Viroid detection

RT-LAMP of potato spindle tuber viroid RNA using SuperScript IV RT-LAMP Master Mix

Introduction

Potato spindle tuber viroid (PSTVd) is a member of the genus *Pospiviroid* in the Pospiviroidae family consisting of only a circular, unencapsidated RNA of 356–361 nucleotides with an extremely complex and stable secondary structure [1]. PSTVd is highly contagious and is readily spread from infected to noninfected plants by mechanical means (e.g., using contaminated tools during agricultural operations), vegetative propagation, insects, and pollen. Depending on strain–host combinations, PSTVd infections can cause severe diseases and even plant death in many crop plants such as potato, tomato, avocado, sweet potato, and pepino, and can also lead to significant yield loss [2,3]. For these reasons, it is important to undertake proper detection methods for disease prevention and surveillance.

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) has emerged as a promising technology for simple, rapid, and sensitive identification of a selected target that can be implemented in medicine, food industries, and agriculture. RT-LAMP technology allows for simple preparation of a reaction and target amplification in 5–30 minutes under isothermal conditions. A typical RT-LAMP reaction employs a set of 4–6 specially designed primers that facilitate formation of cauliflower-like structures, a reverse transcriptase catalyzing the conversion of RNA template into a complementary DNA (cDNA), and a DNA polymerase with strong strand displacement activity, like *Bst* DNA polymerase. RT-LAMP requires no specialized equipment such as a thermal cycler and can be performed simply with a heat block or water bath capable of maintaining a constant temperature (65°C) for incubation of reactions.

Here we demonstrate the use of Invitrogen™ SuperScript™ IV RT-LAMP Master Mix for rapid detection of PSTVd in RNA extracted from infected tomato plants. Average time to obtain signal was less than 10 minutes. The current method demonstrates that viroid RNA can be detected using real-time RT-LAMP with Invitrogen™ SYTO™ 9 stain, visual evaluation of the color change induced by Invitrogen™ SYBR™ Green I stain, and agarose gel electrophoresis with Invitrogen™ E-Gel™ precast gels.

Important notes

RT-LAMP is a highly sensitive detection method, and precautions should be taken to prevent any carryover contamination of new reactions with RT-LAMP products from previous reactions.

- Use separate workspaces as well as specially dedicated equipment and supplies for:
  - RT-LAMP reaction setup and amplification
  - Analysis of RT-LAMP products by endpoint detection
- Using pipette tips with aerosol filters to prepare RNA samples and set up RT-LAMP reactions is strongly recommended.
- Setting up RT-LAMP mixtures in a laminar flow cabinet equipped with a UV lamp is recommended.
- Change gloves frequently (wear fresh gloves after cleaning the laboratory workspace and equipment, before RNA sample preparation, and after RT-LAMP mixture setup).
- Always include negative control reactions, e.g., a no-template control (NTC), to check for background amplification.
- Close tubes or wells with the NTC before adding the RNA template to other reactions, to eliminate the risk of sample cross-contamination.
- Prior to each RT-LAMP experiment, prepare a fresh 50 µM solution of SYTO 9 stain by diluting 1:100 the 5 mM stock solution of SYTO 9 Green Fluorescent Nucleic Acid Stain.
- To visually evaluate RT-LAMP amplicons by agarose gel electrophoresis, 10-fold dilution of RT-LAMP samples stained with SYBR Green I stain (Cat. No. S7567; 10,000X stock) is recommended. If RT-LAMP reactions are not stained, 5-fold dilution is enough to visualize a ladder-like pattern of bands on an agarose gel.

Materials

Reagents

- Applied Biosystems™ MagMAX™ Plant RNA Isolation Kit (Cat. No. A33899)
- Invitrogen™ SuperScript™ IV RT-LAMP Master Mix (Cat. No. A51801)
- Invitrogen™ SYTO™ 9 Green Fluorescent Nucleic Acid Stain (Cat. No. S34854)
- Thermo Scientific™ Water, nuclease-free (Cat. No. R0581)
- Invitrogen™ SYBR™ Green I Nucleic Acid Gel Stain, 10,000X concentrate in DMSO (Cat. No. S7567)
- Invitrogen™ E-Gel™ Low Range Quantitative DNA Ladder (Cat. No. 12373031)
- Invitrogen™ E-Gel™ EX Double Comb Agarose Gels, 2% (Cat. No. A42346)
- Applied Biosystems™ *Power* SYBR™ Green RNA-to-C_T™ *1-Step* Kit (Cat. No. 4389986)
- 70% ethanol solution
- Invitrogen™ DNA*Zap*™ PCR DNA Degradation Solutions (Cat. No. AM9890)
- Invitrogen™ RNase*Zap*™ RNase Decontamination Solution (Cat. No. AM9784)
- Invitrogen™ GeneArt™ synthetic DNA fragment for PSTVd
- Invitrogen™ RNase*Zap*™ RNase Decontamination Wipes (Cat. No. AM9786)

RT-LAMP target and primer set

- Tissue: tomato plant inoculated with PSTVd
- Pathogen: PSTVd
- Strain: Isolate MT38ozon (GenBank Acc. No. KY522736)
- Primer set: Primers targeting PSTVd-specific and conserved region [3]

Methods

Tissue preparation and manual RNA extraction

Tissue was disrupted and total RNA was extracted using the MagMAX Plant RNA Isolation Kit following the manufacturer's instructions, with minor modifications: 200 mg of fresh tomato plant leaves were used instead of 50 mg; and, to prevent degradation of the PSTVd RNA, samples were mixed by gentle tapping and pulse vortexing instead of vortexing for 10 seconds on a high setting.

Fresh young tomato plant leaves (200 mg) were homogenized manually by grinding in liquid nitrogen with a mortar and pestle and used as infected plant material for the magnetic bead–based total plant RNA extraction (Figure 1). Isolated total RNA was quantified on the Invitrogen™ Qubit™ 4 Fluorometer (Cat. No. Q33238) using the Invitrogen™ Qubit™ RNA High Sensitivity (HS) Assay Kit (Cat. No. Q32852). The extracted total RNA from infected and noninfected plant material was stored at –80°C for further use.

RT-LAMP reaction setup

The reaction mix was prepared on ice according to Table 1. RT-LAMP reactions were carried out in single tubes using SuperScript IV RT-LAMP Master Mix in a total volume of 25 µL. Eppendorf™ DNA LoBind™ 1.5 mL nuclease-free and nonstick tubes, along with sterile filtered pipette tips, were used to prevent aerosol contamination and maximize sample recovery for improved reaction performance. When real-time RT-LAMP was performed, SYTO 9 Green Fluorescent Nucleic Acid Stain was added to the reaction mix.

Real-time RT-LAMP

All real-time RT-LAMP reactions were performed in real-time PCR instruments (Applied Biosystems™ QuantStudio™ 6 Flex and 7 Flex Real-Time PCR Systems) with one-step cycling conditions (Table 2). Specificity of the amplification products and reaction kinetics were analyzed using Applied Biosystems™ QuantStudio™ Real-Time PCR Software for QuantStudio 6 Flex and 7 Flex systems. A melt curve analysis was performed to verify the specificity of amplified products.

Endpoint detection

Naked-eye visualization and gel analysis of RT-LAMP products was performed using the same reaction setup as described in Table 1, except that SYTO 9 stain was replaced with nuclease-free water. The RT-LAMP reaction mixture contained 2 µL of total RNA extract from infected tomato plant material. Amplification was carried out at 65°C for 15 minutes on a preheated thermal block. The reaction was terminated by inactivating the polymerase in SuperScript IV RT-LAMP Master Mix at 95°C for 2 minutes. A positive control (PSTVd synthetic DNA fragment) and negative controls (NTC and total RNA extracts from noninfected tomato leaves) were included in the run.

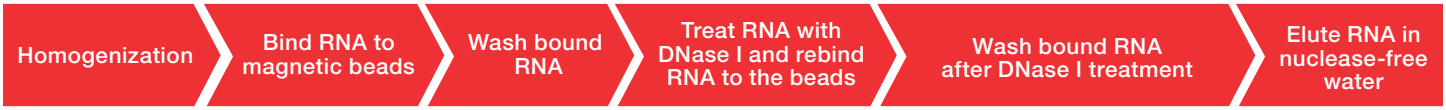

Figure 1. Steps for total RNA extraction from tomato leaves and RNA purification.

Table 1. Reaction setup.

Component	Sample with target RNA	Positive control	No-template control (NTC)	Final concentration
SuperScript IV RT-LAMP Master Mix (2X)	12.5 µL	12.5 µL	12.5 µL	1X
100 µM FIP/BIP primer	0.25 µL each	0.25 µL each	0.25 µL each	1 µM
100 µM F3/B3 primer	0.05 µL each	0.05 µL each	0.05 µL each	0.2 µM
100 µM LoopF/LoopB primer	0.25 µL each	0.25 µL each	0.25 µL each	1 µM
50 µM fresh stock solution of SYTO 9 Green Fluorescent Nucleic Acid Stain*	2.5 µL	2.5 µL	2.5 µL	5 µM
Nuclease-free water	6.9 µL	7.9 µL	8.9 µL	
NTC tubes or wells were closed prior to adding target RNA				
Total RNA from infected or noninfected plant material	2 µL			
Synthetic PSTVd DNA fragment		1 µL		
Total volume	**25 µL**	**25 µL**	**25 µL**	

* For real-time fluorescence detection only.

Table 2. One-step cycling protocol.

Number of cycles	Step	Temperature	Time
30	Amplification	65°C	30 seconds
1	Inactivation	95°C	2 minutes
–	Melt curve	60–95°C	–

Colorimetric naked-eye detection

The RT-LAMP mixture tubes were removed after termination of the reaction, and 1 µL of 10-fold diluted SYBR Green I stain (Cat. No. S7567; 10,000X stock) was added to each reaction tube for color change evaluation by naked eye. To prevent contamination with amplicons from the positive reaction tubes, negative control reactions were stained first. For the same reason, only one tube at a time was opened, and closed quickly after adding the SYBR Green I stain. Positive results were determined by a color change from orange (negative) to yellow-green (positive).

Agarose gel electrophoresis

The results of the naked-eye visualization were further verified by agarose gel electrophoresis using Invitrogen™ E-Gel™ EX Double Comb Agarose Gels with SYBR™ Gold DNA stain (Cat. No. A42346) and the Invitrogen™ E-Gel™ Power Snap Electrophoresis System. For the RT-LAMP reaction samples initially stained with SYBR Green I dye, 10-fold dilution was used (2 µL of the sample mixed with 18 µL of nuclease-free water). Invitrogen™ E-Gel™ Low Range Quantitative DNA Ladder (Cat. No. 12373031) was diluted by adding 5 µL of the ladder to 15 µL of nuclease-free water. The prepared ladder and samples (20 µL) were transferred into wells of an E-Gel EX Double Comb Agarose Gel, starting with the negative control reactions. NTC and negative test samples were loaded into separate E-Gel EX Double Comb Agarose Gels to minimize the risk of contamination.

Real-time RT-PCR reaction setup for determination of viroid load

The reaction mix for real-time RT-PCR was prepared on ice according to Table 3. The amplification reactions were carried out in a total volume of 20 µL. The set of primers used for PSTVd target amplification and quantification has been described by Boonham et al. [4]. Real-time RT-PCR detection and quantification of PSTVd copy number from total infected plant RNA was performed using the Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System, where a thermal cycling protocol was applied (Table 4). The specific target amplification was analyzed by melt-curve analysis using Applied Biosystems™ Design and Analysis 2 Software for the QuantStudio 5 system.

Standard curve

To quantitate viroid RNA copy number in the total RNA extract from the PSTVd-infected tomato plant, a standard curve was obtained by co-amplification of known amounts of synthetic PSTVd DNA fragment control. The copy number/µL for control stock solution was determined from its molecular weight and Avogadro's constant using the copy number calculator in SnapGene™ software. Ten consecutive dilutions (dilution factor 1:2) were prepared containing 128,000 to 250 copies/reaction. The amount of PSTVd RNA in tested samples was obtained by plotting C_q values onto the standard curve.

Table 3. Reaction setup.

Component of the reaction mix	Detection of target RNA	Positive control	No-template control (NTC)	Final concentration
Power SYBR Green RT-PCR Mix (2X)	10 µL	10 µL	10 µL	1X
10 µM forward primer	0.4 µL	0.4 µL	0.4 µL	200 nM
10 µM reverse primer	0.4 µL	0.4 µL	0.4 µL	200 nM
RT enzyme mix (125X)	0.16 µL	0.16 µL	0.16 µL	1X
Nuclease-free water	7.04 µL	8.04 µL	9.04 µL	
NTC tubes or wells were closed prior to adding target RNA				
Total plant RNA extract from infected or noninfected plant material	2 µL			
Synthetic PSTVd DNA fragment		1 µL		
Total volume	**20 µL**	**20 µL**	**20 µL**	

Table 4. Thermal cycling conditions.

Number of cycles	Stage	Step	Temperature	Time
1	Holding	Reverse transcription	48°C	30 min
1	Holding	Activation of the DNA polymerase	95°C	10 min
40	Cycling	Denature	95°C	15 sec
		Anneal and extend	60°C	1 min
1	Melt curve	Denature	95°C	15 sec
		Anneal	60°C	15 sec
		Denature	95°C	15 sec

Results and discussion

Real-time detection of RT-LAMP products

The aim of this study was to demonstrate a quick and reliable method for PSTVd detection, based on RT-LAMP using SuperScript IV RT-LAMP Master Mix. Real-time RT-LAMP reactions were carried out to determine amplification kinetics. Reaction speed and sensitivity were evaluated using different input amounts of total plant RNA (1, 2, and 3 µL).

Results presented in Figure 2 show that amplification signals of the target region were detected in less than 10 minutes, regardless of total plant RNA input. No amplification signals were observed in replicates of the NTC and negative control (total RNA extracts from noninfected tomato leaves) in under 15 minutes post-reaction. The specificity of real-time RT-LAMP amplification products was confirmed by melt curve analysis. The melting temperature (T_m) of the PSTVd amplicons from plant RNA extracts and of a synthetic PSTVd DNA fragment positive control were compared. In tested samples with total RNA from infected plants, melt curve analysis showed a T_m peak that matched that of the synthetic PSTVd DNA fragment positive control, indicating specific amplification of the PSTVd target region. Representative melt peaks are shown in Figure 3.

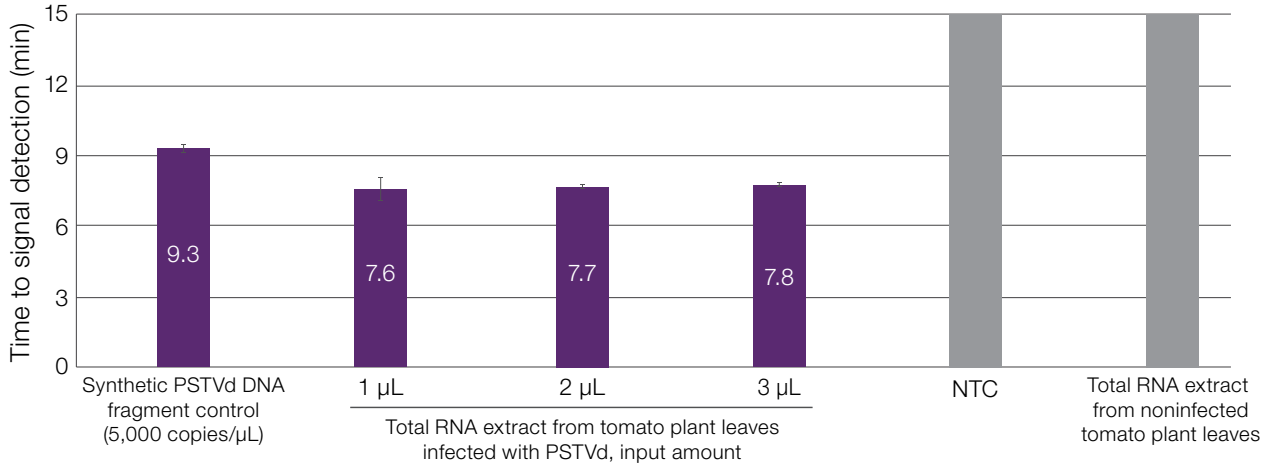

Figure 2. Detection of PSTVd RNA from tomato total RNA extract in less than 10 minutes via real-time RT-LAMP using SYTO 9 stain. Amplification kinetics were demonstrated using different input amounts of plant total RNA. No amplification signals were observed in NTC and negative control replicates. A positive control was used to assess the specificity and sensitivity of the real-time RT-LAMP assay. Error bars represent standard deviation of reaction speed (time to signal) values calculated from multiple technical replicates (n = 4) for each tested sample.

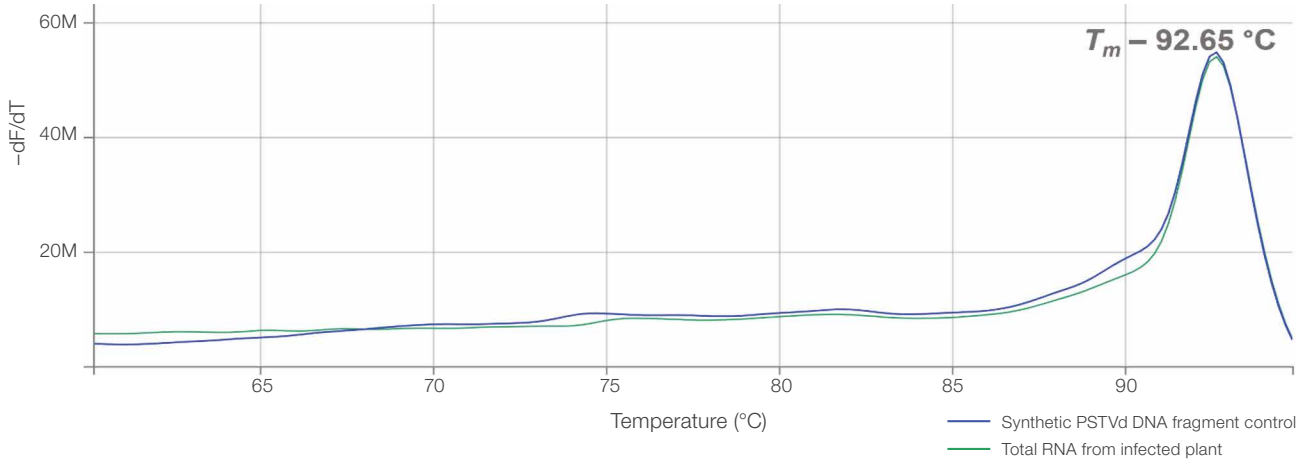

Figure 3. Representative melt curves of real-time RT-LAMP for PSTVd detection. Total RNA extracted from leaves of PSTVd-infected tomato was used as the template for the real-time RT-LAMP test. A synthetic PSTVd DNA fragment was used as a positive control.

Endpoint detection of RT-LAMP products

The RT-LAMP outcome was also assessed by visual inspection of DNA accumulation in the reaction tubes (Figure 4). The specificity of the visual RT-LAMP assay was evaluated by utilizing the total RNA extracted from healthy tomato leaves along with the NTC. A synthetic PSTVd DNA fragment (5,000 copies) was used as a positive control. The change in the color of the solution was observed directly by naked eye following the addition of SYBR Green I stain. An orange color indicated no amplification, whereas a change from orange to yellow-green demonstrated the presence of amplicons. Results were further verified by agarose gel electrophoresis by the ladder-like pattern in the positive samples. Negative and no-template controls did not give any visible fluorescence or ladder-like pattern indicating absence of RT-LAMP amplicons.

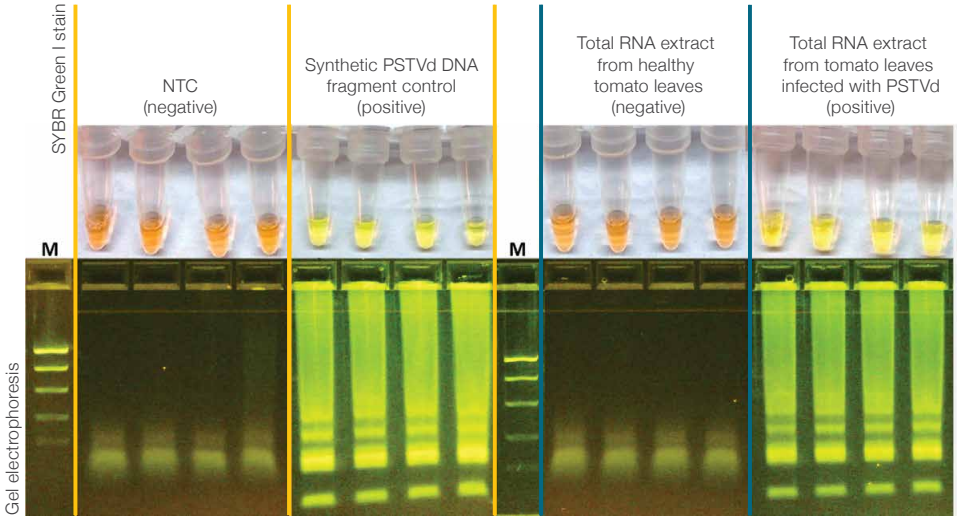

Figure 4. Endpoint detection of RT-LAMP products by visual assessment and electrophoretic analysis. Total RNA extracted and purified from noninfected plant material and from tomato leaves infected with PSTVd was used along with a synthetic PSTVd DNA fragment positive control and a NTC. Representative examples of one PSTVd-negative and one PSTVd-positive RNA sample are shown with 4 replicates. Notable color changes from the original orange color to yellow-green following the addition of 1 µL of the diluted SYBR Green I stain indicate PSTVd detection and presence of RT-LAMP amplicons. The orange color was retained in negative control samples and indicated no amplification. The accuracy of the colorimetric RT-LAMP assay was verified by visualization of a characteristic ladder-like pattern in the E-Gel EX Double Comb Agarose Gel (2%) with SYBR Gold DNA stain. Lane M contains the E-Gel Low Range Quantitative DNA Ladder.

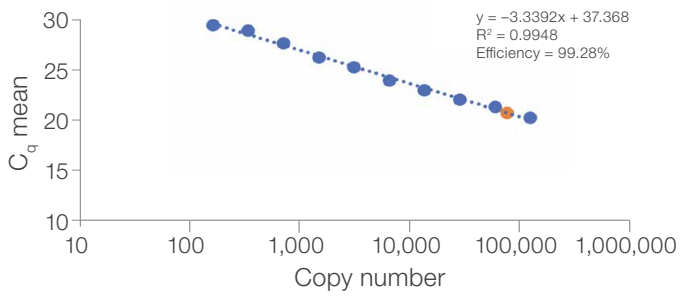

Figure 5. Real-time RT-PCR standard curve for quantification of PSTVd load from total infected tomato plant RNA extract. The standard curve was generated with serial 2-fold dilutions of the synthetic PSTVd DNA fragment, ranging from 128,000 to 250 copies/µL (blue data points representing averages of 3 replicates). Average C_q values from tested samples were plotted onto the standard curve, and the copy number of PSTVd genome was calculated per 2 µL. The average PSTVd RNA copy number from tested samples was calculated from duplicate samples (orange data point).

Estimation of viroid copy number

A known amount of synthetic PSTVd DNA fragment was used in two-fold serial dilutions ranging from 128,000 to 250 copies of PSTVd genome to generate a standard curve in real-time RT-PCR. A standard curve was established by plotting the C_q average values from the dilution series against copy number of synthetic PSTVd DNA fragment, followed by linear regression analysis (Figure 5). The standard curve had a slope of –3.3392 with a regression coefficient (R^2) value of 0.995 and an amplification efficiency of 99.3%. The averaged quantification cycle (C_q) values from tested samples (infected extract total RNA) were plotted onto the standard curve. The copy number of PSTVd genome was calculated per 2 µL to match the RNA input in the RT-LAMP endpoint detection experiment. No amplification was detected in the NTC reactions.

Based on the real-time RT-PCR results, the copy number of PSTVd genome was approximately 80,000 copies/2 µL. As previously mentioned, LAMP differs from PCR in that six primers are used for amplification of a target region. Since PSTVd has a very short RNA genome and six LAMP primers target almost the whole genome, only a full-length RNA can be detected via RT-LAMP. In contrast, RT-PCR requires only two primers targeting a 65 bp region, thus amplifying and detecting even fragmented RNA. Nevertheless, the results described here indicate successful RNA extraction and PSTVd detection from infected tomato leaves via RT-LAMP using SuperScript IV RT-LAMP Master Mix.

Conclusions

SuperScript IV RT-LAMP Master Mix permits fast reaction preparation, outstanding reaction speed, and compatibility with real-time and endpoint detection methods. With SuperScript IV RT-LAMP Master Mix, PSTVd RNA in tomato leaves was reliably detected in less than 10 minutes, regardless of total plant RNA input amount. These advantages indicate that SuperScript IV RT-LAMP Master Mix is well suited for the development of highly sensitive, accurate, and fast RT-LAMP assays for the detection of plant pathogens such as PSTVd.

Acknowledgments

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References

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4. Boonham N, Pérez L, Mendez M et al. (2004) Development of a real-time RT-PCR assay for the detection of potato spindle tuber viroid. *J Virol Methods* 116(2):139–146.

Ordering information

Description	Cat. No.
DNA*Zap* PCR DNA Degradation Solutions	AM9890
UltraPure DNase/RNase-Free Distilled Water	10977049
SuperScript IV RT-LAMP Master Mix (2X)	A51801
SYTO 9 Green Fluorescent Nucleic Acid Stain	S34854
Water, nuclease-free	R0581
RNase*Zap* RNase Decontamination Solution	AM9784
SYBR Green I Nucleic Acid Gel Stain, 10,000X concentrate in DMSO	S7567
Power SYBR Green RNA-to-C_T 1-Step Kit	4389986
RNase*Zap* RNase Decontamination Wipes	AM9786
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E-Gel EX Double Comb Agarose Gels, 2%	A42346
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