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A Real-Time Recombinase Polymerase Amplification Platform for Rapid and Field-Deployable Detection of Major DNA and RNA Viruses in Sweetpotato

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Abstract

Background

Sweetpotato (*Ipomoea batatas*) production is affected by persistent viral infections that accumulate over generations contributing to cultivar decline. Although PCR-based methods are commonly used to detect sweetpotato viruses, they require specialized equipment and laboratory facilities. This study aimed to develop rapid RPA assays for real-time detection of seven major DNA and RNA viruses in sweetpotato.

Methods

We developed a rapid and sensitive isothermal platform using Exo RPA/RT-RPA assays targeting one DNA begomovirus, four RNA potyviruses, and two strains of one RNA crinivirus. An artificial positive control was constructed to contain a synthetic sequence including seven target viral genomic regions for RPA assay development. An internal control targeting the endogenous sweetpotato ADP-ribosylation factor gene was used to assess sample quality for RPA amplification. Assay sensitivity, specificity, and performance were evaluated and compared with conventional PCR-based methods.

Results

All seven Exo RPA/RT-RPAassays produced detectable fluorescence at 39°C within 20–30 min. Depending on the target virus, the provisional analytical detection limits ranged from 10^1 to 10^4 plasmid copies/μL. No cross-reactivity was detected among the seven viral targets. Overall, the Exo RPA/RT-RPA results agreed with the corresponding PCR-based reference methods. In several samples, Exo RT-RPA was more sensitive and consistent in detecting several potyviruses in mixed infections that were not detectable by multiplex RT-PCR.

Conclusions

The singleplex Exo RPA/RT-RPA assays provide a rapid, sensitive, and specific method for detecting major DNA and RNA viruses in sweetpotato. This platform will provide virus screening, disease surveillance, and nursery clean plant propagation.

Introduction

Sweetpotato (*Ipomoea batatas* [L.] Lam), a member of the family Convolvulaceae, is an important root crop in the world due to its high nutritional value, adaptability, and economic importance [1]. Sweetpotato is cultivated in more than 100 countries across tropical and subtropical regions, including Asia, Africa, and the Americas [2]. However, its productivity is severely affected by a wide range of viral pathogens, many of which occur as mixed infections thus complicating accurate diagnosis and disease management [3, 4]. The most economically important viruses infecting sweetpotato worldwide include potyviruses: sweet potato feathery mottle virus (SPFMV, *Potyvirus bataplumei*), sweet potato virus C (SPVC, *Potyvirus cebatatae*), sweet potato virus 2 (SPV2, *Potyvirus duobatatae*), and Sweet potato virus G (SPVG, *Potyvirus gebatatae*), Begomoviruses (Sweepoviruses): sweet potato leaf curl virus (SPLCV, *Begomovirus ipomoeae*), and Criniviruses: sweet potato chlorotic stunt virus (SPCSV, *Crinivirus ipomeae*) West African (SPCSV-WA) and East African (SPCSV-EA) strains. Mixed virus infections can cause substantial yield losses, particularly when synergistic interactions occur [3, 5, 6].

The six viruses targeted in the present study can induce a wide range of symptoms depending on virus species, cultivar susceptibility, mixed infection, and environmental conditions. Typical symptoms include chlorosis, vein clearing, mottle, rugosity, leaf curling, stunting, and reduced root development [7, 8]. Sweetpotato virus disease (SPVD), caused by synergistic co-infection of SPFMV and SPCSV, is particularly damaging and can cause severe yield losses that can exceed 80–90% in susceptible varieties [5]. In addition to yield reduction, viral infection significantly compromises the quality of storage roots by causing malformation, discoloration, cracking, and internal corking, making them unsuitable for both local consumption and

commercial markets [9]. The reduction in marketable yield severely impacts on farmer income, seed distribution systems, and overall food security, especially in regions where sweetpotato serves as a dietary staple. Moreover, the accumulation and perpetuation of viruses through vegetative propagation exacerbate their spread and persistence resulting in cultivar decline [3]. Therefore, it is critically important to develop effective and accessible diagnostic tools to support virus indexing and clean seed programs [10].

The six major viruses represent both DNA and RNA virus groups with different modes of transmission and epidemiology [3, 4]. They are considered the most economically important viruses by the USDA National Clean Plant Network – Sweetpotatoes [10]. These potyviruses have been shown to rapidly reinfect clean sweetpotato planting material under commercial field production conditions in California [11] and are also commonly observed in heritage sweetpotato in Hawaii [12]. For SPCSV, only one incidence of SPCSV-WA occurrence has been reported in the U.S [13]. The current standard method recommended by the NCPN -sweetpotatoes for detecting four potyviruses (SPFMV, SPV2, SPVC and SPVG) is using a multiplex one-step RT-PCR assay [14]. Although this method is sensitive, it is technically complicated and labor intensive, which could easily result in cross contamination from amplified PCR products. These potyviruses are primarily transmitted by aphids in a non-persistent manner and are efficiently maintained and disseminated through infected vegetative cuttings, further complicating disease diagnosis and management [6, 15]. SPLCV, a DNA virus among the group, is a single-stranded DNA virus from the family *Geminiviridae*, genus *Begomovirus*. In addition to SPLCV, there are at least 14-related sweetpotato-infecting begomoviruses, collectively named sweepoviruses [3, 16]. Despite their genetic diversity, certain conserved genomic sequences could be identified for primers used in real-time PCR detection for reliable detection of sweepoviruses in a single reaction [17]. These sweepoviruses are persistently transmitted by whiteflies (*Bemisia tabaci*) and efficiently maintained in clonal planting materials. SPLCV often causes symptomless or mild infections when alone, but it can exacerbate symptoms when in mixed infection with RNA viruses [9]. SPCSV is a phloem-limited, whitefly-transmitted, bipartite positive-sense RNA virus represented by genetically and serologically distinct West African and East African strain groups [15, 18, 19, 20]. Strain-specific detection is important because sequence divergence may prevent a single assay from detecting both strains of the virus with equal efficiency. In real-time RT-PCR, SPCSV strain-specific assays are recommended to distinguish EA and WA strain groups [10]. Even though these PCR-based methods are generally reliable, a combination of conventional multiplex RT-PCR and real-time RT-PCR can be technically complicated and labor intensive, with potential for cross contamination. Therefore, a rapid, simple, and field-deployable method would be necessary to improve detection accuracy, consistency and reliability directly at the point of need.

Early, sensitive, and specific detection of target viruses is essential for virus indexing, clean-plant programs, and distribution of virus-tested propagation material to growers. Molecular methods used for plant-virus diagnosis have progressed from conventional PCR and RT-PCR to multiplex RT-qPCR, isothermal amplification, and portable lateral-flow detection systems [21]. Conventional assays such as ELISA, RT-PCR, and real-time PCR are reliable but generally depend on laboratory instrumentation and trained personnel [22]. To address these limitations, isothermal nucleic acid amplification methods provide alternatives for rapid testing [23]. Among these, Loop-mediated isothermal amplification (LAMP) has been designed to detect three major viruses (SPCSV, SPFMV, and SPLCV) in sweetpotato [24, 25]. In comparison to LAMP, recombinase polymerase amplification (RPA) requires fewer primers, shorter reaction times, and simpler optimization. RPA, combined with reverse transcription for RNA targets and an exonuclease-cleavable fluorescence probe, can provide accurate, rapid, sensitive, and simple diagnostics that are ideal for field deployment [26]. Since its initial description by Piepenburg et al., [27] RPA has been adapted for diverse pathogen-detection applications [28]. Basic RPA assays commonly rely on post-amplification visualization, whereas Exo RPA uses a sequence-specific fluorescence probe that can be monitored in real time with a portable fluorometer. This eliminates gel electrophoresis and reduces the instrumentation required for detection, improving its suitability for field-deployable testing. RPA has emerged as a rapid isothermal alternative to conventional PCR-based diagnostics because amplification can be completed at a constant low temperature using minimal equipment. Previous studies demonstrated the simultaneous detection of DNA and RNA cucurbit viruses using multiplex RT-RPA and showed that RPA products can be detected using portable lateral-flow strips for on-site diagnosis [29, 30]. These findings support the development of field-deployable RPA assays for rapidly screening vegetatively propagated crops such as sweetpotato. Although RPA has been applied to several plant viruses, a unified panel of singleplex Exo RT-RPA assays covering both DNA and RNA viruses in sweetpotato have not been available.

Here, we report on the development and validation of Exo RPA/RT-RPA as a rapid, portable, and field-deployable diagnostic platform for accurate and sensitive detection of six major sweetpotato viruses, including two SPCSV strains. To our knowledge, this is the first study to describe an Exo RPA/RT-RPA detection platform to detect six major sweetpotato viruses. This tool offers a cost-effective and field-deployable diagnostic solution to support virus indexing, clean plant certification programs, and disease management in sweetpotato production systems.

MATERIALS AND METHODS

Sweetpotato virus cultures and sample tissue selection

Sweetpotato (*Ipomoea batatas* [L.] Lam) samples used in this study were obtained from commercial and experimental fields during the growing season in 2025–2026, including 15 virus cultures maintained at the Micropropagation and Repository Unit, North Carolina State University, Raleigh, NC and 17 sweetpotato breeding lines grown in an experimental field at the U.S. Vegetable Laboratory, Charleston, SC. Extracted total nucleic acid preparations from the 15 virus cultures were provided by Christie Almeyda in North Carolina. For the 17 field-collected samples collected in Charleston, both purified total nucleic acid and crude extract were used for developing and validating Exo RPA/RT-RPA. For leaf sampling, two fully expanded young leaves, preferentially the third to fifth leaf from the growing point, together with the corresponding petiole were collected and kept cool on ice or in a refrigerator until processing. Where storage roots were available, a 2–5 g piece containing periderm and adjacent cortex was removed with a sterile scalpel for nucleic-acid extraction. Sample identifiers and available metadata are provided in the corresponding tables and supplementary information.

Total nucleic acid extraction

Total nucleic acid was extracted from sweetpotato leaf or storage-root tissues, where indicated, using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNase treatment was omitted so that viral/genomic DNA, including SPLCV DNA, would be retained together with RNA. Nucleic-acid concentration and purity were assessed with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Virus-free plants and previously characterized virus-positive plants were used as controls. Extracts were kept on ice during reaction setup and stored at − 80°C. Sweetpotato ADP-ribosylation factor (ARF) gene was selected as an internal control to evaluate sample integrity for the presence of amplifiable plant nucleic acid by RT-RPA.

Crude sample preparation for field-deployable testing

For rapid crude-sap preparation, approximately 100 mg of fresh sweetpotato leaf tissue was homogenized using a hand roller, a rubber mallet or a marker pen over a sample extraction bag in 1 mL of either 1× Extraction Buffer General (1:10 w/v) prepared from a commercial 10× concentrate (BIOREBA AG, Reinach, Switzerland) or 1× General Extract Buffer (GEB; catalog no. ACC 00955; Agdia Inc., Elkhart, IN, USA) in a disposable extraction bag. The 1× BIOREBA buffer contained, per liter of distilled water, 2.40 g Tris, 8.00 g NaCl, 20.00 g polyvinylpyrrolidone K25 (molecular weight 24,000), 0.50 g Tween 20, 0.20 g KCl, and 0.20 g Sodium Azide, with the pH adjusted to 7.4 using HCl. Following the manufacturer's instructions, 1× Agdia GEB was prepared by combining 16.5 g of the proprietary GEB powder and 10.0 g of TWEEN® 20 with deionized water to a final volume of 500 mL. The buffer was mixed for 15–30 min until completely dissolved, and its pH was maintained within the recommended range of 7.2–7.8. The extraction procedure required approximately 5 min. After the homogenate was allowed to settle for 1–2 min, 1–2 µL of the clarified supernatant was used directly as the reaction template for Exo RPA/RT-RPA.

Identification of conserved sequence regions through multi sequence alignment of the target viruses and a sweetpotato gene for internal control

Complete and partial nucleotide sequences were retrieved from the NCBI GenBank database and aligned with MAFFT v7 [31] to identify conserved regions for SPLCV, SPFMV, SPV2, SPVC, SPVG, SPCSV-WA, and SPCSV-EA that were suitable for Exo RPA/RT-RPA amplification (**Supplementary Figure S1**). Representative isolates from different geographic origins were included where available to improve sequence coverage and diagnostic inclusivity. For SPCSV-WA and SPCSV-EA, strain-specific conserved

regions in RNA2 were selected to permit differentiation of the two strain groups. In addition, primers targeting the sweetpotato ADP-ribosylation factor (ARF) gene were selected as an internal control for RT-RPA amplification (Table 1).

Primer design for RPA detection of target viruses

From the selected conserved sequence region from each target virus, primers were designed manually following RPA design recommendations (TwistDx, Cambridge, UK) and evaluated using OligoAnalyzer (Integrated DNA Technologies, Coralville, IA, USA). The following criteria were applied: candidate primer length of 30–35 nucleotides, moderate GC content) and limited predicted secondary structure or 3' complementarity. Candidate primer pairs were selected to minimize primer-dimer formation with a conserved sequence region for the Exo probe positioned within the resulting amplicon. Candidate primer and probe regions were screened *in silico* with BLASTn against the NCBI nucleotide database to assess target specificity and potential homology with non-target sequences. The final oligonucleotide sequences used in this study are listed in Table 1.

Table 1
Oligonucleotide primers and probes used for Exo RPA/RT-RPA and PCR validation of major sweetpotato viruses.

Assay Method	Primer Name	Sequence (5'–3')	Source
Exo RPA/RT-RPA primers and probes			
Exo-RPA for SPLCV	SPLCV-RPA-F	GTCATCAATGACGTTATACCAAGCACTGTT	This study
	SPLCV-RPA-R	TCTTCTTTCAATGCCGTTCCTGACATTATC	
	SPLCV-RPA-probe	TATGGGTCTATCCGGCCGCGCAGCGGAA[FAM-dT] C [THF]A[BHQ-1-dT] GACATTTTCAGCGG [C3-Spacer]	
Exo RT-RPA for SPFMV	SPFMV-RPA-R	ACTAGAAATATACATAACCTCTTAGGAATGA	This study
	SPFMV-RPA-F	GACTTCTCAAATGCAATAACCGAGATATAA	
	SPFMV-RPA-probe	GCACTGTAGTTTATACTTATGTTATCTT[FAM-dT] A [THF]TA[BHQ-1-dT] GCCTTTAATTTA [C3-Spacer]	
Exo RT-RPA for SPVG	SPVG-RPA-F	TGATGTTACAAGGAATATACATAACCTCTT	This study
	SPVG-RPA-R	GGTTCTTCGGGACTGAAAGATACGAATTTA	
	SPVG-RPA-Probe	TATGAGGGGTGTGCAGTAAACAATATAT[FAM-dT] GC [THF]CG[BHQ-1-dT] ACCTTTTAATTTC [C3-Spacer]	
Exo RT-RPA for SPVC	SPVC-RPA-F	CTGTATGGAAAAAACTGGACGAACAATTGG	This study
	SPVC-RPA-R	TGGAGATTTTAAAATCCTTTTCCGCTTCAT	
	SPVC-RPA-probe	TGCCACCTCAGATTTTAACGGGCATTTCGA[FAM-dT] A [THF]C[BHQ-1-dT] GGTGGCCCAGTAGCG [C3-Spacer]	
Exo RT-RPA for SPV2	SPV2-RPA-F	AATTATTATGCGGCAACATGGAAACAATTT	This study
	SPV2-RPA-R	GAAAGACTAGCCTCATGCTCAAAGAAAGTC	
	SPV2-RPA-Probe	GCCAAGTATGATATGGAGCAAATAGCTCC[FAM-dT] AC [THF]GC[BHQ-1-dT] GGCATGCTGGAGAGG [C3-Spacer]	
Exo RT-RPA for SPCSV-EA	SPCSV-EA-RPA-F	CGTTTCTCTAATAATGTCACAGTCTATAATC	This study
	SPCSV-EA-RPA-R	GAATACTTATTCCTATTGATCTGGTCTAAAT	
	SPCSV-EA-Probe	AAACATTCAGATATGCTTTTAAGAAGAC[FAM-dT]GA[THF]G[BHQ-1-dT]TAGAGATAAATTGGA[C3-Spacer]	
Exo RT-RPA for SPCSV-WA	SPCSV-WA-RPA-F	GATTTATATTAATTTGTCATGGAAGCTAAGG	This study
	SPCSV-WA-RPA-R	ATTACATCACCACTCTTAGTAATAGATAAAC	
	SPCSV-WA-Probe	CTGGTCTCGATTTCGGTACTACTTTCAG[ROX-dT]AC[THF]A[BHQ-2-dT]ATCGGCGTATGTTGGT[C3-Spacer]	
Internal control ARF for RT-RPA	Inter-ARF-RPA-F	ACGATCTCGATCATTACTGTCAACAACAAA	This study
	Inter-ARF-RPA-R	ATATTGTATAAACTTAAGCTGGGAGAGATTGTTACT	
RT-PCR and qPCR primers and probes			
Multiplex RT-PCR for potyviruses	SPVG-F (1191 bp)	GTATGAAGACTCTCTGACAAATTTTG	Li et al., 2012b

Assay Method	Primer Name	Sequence (5'–3')	Source
Exo RPA/RT-RPA primers and probes			
	SPVC-F (836 bp)	GTGAGAAAYCTATGCGCTCTGTT	
	SPFMV-F (589 bp)	GGATTAYGGTGTTGACGACACA	
	SPV2-F (369 bp)	CGTACATTGAAAAGAGAAACAGGATA	
	SPFCG2-R (four viruses)	TCGGGACTGAARGAYACGAATTTAA	
Real-time PCR for SPLCV	KL07-40 SPLCV.F2	GAGACAGCTATCGTGCC	Ling et al., 2010
	KL07-41 SPLCV.R2	GAAACCGGGACATAGCTTCG	
	KL07-42 SPLCV.P2	FAM-TACACTGGGAATGCTGTCCCAATTGCT-TAMRA	
RT-PCR for Criniviruses SPCSV-EA and SPCSV-WA	SPCSV-f1-WA	ATCGGCGTATGTTGGTGGTA	This study
	SPCSV-r1-WA	GCAGCAGAAGGCTCGTTTAT	
	SPCSV-f2-EA	CGTCTAGATTGTTAGAAATA	
	SPCSV-r2-EA	CGCGGAAGTGTAAGGTATAG	

Exo probe design for Exo RPA/RT-RPA detection

For real-time fluorescence detection, Exo probes compatible with the TwistAmp Exo kit chemistry were designed to hybridize within the amplified region between the forward and reverse primers. Each probe was approximately 45–52 nucleotides in length identified from the conserved sequence region for the target virus. In Exo RPA probe design, the selection of the specific nucleotide to be removed and replaced with a tetrahydrofuran (THF) site relies entirely on finding a local target sequence where two anchor thymine (T) residues can be found near each other (ideally 1 to 5 bases apart). These two thymine nucleotides (dT) were modified to attach the fluorescent label (e.g., FAM) on the upstream thymine before the THF site, followed by the downstream thymine with a dT-quencher (e.g., BHQ-1). Each Exo probe contained at least 30 nucleotides before the THF site and at least 15 nucleotides after it. A C3 spacer was added to the 3' end of the entire probe to prevent extension by the DNA polymerase. The fluorescent reporter for the probes of six of the sweetpotato virus targets was 6-carboxyfluorescein (FAM) along with Black Hole Quencher-1 (BHQ1) as quencher. The fluorescent reporter of the probe for SPCSV-WA was ROX with BHQ-2 as quencher. The final oligonucleotide sequences used in this study are listed in Table 1.

Multiplex RT-PCR for four potyviruses and real-time PCR assay for SPLCV

The standard PCR methods adapted by the NCPN-Sweetpotatoes for virus screening [10] including multiplex RT-PCR assays for four potyviruses described by Li et al [14]. and real-time PCR for SPLCV in Ling et al [17]. were used as reference methods for comparison with the developed Exo RPA/RT-RPA assays. The four potyviruses, SPFMV, SPV2, SPVC, and SPVG, were detected simultaneously using the multiplex one-step RT-PCR primer set described by Li et al [14]. and a OneTaq® One-Step RT-PCR Kit (New England Biolabs, Ipswich, MA, USA). Each 25-μL reaction contained 12.5 μL of 2× OneTaq One-Step Reaction Mix, a mixture of virus-specific forward and reverse primer, including 2.5 μl of 10 μM SPVG-F, 0.4 μl of 10 μM SPVC-F, 2.0 μl of 10 μM SPFMV-F, 0.2 μl of 10 μM SPV2-F and 2.0 μl of 10 μM SPFCG2-R reverse primer, 1 μL of total RNA template and 0.5 μL of OneTaq One-Step Enzyme Mix. The remaining volume was adjusted with nuclease-free water and mixed well. With the protocol

of 50°C for 30 min, 95°C for 2 min; 40 cycles of 94°C for 30 s, 60°C for 30 s, 65°C for 1 min; followed by final incubation at 72°C for 5 min; hold on at 4°C. PCR products were resolved by electrophoresis on a 1.5% agarose gel stained with SYBR Safe (Invitrogen, USA) and visualized under UV illumination using a GelDoc (Invitrogen, USA). The expected amplicon sizes for each virus were verified against a 100 bp DNA ladder (NEB). The four viruses were identified according to their expected amplicon sizes: 1,191 bp for SPVG, 836 bp for SPVC, 589 bp for SPFMV, and 369 bp for SPV2. Because SPLCV is a DNA virus, it was evaluated separately using the probe-based real-time PCR assay described by Ling et al., [17] with the SPLCV-specific primers and probe listed in Table 1. The real-time PCR results served as the reference for evaluating the diagnostic performance of the SPLCV singleplex Exo RPA assay. Positive, negative, and no-template controls were included in each RT-PCR and real-time PCR run.

RT-RPA assays for SPCSV-WA, SPCSV-EA, and ARF

Conventional RT-RPA reactions were performed separately for SPCSV-WA, SPCSV-EA, and the sweetpotato ARF internal control using the TwistAmp® Basic kit (TwistDx Ltd., Cambridge, UK). Each 50 µL reaction contained 29.5 µL rehydration buffer, 2.4 µL each of forward and reverse primers (10 µM), 11.2 µL nuclease-free water, 1 µL SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), 1 µL total nucleic acid or crude extract, and 2.5 µL of 280 mM magnesium acetate. Reactions were incubated at 39°C for 20 min, with brief mixing and centrifugation after 1 min. Products were purified with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), separated on a 2% agarose gel containing SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA), and visualized with a gel-documentation system. Strain-specific positive controls, a healthy sweetpotato control, and a no-template control were included in every run.

Exo RPA/RT-RPA assay setup and amplification conditions

Each viral Exo RPA/RT-RPA assay was performed as an independent singleplex reaction using a TwistAmp® exo kit (TwistDx Ltd., Cambridge, UK). Each 50-µL reaction contained 29.5 µL of rehydration buffer, 2.1 µL each of the forward and reverse primers (10 µM), 0.6 µL of the corresponding Exo probe (10 µM), 11.2 µL of nuclease-free water, 1 µL of reverse transcriptase enzyme, and 1 µL of template. The reaction mixture was added to the lyophilized pellet, and amplification was initiated by adding 2.5 µL of 280 mM magnesium acetate. Reactions were mixed briefly, collected by quick centrifugation, and incubated in an AmpliFire® real-time fluorometer (Agdia Inc., Elkhart, IN, USA). Temperature screening was performed independently for all seven viral targets SPLCV, SPFMV, SPVC, SPV2, SPVG, SPCSV-EA, and SPCSV-WA at temperatures ranging from 35 to 42°C. Based on the overall amplification performance of the seven assays, 39°C was selected as the routine reaction temperature. Fluorescence was recorded for 20–30 min. The SPLCV, SPFMV, SPVC, SPV2, SPVG, and SPCSV-EA assays were monitored in the FAM channel, whereas the SPCSV-WA assay was monitored in the ROX channel. Each assay run included a target-specific artificial plasmid positive control, healthy sweetpotato control, and no-template control. A general schematic workflow depicting singleplex Exo RPA/RT-RPA detection of six sweetpotato viruses is shown in Fig. 1.

Developing an artificial positive control plasmid containing template sequences for all major sweetpotato viruses

Because not all virus cultures were available for analysis and validation, particularly those regulated viruses, such as SPCSV-EA which has not been reported in the U.S. To generate positive controls for assay validation and analytical sensitivity testing, artificial plasmid DNA constructs were developed using the target Exo RT-RPA amplicon sequences of all major sweet potato-infecting viruses. Synthetic DNA fragments corresponding to the validated target regions of SPLCV, SPFMV, SPVC, SPV2, SPVG, SPCSV-EA, and SPCSV-WA were combined into a single synthetic construct and cloned into one pUC57 plasmid vector to generate a common artificial positive control for all seven singleplex Exo RPA/RT-RPA assays (Fig. 2). The concentration of the recombinant plasmid DNA was measured spectrophotometrically. Plasmid copy number and the volumes required for preparing the desired copy-number dilutions were determined using the online Thermo Scientific DNA Copy Number and Dilution Calculator (Thermo Fisher Scientific, Waltham, MA, USA) by entering the measured plasmid concentration and the total length of the recombinant plasmid. Tenfold serial dilutions of the common plasmid control were prepared and used to evaluate target

amplification and determine the analytical sensitivity of each singleplex assay. Because the plasmid contained DNA copies of all seven target regions, to evaluate amplification and fluorescence-detection steps. A 2,000-bp artificial positive-control insert containing assay-target regions from SPLCV, SPFMV, SPVG, SPV2, SPVC, SPCSV-EA, and SPCSV-WA was generated by chemically synthesizing overlapping oligonucleotides and assembling them into a full-length DNA fragment. The assembled insert was cloned into the pUC57-BsaI-Free vector, propagated in *Escherichia coli*, and verified by Sanger sequencing by Gene Universal Inc. (Newark, DE, USA). The seven target regions produced predicted amplicons of 228, 184, 132, 233, 302, 156, and 161 bp, respectively, and their nucleotide sequences and primer- and probe-binding sites are presented (**Supplementary Figure S3**).

Analytical sensitivity of Exo RT-RPA assays

Analytical sensitivity was evaluated independently for each singleplex assay using ten-fold serial dilutions of the appropriate artificial plasmid control from 10^8 to 10^0 copies/µL. One microliter of each dilution was added per reaction, and fluorescence was monitored at 39°C for up to 30 min with the AmpliFire® real-time fluorometer (Agdia, Elkhart, IN, USA). FAM was used for the original five virus targets and SPCSV-EA, and ROX was used for SPCSV-WA. The lowest concentration meeting the predefined positivity criterion was recorded as a provisional endpoint. Because the SPCSV dilution series used DNA plasmids and did not establish replicate-based LoD95 values, those results are reported as preliminary concentration-dependent amplification rather than a formal RNA detection limit.

Analytical specificity of Exo RT-RPA assays

The analytical specificity of the Exo RPA/RT-RPA assays was evaluated using two complementary template systems: (i) total nucleic acid from sweetpotato virus-culture sample SP5 (Table 2), which previously tested by real-time PCR and multiplex RT-PCR to contain SPLCV, SPFMV, SPV2, SPVC, SPVG, and SPCSV-WA; and (ii) an artificial positive-control (APC) plasmid containing the target sequences for the seven viruses, including SPCSV-EA (Fig. 2). Healthy sweetpotato nucleic acid and no-template reactions were included as negative controls. Each viral assay was performed as a separate singleplex reaction using its specific primer–probe set. The assays were evaluated for target-specific amplification and potential cross-reactivity with the other six target-virus sequences in the panel. The conventional RT-RPA for internal control ARF primer pair was evaluated separately using healthy and virus-infected sweetpotato extracts. These experiments assessed analytical exclusivity within the evaluated seven-target virus panel.

Data analysis and assay interpretation

Fluorescence traces were interpreted using the predefined positivity criterion described above. Optimization, analytical sensitivity, and analytical specificity experiments comprised four technical replicate reactions per condition and were repeated on three separate days. Experiments involving biological material included two independent biological samples, each tested in four technical replicate reactions per run. Because the study was designed as an analytical feasibility and method-agreement evaluation, results were summarized descriptively; inferential statistical tests and LoD95 estimates were not calculated.

Results

Analytical sensitivity of Exo RPA assays

To evaluate sensitivity of the newly designed Exo RPA assays, we used serial dilutions of the artificial plasmid control from 10^8 to 10^0 copies/µL to test each of the seven sweetpotato virus targets (Fig. 3). For the original five targets, the provisional endpoints were 10^1 copies/µL for SPVG, 10^2 for SPFMV and SPV2, 10^3 for SPLCV, and 10^4 for SPVC. In separate testing for SPCSV-EA/FAM and SPCSV-WA/ROX, Exo-RPA reactions generated concentration-dependent amplification profiles across the evaluated dilution series, and their non-template controls remained negative (Fig. 3).

Analytical specificity of Exo RT-RPA assays to seven sweetpotato virus targets using total nucleic acid as templates

To evaluate specificity of the newly designed RPA assays, we used a total nucleic acid preparation generated from the virus culture SP5 which was previously determined by PCR testing to contain six viruses in a mixed infection, including SPLCV, SPFMV, SPV2, SPVC, SPVG and SPCSV-WA, each virus-specific singleplex Exo RT-RPA assay amplified its corresponding target without detectable amplification from the other viruses or the healthy control (Fig. 4). Since we did not have a natural virus culture for SPCSV-EA, we spiked the artificial positive plasmid DNA into the SP5 preparation as template to evaluate its specificity, Exo RT-RPA produced one single positive signal for SPCSV-EA with cross reaction to any other viruses (Fig. 4). SPCSV-EA produced signal only in its FAM singleplex reaction, whereas SPCSV-WA produced signal only in its ROX singleplex reaction; no cross-reactivity was observed between the two strain groups or with SPLCV, SPFMV, SPV2, SPVC, and SPVG.

As an independent confirmation of assay specificity, each primer–probe set was also evaluated using the corresponding target sequence in the APC plasmid. Each assay produced a signal only when its target-specific primers and probe were used, and no nonspecific or contaminating signals were detected in the other reactions. In particular, SPCSV-EA generated a signal only in its FAM-labeled singleplex reaction, whereas SPCSV-WA generated a signal only in its ROX-labeled singleplex reaction. No cross-reactivity was observed between the two SPCSV strain groups or with SPLCV, SPFMV, SPV2, SPVC, or SPVG. Together, the mixed-infection sample and APC-plasmid experiments independently confirmed the analytical specificity of the seven Exo RPA/RT-RPA assays.

Conventional RT-RPA validation of SPCSV-EA and SPCSV-WA

Since SPCSV is rarely identified in the U.S., only a single occurrence of SPCSV-WA was reported in North Carolina [13]. Therefore, RPA assays were developed using the total nucleic acid from an infected sweetpotato sample, whereas the SPCSV-EA assay was developed using artificial positive-control plasmid DNA as the template. Conventional RT-RPA detection of SPCSV-EA and SPCSV-WA was validated through primer testing, temperature optimization, and sample evaluation (Fig. 5). Specific detection was confirmed using sweetpotato samples containing SPLCV, SPFMV, SPV2, SPVC, SPVG, or SPCSV-WA, together with the artificial positive-control plasmid containing the SPCSV-EA target sequence. Temperature optimization identified 39°C as the optimal incubation temperature for both conventional SPCSV-EA and SPCSV-WA RT-RPA assays. RPA generated a specific reaction to SPCSV-EA artificial plasmid template whereas RPA detected SPCSV-WA on artificial as well as natural samples from virus cultures. Five of the 15 North Carolina virus culture samples were positive for SPCSV-WA. As expected, none of the field collected samples were positive for SPCSV-EA. These strain assignments by conventional RPA agreed with the independent singleplex Exo RT-RPA assays, in which SPCSV-WA was monitored using ROX and SPCSV-EA using FAM.

Optimization experiments were performed independently for each singleplex assay. For SPCSV-EA and SPCSV-WA, temperatures from 35 to 42°C and reaction times of 20, 25, and 30 min were evaluated. The strongest and most consistent fluorescence development was observed at 39°C. Strong SPCSV signals were generally detected within 15–20 min, while monitoring to 25–30 min accommodated lower-titer templates. Accordingly, 39°C with a 20–30-min monitoring window was selected for the seven-virus singleplex workflow (Fig. 5).

Developing an internal control using ADP-ribosylation factor (ARF) gene for RPA

To assess the quality and integrity of nucleic acid preparations in both purified nucleic acid and crude extract that were suitable for RT-RPA amplification, we evaluated ARF primers on field collected samples. The ARF primer pair produced the expected conventional RT-RPA amplicon in all 32 samples, including total nucleic acid preparations or crude extract from 17 field samples collected in the sweetpotato breeding program in Charleston, South Carolina, as well as extracted total nucleic acid from 15 virus culture samples from North Carolina (Fig. 6). These tests confirmed the presence of amplifiable plant nucleic acid and supporting its use to monitor potential false-negative results and reaction stability.

Validation of Exo RPA/RT-RPA using standard PCR-based detection of seven virus targets on 15 virus culture samples

Fifteen sweetpotato virus culture samples (SP1–SP15), including SP15 as the healthy control, were compared using individual Exo RPA/RT-RPA assays (Fig. 7) and the corresponding reference methods: multiplex RT-PCR for SPFMV, SPV2, SPVC, and SPVG (**Supplementary Figure S2**), real-time PCR for SPLCV and conventional RPA for SPCSV-EA and SPCSV-WA. A complete agreement was observed across all five virus targets in 15 samples (Table 2), corresponding to 75 of 75 virus-by-sample comparisons (100% agreement). No discordant results were observed between Exo RPA/RT-RPA and the PCR-based reference methods in this sample set. An additional 17 sweetpotato field samples (V26-82–V26-98) were subsequently evaluated using the same Exo RPA/RT-RPA assays and corresponding reference methods (Table 2). These samples were also tested using conventional RPA/RT-RPA assays to provide additional validation of the detection results. Together, the 15 virus-culture samples and 17 field samples constituted a 32-sample validation panel.

Table 2
Comparative evaluation of major sweetpotato viruses using Exo RPA/RT-RPA and standard PCR tests on 32 field samples

Virus	SPLCV		SPFMV		SPV2		SPVC		SPVG		SPCSV-EA		SPCSV-WA		ARF
Method	**qPCR**	**RPA**	**PCR**	**Exo RPA**	**PCR**	**Exo RPA**	**PCR**	**Exo RPA**	**PCR**	**Exo RPA**	**RPA**	**Exo RPA**	**RPA**	**Exo RPA**	**RPA**
Sweetpotato virus cultures from North Carolina															
SP1	+	+	–	–	–	–	–	–	+	+	–	–	+	+	+
SP2	+	+	+	+	–	–	–	–	+	+	–	–	–	–	+
SP3	+	+	–	–	–	–	–	–	+	+	–	–	+	+	+
SP4	+	+	–	–	–	–	–	–	+	+	–	–	+	+	+
SP5	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+
SP6	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+
SP7	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+
SP8	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
SP9	+	+	–	–	–	–	+	+	–	–	–	–	–	–	+
SP10	–	–	+	+	–	–	–	–	–	–	–	–	–	–	+
SP11	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+
SP12	–	–	+	+	–	–	–	–	–	–	–	–	–	–	+
SP13	–	–	–	–	–	–	+	+	–	–	–	–	–	–	+
SP14	+	+	–	–	–	–	+	+	+	+	–	–	–	–	+
SP15	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Field-collected sweetpotato samples in South Carolina															
V26-82	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
V26-83	–	–	–	–	+	+	–	–	–	–	–	–	–	–	+
V26-84	+	+	–	–	+	+	–	–	–	–	–	–	–	–	+
V26-85	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+
V26-86	+	+	–	–	+	+	–	–	–	–	–	–	–	–	+
V26-87	+	+	–	–	+	+	–	–	–	–	–	–	–	–	+
V26-88	+	+	–	–	+	+	+	+	–	–	–	–	–	–	+
V26-89	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+
V26-90	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+
V26-91	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+
V26-92	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
V26-93	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+
V26-94	+	+	–	–	+	+	–	–	–	–	–	–	–	–	+
V26-95	+	+	–	–	+	+	–	–	–	–	–	–	–	–	+
V26-96	+	+	+*	+	+	+	+*	+	+	+	–	–	–	–	+

Virus	SPLCV		SPFMV		SPV2		SPVC		SPVG		SPCSV-EA		SPCSV-WA		ARF
Method	**qPCR**	**RPA**	**PCR**	**Exo RPA**	**PCR**	**Exo RPA**	**PCR**	**Exo RPA**	**PCR**	**Exo RPA**	**RPA**	**Exo RPA**	**RPA**	**Exo RPA**	**RPA**
Sweetpotato virus cultures from North Carolina															
V26-97	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+
V26-98	+	+	+*	+	+	+	+*	+	+*	+	–	–	–	–	+

*Note: There were a total of 32 field samples, including 15 virus cultures from North Carolina and 17 field sweetpotato samples from South Carolina in 2026. In two field samples (V26-96 and V26-98), sensitivity of the conventional multiplex RT-PCR failed to detect several potyviruses (Supplementary Figure S2) which were positive when testing using singleplex RT-PCR for that virus or by newly designed Exo-RPA assays. Both field samples V26-96 and V26-98 contained four potyviruses in mixed infection, however the multiplex RT-PCR detected only two viruses (SPV2 and SPVG) in V26-96 and one virus (SPV2) in V26-98. The newly developed Exo RT-RPA detected all four potyviruses, which were confirmed using virus-specific singleplex RT-PCR, and conventional RT-RPA.

Discussion

Sweetpotato virus diseases exert a significant impact on global sweetpotato production, causing yield losses of approximately 20–30%, depending on cultivar susceptibility and the severity of mixed infections [3]. Because no chemical treatments are available to directly control plant viral infections, one of the most effective management strategies for vegetatively propagated crops such as sweetpotato is the use of virus-tested, pathogen-free planting materials [32]. Therefore, early and accurate detection of infected plants is critical for seed certification programs and for preventing the dissemination of viruses through propagation and trade.

Previous studies have employed multiplex RT-PCR assays for the simultaneous detection of major sweetpotato viruses, including SPFMV, SPCSV, SPVC, SPVG, and SPV2 [33, 34]. Although multiplex RT-PCR offers excellent sensitivity and specificity, it requires thermal cycling, skilled personnel, and laboratory infrastructure, factors that can limit its suitability for routine field diagnostics, particularly in resource-limited regions. RPA, first described by Piepenburg et al., [27] has emerged as a promising isothermal nucleic acid amplification technology that enables rapid pathogen detection at a low and constant temperature (approximately 37–42°C), generally within 20–30 min, thereby reducing dependence on conventional thermal cycling.

In Hungary, surveys revealed the presence of seven major sweetpotato viruses: SPCSV, SPFMV, SPV2, SPVC, SPVG, SPLCV, and sweet potato pakakuy virus (SPPV), with SPLCV reported for the first time [35]. The occurrence of mixed and asymptomatic infections further highlights the limitations of symptom-based diagnosis alone. Additionally, limited availability of certified, pathogen-free propagation material can contribute to virus accumulation and cultivar degeneration. Although ELISA and RT-PCR remain reliable laboratory-based techniques, they can be time-consuming and dependent on laboratory infrastructure [36]. In contrast, Exo RT-RPA provides a rapid and potentially field-deployable alternative for detecting RNA viruses within approximately 30 min [37]. When combined with virus-elimination and clean-seed programs, such rapid molecular approaches could contribute to early virus detection and sustainable sweetpotato production.

The results of the present study demonstrate the potential of seven independent singleplex Exo RPA/RT-RPA assays for rapid detection of major sweetpotato virus targets. An internal control using ARF was shown to be suitable for conventional RT-RPA to assess the quality and integrity of nucleic acid template in all 32 samples, including both purified total nucleic acid and crude-sap templates. These results support ARF as a conventional RT-RPA process-control candidate for identifying extraction failure or inhibition that could contribute to a false-negative result [38].

Analytical specificity testing showed target-specific amplification without detectable cross-reaction among the seven evaluated virus targets using natural samples infected with multiple viruses or spiked artificial plasmid control template for SPCSV-EA. SPCSV-EA/FAM and SPCSV-WA/ROX were differentiated in their respective singleplex reactions. The current results

demonstrate exclusivity within the evaluated virus panel rather than broad specificity against all sweetpotato-infecting viruses. Additional geographically diverse EA- and WA-positive isolates are needed because geographic strain names do not define their present distributions and sequence diversity of virus isolates may also affect inclusivity.

Creating an artificial plasmid with synthesized DNA consisting of a series of target virus sequences in a single molecule for positive control is a novel idea which has greatly improved the efficiency in developing the Exo RPA technology for detection of multiple sweetpotato viruses. This technology could be applied to include many other viruses where natural virus cultures are not readily available. This plasmid DNA can be easily stored for a long term or shared with other collaborating labs without concern of regulatory restriction in movement than that for exchanging living virus cultures.

Comparison of the Exo RPA/RT-RPA assays with the PCR-based reference methods using the SP1–SP15 virus culture sample set showed complete agreement across the 75 virus-by-sample comparisons. SP15 served as the healthy control and was negative for all five virus targets by both the reference methods and Exo RPA/RT-RPA. These results demonstrate concordance between the two detection approaches within the sample set evaluated. Broader validation using larger numbers of positive and negative samples from diverse geographical locations and infection backgrounds would be beneficial before definitive estimates of diagnostic sensitivity and specificity can be established.

The reproducibility observed in this study was further supported by the experimental replication design. Optimization, analytical sensitivity, and analytical specificity experiments were conducted using four technical replicate reactions per condition and were independently repeated on three separate days. For experiments involving biological material, two independent biological samples were evaluated, with each biological sample tested in four technical replicate reactions during each experimental run. This design provided an assessment of within-run consistency and day-to-day reproducibility. Nevertheless, these experiments should not be considered an inter-laboratory ring trial because testing was not conducted independently across multiple laboratories. Future inter-laboratory validation would be valuable for assessing assay reproducibility among different operators, instruments, and laboratory environments.

In this study, we developed and evaluated seven virus-specific singleplex Exo RPA/RT-RPA assays alongside established standard PCR reference methods. Multiplex RT-PCR was used only as the comparative method for SPFMV, SPV2, SPVC, and SPVG; real-time PCR was used for SPLCV; and strain-specific conventional RT-RPA were used for SPCSV-WA and SPCSV-EA. The ARF primer pair was validated separately by conventional RT-RPA only and was not evaluated by Exo RT-RPA. Formal diagnostic sensitivity, diagnostic specificity, confidence intervals, replicate-based RNA LoD95 values, and future development of an ARF Exo probe remain priorities for a larger blinded validation.

Compared with loop-mediated isothermal amplification (LAMP), RPA generally requires fewer primers per target, operates at a lower temperature, and can provide shorter reaction times and relatively simple assay optimization. Although LAMP has been widely adopted for plant-pathogen detection and specifically for the detection of sweetpotato viruses [24, 25], it typically requires multiple primers for each target [39]. RPA provides an alternative approach with rapid reaction kinetics and potential adaptability to field-deployable diagnostic workflows. Previous studies have successfully integrated RPA with lateral-flow strips for visual detection of *Tomato spotted wilt virus* [39] and demonstrated dual detection of *Sugarcane mosaic virus* and *Maize chlorotic mottle virus* [40]. The singleplex Exo RPA/RT-RPA assays developed here extend these applications by providing fluorescence-based detection of seven important sweetpotato viruses under a common reaction temperature.

The use of crude leaf extracts further provides a potential route toward simplified sample preparation for field-deployable applications. However, because a systematically paired comparison between crude extracts and purified nucleic acid was not conducted across the complete validation set, the present results do not establish equivalence between the two sample-preparation approaches. Potential effects of plant-derived inhibitors on analytical sensitivity and detection time therefore require further investigation. The conventional RT-RPA ARF assay provides an initial extraction and reaction-quality check, but a validated ARF Exo probe will be needed before ARF can function as a real-time Exo RT-RPA internal control.

The present singleplex Exo RPA/RT-RPA platform builds upon earlier efforts to simplify and decentralize plant-virus diagnosis. A one-step multiplex RT-PCR assay previously enabled the simultaneous detection of DNA and RNA viruses in cucurbit samples

but still required conventional thermal cycling and post-amplification gel electrophoresis.[41] Multiplex RT-RPA subsequently reduced the equipment and temperature-control requirements for detecting mixed virus infections [29], while multiplex RT-qPCR provided sensitive, probe-based detection from plant tissue and individual whiteflies [42]. More recently, an RPA-lateral-flow assay demonstrated the feasibility of visually detecting a plant virus directly at the sampling location [30]. Together, these studies demonstrate the progression toward rapid, portable, and field-deployable plant-virus diagnostics and provide a strong basis for the seven-target sweetpotato Exo RPA/RT-RPA platform developed in the present study.

In conclusion, this study developed seven independent singleplex Exo RPA/RT-RPA assays for the rapid detection of SPLCV, SPFMV, SPV2, SPVC, SPVG, SPCSV-WA, and SPCSV-EA. To our knowledge, this is the first report describing an Exo RPA/RT-RPA detection platform that encompasses these seven major sweetpotato virus targets, including the East African and West African strain groups of SPCSV. Among the 32 samples evaluated using the complete assay panel, five North Carolina ring-test samples tested positive for SPCSV-WA, whereas the remaining 27 samples were negative. SPCSV-EA was not detected in any of the 32 samples. The ARF endogenous internal control produced the expected amplicon by conventional RT-RPA in all 32 samples, including the 17 Charleston samples evaluated using both purified total nucleic acid and crude sap. The ARF primer pair therefore provided a useful conventional RT-RPA sample-validity control for assessing sample integrity, nucleic-acid recovery, potential false-negative results, and reaction stability.

The ability to complete viral detection within 20–30 min at 39°C using a portable fluorometer demonstrates the potential of this assay platform for rapid on-site diagnosis of sweetpotato viruses. Its simple isothermal workflow, short assay time, portable instrumentation, and straightforward fluorescence interpretation could make the technology useful for plant-diagnostic laboratories, extension agents, crop consultants, seed-production programs, and trained growers. Following appropriate training and further field validation, users with limited molecular-diagnostic experience may be able to perform the assay near production fields and rapidly identify infected planting material. Early on-site detection could support the timely removal of infected plants, reduce the distribution of virus-infected propagative material, and improve the production of virus-tested sweetpotato stocks. Nevertheless, broader validation using confirmed SPCSV-EA-positive samples, geographically diverse SPCSV isolates, RNA-based analytical standards, larger sample populations, and formal diagnostic-sensitivity and diagnostic-specificity analyses are required before the assays can be recommended for routine certification or regulatory testing. Future development and validation of an ARF Exo probe would further strengthen the platform by incorporating an endogenous internal control directly into the real-time fluorescence workflow.

Abbreviations

APC, artificial positive control; ARF, ADP-ribosylation factor; Exo RPA, exonuclease-probe recombinase polymerase amplification; Exo RT-RPA, exonuclease-probe reverse transcription recombinase polymerase amplification; NTC, no-template control; RPA, recombinase polymerase amplification; RT-RPA, reverse transcription recombinase polymerase amplification; SPCSV-EA, sweet potato chlorotic stunt virus East African strain group; SPCSV-WA, sweet potato chlorotic stunt virus West African strain group.

Declarations

Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Conflict of Interest

The authors declare no competing financial interest.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Figures

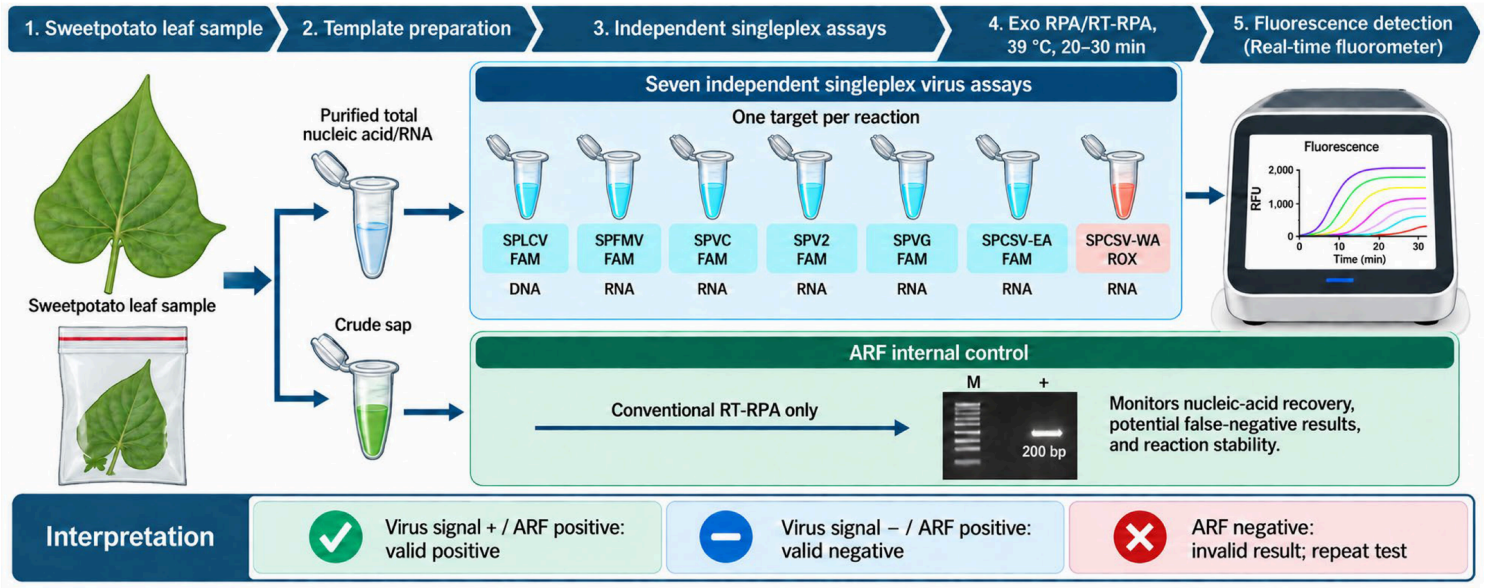


Figure 1

Schematic workflow for singleplex Exo RPA/RT-RPA detection of six major sweetpotato viruses. Purified total nucleic acid, RNA, or crude sap is prepared from sweetpotato leaf tissue and tested in seven independent singleplex reactions targeting sweet potato leaf curl virus (SPLCV), sweet potato feathery mottle virus (SPFMV), sweet potato virus C (SPVC), sweet potato virus 2 (SPV2), sweet potato virus G (SPVG), and the East African (EA) and West African (WA) strains of sweet potato chlorotic stunt virus (SPCSV). SPLCV, SPFMV, SPVC, SPV2, SPVG, and SPCSV-EA are detected in the FAM channel, whereas SPCSV-WA is detected in the ROX channel. Reactions are incubated at 39 °C for 20–30 min and monitored using a portable real-time fluorometer. The approximately 200-bp ARF product is evaluated by conventional RT-RPA and serves as an internal control for nucleic-acid recovery, false-negative monitoring, and reaction stability. A virus-negative result is considered valid only when the ARF control is positive.

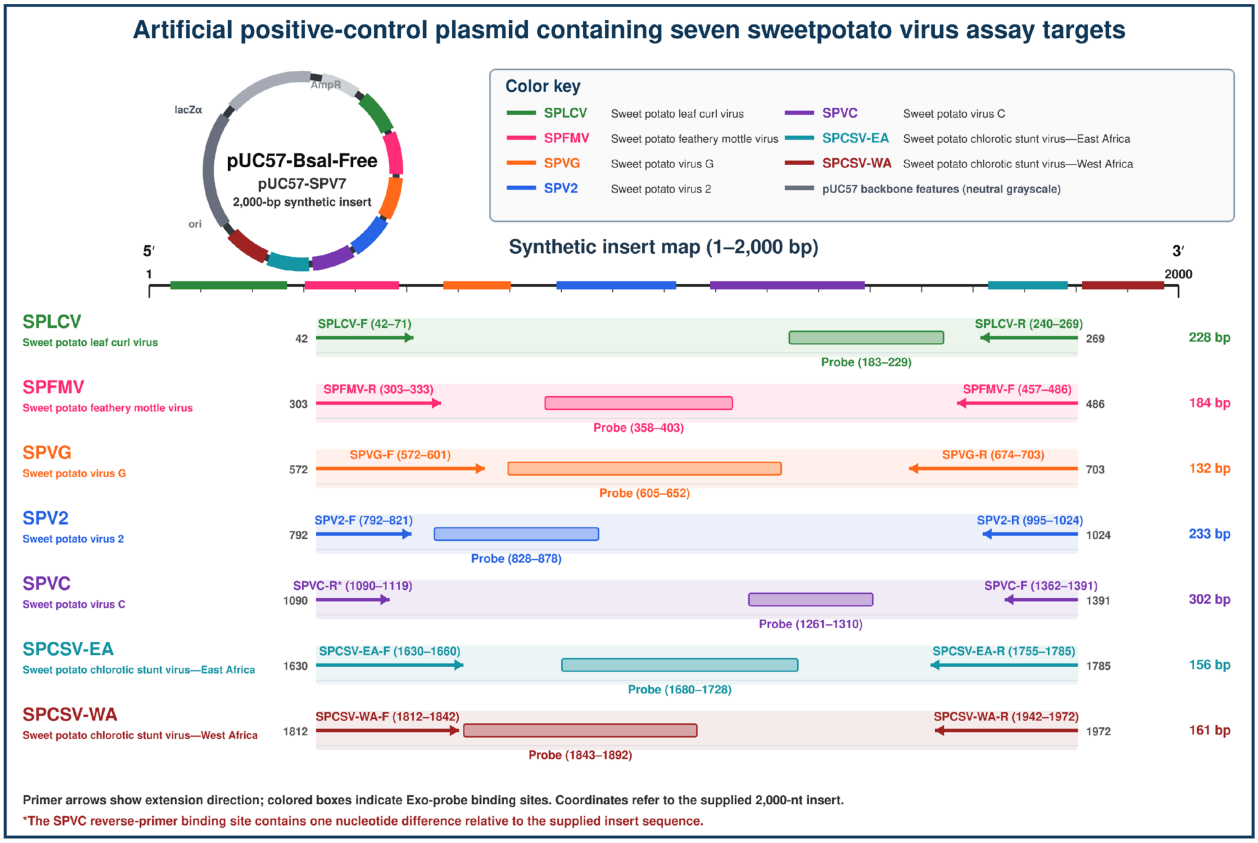


Figure 2

Schematic organization of the artificial positive-control construct and locations of the seven virus-specific RPA primer and Exo-probe sets. Target regions from SPLCV, SPFMV, SPVG, SPV2, SPVC, SPCSV-EA, and SPCSV-WA were assembled in a pUC57 plasmid. The linear insert map shows the positions and orientations of each forward primer, Exo probe, and reverse primer; the accompanying table lists the oligonucleotide sequences and expected amplicon sizes. Probe symbols identify the reporter-labeled thymidine, tetrahydrofuran abasic-site mimic (THF), quencher-labeled thymidine, and 3' C3 spacer.

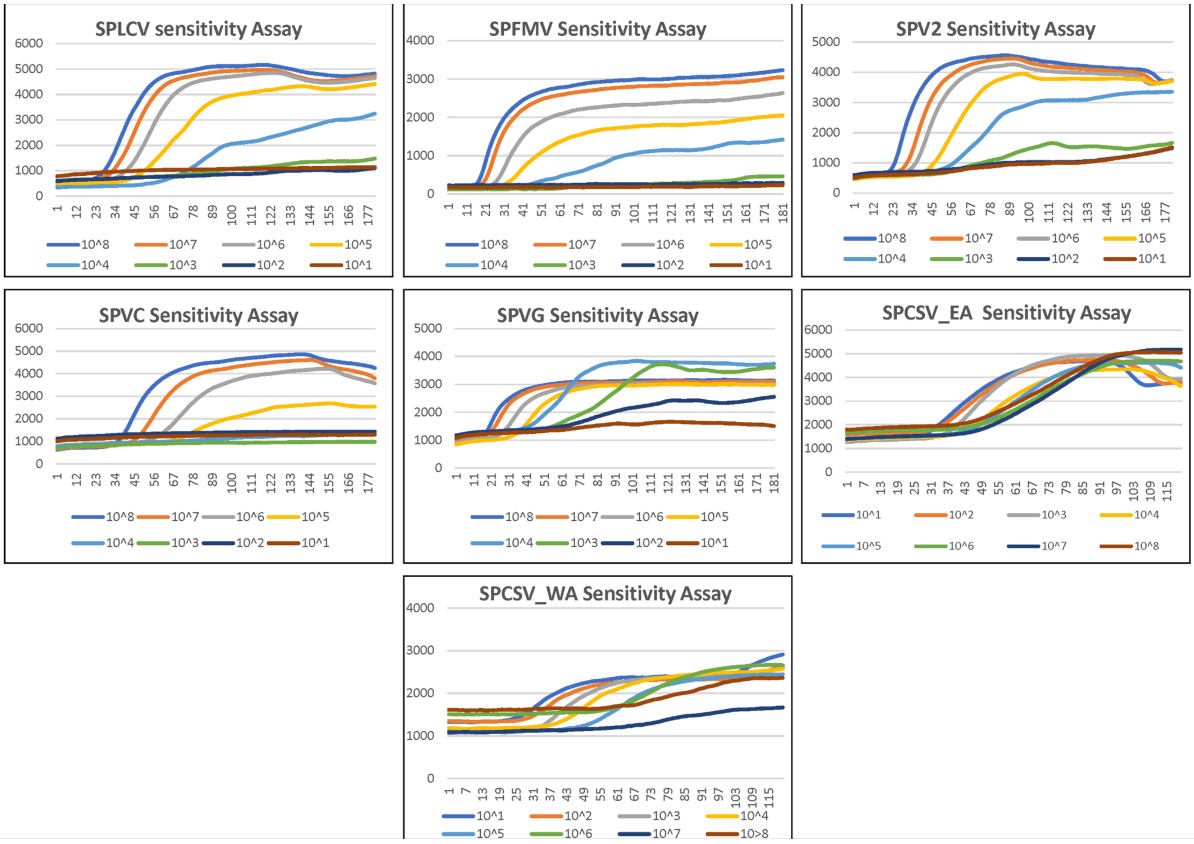


Figure 3

Analytical sensitivity of the seven singleplex Exo RPA/RT-RPA assays for major sweetpotato viruses. Real-time fluorescence amplification profiles are shown for SPLCV, SPFMV, SPV2, SPVC, SPVG, SPCSV-EA, and SPCSV-WA using tenfold serial dilutions of the corresponding artificial positive-control templates. SPLCV, SPFMV, SPV2, SPVC, SPVG, and SPCSV-EA were monitored in the FAM channel, whereas SPCSV-WA was monitored in the ROX channel. Fluorescence was recorded during incubation at 39 °C; template concentrations are identified in the individual panel legends.

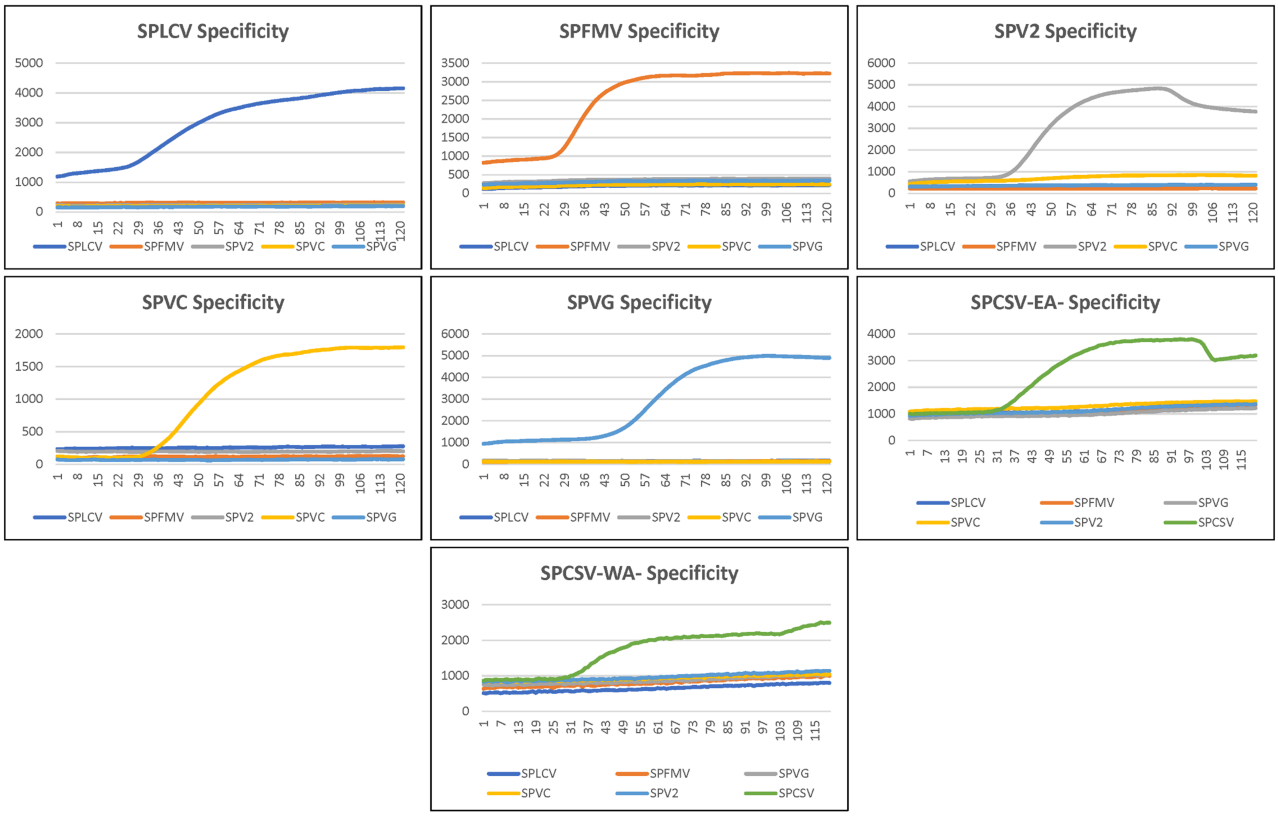


Figure 4

Analytical specificity of seven singleplex Exo RPA/RT-RPA assays. Each assay was challenged with target and non-target sweetpotato virus templates. Target-dependent fluorescence was observed for SPLCV, SPFMV, SPV2, SPVC, SPVG, SPCSV-EA, and SPCSV-WA, with no substantial amplification from the non-target templates included in the respective panels. The results demonstrate analytical exclusivity within the tested seven-virus panel.

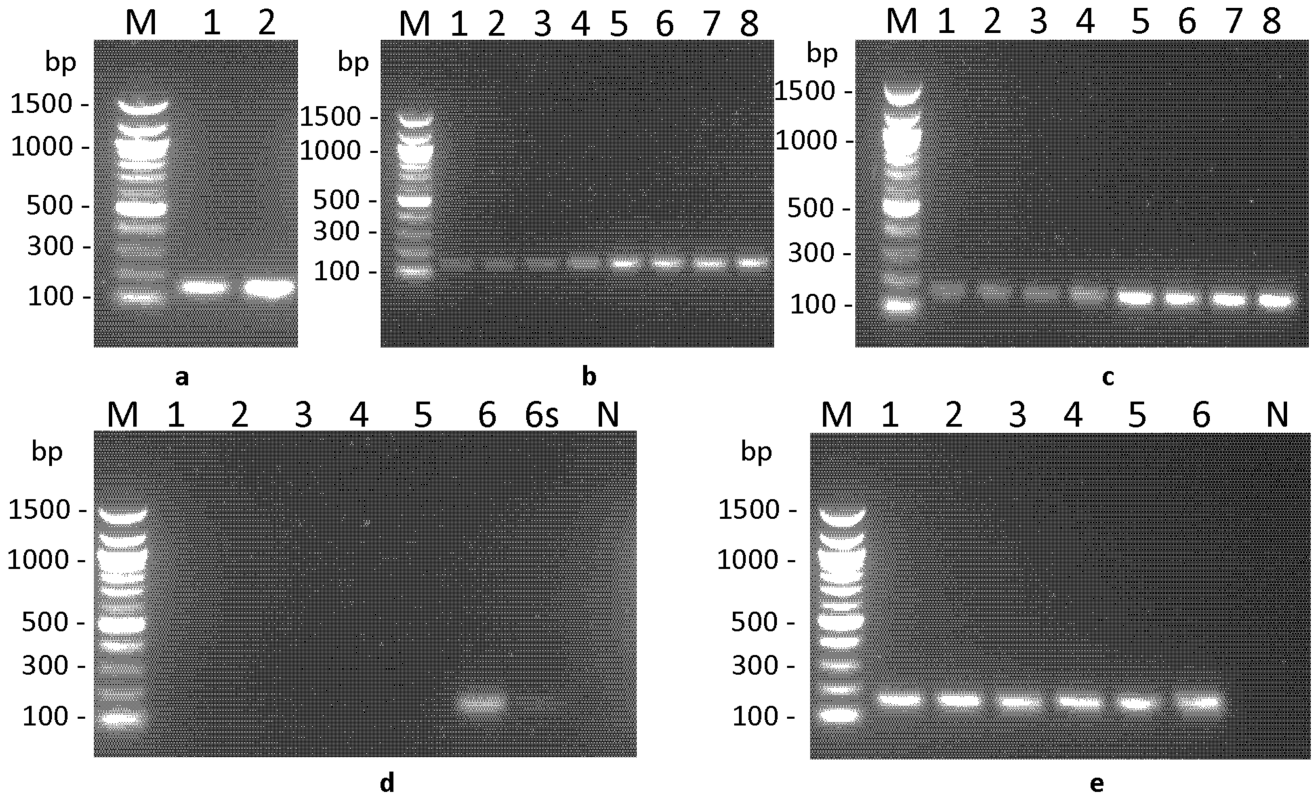


Figure 5

Conventional RT-RPA validation of the SPCSV-EA and SPCSV-WA assays using total nucleic acid from SPCSV-WA-infected sweetpotato samples and artificial positive-control (APC) plasmid DNA as templates. (a) Primer validation for SPCSV-EA using APC plasmid DNA and for SPCSV-WA using total nucleic acid from an infected sweetpotato sample. (b) Temperature optimization of the SPCSV-EA assay at 35–42 °C, with lanes 1–8 corresponding to 1 °C increments. (c) Temperature optimization of the SPCSV-WA assay at 35–42 °C, with lanes 1–8 corresponding to 1 °C increments. (d) Testing of SPCSV-EA: lane 6, positive control; lane 6s, positive-control sample spillage; N, no-template negative control. (e) Testing of SPCSV-WA using APC plasmid DNA as the template. M, DNA molecular-size marker; numbered lanes, tested samples.

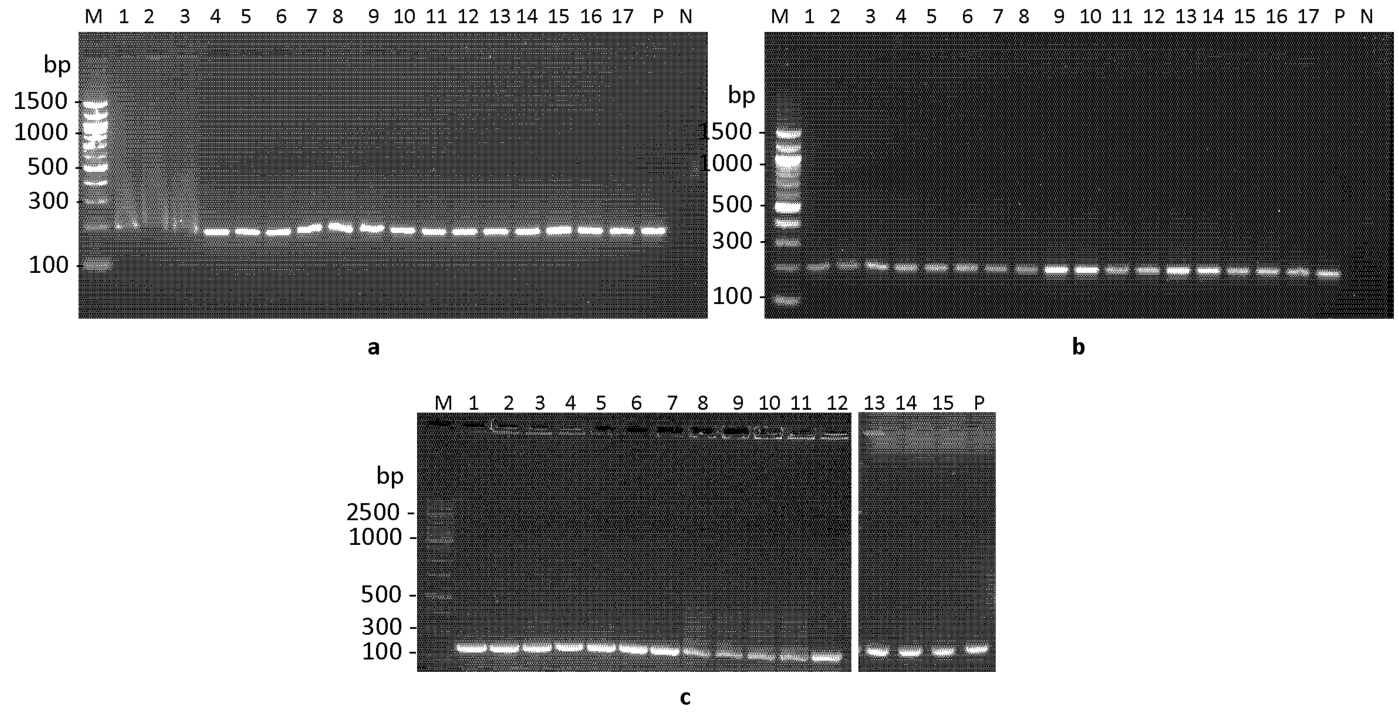


Figure 6

RT-RPA validation of the ARF internal control. The ADP-ribosylation factor (ARF) internal control was tested using (a) extracted total nucleic acid from 17 sweetpotato samples collected in Charleston, South Carolina; (b) crude extract from the same 17 Charleston samples; and (c) extracted total nucleic acid from 15 North Carolina virus culture samples. The expected 200-bp ARF product was detected by conventional RT-RPA. M, DNA molecular-size marker; P, positive control; N, no-template negative control.

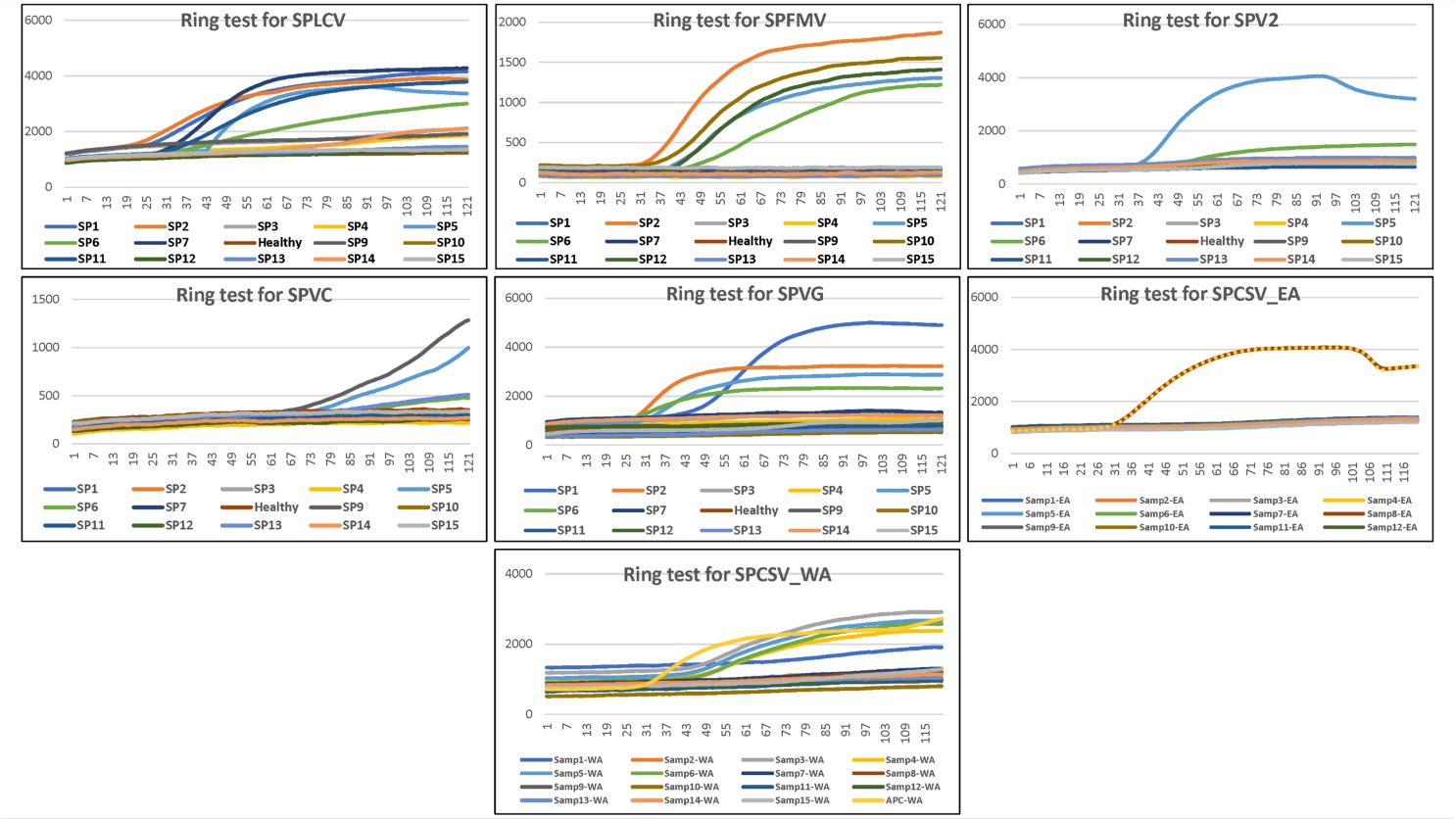


Figure 7

Comparative detection of seven sweetpotato viruses in the 15 virus culture panel using singleplex Exo RPA/RT-RPA. Real-time fluorescence profiles are shown separately for SPLCV, SPFMV, SPV2, SPVC, SPVG, SPCSV-EA, and SPCSV-WA. Samples SP1–SP15 were tested individually, together with the controls identified in the panel legends. Each virus was evaluated in an independent singleplex reaction; SPCSV-WA was detected on the ROX channel, and the other six targets were detected on the FAM channel.

Supplementary Files

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- FinalSPRPAMSSupportingInformation11.docx