National Diagnostic Protocol

Potato spindle tuber viroid

PSTVd

NDP 7 V2

To be used with ISPM 27 DP7:

Potato spindle tuber viroid **(2015)**

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Purpose

National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists it should be used in preference to NDPs, unless it is shown that the NDP has improved procedures for Australian conditions. NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

https://www.ippc.int/core-activities/standards-setting/ispms

Process

NDPs are facilitated and endorsed by the Subcommittee on Plant Health Diagnostics (SPHD). SPHD reports to Plant Health Committee and is Australia's peak technical and policy forum for plant health diagnostics.

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at https://www.plantbiosecuritydiagnostics.net.au/work/subcommittee-on-plant-health-diagnostics/

NDPs are living documents. They are updated every 5 years or before this time if required (i.e. when new techniques become available).

Document status

This version of the National Diagnostic Protocol (NDP) for potato spindle tuber viroid is current as at the date contained in the version control box below.

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Further information

Inquiries regarding technical matters relating to this protocol should be sent to: sphd@agriculture.gov.au

Contents

1 INTRODUCTION

Potato spindle tuber viroid (PSTVd) is most commonly found affecting solanaceous crops such as potato and tomato.

Many strains of PSTVd have been reported that cause symptoms ranging from mild to severe. Severe strains of PSTVd in potato can result in loss of tuber yield of up to 65% (Salazar, 1989). Outbreaks of the disease in commercial tomato crops have resulted in the destruction of the crop (Mumford et al., 2003).

Viroids such as PSTVd have a simple structure consisting of an unencapsidated (no coat protein), single-stranded, circular ribonucleic acid (RNA), ranging from 246-375 nucleotides in length. Replication occurs in plant cell nuclei, using the hosts' cell constituents. Viroids can cause varied syndromes in plants due to the direct interaction of the viroid RNA with host cell constituents (Xiong, 2002).

PSTVd is a highly contagious disease, transmitted between plants by touch. The use of cutting or pruning tools, contaminated machinery or any form of physical contact between plants can result in disease transmission. PSTVd has been transmitted by the aphid *Myzus persicae* from plants that are co-infected with Potato leafroll virus (Querci et al., 1997; Salazar et al., 1995). In potatoes the most important means of PSTVd spread from one generation to the next is via infected potato tubers. PSTVd is transmitted through pollen and true seed and can retain its infectious activity in seed for long periods (Timmermann et al., 2001). It is transmitted through potato and tomato true seed at varying rates depending on the host cultivar, the presence of PSTVd in one or both of the botanical parents, and the strain of PSTVd present (Salazar, 1989).

1.1 Host range

Solanaceous crops, potato (*Solanum tuberosum*), tomato (*Lycopersicum esculentum*) and eggplant (*Solanum melongena*), are natural hosts of PSTVd.

PSTVd has been detected in avocado (*Persea americana*) (Querci et al., 1997), in pepper (*Capsicum annuum*) (Lebas et al., 2005), sweet cucumber (*Solanum muricatum*) and an Australian native *Solanum* spp. (Behjatnia et al., 1996). The viroid has also been reported on Cape gooseberry (*Physalis peruviana*) (Ward et al., 2010; Verhoeven et al., 2009).

From the CABI data sheet (http://www.cabi.org/isc/datasheet/43659 accessed 20 Feb 2017), symptomless infections have been reported from *Brugmansia* spp., *Chrysanthemum* sp., *Calibrachoa* sp., *Cestrum* spp., *Dahlia* sp., *Datura* sp. *Lycianthes rantonnei*, *Petunia* sp., *Physalis peruviana*, *Solanum pseudocapsicum*, *Streptosolen jamesonii*, *Solanum jasminoides*, *Solanum muricatum*, sweet potato (*Ipomoea batatas*) and wild *Solanum* spp. (Salazar, 1989; Owens et al., 1992; Querci et al., 1995; Behjatnia et al., 1996; Di Serio, 2007; Verhoeven et al., 2008a, b, 2009, 2010b; Lemmetty et al., 2011; Luigi et al., 2011; Mertelik et al., 2010; Verhoeven, 2010; Tsushima et al., 2011).

Experimentally, 94 plant species in 31 families have been shown to be susceptible (Jeffries 1998).

2 TAXONOMIC INFORMATION

Family: Pospiviroidae

Genus: Pospiviroid

Common name: *Potato spindle tuber viroid* (PSTVd)

3 DETECTION

3.1 Symptoms

Figures 1 – 15 illustrate symptoms of PSTVd in potato and tomato.

Hosts may be symptomless or produce symptoms depending on PSTVd strain, host cultivar and environmental conditions.

Foliar symptoms of potato may include an upright and stunted growth habit with an erect stem and shortened internodes between leaflets resulting in an overlapping appearance. The leaflets are often smaller in size with a rugose appearance. Infected potato tubers are often elongated with pointed ends, have deeper eyes than normal and the tuber skin is often cracked. On severely affected tubers, knobs and swellings may appear.

In tomatoes there is a long latent period of 4 to 5 weeks (or longer, depending on the PSTVd strain and environmental conditions) before symptoms are expressed. In mature tomato plants infected with severe strains of PSTVd, symptoms include purpling and yellowing of the leaflets, shortening of the leaflet internodes, leaf epinasty (downward bending of leaves or other plant parts, resulting from excessive growth of the upper side), thickening, distortion of the leaves, and leaf brittleness. Spindly shoot growth can occur, flowers may abort and the fruit can be dark green in colour.

Website for descriptions and photos: www.dpvweb.net/dpv/showadpv.php?dpvno=66,

Figure 1. A potato plant infected with PSTVd. Note the erect appearance of the plant and overlapping leaflets (© Beltsville Agricultural Research Centre, USDA).

Figure 2. Foliage symptoms in potato cv. Irish Cobbler (right), healthy plant (left) (© US Department of Agriculture).

Figure 3. PSTVd-infected potato showing a stiff upright growth habit (© T.A. Zitter).

Figure 4. Tuber symptoms in potato (Upper row) cv. Saco; (lower row) cv. Kennebec. (left) healthy; (centre) infected with the type strain; (right) infected with the unmottled curly dwarf strain. Note the elongated shape of the tuber and the pronounced, deeper eyes on the PSTVd-infected tubers (© US Department of Agriculture).

Figure 5. Tuber symptoms in potato. Healthy (left) and tubers infected with PSTVd (four on right) (© Department of Agriculture and Food, WA).

Figure 6. Potato tubers on right showing mild symptoms of PSTVd infection. Infected tubers are longer than normal with numerous conspicuous eyes (© H.D. Thurston).

Figure 7. Symptoms of PSTVd infection in tomato (© Grodan).

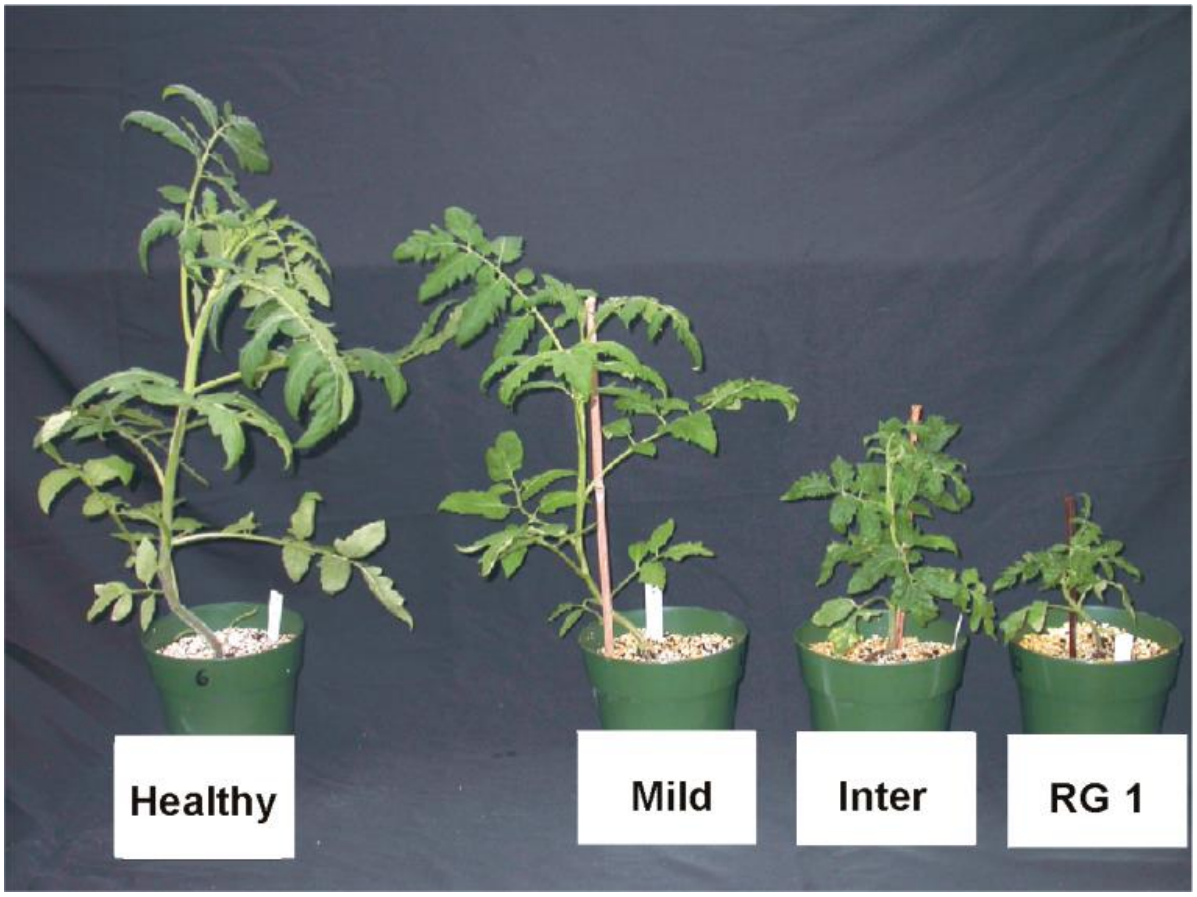

Figure 8. Foliage symptoms of PSTVd infection in tomato cv. Rutgers. Plants were inoculated on the cotyledons with PSTVd strains causing either mild, intermediate, or severe (RG1) symptoms before emergence of the first true leaf and then photographed approximately four weeks after inoculation. (Courtesy R.A. Owens, copyright-free).

Figure 9. Mild symptoms of PSTVd in the top of a 2 month old tomato plant (© VegFed, NZ).

Figure 10. PSTVd well established in a mature tomato plant showing 'purpling' on leaves (© VegFed, NZ).

Figure 11. Well-established PSTVd infection in the head of a mature tomato plant (© VegFed, NZ).

Figure 12. PSTVd showing as 'full blown yellow' throughout the tomato plant (© VegFed, NZ).

Figure 13. Severe symptoms of PSTVd are more evident in older tomato plants (© VegFed, NZ).

Figure 14. PSTVd symptoms evident only on the lower leaves, the upper leaves are symptomless (© VegFed, NZ).

Figure 15. PSTVd symptoms – leaf curling and lesions on young plants (© MPI, NZ).

4 IDENTIFICATION

Viroids can be difficult to diagnose. They cannot be detected using standard virological methods such as enzyme-linked immunosorbent assay (ELISA) used to detect specific proteins, as viroids do not encode a coat-protein (Xiong, 2002). Due to their limited size, viroids are too small to be visualised with an electron microscope (Büchen-Osmond, 2002). The methods currently available for the detection of PSTVd are visual symptoms (if present) and molecular techniques such as hybridisation assays and reverse transcriptase-polymerase chain reaction (RT-PCR), which will detect the viroid RNA molecule and reverse polyacrylamide gel electrophoresis (R-PAGE).

In the laboratory, PSTVd can be confirmed (or negated) using a three-phased RT-PCR test and subsequent sequencing of PCR products. The presence of visual symptoms cannot be relied on.

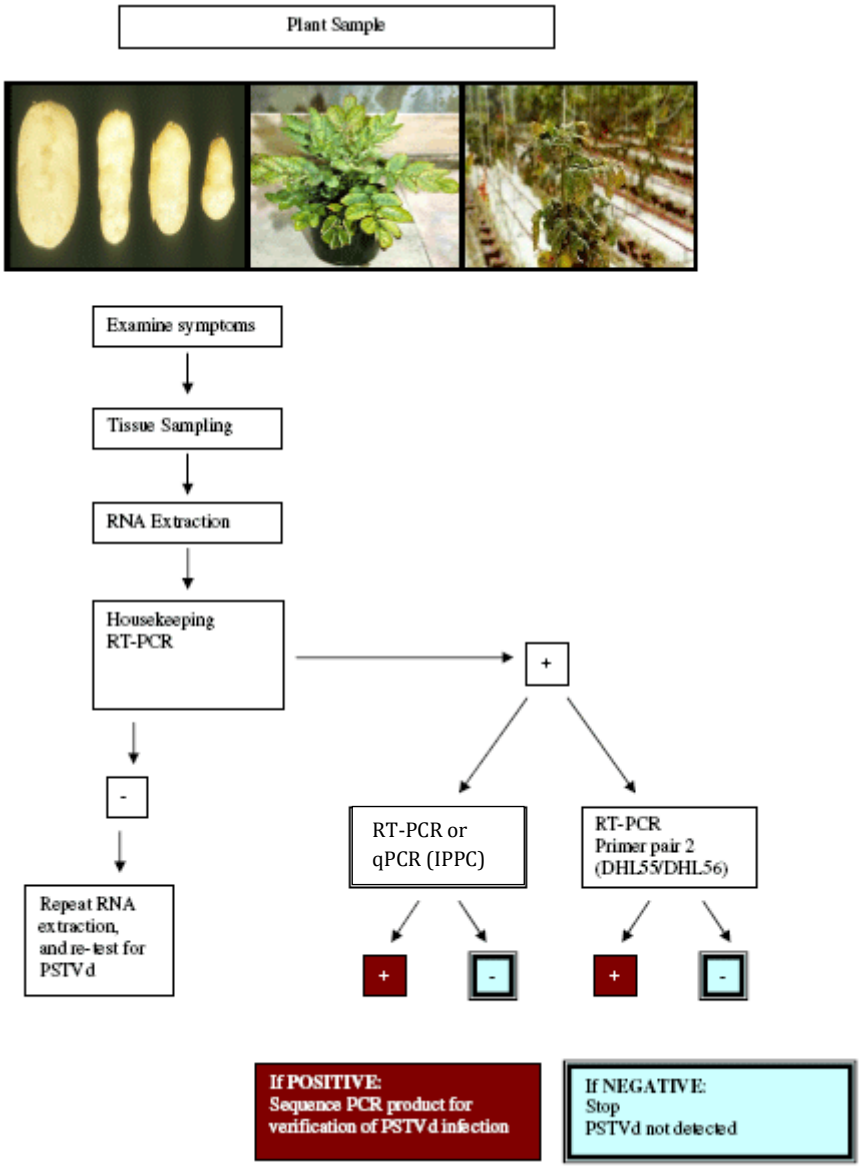

Figure 16. Diagnostic flow chart for PSTVd.

4.1 Molecular methods

The methods listed in this protocol are still valid, however the IPPC protocol (ISPM 27- DP7) recommend the use of different primers for the detection of PSTVd by conventional PCR and for internal control. These include Pospi1 & Vid primers of Verhoven et al., (2004- Eur J Pl Path 110: 823-831); or alternatively use of the Shamloul et al (1997- Can J Pl Path 19:89-96) primers in combination with Vid primers. The Nad5 primers from Menzel et al (2002- J Virol Meth 99: 81-92) (or COX primers) are recommended as internal control assay in ISPM 27 DP7.

It is recommended that either the IPPC protocol be followed, or a combination of the IPPC (RT-qPCR or Verhoeven et al 2004) and DHL primers outlined below.

Other methods from V1 of this NDP are now in the appendix.

4.2 Extraction of total RNA

Laboratory standard protocols can be used to extract total RNA [e.g. Rneasy® Plant Mini Kit (Qiagen)]

4.3 Reverse transcription polymerase chain reaction (RT-PCR) using DHL primers

Primer pair DLH55 (F)/DLH56(R). The DHL55-DLH56 primer pair anneals to a different region of the PSTVd genome compared to the TG21-CT20 primer pair. DHL55(F)/DLH56(R) are designed to amplify a 354 base pair region of the genome (Hailstones et al., 2003).

Equipment required

1. 0-2 µl, 2-20 µl, 20-200 µl, and 200-1000 µl pipettes and sterile tips
2. 0.2 or 0.5 ml sterile PCR tubes
3. Bulb spinner or centrifuge
4. Disposable gloves
5. Freezer
6. Gel electrophoresis tanks, rigs and racks
7. DNA Molecular Weight markers
8. Ice
9. Leather gardening gloves or oven-mitt
10. Sterile microcentrifuge tubes to store reagents
11. Microwave
12. Power pack
13. Thermocycler
14. UV trans illuminator with camera

Reagents

1. Primers

The primer is used at a concentration of 10 µM (Table 1).

Table 1 DHL Primer required for the detection of PSTVd (Hailstones et al 2003

Primer	Sequence (5'-3')	Anneal temp.	PCR product size
DHL55(F)[2]	GGGGAAACCTGGAGCGAAC	55°C	354 bp
DLH56(R)[2]	CCTGAAGCGCTCCTCCGAGC		

2. PCR controls

1. Positive control - RNA extract from plant tissue infected with PSTVd. Alternatively a "plasmid control" that has the PSTVd genome insert cloned into the plasmid (pGEMT).
2. Negative plant control - RNA extract from uninfected plant tissue, preferrably of the same species as that used for the positive control.
3. Negative buffer control - an aliquot of the PCR Master Mix without template.
4. The inclusion of a plant internal control (primers MDH-H968/MDH-C1163), is recommended to eliminate the possibility of false negative results. Amplification of a band of the correct size (196 bp) confirms that RNA has been successfully extracted and that the RNA is PCR competent. Failure to produce an amplicon of expected size indicates extraction failure, nucleic acid degradation or the presence of PCR inhibitors. In this case, RNA would need to be either re-extracted or diluted to dilute out inhibitors.

3. 5x TBE buffer

	Per litre
Tris $H_2NC(CH_2OH)_3$ (Merck #103157P)	54.0 g
Boric acid H_3BO_3 (Sigma # 11611)	27.5 g
0.5 M EDTA ($C_{10}H_{16}N_2O_8$)	20 mL

Store at room temperature.

4. 2% Agarose gel with ethidium bromide

2.0 g DNA grade agarose (Invitrogen #10975-035) per 100 ml 1 x TBE with 30 µl ethidium bromide (1 mg/ml) melted. Store at room temperature.

Note: *New alternative chemicals such as SYBR Safe may used as an alternative to ethidium bromide but have not been verified in this protocol.*

5. 100x TE buffer

	Per 100 ml
Tris-Cl pH 8.0	50 ml
0.5M EDTA pH 8.0 (Sigma # 43,178-8)	20 ml

Store at room temperature.

6. 6x loading dye

	Per 100 ml
1xTE	10 ml
Glycerol (Sigma 200-289-5)	50 ml
Bromophenol blue (Sigma 263-653-2)	trace (0.2%)

Store at room temperature.

Method - One-step RT-PCR detection of PSTVd

Note: *this procedure was written for the SuperScript III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase" kit (Invitrogen™, Cat No. 12574-026). Parameters may vary if using a different kit.*

Use one-step RT-PCR reagents as specified by the manufacturer.

1. Label sterile PCR tubes
2. Prepare "Master Mix" on ice in a sterile microcentrifuge tube. A standard 25 µl one-step RT-PCR reaction usually contains:

   12.5 µl 2x buffer
   1.25 µl Forward primer (10µM)
   1.25 µl Reverse primer (10µM)
   0.5 µl RT/Taq
   8.5 µl Nuclease-free water
   (1 µl RNA extract - Template)
   To prepare the "Master Mix", multiply the above volumes by the number of samples (including controls) to be tested. Add 24 µl of Master Mix to each PCR tube.
3. Add 1 µl of each template (total RNA extract) to each corresponding PCR tube.
4. Cycle the tubes with the following PCR conditions: 1 cycle [48°C for 45 min], 1 cycle [94°C for 2 min], 35 cycles [92°C for 30 secs, Annealing temperature as in Table 1 for 30 secs, 72°C for 30 sec], 1 cycle [72°C for 5 mins] and 15°C hold.
5. Mix 10 µl each PCR sample with 2 µl of 6x gel loading dye.
6. Load samples onto a 2% agarose gel containing Ethidium bromide.
7. Electrophorese in 0.5 X TBE at 100V for 45 minutes or until the Bromophenol blue front has migrated half way down the length of the gel.
8. Visualise and photograph gel on UV transilluminator.

Method - Two-step RT-PCR detection of PSTVd

Use two-step RT-PCR reagents as specified by the manufacturer.

Note: *this procedure was written for the Invitrogen RT-Taq kit. Parameters may vary if using a different kit.*

Step 1

1. Label sterile PCR tubes
2. On ice, to each tube add 8 µl H_2O, 2 µl Reverse primer ((R) in Table 1), 1 µl RNA template (do not forget to include the three controls).
3. Heat PCR tubes at 94°C for 5 mins
4. Place tubes into ice immediately.

Step 2

1. Prepare "RT Master Mix" on ice in a sterile microcentrifuge tube. A standard RT Master Mix contains:
    - 4 µl 5x buffer
    - 2 µl DTT
    - 1 µl dNTPs
    - 1 µl Rnasin
    - 1 µl Superscript

    Multiply volumes by number of samples (including controls) to be tested.
2. Add 9 µl of Master Mix to each PCR tube used in Step 1.
3. Heat PCR tubes at 42°C for 50 mins.
4. Store tubes on ice while preparing PCR reaction mix or store tubes indefinitely at -20°C.

Step 3

1. Prepare "PCR Master Mix" on ice in a sterile microcentrifuge tube. A standard 25 µl PCR reaction usually contains:

    - 13.5 µl H_2O
    - 2.5 µl 10x buffer
    - 1.5 µl $MgCl_2$
    - 2.5 µl dNTPs
    - 2 µL Forward primer (10 µM)
    - 2 µl Reverse primer (10 µM)
    - 0.1 µl HotStart Taq

2. Multiply volumes by number of samples (including controls) to be tested.
3. Add 24 µl of Master Mix to each PCR tube.
4. Add 1 µl of each reaction from Step 2 to each corresponding PCR tube.
5. Cycle the tubes with the following PCR conditions: 1 cycle [92°C for 2 min], 35 cycles [92°C for 1 min, Annealing temperature as in Table 1 for 1 min, 72°C for 1 min], 1 cycle [72°C for 5 mins] and 15°C hold.
6. Mix 10 µl each PCR sample with 2 µl of 6X loading dye.
7. Load samples onto a 2% agarose gel containing Ethidium bromide.
8. Electrophorese in 1 X TBE at 100V.
9. Visualise and photograph gel on UV transilluminator.

4.3.1 .DNA Sequencing of PCR Products

Sequence DNA of PCR products using standard protocols or outsource to a sequencing laboratory that uses established protocols.

5 CONTACTS FOR FURTHER INFORMATION

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8 APPENDIX

8.1 DNA methods from NDP7 V1.

8.1.1 Total RNA extraction from fleshy plant material

Equipment required

1. 2-20 µl, 20-200 µl, and 200-1000 µl pipette and sterile tips
2. Autoclave
3. Autoclave bags
4. Balance (at least 2 decimal places)
5. Disposable gloves
6. Microcentrifuge
7. Sterile microcentrifuge tubes
8. Paper towel
9. Sharps container
10. Sterile scalpel blades and scalpel blade handle
11. Waterbath or heatblock set at 70^{o}C
12. Weighboats

ALSO

- Autoclaved mortar and pestle
- Fume hood
- Sterile sand

OR

- Fastprep homogeniser (Bio101)
- Glass beads <5 mm in diameter
- Screw cap tubes and lids

Reagents required

1. MacKenzie buffer - from MacKenzie *et al*. (1997)

Chemical used	Amount	Final Conc.	Supplier	Catalogue No.
Guanidine thiocyanate ($CH_5N_3 \cdot CHNS$)	23.64 g	4 M	SIGMA	G9277
3M Sodium acetate ($C_2H_3NaO_2$)	3.33 ml	0.2 M	SIGMA	229873
0.5M EDTA ($C_{10}H_{16}N_2O_8$)	2.5 ml	25 mM	MERCK	108417
PVP-40 (Polyvinylpyrrolidone)	1.25 g	2.5% (w/v)	SIGMA	PVP40T

Add sterile distilled water to final volume of 50 ml, store at room temperature

Fresh MacKenzie buffer should be prepared every 3-6 months.

2. β-mercaptoethanol (C_2H_6OS)
3. 20%N-Lauroylsarcosine solution (w/v)
4. RNeasy Plant Mini Kit (Qiagen Cat. No. 74904)
5. 100% Ethanol

Method

The RNA extraction method is based on that described by MacKenzie *et al*. (1997). All steps are carried out at room temperature:

1a. If using a mortar and pestle to homogenise samples:

1. Determine the number of samples and label plastic tubes accordingly.
2. Use new clean gloves and scalpel blades for each sample.
3. Cut each new sample on fresh paper towel on the bench.
4. Weigh out 400 mg of plant sample.
5. Place sample in mortar.
6. Add 1980 µl of MacKenzie buffer.
7. Add 20 µl of β-mercaptoethanol in the fumehood.

Note. *If a fume hood is unavailable β – mercaptoethanol can be omitted but the quality of the extract from some plant species may be affected.*

8. Homogenise in fume hood.
9. Pipette 1.0 ml of the mixture into a labelled microcentrifuge tube (you may need to cut the end of the pipette tip if the slurry is too thick).
10. Continue to step 2.

Note: *"Homex" grinder (Bioreba) and grinding bags (Agdia or Bioreba) or hammer and grinding bags (Agdia or Bioreba) may be used and have been validated in ISPM27 DP7*

1b. If using the Fastprep homogeniser:

1. Determine the number of samples and label plastic screw cap tubes accordingly.
2. Place 2 glass beads in each tube.
3. Use new clean gloves and scalpel blades for each sample.
4. Cut each new sample on fresh paper towel on the bench.
5. Weigh out 175-200 mg of plant sample and place sample in appropriate tube.
6. Add 990 µl of MacKenzie buffer.
7. Add 10 µl of β-mercaptoethanol in the fumehood.

Note. *If a fume hood is unavailable β – mercaptoethanol can be omitted but the quality of the extract from some plant species may be affected.*

8. Close tubes.
9. Place tubes in Fastprep homogeniser, set speed at 4.0 and run 3 times for 20 seconds, checking tubes in-between each run to make sure that tubes are not leaking or hot.
10. Continue to step 2.

2. Carefully read the RNeasy® Mini Handbook.

3. Add 100 µl of 20% Sarkosyl to each tube and mix.

4. Incubate tubes at 70°C for 10 minutes.

5. Spin tubes in microcentrifuge for 1 minute at 13,000 rpm.

6. Continue with step 4 of the "Rneasy Mini Protocol for Isolation of Total RNA from Plant Cells and Tissues and Filamentous Fungi" on page 75 of the RNeasy® Mini Handbook and follow as per manufacturer's instructions.

8.1.2 Detection of Potato spindle tuber viroid in total RNA extracts using reverse transcription polymerase chain reaction (RT-PCR).

For the reliable detection of PSTVd, total RNA extracts should be assayed using three PCR tests. The three PCR tests to be run are;

- **Primer pair TG21 (F)/CT20(R)**. Detect the pathogen using the primer pair TG21(F)/CT20(R) that will amplify a 258 base pair region of the PSTVd genome (Constable and Moran, 1996).
- **Primer pair DLH55 (F)/DLH56(R)**. The DHL55-DLH56 primer pair anneals to a different region of the PSTVd genome compared to the TG21-CT20 primer pair. DHL55(F)/DLH56(R) are designed to amplify a 354 base pair region of the genome (Hailstones et al., 2003).
- **Internal control gene (Primer pair MDH-H968(F)/MDH-C1163(R))**. The MDHH968/MDH-C1163 primer pair are designed to amplify a 196 bp region of the plant mRNA encoding malate dehydrogenase (MDH) gene. This plant gene is highly conserved among plants and PCR amplification of the MDH mRNA is used as an internal PCR control to a) determine the quality of the RNA extract and b) determine whether the RNA extract contains inhibitors that will interfere with the activity of the reverse transcriptase and Taq DNA polymerase enzymes. This PCR is particularly important when confirming the absence of PSTVd in the test sample.

It is necessary to use two sets of PSTVd specific primer pairs to confirm the presence of PSTVd as there are no other convenient means of confirming the presence of PSTVd. However, dot blot hybridisation assays using a PSTVd specific cDNA probe could be an alternative test, but we believe it is simpler to use two primer sets that anneal to different regions of the viroid genome.

This PCR protocol for the detection of PSTVd will require three separate PCR tests, using the three pairs of primers.

Equipment required

15. 0-2 µl, 2-20 µl, 20-200 µl, and 200-1000 µl pipettes and sterile tips
16. 0.2 or 0.5 ml sterile PCR tubes
17. Bulb spinner or centrifuge
18. Disposable gloves
19. Freezer
20. Gel electrophoresis tanks, rigs and racks
21. DNA Molecular Weight markers
22. Ice
23. Leather gardening gloves or oven-mitt
24. Sterile microcentrifuge tubes to store reagents
25. Microwave
26. Power pack
27. Thermocycler
28. UV transilluminator with camera

Reagents

1. Primers

For the detection of PSTVd three specific primers sets need to be used (Table 1). Each primer is used at a concentration of 10 μM.

Table 2 Primers required for the detection of PSTVd

Primer	Sequence (5'-3')	Anneal temp.	PCR product size
TG21(F)[1]	TGTGGTTCACACCTGACCTCC	55°C	258 bp
CT20(R)[1]	CTTCAGTTGTTTCCACCGGG		
DHL55(F)[2]	GGGGAAACCTGGAGCGAAC	55°C	354 bp
DLH56(R)[2]	CCTGAAGCGCTCCTCCGAGC		
MDH-C1163(F)[3]	GCATCTGTGGTTCTTGCAGG	54°C	196 bp
MDH-H968(R)[3]	CCTTTGAGTCCACAAGCCAA		

1. Constable and Moran (1996), 2. Hailstones *et al.* (2003), 3. Nassuth *et al*. (2000).

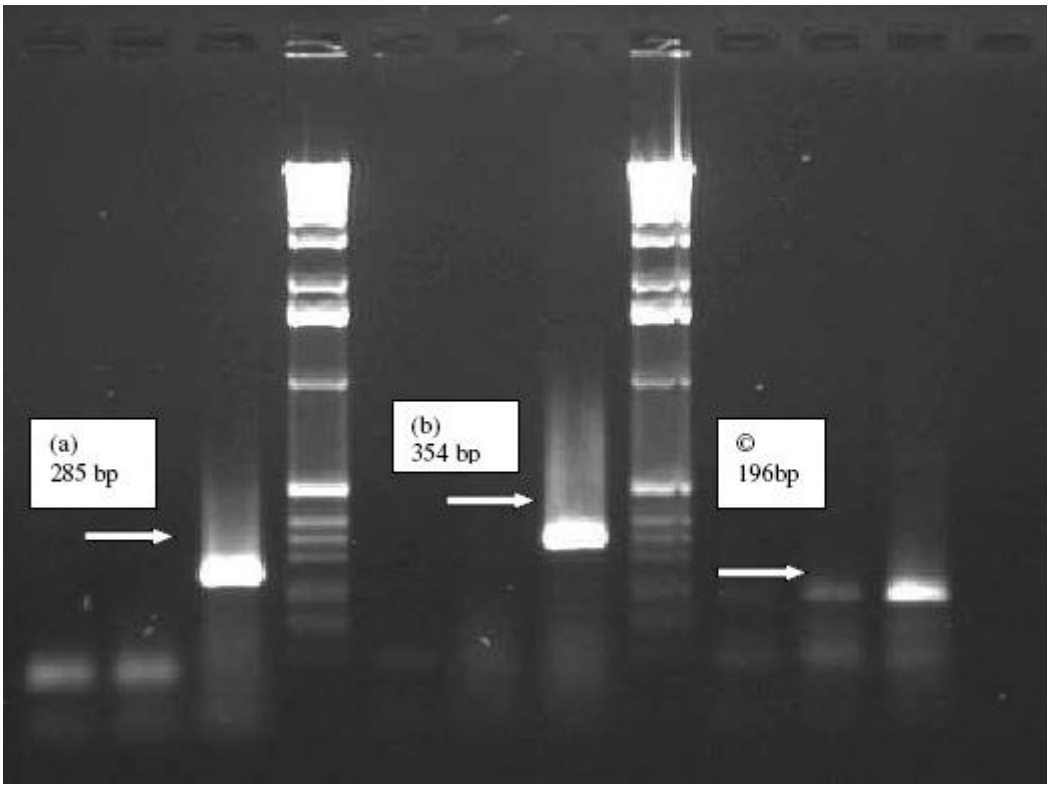

Figure 17. An electrophoresis gel showing bands generated by the three sets of primers used for the detection of PSTVd. This gel has a buffer, healthy and positive control from left to right for each of the three primer sets (a,b). Each reaction is separated by a DNA molecular weight marker X, 0.07-12.2 kbp, (a) 258 bp band as generated by the primer pair TG21/CT20, (b) 354 bp band as generated by the primer pair DHL55/DHL56, (c) 196 bp band as generated by the primer pair MDH-C1163/MDH-H968.

2. PCR controls

5. Positive control - RNA extract from plant tissue infected with PSTVd. Alternatively a "plasmid control" that has the PSTVd genome insert cloned into the plasmid (pGEMT).
6. Negative plant control - RNA extract from uninfected plant tissue of the same species as that used for the positive control.
7. Negative buffer control - an aliquot of the PCR Master Mix without template.
8. The inclusion of a plant internal control (primers MDH-H968/MDH-C1163), is recommended to eliminate the possibility of false negative results. Amplification of a band of the correct size (196 bp) confirms that RNA has been successfully extracted and that the RNA is PCR competent. Failure to produce an amplicon of expected size indicates extraction failure, nucleic acid degradation or the presence of PCR inhibitors. In this case, RNA would need to be either re-extracted or diluted to dilute out inhibitors.

3. 5x TBE buffer

	Per litre
Tris $H_2NC(CH_2OH)_3$ (Merck #103157P)	54.0 g
Boric acid H_3BO_3 (Sigma # 11611)	27.5 g
0.5 M EDTA ($C_{10}H_{16}N_2O_8$)	20 mL

Store at room temperature.

4. 2% Agarose gel with ethidium bromide

2.0 g DNA grade agarose (Invitrogen #10975-035) per 100 ml 1 x TBE with 30 µl ethidium bromide (1 mg/ml) melted. Store at room temperature.

Note: *New alternative chemicals such as SYBR Safe may used as an alternative to ethidium bromide but have not been verified in this protocol.*

5. 100x TE buffer

	Per 100 ml
Tris-Cl pH 8.0	50 ml
0.5M EDTA pH 8.0 (Sigma # 43,178-8)	20 ml

Store at room temperature.

6. 6x loading dye

	Per 100 ml
1xTE	10 ml
Glycerol (Sigma 200-289-5)	50 ml
Bromophenol blue (Sigma 263-653-2)	trace (0.2%)

Store at room temperature.

Method - One-step RT-PCR detection of PSTVd

Note: *this procedure was written for the SuperScript III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase" kit (Invitrogen™, Cat No. 12574-026). Parameters may vary if using a different kit.*

This method is to be repeated for each set of the three sets of primers used. Use one-step RT-PCR reagents as specified by the manufacturer.

9. Label sterile PCR tubes
10. Prepare "Master Mix" on ice in a sterile microcentrifuge tube. A standard 25 µl one-step RT-PCR reaction usually contains:

    12.5 µl 2x buffer
    1.25 µl Forward primer (10µM)
    1.25 µl Reverse primer (10µM)
    0.5 µl RT/Taq
    8.5 µl Nuclease-free water
    (1 µl RNA extract - Template)
    To prepare the "Master Mix", multiply the above volumes by the number of samples (including controls) to be tested. Add 24 µl of Master Mix to each PCR tube.

11. Add 1 µl of each template (total RNA extract) to each corresponding PCR tube.
12. Cycle the tubes with the following PCR conditions: 1 cycle [48°C for 45 min], 1 cycle [94°C for 2 min], 35 cycles [92°C for 30 secs, Annealing temperature as in Table 1 for 30 secs, 72°C for 30 sec], 1 cycle [72°C for 5 mins] and 15°C hold.
13. Mix 10 µl each PCR sample with 2 µl of 6x gel loading dye.
14. Load samples onto a 2% agarose gel containing Ethidium bromide.
15. Electrophorese in 0.5 X TBE at 100V for 45 minutes or until the Bromophenol blue front has migrated half way down the length of the gel.
16. Visualise and photograph gel on UV transilluminator.

Method - Two-step RT-PCR detection of PSTVd

This method is to be used for each set of primers, to run the three PCR tests necessary for an accurate and valid diagnosis of PSTVd. Use two-step RT-PCR reagents as specified by the manufacturer.

Note: *this procedure was written for the Invitrogen RT-Taq kit. Parameters may vary if using a different kit.*

<u>Step 1</u>

5. Label sterile PCR tubes
6. On ice, to each tube add 8 µl H_2O, 2 µl Reverse primer ((R) in Table 1), 1 µl RNA template (do not forget to include the three controls).
7. Heat PCR tubes at 94°C for 5 mins
8. Place tubes into ice immediately.

Step 2

2. Prepare "RT Master Mix" on ice in a sterile microcentrifuge tube. A standard RT Master Mix contains:
    - 4 µl 5x buffer
    - 2 µl DTT
    - 1 µl dNTPs
    - 1 µl Rnasin
    - 1 µl Superscript

    Multiply volumes by number of samples (including controls) to be tested.

5. Add 9 µl of Master Mix to each PCR tube used in Step 1.
6. Heat PCR tubes at 42°C for 50 mins.
7. Store tubes on ice while preparing PCR reaction mix or store tubes indefinitely at -20°C.

Step 3

10. Prepare "PCR Master Mix" on ice in a sterile microcentrifuge tube. A standard 25 µl PCR reaction usually contains:

    - 13.5 µl H_2O
    - 2.5 µl 10x buffer
    - 1.5 µl $MgCl_2$
    - 2.5 µl dNTPs
    - 2 µL Forward primer (10 µM)
    - 2 µl Reverse primer (10 µM)
    - 0.1 µl HotStart Taq

11. Multiply volumes by number of samples (including controls) to be tested.
12. Add 24 µl of Master Mix to each PCR tube.
13. Add 1 µl of each reaction from Step 2 to each corresponding PCR tube.
14. Cycle the tubes with the following PCR conditions: 1 cycle [92°C for 2 min], 35 cycles [92°C for 1 min, Annealing temperature as in Table 1 for 1 min, 72°C for 1 min], 1 cycle [72°C for 5 mins] and 15°C hold.
15. Mix 10 µl each PCR sample with 2 µl of 6X loading dye.
16. Load samples onto a 2% agarose gel containing Ethidium bromide.
17. Electrophorese in 1 X TBE at 100V.
18. Visualise and photograph gel on UV transilluminator.