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ORIGINAL ARTICLE

A rapid and sensitive RPA-Cas12a assay for detection of banana bunchy top virus (*Babuvirus musae*)

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Abstract

Banana bunchy top virus (BBTV, *Babuvirus musae*) is one of the most damaging viral threats to banana production, yet frontline diagnostics rely on PCR, ELISA or proprietary assays that are poorly suited to low resource deployment or associated with high per test cost. We developed a recombinase polymerase amplification (RPA)-Cas12a assay that achieves high sensitivity using crude extracts with fluorescence or lateral flow readouts. Using RPA primers to BBTV *DNA-R* gene and a Cas12a crRNA targeting a conserved site, we developed the first CRISPR-based diagnostic assay for BBTV. The assay was configured in a one-tube workflow in which the RPA and Cas12a components are separated until amplification is complete, reducing contamination risk. With a *DNA-R* gBlock standard, the one-tube assay achieved a limit of detection of 5.11 aM (~3 copies/µl) in both formats, which is around 250-fold more sensitive than a commercially available fluorescence RPA and about fourfold more sensitive than an available RPA-based lateral flow. The RPA-Cas12a assay reliably detected BBTV in both fresh and dried leaf material, across isolates from both genetic subgroups, and showed no cross-reactivity with common banana viruses. A two-step high sensitivity workflow further reduced the limit of detection to near single copy levels. This work establishes a generic RPA-Cas12a framework for plant virus diagnostics and an immediately deployable assay for BBTV surveillance, eradication and clean planting material programmes.

Keywords CRISPR · Cas12a · RPA · Banana bunchy top virus · Plant virus diagnostics · Field-deployable

Introduction

Banana (*Musa* spp.) is both a staple food and a significant commercial crop and is a vital source of income for millions of smallholder farmers throughout the tropics and subtropics (Kumar et al. 2015; Scott 2021). Banana bunchy top virus (BBTV, *Babuvirus musae*) is one of the most destructive pathogens of bananas and causes severe stunting, rosetting, upcurling and marginal chlorosis of leaves. These symptoms often occur in conjunction with discontinuous dark green streaking on the leaf lamina, midrib and petiole. Infection ultimately results in failure of marketable fruit production (Kumar et al. 2015; Rahayuniati et al. 2021; Thomas 2019). Effective management of BBTV relies on early detection and removal of infected plants, strict movement controls and clean planting material schemes. These measures, in turn, are strengthened by rapid, sensitive and specific diagnostics that can be deployed close to the point of sample collection (Omondi et al. 2020; Kumar et al. 2015; Thomas 2019).

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BBTV is a multipartite circular single-stranded DNA (ssDNA) virus in the family Nanoviridae and is transmitted in a persistent, circulative manner by the banana aphid, *Pentalonia nigronervosa* (Thomas et al. 2021), and the closely related cardamom aphid, *Pentalonia caladii* (Greenwell 2012). The origin of BBTV is thought to be Southeast Asia (Thomas 2019) but BBTV is now also established across large parts of the Pacific region, the Indian subcontinent, Australia and parts of Africa (Stainton et al. 2015). Once introduced to an area, the virus can spread rapidly within and between plantations. In heavily affected areas, BBTV can cause near total yield loss, necessitating destruction of infected mats, replanting and long-term movement restrictions on planting material. These impacts fall disproportionately on smallholder producers, undermining household income, local employment and regional food security (Retkute & Gilligan 2025; Cook et al. 2012).

Current frontline laboratory diagnostics for BBTV are based largely on PCR, ELISA and commercial recombinase polymerase amplification (RPA) kits (Babu et al. 2018; Chen & Hu 2013; Geering & Thomas 1996; Kapoor et al. 2017; Thomas & Dietzgen 1991). These assays can provide high sensitivity and specificity, but they require either laboratory infrastructure, specialist equipment, longer timeframes or refrigerated supply chains that can limit their uptake in resource-limited settings. Commercial RPA kits that couple isothermal amplification with fluorescent or lateral flow readouts have expanded the possibilities for field diagnostics, but they still rely on proprietary chemistry and target-specific probes and are associated with significant cost (Babu et al. 2018; Mota et al. 2022; Zhang et al. 2024).

Clustered regularly interspaced short palindromic repeats (CRISPR)–based diagnostics provide an opportunity to address some of these limitations (Aman et al. 2020a, 2020b; Mota et al. 2022). LbCas12a from Lachnospiraceae bacterium ND2006 (referred to here as Cas12a) exhibits target-activated indiscriminate cleavage of single-stranded DNA (ssDNA), enabling highly sensitive detection when combined with an upstream amplification step and a generic ssDNA reporter (Broughton et al. 2020; Chen et al. 2018). Upon binding and recognising its specific double-stranded DNA target via a CRISPR RNA (crRNA), the Cas12a ribonucleoprotein (RNP) is activated and cleaves nearby reporter ssDNA molecules, generating either a fluorescence signal or a visible band on a lateral flow strip (Razavi et al. 2026; Chen et al. 2018).

In the fluorescence format, cleavage of a FAM-quencher ssDNA reporter produces an increase in fluorescence that can be monitored in real time on a fluorometer for a semi-quantitative readout or visualised at endpoint with a blue-light torch in very low-resource settings (Ding et al. 2020; Broughton et al. 2020; Chen et al. 2018). In the lateral flow format, cleavage of a dual-labelled FAM-biotin ssDNA reporter alters its capture on a Milenia HybriDetect dipstick, producing an interpretable positive or negative banding pattern (Marqués et al. 2022; Bai et al. 2019; Milenia Biotec GmbH 2024; Broughton et al. 2020).

This modular architecture allows the same core Cas12a detection chemistry to be reused across different assays by changing only the crRNA sequence, while the use of generic Cas12a enzyme and ssDNA reporters simplifies assay development, manufacture and logistics (Aman et al. 2020b; Broughton et al. 2020; Chen et al. 2018).

As Cas12a detection assays often rely on an upstream amplification step that typically have different optimal conditions from the detection step, fully mixed 'one-pot' reactions containing both amplification and Cas12a detection reagents can underperform due to suboptimal buffering and premature Cas12a activity that cleaves template, amplicon or primers before amplification is complete (Zhao et al. 2023). Many assays therefore adopt a closed-tube, sequential 'one-tube' format in which amplification and detection are physically separated then combined after amplification, avoiding post-amplification tube opening and the associated contamination risk that would arise if Cas12a was manually added to the amplified product (Wei et al. 2025; Li et al. 2022). Separation can be achieved with viscous or hydrophobic barriers (for example glycerol or mineral oil), meltable wax partitions or spatial compartmentalisation within the vessel (Wang et al. 2025; Chen et al. 2020; Liu et al. 2024, 2025).

The simplest and most common approach is the cap-droplet configuration: Amplification (for example RPA or LAMP) proceeds in the tube body while a small droplet containing Cas12a, crRNA, reporter and detection buffer is held in the cap by surface tension, and then, centrifugation or flicking merges the reactions to initiate Cas12a detection (Li et al. 2022; Yang et al. 2025; Bi et al. 2025). This staged design minimises handling and contamination risk while preserving performance by allowing amplification to complete before nuclease activation.

Plant virus diagnostics using Cas12a are emerging, but there remains a need for well characterised assays that have been benchmarked against existing commercial tests, validated on diverse global isolates and field-tested on naturally infected plants (Jaybhaye et al. 2024). For BBTV in particular, an ideal assay would combine the speed and portability of RPA with the sensitivity and flexibility of Cas12a, operate with crude extracts and provide both fluorescence and lateral flow readouts suitable for field and laboratory applications.

Here we report the development and characterisation of an RPA-Cas12a assay for BBTV targeting the conserved *DNA-R* gene, which encodes the replication-associated protein. Existing RPA primers were leveraged to streamline development and ensure compatibility with established amplification chemistry, and a Cas12a crRNA was designed in a conserved region identified from alignment of 54 BBTV

DNA-R sequences spanning the major global diversity of BBTV. We configured the assay in a one-tube, low-resource format suitable for field deployment and compared its analytical sensitivity with commercial kits. We further assessed performance on dried and fresh banana leaf material, evaluated specificity against a panel of common banana viruses and established a two-step, high-sensitivity workflow for laboratory use that achieves a sub-attomolar limit of detection. Together, these data demonstrate that RPA-Cas12a can provide a rapid, sensitive and flexible platform for BBTV diagnostics with clear potential for integration into plant health surveillance and certification programmes.

Material and methods

Sample collection and preparation

A BBTV-free healthy banana plant was maintained under containment conditions at Elizabeth Macarthur Agricultural Institute, Menangle, NSW. Fresh BBTV-infected samples were collected from Nambour, Queensland, Australia. BBTV isolates from overseas were imported under permit as dried leaf cultures and analysed in a certified laboratory in Brisbane, Queensland. Dried leaf samples of other banana viruses were sourced from the Queensland DPI Virus Isolate Collection. Dried and fresh samples were processed as outlined in Fig. S1. Extracts were stored at 4 °C for up to a week and at − 80 °C for long-term storage.

Serial dilution and controls

For RPA-Cas12a testing, healthy banana leaf tissue underwent crude extraction by the method outlined in Fig. S1. The supernatant from the healthy banana tissue was used as both a healthy banana control (HP) and as diluent to generate a 1:4 serial dilution series of BBTV_*DNA-R*_gBlock. See Table S1 for dilution series concentrations.

For commercial RPA kit testing with AmplifyRP XRT for BBTV fluorescence kit (Agdia; cat. no. XCS 24700/0008) and AmplifyRP Acceler8 for BBTV lateral flow kit (Agdia; cat. no. ACS 24700/0008), diluent was prepared according to the manufacturer's instructions by grinding 60 mg uninfected banana leaf tissue in 600 µl AMP1 extraction buffer in an extraction bag. This extract was used to generate an equivalent 1:4 serial dilution series of BBTV_*DNA-R*_gBlock.

Commercial test kits

AmplifyRP XRT BBTV fluorescence and AmplifyRP Acceler8 BBTV lateral flow assays were performed according to the manufacturer's instructions (Agdia Inc. 2020, Agdia Inc. 2022). For the AmplifyRP XRT kit, this included a vortex and spin at 4 min into incubation, which is the cause of the sudden drop in fluorescence at the 4 min timepoint during monitoring. AmplifyRP XRT reactions were run on a Genie HT isothermal real-time fluorometer (OptiGene).

Assay design

All nucleic acid sequences and their supplier are listed in Table S2.

The amplification step was based on previously published RPA primers targeting the *DNA-R* gene of BBTV to maximise compatibility with existing diagnostic workflows and streamline assay development (Kapoor et al. 2017). Primers BBTV_F2 and BBTV_R2 amplify a 163 bp region of BBTV DNA-R. To assess conservation of this target region, 54 *DNA-R* sequences were downloaded from NCBI, excluding sequences submitted or published before 1993. Accession numbers are listed in Fig. S2. Sequences were aligned using MAFFT v7.490 with default parameters (Katoh and Standley 2013). The alignment confirmed that the RPA target region was well conserved across Pacific Indian Ocean (PIO) and South-East Asian (SEA) BBTV genetic subgroups (Fig. S2) (Yu et al. 2012; Chakraborty et al. 2023). Nucleotide ambiguities were localised and highlighted in the schematic alignment in Fig. 1a.

A BBTV-specific LbCas12a crRNA (BBTV_crRNA) was designed using the CRISPOR online tool (Haeussler et al. 2016), with PAM settings TTT(A/C/G)−21 bp-Cas12a (Cpf1) and off-target analysis against the banana genome (ASM31385v2). The crRNA was selected within a fully conserved region of the aligned BBTV *DNA-R* sequences (Fig. S2), adjacent to a canonical Cas12a PAM (5′-TTTA), with no predicted off-target mismatches in the banana genome. A 263 bp synthetic fragment (BBTV_*DNA-R*_gBlock), extending 50 bp beyond each end of the RPA amplicon, was used as a quantified standard for sample preparation optimisation and limit-of-detection experiments. The BBTV_DNA-R_gBlock was quantified using the Qubit 1X dsDNA High Sensitivity Kit (Thermo Fisher Scientific, cat. no. Q33231).

A one-tube RPA-Cas12a workflow was used to reduce the risk of post-amplification contamination and improve suitability for low-resource settings (Fig. 1b). Detailed reagent compositions and protocols are provided in "RPA-Cas12a reagents" to "One-tube RPA-Cas12a lateral flow assay". Briefly, the RPA master mix and sample extract were combined at the bottom of the reaction tube, while the pre-assembled Cas12a RNP and ssDNA reporter were held separately as a droplet on the inner surface of the tube cap by surface tension. The reaction was incubated to allow isothermal amplification and then briefly flicked or centrifuged to introduce the Cas12a RNP and reporter into the amplified product. Cas12a-mediated detection then proceeded

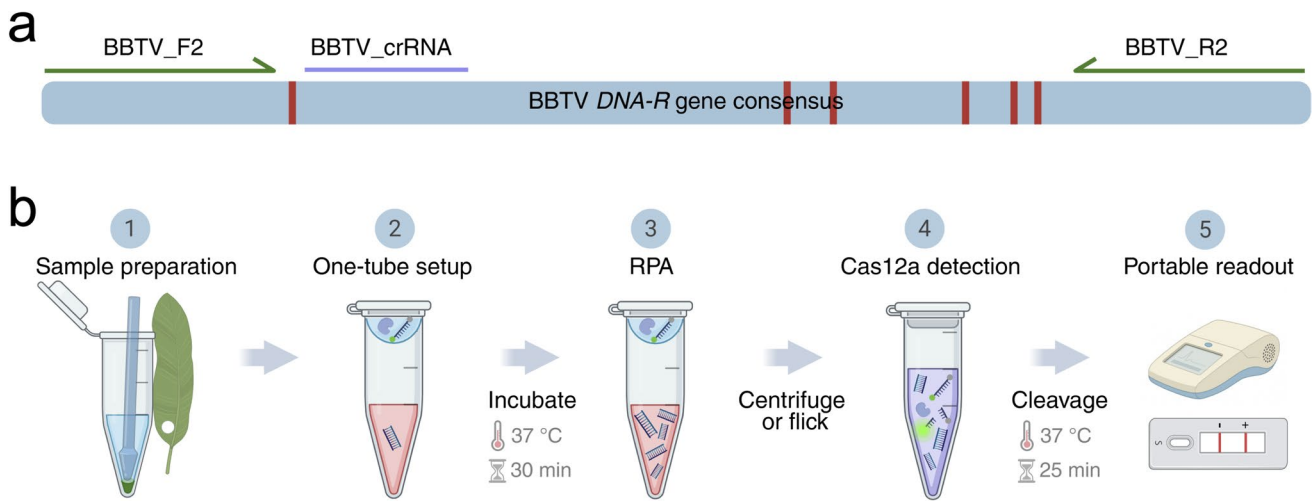

Fig. 1 RPA Cas12a reagent design and one-tube low resource workflow. **a** Published RPA primers from Kapoor et al. (2017) were used to define the BBTV target region. Fifty-four *DNA-R* sequences retrieved from NCBI were aligned (Fig. S2); nucleotide ambiguities in the alignment are highlighted in red and a crRNA was designed within a conserved region. **b** Schematic of the one-tube RPA-Cas12a workflow. (1) Crude sample extract was added to (2) an RPA reaction mix at the bottom of the tube, while Cas12a RNP and reporter were held in a droplet in the lid. (3) RPA was performed for 30 min at 37 °C to amplify target DNA; after which, the droplet was centrifuged or the tube was flicked in the absence of a centrifuge to combine it with the RPA reaction. (4) Cas12a targeted and collateral cleavage occurred at 37 °C for 25 min. (5) Detection was read by fluorometer or on a lateral flow strip. Created with BioRender.com

without opening the tube. Fluorescence was monitored in real time using a Genie III portable isothermal fluorometer (OptiGene), while a biotin-labelled reporter was used for lateral flow detection.

Fluorescence and lateral flow reporters were based on the 5′-TTATTATT sequence described by Broughton et al. (2020). All fluorescence assays were performed in triplicate, whereas lateral flow assays were performed as single measurements.

RPA-Cas12a reagents

RPA was performed with TwistAmp Basic kit (TwistDx, cat. no. TABASO3KIT) with each pellet rehydrated in 29.5 µl rehydration buffer, vortexed and pulse centrifuged. All reactions were prepared in Genie 8-well strip tubes (OptiGene). For each 20 µl reaction, 12 µl of RPA master mix was prepared on ice containing 7 µl rehydrated RPA reagent, 1 µl BBTV_F2 (10 µM), 1 µl BBTV_R2 (10 µM), 2 µl nuclease-free water and 1 µl MgOAc (280 mM).

Cas12a detection used pre-assembled mixes for fluorescence (Cas_FL mix) or lateral flow (Cas_LFA mix). Each 4 µl Cas_FL mix aliquot contained 0.3 µl LbCas12a (12 µM, New England Biolabs, cat. no. M0653T), 0.24 µl BBTV-specific crRNA (15 µM), 2 µl 10 × NEBuffer r2.1 (New England Biolabs, cat. no. B6002S), 0.75 µl FAM ssDNA reporter (40 µM) and 0.71 µl nuclease-free water. Each 4 µl Cas_LFA aliquot contained 0.3 µl LbCas12a (12 µM), 0.24 µl BBTV-specific crRNA (15 µM), 2 µl 10 × NEBuffer r2.1, 0.25 µl FAM-labelled lateral flow reporter (40 µM) and 1.21 µl nuclease-free water. Cas mixes were prepared as master mixes on ice and dispensed as 4 µl aliquots.

One-tube RPA-Cas12a fluorescence assay

For each reaction, 12 µl RPA master mix and 4 µl template (gBlock standard, plant extract or DNA/RNA Shield Direct-Detect as NTC) were combined in the tube base. A 4 µl Cas_FL mix aliquot was pipetted onto the inner lid surface of the reaction tube without contacting the RPA mix, and tubes were closed without vortexing or spinning to keep the phases separated.

Tubes were incubated for 30 min in a Genie III or Genie HT isothermal real-time fluorometer (OptiGene), with the tube base held at 37 °C and the lid held at 42 °C (lid temperature is set by default 5 °C higher than the base). Tubes were then briefly pulse-centrifuged at 2000 × *g*, where available, or flicked in the absence of a centrifuge to combine the Cas_FL mix in the cap with the RPA product, and immediately returned to the fluorometer at 37 °C. FAM fluorescence was recorded for 25 min; fluorescence trajectories over the 25 min after Cas_FL mix addition were used for analysis.

One-tube RPA-Cas12a lateral flow assay

The one-tube lateral flow format used the same assembly, with Cas_LFA mix instead of Cas_FL mix. For each reaction, 12 µl RPA master mix and 4 µl template were placed in the tube base and 4 µl Cas_LFA mix was placed in the lid. Tubes were incubated at 37 °C for 30 min and then briefly

pulse-centrifuged at 2000 × *g*, where available, or flicked in the absence of a centrifuge to mix the phases and returned to 37 °C for a further 25 min.

After incubation, 40 µl 1 × NEBuffer r2.1 was added to each tube and mixed thoroughly. A HybriDetect universal lateral flow strip (Milenia Biotec, cat. no. MGHD 1) was inserted into the reaction and allowed to develop at room temperature for up to 10 min. Reactions were scored positive when a distinct positive (+) band appeared, either with or without the negative (−) band, within the development period.

Two-step high-sensitivity RPA-Cas12a fluorescence assay

The high-sensitivity format used the same RPA master mix and Cas_FL mix formulations but with separate amplification and detection steps. For each reaction, 12 µl RPA master mix and 4 µl template were combined, mixed and pulse centrifuged at 2000 × *g* and then incubated at 37 °C for 30 min. Tubes were briefly vortexed and pulse centrifuged at 4 and 8 min into incubation to enhance mixing.

At 30 min, tubes were pulse centrifuged at 2000 × *g* and opened in a clean area, and 4 µl Cas_FL mix was added directly to each 16 µl RPA reaction. Tubes were vortexed, pulse centrifuged and transferred to the fluorometer at 37 °C. FAM fluorescence was recorded for 25 min in a Genie III or HT fluorometer at 37 °C, but again tubes were briefly vortexed and pulse centrifuged at 4 and 8 min of the incubation to enhance mixing of amplicons with the Cas_FL mix. Endpoint values at 25 min after Cas_FL mix addition were used to determine reaction positivity and limit of detection.

Data analysis

Fluorescence data were analysed and visualised in GraphPad Prism (v10.4.1, Dotmatics). For RPA-Cas12a assays run on the Genie III or HT fluorometer, a conservative positivity threshold of 100,000 arbitrary fluorescence units was defined as approximately twice the mean no template control (NTC) signal (51,646). This threshold exceeds the commonly used mean plus 3 standard deviation criterion (which would equate to 59,074 arbitrary fluorescence units) and was selected to minimise the risk of false-positive detections.

Results

Crude DNA extraction optimisation

To identify a crude extraction method that was compatible with RPA-Cas12a, we compared five solutions for preparation of banana leaf extracts: DNA/RNA Shield DirectDetect (Zymo Research), DNA Extract All Reagents Kit (Thermo Fisher Scientific), Tris–EDTA (TE) buffer, water and PBS. DNA/RNA Shield DirectDetect consistently produced the highest fluorescence signal and lowest variability in the RPA-Cas12a assay (Fig. S3) and was therefore used in the sample preparation workflow (Fig. S1).

Analytical sensitivity and comparison to commercial RPA kits

We evaluated the analytical sensitivity of the RPA-Cas12a assay using a 1:4 serial dilution series of the BBTV_*DNA-R*_gBlock spanning the femtomolar to sub-attomolar range, tested in both fluorescence and lateral flow formats. A conversion table for molarity, mass and copy number for this dilution series is provided in Table S1.

In the one-tube configuration, the RPA-Cas12a assay consistently detected the target down to 5.11 aM (~ 3 copies/µl) across three independent replicates in the fluorescence format (Fig. 2a) and at the same concentration in a single measurement using the lateral flow format (Fig. 2b). At 5.11 aM, fluorescence trajectories showed clear separation from the no template control (NTC), with mean endpoint signals at 25 min being 8.8-fold higher than the NTC. Based on these findings, we set a positivity threshold of 100,000 fluorescence units for a Genie isothermal real-time fluorometer, corresponding to approximately twice the mean endpoint signal of the NTC. Below 5.11 aM, fluorescence curves converged with the NTC and lateral flow strips produced only faint subjectively positive bands, indicating loss of reliable detection at lower template levels.

To contextualise this performance, we benchmarked the assay against two commercial BBTV RPA kits that target the same BBTV *DNA-R* region and are likely derived from the same published BBTV RPA primer set by Kapoor et al. (2017). We therefore used the same BBTV_*DNA-R*_gBlock standard, preparing samples according to the manufacturers' instructions and spiking to create the same dilution series. The AmplifyRP XRT fluorescence kit (Agdia) reliably detected down to 1.31 fM (~ 787 copies/µl, Fig. 2c) for all three replicates; below this concentration, amplification curves overlapped with the NTC and positives could not be called with confidence. The AmplifyRP Acceler8 lateral flow kit (Agdia) detected down to 20.4 aM (~ 12 copies/µl, Fig. 2d), with test band visible at this concentration but absent in lower dilutions. In these side-by-side experiments, the one-tube RPA-Cas12a fluorescence assay therefore had an approximately 250-fold lower limit of detection than the AmplifyRP XRT fluorescence assay, and the one-tube lateral flow assay was about fourfold more sensitive than the AmplifyRP Acceler8 lateral flow assay.

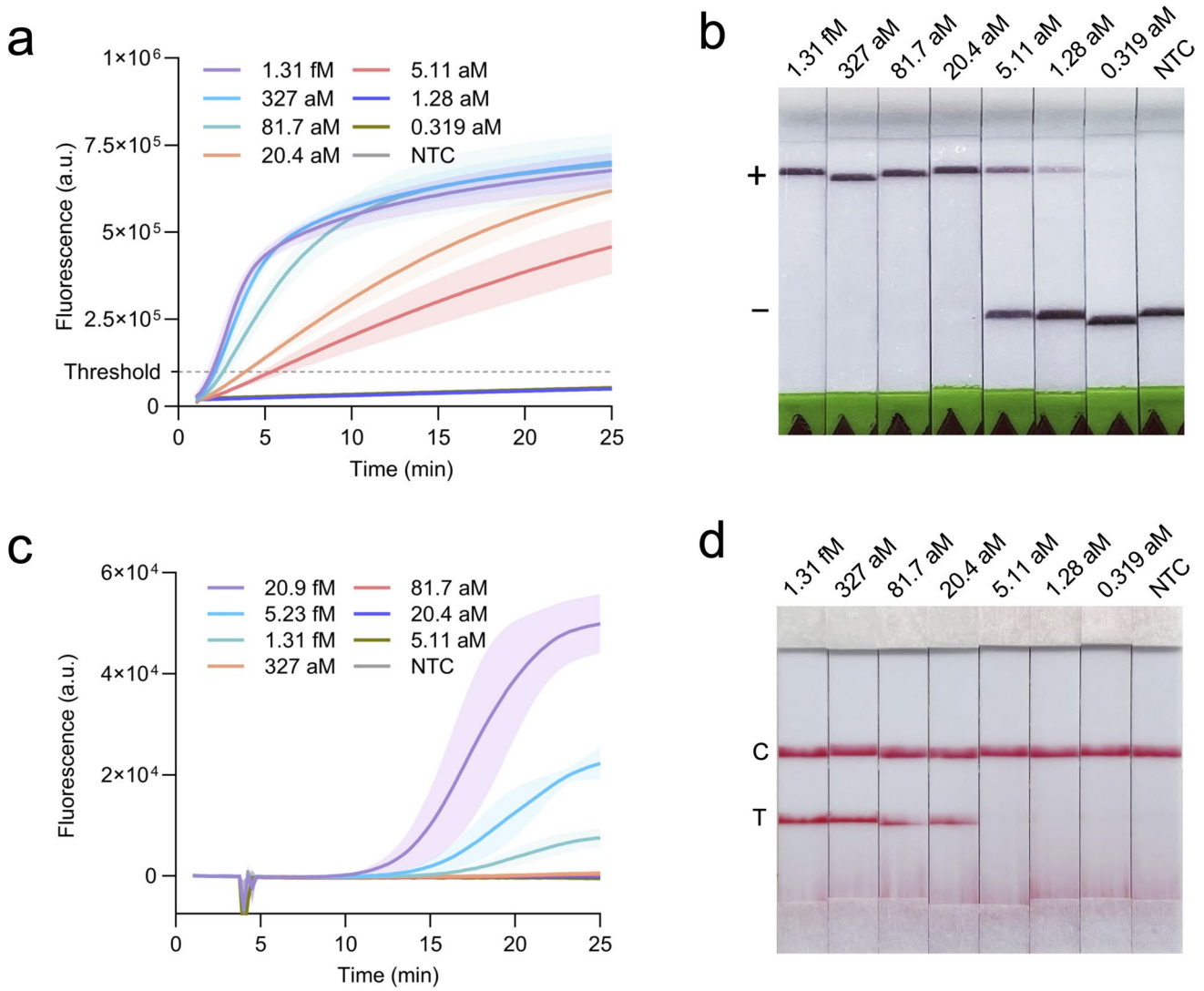

Fig. 2 Limit of detection for RPA-based fluorescence and lateral flow assays for BBTV_*DNA-R*_gBlock. **a**, **b** One-tube RPA-Cas12a assays with reliable detection down to 5.11 aM (~3 copies/µl) in fluorescence and lateral flow formats. **c** AmplifyRP XRT BBTV fluorescence kit (Agdia) with detection down to 1.31 fM (~787 copies/µl). **d** AmplifyRP Acceler8 BBTV lateral flow kit (Agdia) with detection down to 20.4 aM (~12 copies/µl). Curves show means of triplicate reactions with shaded regions indicating standard deviation; lateral flow assays were run as single measurements. For RPA-Cas12a, + band indicates cleaved reporter and BBTV *DNA-R* detection and the – band indicates intact reporter; for AmplifyRP Acceler8, T and C indicate test and control bands, respectively

Detection of globally diverse BBTV isolates from dried leaf material

To assess inclusivity across a range of BBTV genetic diversity and evaluate performance on field-preserved plant material, we applied the RPA-Cas12a assay to dried banana leaf samples collected from naturally infected plants in multiple countries. The panel comprised seven BBTV isolates from Australia, Tonga, Benin, Mozambique, Indonesia, Vietnam and the Philippines, which cover both PIO and SEA genetic subgroups (Rahayuniati et al. 2021; Stainton et al. 2015; Yu et al. 2012). Crude extracts were prepared using the workflow shown in Fig. S1 and used as input to the assay.

The fluorescence assay generated signals for all seven BBTV isolates, with amplification curves rising well above the NTC, healthy plant (HP) control and the positivity threshold and showing broadly similar kinetics across isolates (Fig. 3a). Endpoint fluorescence at 25 min after Cas12a RNP and reporter addition was at least 8.2-fold higher than the NTC and clustered within a relatively narrow range across isolates, consistent with comparable assay performance despite differing geographic origins and potential variation in template quality associated with dried material. Corresponding lateral flow tests produced clear positive bands for all BBTV samples and remained negative for HP and NTC controls (Fig. 3b).

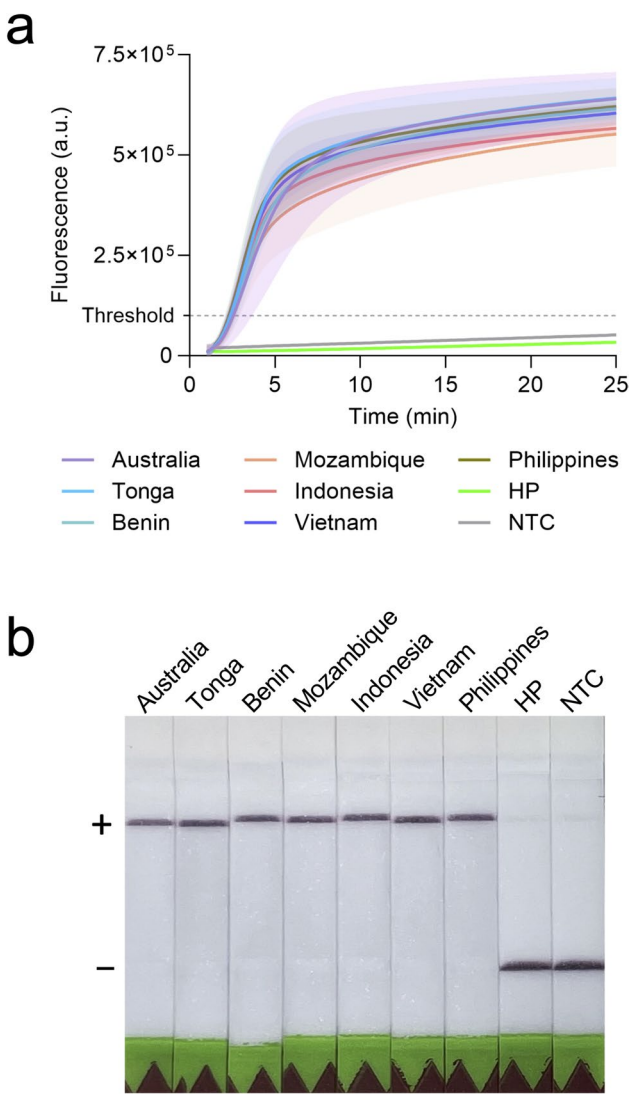

Fig. 3 One-tube RPA-Cas12a BBTV assay performance on isolates from dried leaf material. Seven BBTV isolates, spanning the Pacific Indian Ocean (PIO) and South-East Asian (SEA) genetic subgroups and collected from naturally infected banana plants worldwide, were tested. **a** Fluorescence assay and **b** lateral flow assay showed consistent positive results across all isolates. HP indicates a healthy banana control and NTC a no template control. Fluorescence curves show means of triplicate reactions, with shaded regions indicating standard deviation; lateral flow assays were run as single measurements, + band indicates cleaved reporter and BBTV detection and – band binds intact reporter

Specificity against other banana viruses

Analytical specificity was evaluated using a panel of eight banana viruses commonly encountered in diagnostic laboratories: banana streak OL virus (BSOLV), banana streak IM virus (BSIMV), banana streak GF virus (BSGFV), banana streak MY virus (BSMYV), banana streak cavendish virus (BSCAV), banana mild mosaic virus (BanMMV), cucumber mosaic virus (CMV) and BBTV. Dried leaf material from infected banana plants was processed by crude extraction (Fig. S3), and each extract was tested in the RPA-Cas12a fluorescence and lateral flow formats.

In both formats, only the BBTV-positive sample yielded a detectable signal (Fig. 4). For BSOLV, BSIMV, BSGFV, BSMYV, BSCAV, BanMMV, CMV and HP, fluorescence trajectories remained at the level of the NTC throughout the reaction and well below the 100,000-fluorescence unit threshold, and lateral flow strips showed only the negative band for all samples other than BBTV. This indicates that, within this test panel, the RPA primers and Cas12a crRNA provided high analytical specificity for BBTV.

Application to fresh field-collected samples

We next tested the RPA-Cas12a assay on fresh field-collected material from naturally infected banana plants with differing symptom expression. In the first experiment, the youngest leaf was sampled from each of three plants in a BBTV-affected plantation. Two plants (P1 and P2) displayed typical BBTV symptoms, including dark green streaks on the midrib and marginal chlorosis on the leaf blade, whereas the leaf sampled from the third plant (P3) exhibited only mild streaking localised to the leaf sheath (Fig. 5a and Fig. S4). Crude extracts from leaf tissue were prepared and assayed by RPA-Cas12a.

BBTV was reliably detected in all three plants (Fig. 5b, c). P1 and P2 produced strong fluorescence signals and clear lateral flow positive bands, consistent with high viral loads in symptomatic leaves. The leaf from P3, despite its milder sheath-restricted symptoms, also yielded fluorescence curves that rose well above HP, NTC and the positivity threshold, and a positive lateral flow result.

In a second experiment, we investigated within-plant variation in BBTV detection. Four leaves (L1–L4) were collected from a single recently infected plant. The youngest leaves (L1 and L2) exhibited only very subtle streaking, apparent only to an experienced plant pathologist, whereas L3 and L4 were macroscopically asymptomatic (Fig. 5d). Using the same crude extraction protocol, BBTV was detected in L1 and L2 by both fluorescence and lateral flow. For L3, the fluorescence assay detected BBTV above the threshold in one of three technical replicates, while the other two replicates overlapped with HP and NTC and remained below the positivity threshold; lateral flow results for L3 were negative. BBTV was not detected in L4 in any format (Fig. 5e). These data are consistent with a gradient of viral load within the plant, with higher titres in younger symptomatic leaves and lower or undetectable levels in older asymptomatic leaves.

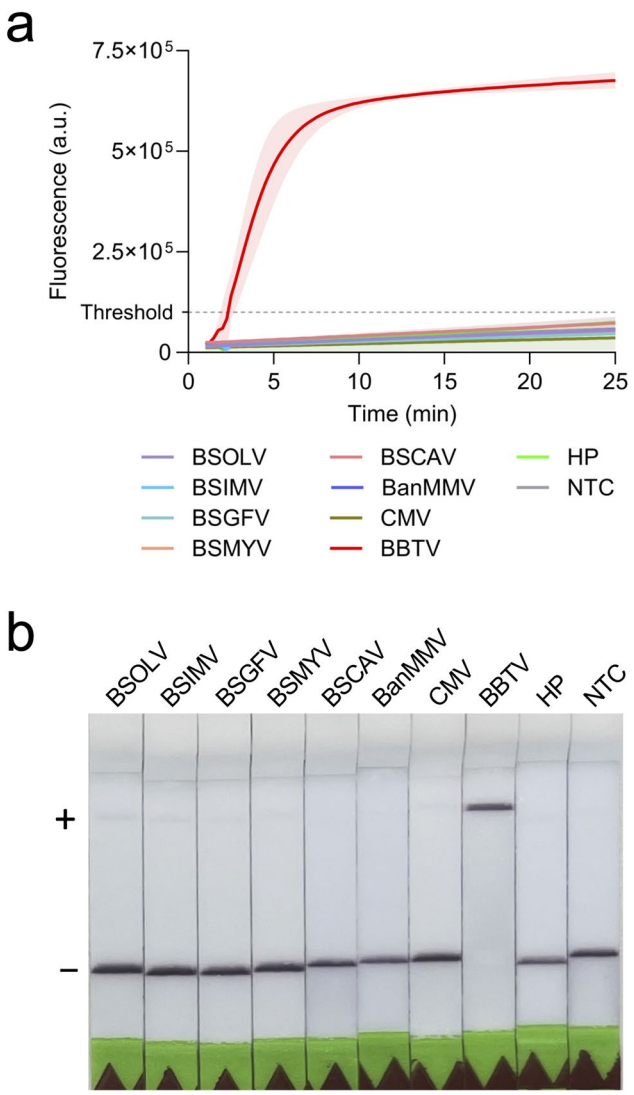

Fig. 4 One-tube RPA Cas12a BBTV assay performance on diverse banana virus isolates from dried leaf material. Eight viruses from dried banana leaf material were tested: banana streak OL virus (BSOLV), banana streak IM virus (BSIMV), banana streak GF virus (BSGFV), banana streak MY virus (BSMYV), banana streak cavendish virus (BSCAV), banana mild mosaic virus (BanMMV), cucumber mosaic virus (CMV) and banana bunchy top virus (BBTV). **a** Fluorescence assay and **b** lateral flow assay did not have evidence of cross reactivity with other viruses. HP indicates a healthy banana control and NTC a no template control. Curves in (**a**) show means of triplicate reactions, with shaded regions indicating standard deviation; lateral flow assays in (**b**) were run as single measurements, + band indicates cleaved reporter and BBTV detection and – band binds intact reporter

Two-step high-sensitivity workflow for laboratory use

Although the one-tube format is advantageous for field or low-resource environments, we hypothesised that the presence of Cas12a RNP and reporter during the amplification step might limit maximal sensitivity in some contexts, for example when testing tissue cultures where maximum sensitivity is desirable. We therefore evaluated a two-step workflow in which RPA is performed first, allowing intermittent agitation because the Cas12a RNP plus reporter mix is not present in the tube lid. After the amplification step was complete, Cas12a detection was initiated by adding the Cas12a RNP and reporter mix by pipette (Fig. 6).

In this configuration, RPA reactions were incubated alone and briefly agitated by vortexing at 4 and 8 min to enhance mixing and mass transfer for very low-abundance target DNA. At 30 min, tubes were centrifuged, opened in a controlled laboratory environment, and a Cas12a RNP plus reporter mix was added by pipette. The reactions were then incubated and monitored for fluorescence as before for a further 25 min and again agitated at 4 and 8 min to enhance mixing. This decoupled format yielded a marked improvement in analytical sensitivity. The limit of detection was reduced from 5.11 aM (~3 copies/µl) in the one-tube format to positive detections in 2 out 3 replicates at 1.28 aM (~0.8 copies/µl) and 1 out of 3 replicates at 0.319 aM (~0.2 copies/µl), consistent with random loss of signal in some reactions at or below single-copy template levels (Fig. 6).

Discussion

In this study, we developed and characterised an RPA-Cas12a assay for BBTV that combines high analytical sensitivity and specificity with flexible readouts and operational simplicity. By building on an existing RPA primer pair by Kapoor et al. (2017) and incorporating a Cas12a crRNA targeted to a highly conserved region of the *DNA-R* gene amplicon, we created an assay that detects BBTV isolates from both genetic subgroups, performs well on both dried and fresh plant material and exhibits no detectable cross-reactivity with other common banana viruses.

A key feature of this work is the benchmarking of RPA-Cas12a against established commercial RPA kits. When tested on a serially diluted BBTV *DNA-R* synthetic standard, the one-tube RPA-Cas12a assays showed substantially lower limits of detection than the AmplifyRP XRT BBTV fluorescence and AmplifyRP Acceler8 BBTV lateral flow RPA kits evaluated (Fig. 2). This gain in sensitivity is particularly relevant for early infections, low titre tissues and surveillance scenarios where viral loads may be close to the threshold of detection (Jebakumar et al. 2025). The improved performance is likely due to the signal amplification inherent in Cas12a-mediated collateral cleavage, where a single target molecule can result in cleavage of many reporter molecules, in contrast to conventional RPA assays in which signal relates more directly with target copy number and consistent

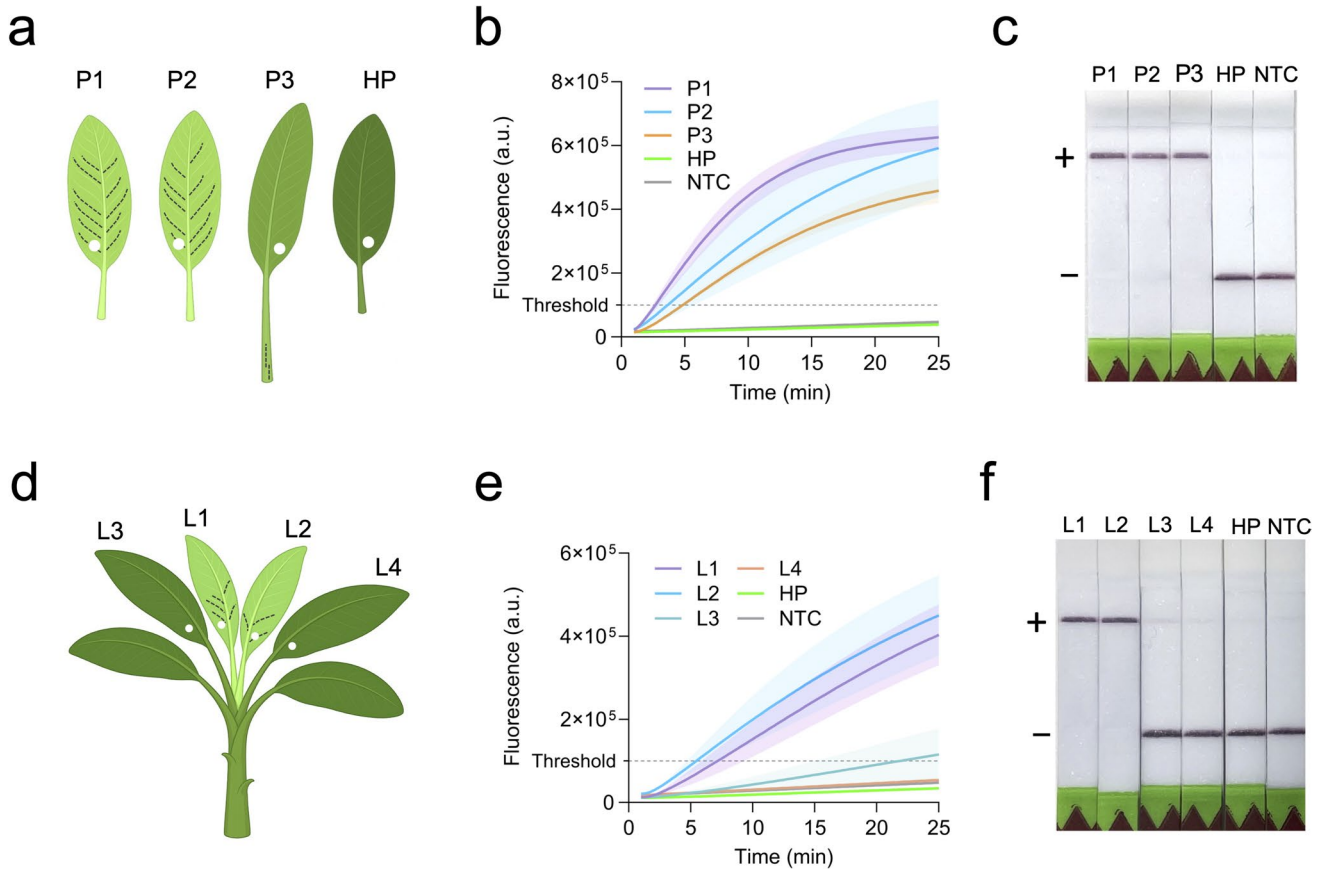

Fig. 5 One-tube RPA-Cas12a BBTV assay applied to fresh field-collected samples from naturally infected banana plants. **a** Leaves from three plants were sampled: plants P1 and P2 showed typical BBTV symptoms on the leaf sampled, whereas the leaf sampled from P3 displayed mild symptoms restricted to the leaf sheath (see Fig. S4). BBTV was detected in all three plants (P1–P3) by fluorescence (**b**) and lateral flow (**c**). **d** Four leaves (L1–L4) were collected from a single plant at early stage of infection; the youngest leaves (L1 and L2) showed mild symptoms, whereas L3 and L4 were asymptomatic. **e** BBTV was reliably detected in L1 and L2, detected in one of three technical replicates in the fluorescence assay for L3, and not detected in L4. **f** BBTV was detected in L1 and L2 by lateral flow assay. HP indicates a healthy banana control and NTC a no template control. Curves in (**b**) and (**e**) show means of triplicate reactions, with shaded regions indicating standard deviation; lateral flow assays in (**c**) and (**f**) were run as single measurements, + band indicates cleaved reporter and BBTV detection and – band binds intact reporter

with the sensitivity gains reported for other CRISPR-based detection systems (Feng et al. 2023; Lv et al. 2021; Xiong et al. 2020).

For the lateral flow assay, we reduced the ssDNA reporter (LFA_reporter) concentration to one third of the concentration of fluorescence reporter (FL_reporter) used in the fluorescence assay. This adjustment improved positive/negative discrimination at low target levels by increasing the proportion of reporter cleaved and reducing background signal, consistent with previous Cas12a-based lateral flow optimisation studies (Lei et al. 2023; Wu et al. 2020). Excess uncut quenched reporter is unlikely to materially affect fluorescence signal readouts.

The inclusivity of the assay across global BBTV diversity is underpinned by the initial sequence alignment of 54 *DNA-R* variants (Fig. S2). By deliberately selecting a crRNA target site within a highly conserved region and retaining the validated RPA primer positions from Kapoor et al. (2017), we minimised the risk that target-site variation would compromise detection. The successful detection of isolates from Australia, the Pacific and multiple Asian and African countries supports this design strategy and indicates broad compatibility with both the PIO and SEA BBTV genetic subgroups currently recognised (Stainton et al. 2015).

High analytical specificity is equally important for a frontline diagnostic. The negative results obtained for BSOLV, BSIMV, BSGFV, BSMYV, BSCAV, BanMMV and CMV banana isolates indicate that the combined primer and crRNA design confers excellent discrimination. This is consistent with the dual specificity inherent in RPA-Cas12a diagnostics, where both the amplification primers and the Cas12a RNP must recognise their cognate target sequences for a positive signal to be generated (Ji et al. 2025). This dual requirement is a key advantage over some isothermal assays,

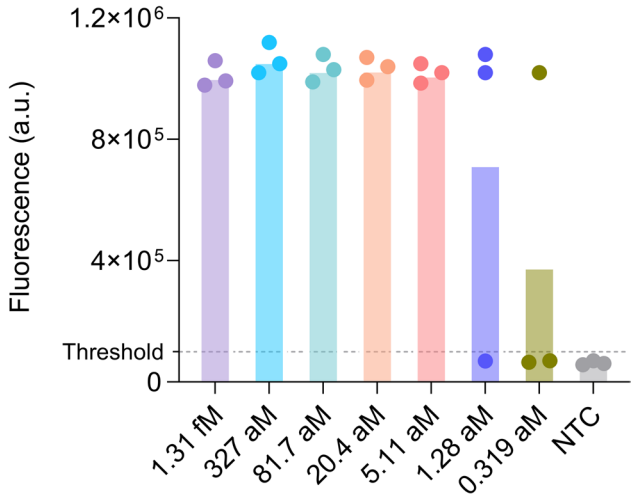

Fig. 6 Two-step high-sensitivity RPA-Cas12a workflow for laboratory use on serial dilution of BBTV_*DNA-R*_gBlock. In this format, the RPA step is run alone for 30 min at 37 °C with brief agitation at 4 and 8 min. After RPA, tubes are opened and Cas12a RNP and reporter are added by pipette, followed by a further 25-min incubation with agitation at 4 and 8 min. This two-step configuration increased analytical sensitivity towards sub-attomolar levels, with positive reactions at 1.28 aM (~0.8 copies/µl) in 2 of 3 replicates and at 0.319 aM (~0.2 copies/µl) in 1 of 3 replicates. Bars show mean endpoint fluorescence of triplicate reactions, and solid dots indicate individual replicate values at 25 min after Cas12a addition. NTC denotes the no template control

where non-specific amplification can contribute to false-positive results (Lobato & O'Sullivan 2018; Xu et al. 2023).

The ability to operate directly on crude extracts from dried and fresh leaf tissue without upstream nucleic acid purification has important low resource deployment. DNA/RNA Shield DirectDetect was the best performing and most consistent extraction solution (Fig. S3 and S3), which we hypothesise may be due in part to the inclusion of glycerol in the proprietary solution, previously reported to enhance the efficiency of RPA-Cas12a detection reactions (Lin et al. 2022; Wang et al. 2025). In addition, DNA/RNA Shield DirectDetect provides nucleic acid stabilisation at ambient temperature until suitable cold storage is available, facilitating sample collection and archiving (Zymo Research Corp. 2025, Kitamura et al. 2023).

Dried leaf samples are widely used in plant health surveillance and diagnostic networks, especially where long-distance transport or storage is required (Shivas et al. 2023). Our results show that the RPA-Cas12a assay maintains high sensitivity on such material, demonstrating its applicability both for confirmatory testing in central laboratories and for retrospective analysis of archived specimens. The assay also detected BBTV in fresh field-collected material from plants with typical symptoms and difficult-to-identify early infection symptoms, indicating compatibility with field sampling and rapid decision-making.

The within-plant gradient observed in Fig. 5c–e, where BBTV was reliably detected in young symptomatic leaves but only sporadically detected or undetectable in older asymptomatic leaves, is consistent with the known movement of BBTV in infected plants and its absence in leaves formed before infection (Thomas 2019). This pattern underscores the importance of sampling the youngest, preferably symptomatic leaves for diagnostics and avoiding older leaves. While routine surveillance relies on visual detection of symptomatic plants to efficiently screen whole plantations on a regular basis, the assay could support more informed plant removal decisions when symptoms are unclear or during biosecurity incursions.

From an implementation perspective, the two workflows described here offer complementary strengths. The one-tube format is designed for simplicity, minimal equipment and reduced contamination risk. It is well suited to near-field or in-field use, plant clinic laboratories with limited infrastructure and mobile diagnostic units (Kaminski et al. 2021). RPA-Cas12a reagents can be lyophilised to provide short term shelf stability (Huang et al. 2025; Hao et al. 2024). The closed-tube configuration, in which amplification and detection reagents are physically separated until the end of the amplification period, enables users to carry out the entire assay with a single heating device and basic handling skills (Huang et al. 2025; Aman et al. 2020b; Ding et al. 2020). The availability of both fluorescence and lateral flow outputs provides flexibility: Fluorescence can be used with portable fluorometers for real-time semi-quantitative assessment, while lateral flow strips provide an intuitive binary readout without instrumentation.

The two-step high-sensitivity workflow trades some of this simplicity for increased analytical performance. By separating the reaction set up so RPA and Cas12a are added in separately by pipetting and exploiting intermittent agitation during amplification and detection, we achieved a limit of detection at sub-attomolar levels, at near single-digit copy numbers per reaction. This level of sensitivity approaches that of well optimised qPCR assays and may be desirable for certain applications, such as testing high-value germplasm, early-stage surveillance in new regions, sample bulking or resolving ambiguous results from other tests. However, it requires opening tubes and adding reagents in a laboratory environment, with associated contamination risks. Accordingly, the one-tube workflow is likely to be most useful for routine diagnostics, whereas the two-step workflow is better suited to confirmatory testing or research applications.

There are, nonetheless, limitations. One limitation relates to how we benchmarked performance against other diagnostics. Benchmarking was restricted to two commercial RPA kits, AmplifyRP Acceler8 and AmplifyRP XRT for BBTV, using the BBTV_DNA-R_gBlock standard, and did not include serially diluted infected plant material

because of kit costs. The proprietary nature of these assays and the use of a Genie HT isothermal fluorometer rather than the dedicated AmplifyRP XRT reader mean that some bias in the comparison cannot be excluded. Nevertheless, our observed AmplifyRP Acceler8 performance was broadly consistent with the manufacturer's claim of detection down to 5 copies/µl, whereas the AmplifyRP XRT assay was less sensitive in our hands than the 10 copies/µl reported by Agdia (Agdia Inc. 2020, Agdia Inc. 2022). We also did not benchmark the assay against ELISA or other published BBTV detection methods (Geering & Thomas 1996; Chandrasekar et al. 2011; Peng et al. 2012), nor evaluate *Pentalonia* sp. aphids. Future validation should therefore compare the assay against established laboratory pipelines and assess its suitability for vector-based surveillance.

Although our diversity panel covered major BBTV lineages, ongoing surveillance will be needed to ensure that emerging variants remain compatible with the assay and that specificity is maintained against viruses not assessed here. The modularity of Cas12a-based diagnostics means that, if required, updated crRNAs can be incorporated relatively quickly if required, and we have identified eight additional LbCas12a crRNA recognition sites with suitable PAM sequences within the current RPA amplicon. As the current assay targets a single genomic region, multiplexing with additional BBTV targets or internal controls could further enhance reliability, particularly in heavily degraded samples.

More broadly, the design principles established here are transferable to other plant viruses. Reusing validated RPA primers or converting PCR protocols to RPA format, aligning diverse target sequences to identify conserved Cas12a recognition sites and configuring one-tube and two-step workflows allows rapid development of new highly sensitivity and specific diagnostics. For banana in particular, there is scope to develop a panel of Cas12a-based assays that collectively cover multiple economically important viruses. Such assays could support faster outbreak response, more informed surveillance, and more efficient certification of clean planting material.

In conclusion, this is the first reported CRISPR-based diagnostic for BBTV, and the RPA-Cas12a assay described here provides a rapid, sensitive and specific method for BBTV detection. It demonstrates improved analytical sensitivity relative to existing commercial RPA kits, performs effectively on both dried and fresh banana leaf material and offers operational flexibility through both one-tube and two-step workflows. These characteristics make it a strong candidate for integration into BBTV management programmes, particularly where rapid, low-resource testing is needed.

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Data availability Data supporting the findings of this study are provided in the article or Supporting Information.

Declarations

Conflict of interest The authors declare no competing interests.

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