and the error bars represented the means ± SD.

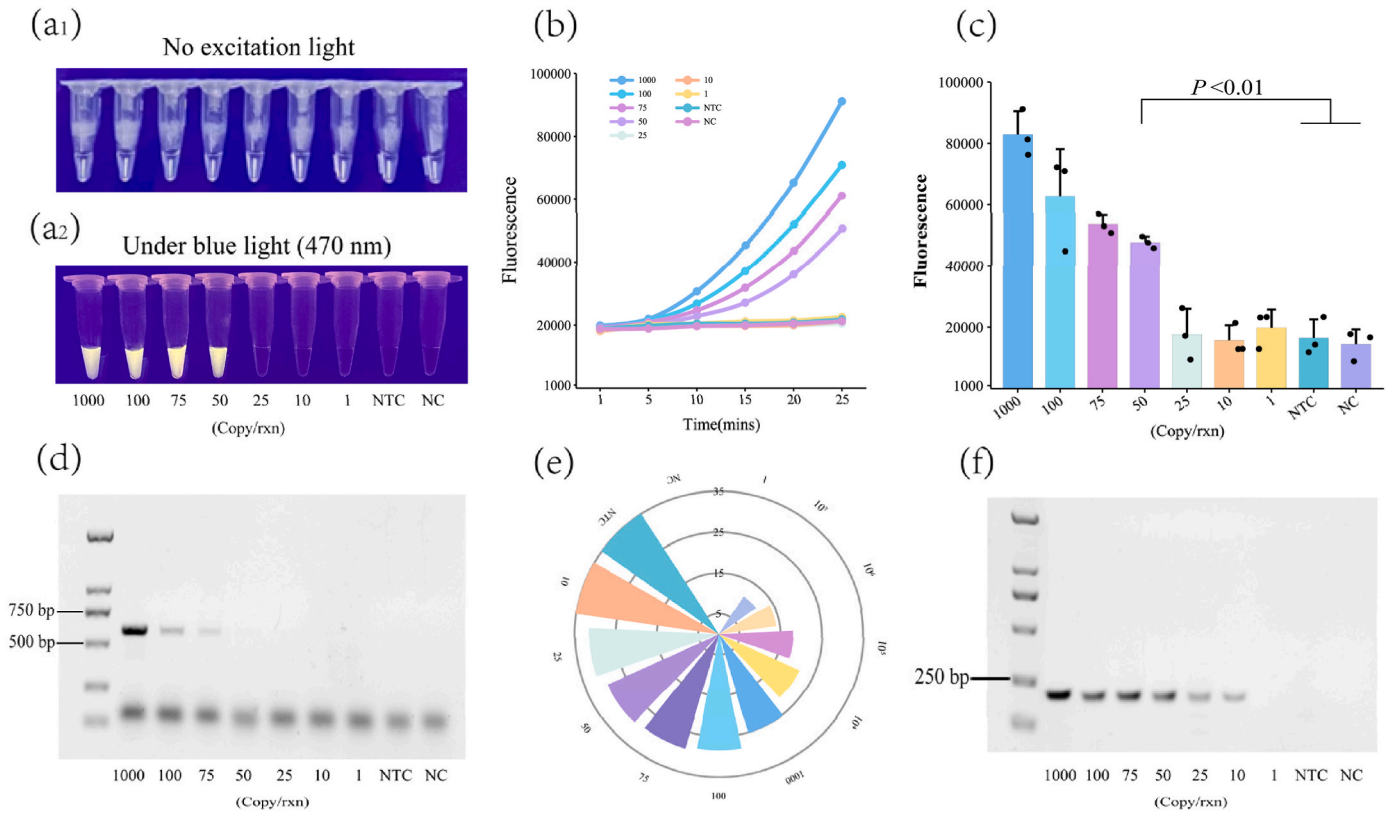


Fig. 3. Sensitivity comparison of RPA-CRISPR/Cas12a, conventional PCR, and qPCR for SCSMV plasmid. (a_1, a_2) Results were visualized following the reaction. The numbers 1,000, 100, 75, 50, 25, 10, and 1 denote the copy number of plasmids was 1,000, 100, 75, 50, 25, 10, and 1 in each reaction, respectively. NTC refers to the negative control, while NC indicates the blank control. (b) Real-time fluorescence profiles of the CRISPR cutting probes were recorded 15 min post-RPA amplification. (c) The endpoint fluorescence values were measured. The experiment was conducted in triplicate, and the error bars represented the means ± SD. (d) PCR sensitivity electrophoresis detection results. The DNA ladder is 2000 bp. (e) Rose chart depicting CT values. The outermost value corresponds to the associated copy number. The values of 5, 15, 25, and 35 located on the circle represent the CT values. (f) Results of qPCR sensitivity as determined by electrophoretic detection.

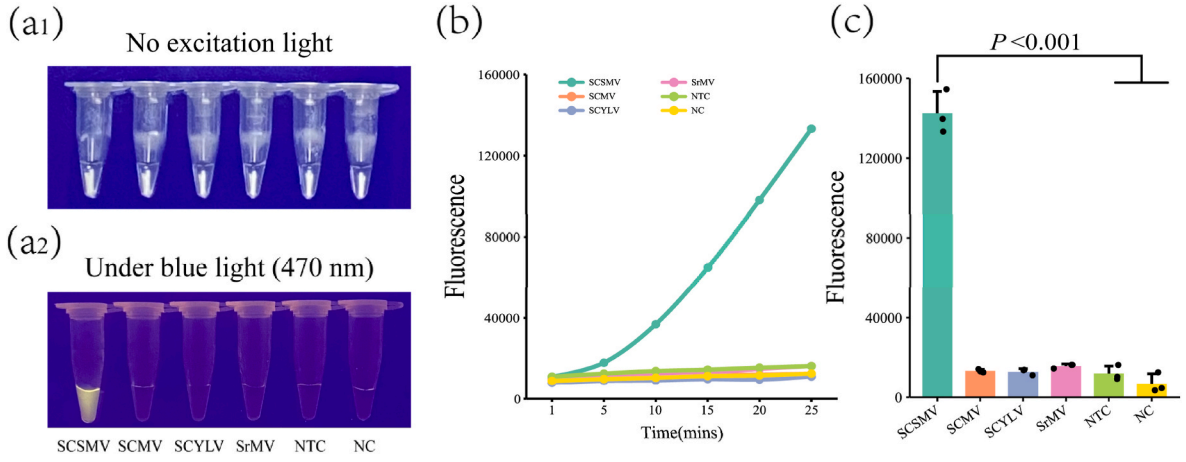


Fig. 4. Specific detection. (a_1, a_2) Results were visualized following the reaction. (b) Real-time fluorescence profiles of the CRISPR cutting probes were recorded 15 min post-RPA amplification. (c) The endpoint fluorescence values were measured. The experiment was conducted in triplicate, and the error bars represented the means ± SD.

fluorescence curve revealed a rapid increase in fluorescence for SCSMV over time, in contrast to the minimal changes observed in the other test tubes (Fig. 4b). Furthermore, the endpoint fluorescence value for SCSMV was significantly higher than those of the other samples and the control group (Fig. 4c). These results demonstrate that the RPA-CRISPR/Cas12a detection system exhibits excellent specificity for detection of SCSMV, and not disturbed by other non-target viruses tested.

3.4. Practical application of RT-RPA-CRISPR/Cas12a

RT-RPA-CRISPR/Cas12a detection was conducted on RNA crude extracts from 40 different accessions of sugarcane. The findings demonstrated that the fluorescence readings of nine samples rose quickly as the reaction time was extended, and pronounced fluorescence was observed in these samples upon completion of the reaction. The

samples exhibiting this fluorescence were FN14-15, FN14–38, GT29, GT42, YT93-159, YZ01-1413, YZ03-103, YZ03-194, and YZ05-51, respectively. In contrast, other samples displayed no significant fluorescence when compared to negative and blank controls (Fig. 5a, b, c, d). Subsequently, PCR was performed on the 40 RNA samples from different sugarcane accessions, revealing that nine samples amplified target bands, which were consistent with the positive samples detected by RT-RPA-CRISPR/Cas12a (Fig. 5e). Furthermore, qPCR testing of all 40 samples yielded CT values ranging from 21.45 to 28.11 for FN14-15, FN14–38, GT29, GT42, YT93-159, YZ01-1413, YZ03-103, YZ03-194, and YZ05-51, which were significantly lower than those of the other samples. Negative and blank controls were included in this analysis (Fig. 5f). To ensure the accuracy of the qPCR detection, all 40 qPCR samples, along with the negative and blank controls, were analyzed using agarose gel electrophoresis. Examination of the gel revealed that the amplification results for the target bands were consistent with those obtained from PCR, with nine target bands amplified across all samples (Fig. 5g). However, analysis of other samples on the gel indicated that non-specific bands were amplified near the size of the target band. Subsequent gel recovery sequencing analysis confirmed that these non-specific bands were primer dimers rather than actual target bands. Therefore, the qPCR detection results for the 40 sugarcane samples from different accessions were entirely consistent with those obtained from PCR and RT-RPA-CRISPR/Cas12a, demonstrating that RT-RPA-CRISPR/Cas12a is a viable method for the detection of actual samples.

4. Discussion

Molecular biological assay, due to its good sensitivity and accuracy, is considered as the gold standard for gene detection [68]. RT-PCR and RT-qPCR are the two most commonly employed detection methods for the RNA virus, such as SCSMV, while they must be conducted in laboratory, necessitate expensive equipment and skilled operational techniques, and the results cannot be visually observed [69], including a one-step RT-qPCR for SCSMV detection [6] which achieved a detection limit of 100 copies of virus RNA and 2 pg of total RNA and demonstrated sensitivity nearly 100 times greater than that of conventional RT-PCR. Additionally, the method of RT-LAMP developed by Li et al. for SCSMV. This method exhibited sensitivity 100 times higher than RT-PCR at 60 °C–63 °C, with a minimum detectable RNA concentration of 167.9×10^{-2} pg [70], and obtained the results observable to the naked eye. However, it still need to prepare total RNA from sugarcane leaves and spends more time compared to our method in spite of a less time compared to the methods of RT-PCR and RT-qPCR. A RT-RPA method established for detecting SCSMV exhibits a high sensitivity that is 100 times greater than that of RT-PCR, along with a less amplification time (5–20 min) at a loose tempreture from 26 °C to 38 °C. However, it still requires refined RNA extracted from sugarcane leaves as a template [71]. A loose reaction temperature and shorter amplification time suggest that RPA is a superior technological choice compared to LAMP. Nonetheless, the use of RPA may lead to non-specific amplification [72],

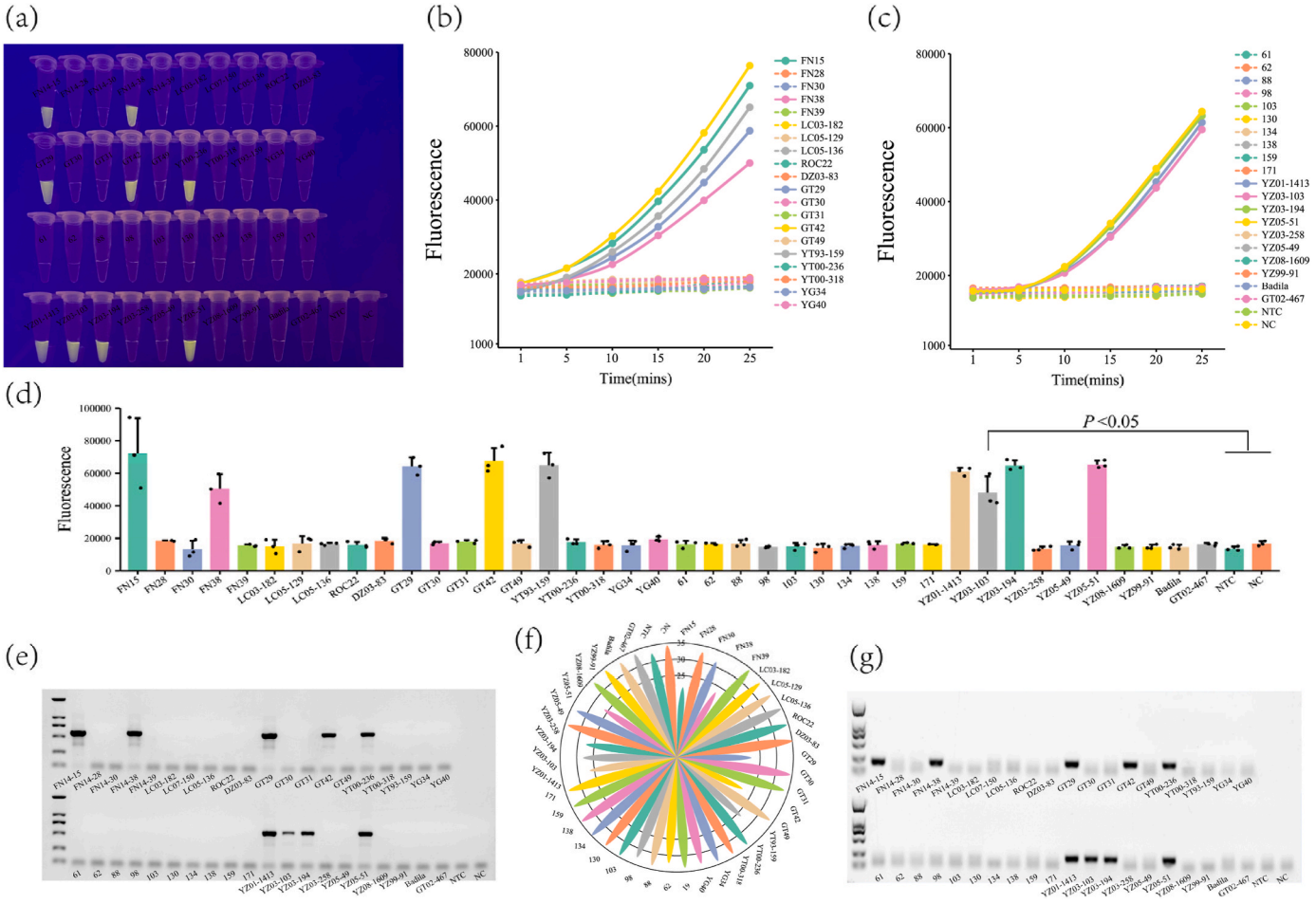


Fig. 5. The results of RPA-CRISPR/Cas12a were consistent with those of PCR and qPCR. (a) Results were visualized following the reaction. Sample placement sequence is attached below. NTC: negative control. NC: blank control. (b, c) Real-time fluorescence profiles of the CRISPR cutting probes were recorded 15 min post-RPA amplification. (d) The endpoint fluorescence values were measured. The experiment was conducted in triplicate, and the error bars represented the means ± SD. (e) The results of the PCR electrophoresis tests are presented. (f) Rose chart of CT values. The outermost ring represents the sample names, and the values of 25, 30, and 35 are indicated on the circle as CT values. (g) The results of the qPCR electrophoresis tests are presented.

as the presence of false positives is an inherent limitation of this technology. This issue is particularly pronounced given the large and highly heterozygous nature of the sugarcane genome [73], where the target sequence may closely resemble sequences within the host's genome, thus increasing the likelihood of false positives. In comparison to traditional RT-PCR and RT-qPCR methods, the technical operations and equipment required for LAMP and RPA detection methods are relatively straightforward. However, these methods still necessitate the extraction of nucleic acids from plant tissues and face challenges such as difficulties in primer design and aerosol contamination in LAMP detection [74]. To address these shortcomings, Xiong et al. and Lv et al. [75,76] innovatively developed a novel technology by combined RPA with CRISPR/Cas12a. This approach enhances detection specificity by utilizing crRNA to identify the target sequence, thereby improving detection sensitivity and reducing false positives associated with RPA. Furthermore, the Cas12a protein exhibits non-specific cutting activity only after it has been recognized and bound to the target DNA, resulting in a high specificity. This technology has been successfully applied to the detection of pathogenic viruses, such as *Rice stripe mosaic virus* (RSMV) [56], *Respiratory syncytial virus* [77], *Phytophthora sojae* [78], *Hematopoietic necrosis virus* [79], and recently SCYLV [67]. The RPA-CRISPR/Cas12a system, characterized by its accuracy, speed, and simplicity, does not rely on traditional virus detection methods that require precise experimental procedures and expensive equipment. Furthermore, it reduces the incidence of false positive results. The system only requires a constant temperature water bath pot and a fluorescence detector, or a UV flashlight priced at less than 20 yuan, to interpret the results. Consequently, compared with RT-PCR [23] and RT-qPCR [6], the detection system developed in here has the remarkable advantages of visual detection results, simple equipment and easy operation, and so is for ELISA [20] or LAMP [70] developed for detection of SCSMV.

In this study, we designed and synthesized specific crRNA and RPA primers targeting the conserved region of SCSMV, which is critical for ensuring the high specificity and accuracy of the RT-RPA-CRISPR/Cas12a detection method. More importantly, our method exhibited no cross reaction with the three common pathogenic viruses affecting sugarcane. Unlike most of other RPA-CRISPR/Cas12a assay methods [80–82], which utilize the classical PAM region (TTTN), this study employs a suboptimal PAM region for several reasons. In the one-tube reaction, a competitive interaction exists between the systems of CRISPR/Cas12a cleavage and the RPA isothermal amplification. The strong binding affinity of the classical PAM region to the Cas protein can skew this competition in favor of Cas protein cleavage, thereby reducing the availability of isothermal amplification substrates and halting amplification, ultimately leading to signal delay and decreased sensitivity [65]. Consequently, utilizing the one-tube method with a classical PAM region is not advisable. By opting for a suboptimal PAM region, it our developed SCSMV detection system can enhance the fluorescence signal, reduce detection time, and improve both sensitivity and specificity in the one-tube reaction, similar to the previous reports [83–85]. We demonstrated that the sensitivity of the SCSMV plasmid detection reached 50 copies/μL, which is comparable to RT-qPCR and superior to RT-PCR. Notably, a variety of different genotypes of sugarcane leaf tissue samples were used, with no cross-reactivity observed with other pathogens, thereby confirming the specificity and accuracy of the RPA-CRISPR/Cas12a detection method. The reaction results were visually assessed and found to be comparable to the detection of *Heterodera avenae* [86], RSMV [56] and SCYLV [67]. It is important to emphasize that all reported SCSMV detection methods to date necessitate complex sample processing, including RNA extraction [5,18,20,21,23,87]. However, this study employs a detection template using crude extract from sugarcane leaves, circumventing the laborious RNA extraction process, which has not been previously utilized in sugarcane SCSMV detection systems. Notably, the RPA reaction can be completed within 20 min, and the preparation of the crude leaf extract takes only 25 min. Consequently, the entire experimental process, from crude extract preparation to detection result observation, needs just 50 min, and comparable to those observed in SCYLV detection using RPA-CRISPR/Cas12a technology [67]. Recently, Wang et al. developed a method, integrating RAA with CRISPR/Cas12a, proved to be more sensitive, achieving 2.5 copies/μL for detection of SrMV in sugarcane and 2.6 copies/μL for RSMV in rice [56]. Subsequently, this method was employed to detect *Maize chlorotic mottle virus* (MCMV) within 45 min, achieving a sensitivity of 10^{-5} compared to RT-PCR [88]. Besides, this method was also used for detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), achieving a sensitivity of 30 copies [89], and for detecting *Tomato mosaic virus* within 30 min and a sensitivity of 172 ag/μL, which is 10,000 times more sensitive than RT-PCR [90]. However, all the aforementioned studies employing RAA-CRISPR/Cas12a needs a cumbersome RNA extraction process, indicates more complicated operations and additional RNA extraction time, and thus resulting in longer time requirement.

In the process of establishing detection technology in this study, another noteworthy technical improvement is the separation of the RPA and CRISPR/Cas12a reaction systems prior to the reaction. The RPA component is added to the centrifuge tube, while the CRISPR/Cas12a component is placed on the cover of the centrifuge tube. Following incubation at 42 °C in a water bath pot, slight centrifugation is performed to facilitate the mixing of the two components. This method effectively avoids competition between the two systems within the same reaction environment, allowing for the one-tube reaction to be realized and achieving the desired reaction outcomes. This approach addresses issues encountered in the previous RPA-CRISPR/Cas12a one-tube reaction systems, where competition between RPA and CRISPR/Cas12a led to delayed or absent fluorescence signals, ultimately resulting in false negatives [65]. Although the method established in this study has obvious advantages, other newly developed technology showed even greater advantages. For example, an Autocatalytic Cas12a Circular DNA Amplification Reaction (AutoCAR) system, belonging to amplification-free CRISPR/Cas12a system, can detect 1 aM (<1 copy/μL) level of DNA and reaction finished in 15 min at room temperature without additional amplification [91]. Finally, in order to compare the method developed here with others and refer to them more clearly, different technical methods for detection of SCSMV were summarized (Table 2).

5. Conclusion

We established a technical system for detecting SCSMV causing mosaic disease in sugarcane. The method is based on the principles of RPA-CRISPR/Cas12a and has remarkable advantages of visual detection results, simple equipment, easy operation, specificity and robust stability. By improving the operational method, i.e. respective reactions of RPA in tube and CRISPR/Cas12a in tube cover, we achieved one-tube isothermal amplification while avoiding false negatives caused by competition between the two processes. This system exhibits satisfactory specificity with a sensitivity of 50 copies/μL, outperforming conventional RT-PCR while achieving comparable performance to RT qPCR, and no sample RNA preparation is required. Its specificity, sensitivity, and stability were validated by detecting other non-target sugarcane viruses and a batch of field samples using leaf crude extracts.

CRediT authorship contribution statement

Shuai Gao: Writing – original draft, Methodology, Formal analysis, Data curation. **Jinlong Guo:** Supervision, Funding acquisition. **Ting Wang:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Liping Xu:** Writing – review & editing, Resources, Funding acquisition, Conceptualization, Supervision.

Table 2
Summary and comparison of different technologies for detection of *Sugarcane streak mosaic virus* (SCSMV).

Technology	Main instrument	Detection measure	Detection type	Limit of detection	References
RT-PCR	PCR thermocycler	Electrophoresis	Total RNA	200 pg	[6]
				167.9×10^{-3} ng	[70]
				171×10^{-4} ng/μL	[71]
			SCSMV plasmid	75 copies/μL	This study
RT-qPCR (TaqMan)	Real-time PCR instrument	Real-time fluorescence	Total RNA	2 pg	[6]
RT-qPCR (SYBR Green)	Real-time PCR instrument	Real-time fluorescence	Total RNA	1 pg	[19]
			SCSMV plasmid	10 copies/μL	This study
RT-LAMP	Thermocycler	Electrophoresis	Total RNA	167.9×10^{-5} ng	[70]
	Water bath pot	Electrophoresis and fluorescence	Total RNA	5 pg	[48]
RT-RPA	Thermocycler	Electrophoresis	Total RNA	171×10^{-6} ng/μL	[71]
RPA-CRISPR/Cas12a	Water bath pot and blue light spectrometer	Blue light spectrometer	SCSMV plasmid	50 copies/μL	This study

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2025.127888.

Data availability

Data will be made available on request.

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