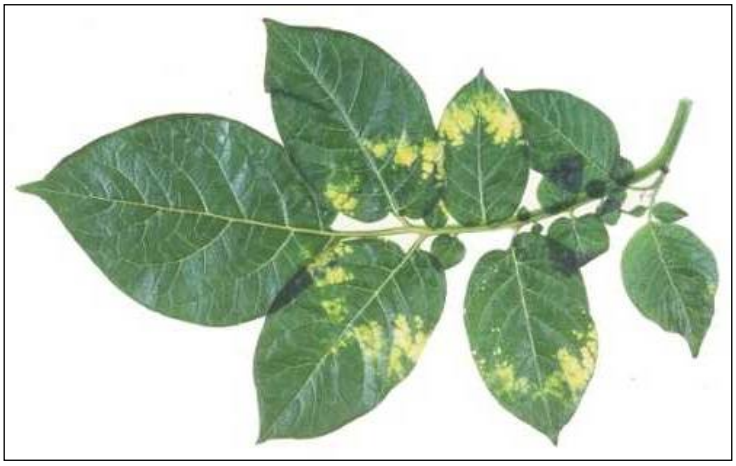
National Diagnostic Protocol for Potato Mop-Top Virus



PEST STATUS	Not present in Australia
PROTOCOL NUMBER	NDP 15
VERSION NUMBER	V1.2
PROTOCOL STATUS	Endorsed
ISSUE DATE	November 2011
REVIEW DATE	2016
ISSUED BY	SPHDS

Australian Government
Department of Agriculture

Prepared for the Subcommittee on Plant Health Diagnostic Standards (SPHDS)

This version of the National Diagnostic Protocol (NDP) for Potato mop top virus is current as at the date contained in the version control box on the front of this document.

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

The most current version of this document is available from the SPHDS website:
http://plantbiosecuritydiagnostics.net.au/resource-hub/priority-pest-diagnostic-resources/

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1 Introduction

PMTV produces internal and external potato tuber symptoms, seriously affecting tuber quality and reducing yield (Germundsson *et al.*, 2002). Infection with PMTV causes some quantitative yield loss but the qualitative losses are more important and can lead to total crop rejection by supermarkets and processors (Jones and Harrison, 1972; Mumford *et al.*, 2000).

1.1 Host range

PMTV affects potato (*Solanum tuberosum*).

Weeds from the Chenopodiaceae and Solanaceae are susceptible (*ICTVdB - The Universal Virus Database*, version 4. http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/, Anderson *et al* 2002).

1.2 Transmission

The plasmodiophoromycete *Spongospora subterranea* f. sp. *subterranea* is the only known vector of PMTV (Helias *et al*., 2003; Sokmen *et al.*, 1998). *S. subterranea* transmits the virus to potato roots via viruliferous fungal zoospores (Sokmen *et al.*, 1998). The vector of PMTV is also the causal agent of powdery scab, and has been associated with potato production since the mid-1800s (Büchen-Osmond, 2001 onwards). PMTV can persist in the soil for many years in the resting spores of its vector (Helias *et al*., 2003), and soils therefore can remain infective for extended periods of time (Sokmen *et al.*, 1998). It has been shown that PMTV can persist for at least two years in the resting spores of *S. subterranea* and, PMTV was also detected in field soil 12 years after the last potato crop had been grown in that soil (Jones and Harrison, 1972). Soil type does not influence the occurrence of PMTV, as the virus has been detected in soil types ranging from light sands to heavy loams (Jones and Harrison, 1972).

A number of plant families are susceptible to infection by powdery scab. Under greenhouse conditions plant species within the *Solanaceae*, *Chenopodiaceae* and *Cruciferae* were found to be hosts of *S. subterranea* (Jones and Harrison, 1972). However, Johnson (2004) reported that only potato and black nightshade (*Solanum nigrum*) produced long-lived fungal resting spores of *S. subterranea*.

2 Taxonomic Information

PMTV is the type member of the *Pomovirus* genus (Büchen-Osmond, 2001 onwards; Cerovska *et al*., 2003; Tidona and Darai, 2002), and has straight, tubular, rigid particles, measuring 18-20 nm in diameter and 100-300 nm in length (Figure 1) (Cerovska *et al*., 2003; Tidona and Darai, 2002). Discrepancies in the reported length of the viral particle are most probably due to the fragility of the particles, which readily disintegrate (Cerovska *et al*., 2003). PMTV occurs in the hosts' cytoplasm (Tidona and Darai, 2002), and is unevenly distributed and present at low concentration in infected tissues (Helias *et al*., 2003).

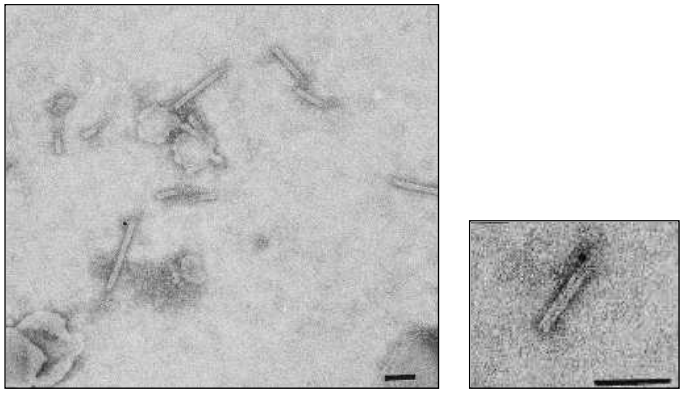


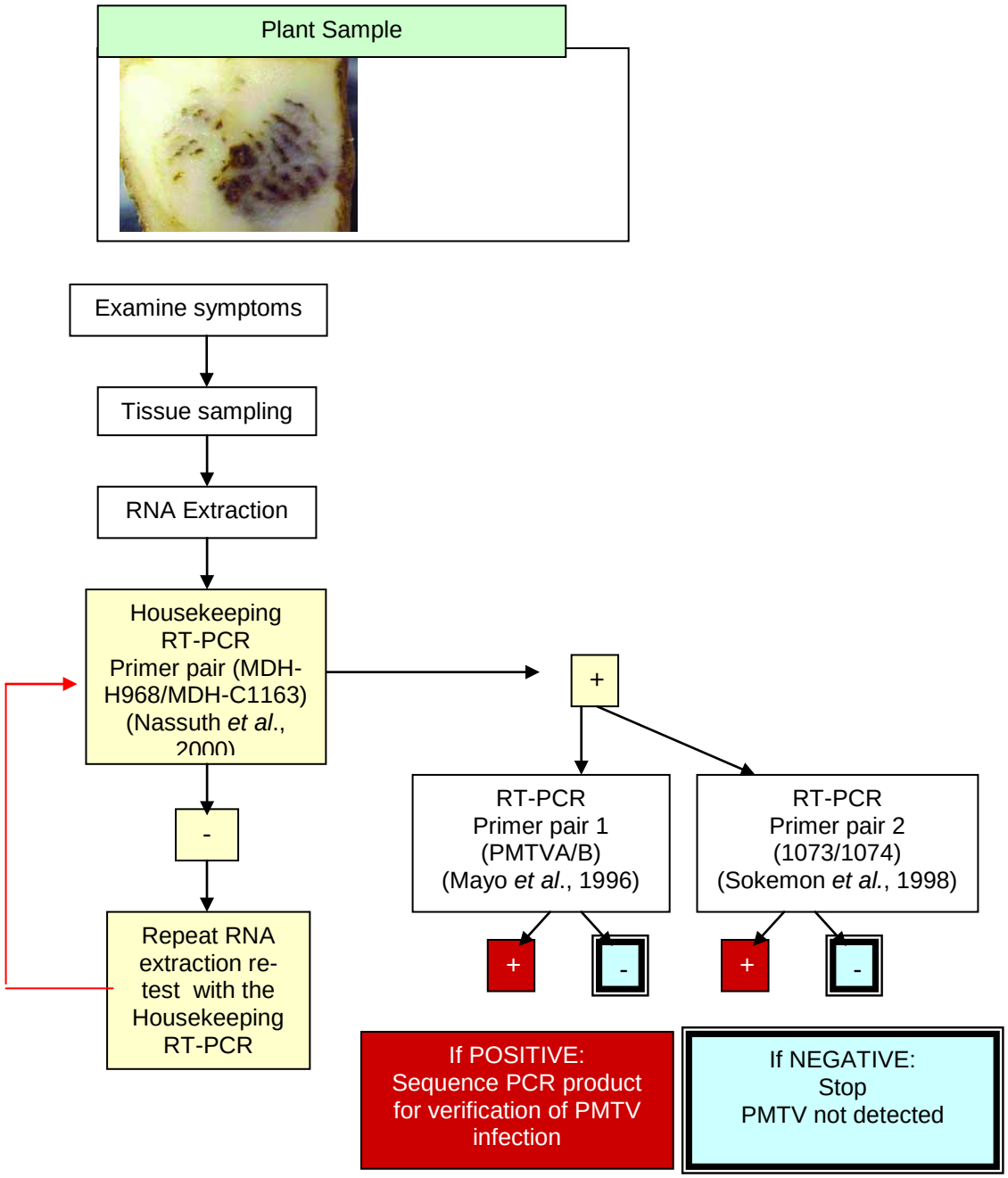
Figure 1. PMTV particles (left), uncoiling of the PMTV particle (right). In each electron micrograph, the bar represents 100 nm (Cowan *et al*., 1997).

The PMTV genome consists of three positive-sense, single-stranded RNA molecules; RNA1 (6 kb), RNA2 (3.2 kb) and RNA3 (2.5 kb) (Helias *et al*., 2003; Mayo *et al.*, 1996; Sokmen *et al.*, 1998; Tidona and Darai, 2002). RNA 1 encodes a methyltransferase, a helicase, and the viral RNA dependent RNA polymerase. RNA 2 encodes the triple gene block and an 8K, cysteine-rich protein of unknown function. RNA 3 encodes coat protein and readthrough protein (Nielsen and Nicolaisen, 2003). Comparisons of the PMTV coat protein nucleotide sequence of various isolates has shown that the coat protein open reading frame is highly conserved (Helias *et al*., 2003).

3 Detection

Please note, that the PMTV detection system outlined in this manual is based on the RT-PCR protocols published by Sokmen *et al.* (1998) and Mayo *et al.* (1996).

3.1 Diagnostic flow chart



3.2 Symptom description

PMTV symptoms on potato include 'mop-top' stunting of foliage (Figure 2), yellow blotching (Figure 3) and yellow V-shaped markings (Figure 4) on potato leaves, and sporadic shortening of internodes (Figure 5) (Calvert, 1968; Mayo *et al.*, 1996; Scott *et al.*, 1994).

PMTV causes a wide range of symptoms in potato haulms (stems) and tubers depending on viral strain, environmental conditions and cultivar susceptibility (Kurppa, 1989). Studies have shown that PMTV symptom expression can vary in the same potato variety, and several virus isolates differing in virulence have been reported (Harrison and Jones, 1970; Mayo *et al.*, 1996; Nielsen and Nicolaisen, 2003).

Tubers infected with PMTV often display rust coloured, necrotic arcs, flecks, rings or lines through the tuber flesh (Figure 6, 11, 12, 13). The brown necroses often penetrate into the tuber's flesh almost without interruption (Figure 14). These symptoms are known as "spraing", or "corky ring-spot". Spraing symptoms (brown lines and arcs in the tuber flesh) in susceptible cultivars are more prevalent in cool weather (around 15^{o}C) (Sokmen *et al.*, 1998). External symptoms include cracking (Figure 7, 8), raised necrotic rings (Figure 9) and raised necrotic lines (Figure 10). Tuber symptoms may be absent at harvest, but can develop during storage. Visual diagnosis based on spraying induced by PMTV is difficult as *Tobacco rattle virus* (TRV), *Potato virus Y* NTN strain (PVY^{NTN}) and internal rust spot can also induce similar symptoms (Figures 14-17).

Figure 2. Healthy potato haulm on the left and PMTV infected potato on right showing "mop top" symptoms (© Scottish Agricultural Science Agency).

Figure 3. Yellow blotching on leaves (© Scottish Agricultural Science Agency).

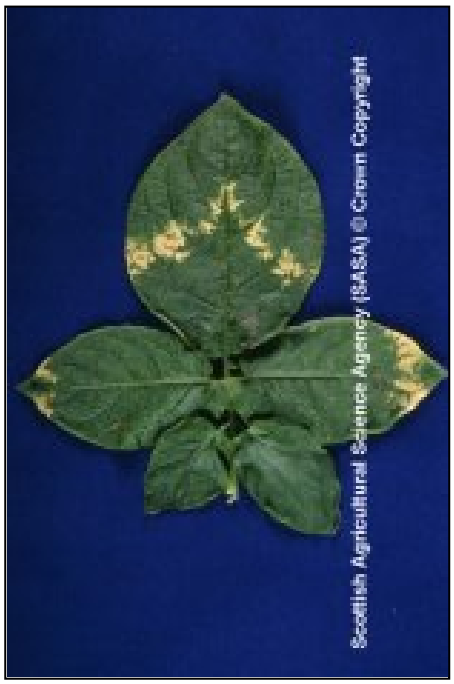


Figure 4. Chlorotic V-shaped markings in the leaflets (© Scottish Agricultural Science Agency)

Figure 5. Sporadic shortening of internodes (source: http://www.unece.org/trade/agr/standard/potatoes/PestPicturesE.htm).

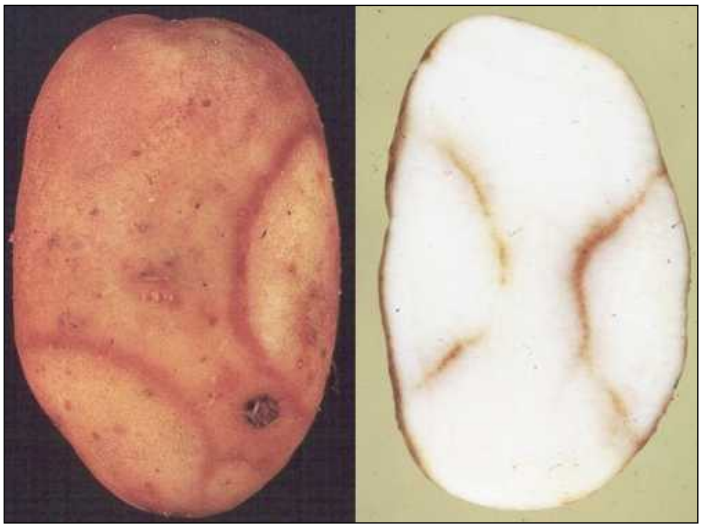


Figure 6. External (left) and internal (right) PMTV spraing symptoms in naturally infected potato tubers cv. Arran Pilot (© Scottish Agricultural Science Agency).

Figure 7. Cracked and distorted naturally infected tubers of potato cv. Alpha produced in second year of infection (© Scottish Agricultural Science Agency).

Figure 8. Tuber cracking and scaring (© Scottish Agricultural Science Agency).

Figure 9. Raised necrotic rings on tuber (© Scottish Agricultural Science Agency).

Figure 10. Raised necrotic lines (© Scottish Agricultural Science Agency).

Figure 11. Multiple necrotic concentric arcs in tuber flesh (© Johnson, University of Maine).

Figure 12. Necrotic arcs in tuber flesh (© Johnson, University of Maine).

Figure 13. Necrotic arcs on potato crisps, UK (Source: Rodoni, DPI Victoria).

3.2.1 Diseases causing symptoms similar to *Potato mop-top virus*

There are several viruses which produce symptoms similar to PMTV. *Tobacco rattle virus* (TRV) can cause yellow blotches on leaves (Figure 14) and necrotic arcs in tubers (Figure 15) similar to PMTV. The tuber necrosis strain of *Potato virus Y* (PVY^{NTN}) causes necrotic rings to appear on potato tubers (Figure 16). The physiological disorder, "internal rust spot" can also cause tuber symptoms (Figure 17) easily confused with PMTV.

Figure 14. Potato showing leaf yellowing symptoms of infection by TRV (Source: British potato council).

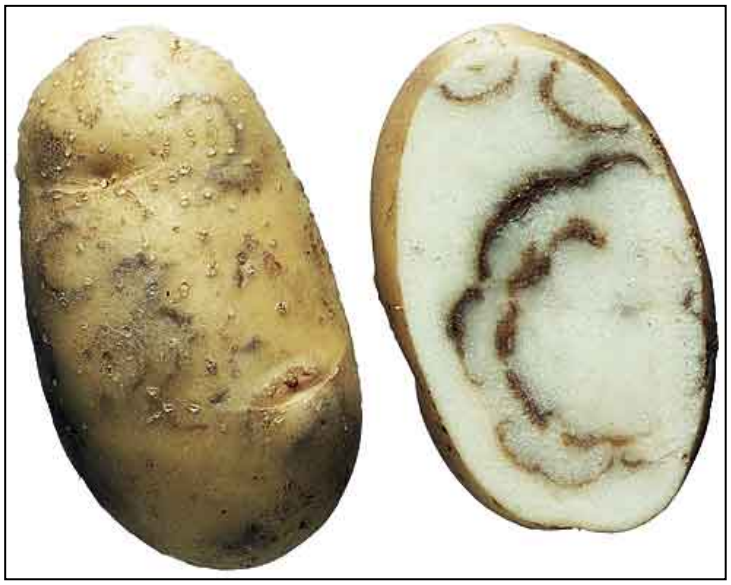


Figure 1. Potato tuber showing necrotic arcs in the flesh caused by infection by TRV (Source: British potato council, http://www.potato.org.uk/department/knowledge_transfer/pests_and_diseases/ref.html?item=27.)

Figure 2. Tubers infected with Potato Tuber Necrotic Ring Spot Disease (PVY^{NTN})(Source: Dr Brendan Rodoni, DPI-Victoria).

Figure 17. Tuber symptoms caused by internal rust spot (Source: British potato council).

3.3 Sample Collection

Leaf and tuber samples should not have free water on their surfaces. Samples should not be collected during hot weather and all samples should be cooled and transported to a testing facility in a cool environment if possible.

3.3.1 Leaf sampling

Record plant symptoms. Older symptomatic leaves around the middle of the haulm (stem) (Figure 3, 4, 6), and leaves showing unusual symptoms must be sampled. Store plant material at 4^{o}C or at –20^{o}C until required.

3.3.2 Tuber sampling

Tubers removed from cold storage should be incubated at 20^{o}C for four weeks prior to testing. This incubation has shown to double the sensitivity of detection (Sokmen *et al.*, 1998). If fresh tubers are used negative test results should be treated with caution due to the low levels of virus in the tuber. Stolon and rose ends of the tuber (Figure 18) must be included in each sample. PMTV does not infect all the tubers produced by an infected mother plant and therefore only a variable proportion of the progeny plants generated from the tuber seed are infected with PMTV in the following year (Sokmen *et al.*, 1998).

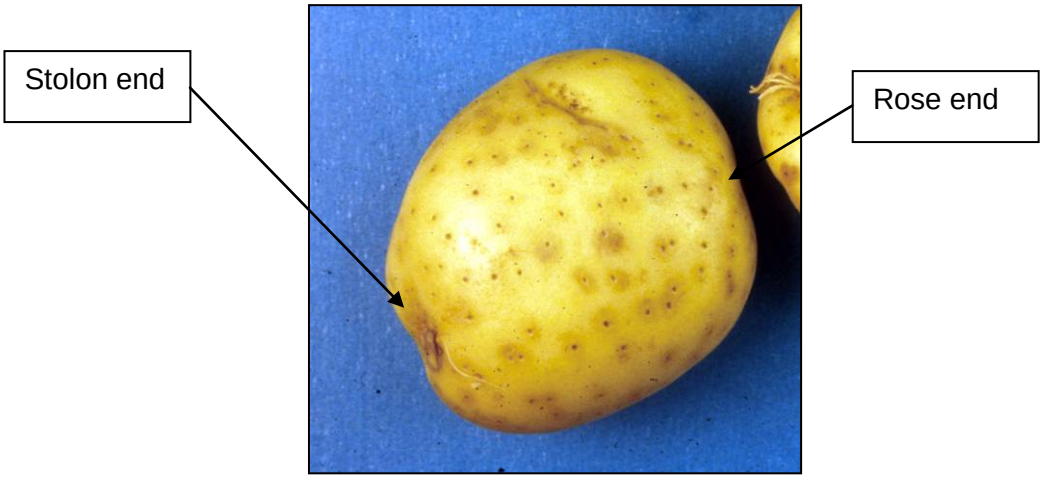


Figure 18. Stolon and rose end of tuber (Source: B Hall, SARDI)

4 Identification

4.1 Molecular methods

4.1.1 Total RNA extraction

Wear disposable gloves and a lab coat at all times. Have an autoclave bag ready to dispose of all plant material, tips, tubes, gloves and paper towel that have come into contact with any suspect plant material. A footbath containing disinfectant located at the doorway of the laboratory must be used when exiting the lab.

4.1.1.1 Equipment required

1. 2-20 μL, 20-200 μL, and 200-1000 μL micropipettes and sterile tips
2. Autoclave
3. Autoclave bags
4. Balance (at least 2 decimal places)
5. Disposable gloves
6. Disposable plastic pasteur pipettes
7. Microcentrifuge
8. RNeasy® Plant Mini Kit (Qiagen™)
9. Sterile microcentrifuge tubes
10. Paper towel
11. Sharps container
12. Sterile scalpel blades and scalpel blade handle
13. Waterbath or heatblock set at 70°C
14. Weighboats

ALSO

1a: Autoclaved mortar and pestle, Fume hood, sterile sand

OR

1b: Qiagen Tissue Lyser(Qiagen™), 2 ml snap-lock tubes, Stainless steel beads, Liquid Nitrogen

OR

1c: Homex tissue macerater, Homex bags (Bioreba AG / BioSys), Plastic disposable pasteur pipettes

4.1.1.2 Reagents required

1. MacKenzie buffer (MacKenzie *et al.,* 1997)

Chemical	Amount	Final Concentration
Guanidine thiocyanate ($CH_5N_3 \cdot CHNS$)	23.64 g	4 M
3M Sodium acetate ($C_2H_3NaO_2$)	3.33 ml	0.2 M
0.5M EDTA ($C_{10}H_{16}N_2O_8$)	2.5 ml	25 mM
PVP-40 (Polyvinylpyrrolidone)	1.25 g	2.5% (w/v)

Add sterile distilled water to final volume of 50 ml

Store at room temperature

Please note, fresh MacKenzie buffer should be prepared every 3-6 months

2. β-mercaptoethanol (C_2H_6OS)
3. 20% N-Lauroylsarcosine solution (w/v)
4. 100% Ethanol

4.1.1.3 Method

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

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

1a-1. Determine the number of samples and label plastic tubes accordingly.

1a-2. Use new clean gloves and scalpel blades for each sample.

1a-3. Cut each new sample on fresh paper towel on the bench.

1a-4. Weigh out 400 mg of plant sample (leaf or tuber).

1a-5. Place sample in mortar.

1a-6. Add 1980 µl of MacKenzie buffer.

1a-7. Add 20 µl of β-mercaptoethanol in the fumehood.

1a-8. Homogenise in fume hood.

1a-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).

1a-10. Continue to step 2.

1b. If using the Qiagen™ Tissue Lyser:

1b-1. Determine the number of samples and label the 2 ml snap-lock tubes accordingly.

1b-2. Use new clean gloves and scalpel blades for each sample.

1b-3. Cut each new sample on fresh paper towel on the bench.

1b-4. Weigh out 100 mg of plant sample (leaf or tuber) and place sample in the appropriate tube.

1b-5. Add 990 µl of MacKenzie buffer.

1b-6. Add 10 µl of β-mercaptoethanol in the fumehood.

1b-7. Close tubes.

1b-8. Place tubes in the Adaptor Set, in the Qiagen™ Tissue Lyser. Homogenise for 1 min at 30 Hz.

1b-9. Rotate tubes within the Adaptor Set, so that tubes in the centre are moved to the outside.

1b-10. Continue to step 2.

1c. If using the Homex tissue macerater:

1c-1. Determine the number of samples and label plastic tubes accordingly.

1c-2. Use new clean gloves and scalpel blades for each sample.

1c-3. Cut each new sample on fresh paper towel on the bench.

1c-4. Weigh out 200 mg of plant sample (leaf or tuber).

1c-5. Place sample in Homex bag.

1c-6. Add 1980 µl of MacKenzie buffer.

1c-7. Add 20 µl of β-mercaptoethanol in the fumehood.

1c-8. Macerate tissue with the Homex.

1c-9. With a plastic disposable pasteur pipette, transfer 1.0 ml of the mixture into a labelled microcentrifuge tube.

1c-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" in the RNeasy® Mini Handbook and follow as per manufacturer's instructions.

4.1.2 Detection of *PMTV* in total RNA extracts using one step RT-PCR

For the reliable detection of PMTV, total RNA extracts are subjected to three RT-PCR tests, as outlined below, with primer sequences and annealing temperatures listed in Table 1.

1. **Primer pair PMTVA (R) / PMTVB (R).** This primer pair will detect the pathogen by amplifying approximately 550bp of the PMTV coat protein gene on RNA 3 (Mayo *et al.*, 1996).
2. **Primer pair 1073 (F) / 1074 (R).** This primer pair anneals to RNA 2 of the PMTV genome and amplifies 283bp of the 3' end of the triple-gene-block and the 8K protein genes (Sokmen *et al*., 1998).
3. **House-keeping gene (Primer pair MDH-H968 (F) / MDH-C1163 (R)**. The MDH-H968/MDH-C1163 primer pair are designed to amplify a 196 bp region of the plant mRNA encoding malate dehydrogenase (MDH) gene (Nassuth *et al*., 2000). This gene is highly conserved among plants and therefore RT-PCR amplification of the MDH mRNA is used as an internal RT-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

4.1.2.1 Equipment required

1. 0-2 μL, 2-20 μL, 20-200 μL, & 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. Crushed ice
9. Sterile microcentrifuge tubes to store reagents
10. Microwave
11. Power pack
12. Thermocycler
13. UV transilluminator with camera

4.1.2.2 Reagents

1. Primers

For the detection of PMTV three specific primers sets are required (Table 1). Each primer is used as a stock solution at a concentration of 10 μM.

Table 1 Primers required for the detection of PMTV

Primer	Sequence (5'-3')	Target	Anneal temp.	PCR product size
PMTVA (F)[1]	CTA TGC ACC AGC CCA GCG T	PMTV RNA-3, nucleotide 268 to 801	50°C	533 bp
PMTVB (R)[1]	TCT CGG ATA CCA CCC TT			
1073 (F)[2]	AGT AGC AAG TAC GCC CTG TG	PMTV RNA-2, nucleotide 2419 to 2702	50°C	283 bp
1074 (R)[2]	CCG AAT TCC TGT AAG CAC TAA CAC			
MDH-H968(F)[3]	GCA TCT GTG GTT CTT GCA GG	mRNA encoding malate dehydogenase (MDH)	54°C	196 bp
MDH-C1163 (R)[3]	CCT TTG AGT CCA CAA GCC AA			

[1](Mayo *et al.,* 1996), [2](Sokmen *et al.,* 1998), [3](Nassuth *et al*., 2000)

2. PCR Controls

1. Positive control - RNA extract from plant tissue infected with PMTV.

   - Alternatively a "plasmid control" that has the target PMTV sequence cloned into the plasmid. (Positive controls available from author – DPI Victoria: see contacts)

2. Negative plant control - RNA extract from uninfected plant tissue of the same species as that used for the positive control.
3. Negative buffer control - an aliquot of the RT-PCR "Master Mix" without template.
4. The house keeping RT-PCR, using primers MDH-H968/MDH-C1163, reduces the risk of false negative results. The generation of a band confirms the presence of RNA in the extract, and that the RNA extract does not contain inhibitors. Failure to produce an amplicon of expected size (196 bp) indicates that either dilution of the RNA extract or re-extraction of RNA from the sample is required.

3. RT-PCR reagents

1. One-step RT-PCR kit:
   - Invitrogen® SuperScript™ One-Step RT-PCR with Platinum® Taq, Catalogue No. 12574-026, is recommended.
   - Qiagen One step RT –PCR kit (Cat. No. 210210) was successfully used in verification of protocol while following the manufacturer's instructions.,
2. Nuclease-free water

4. 5x TBE Buffer

	Per 1 litre
Tris ($C_4H_{11}NO_3$)	54 g
Boric acid (H_3BO_3)	27.5 g
0.5M EDTA ($[CH_2.N(CH_2.COOH).CH_2COONa]_2.2H_2O$) pH 8.0	20 ml

Store at room temperature.

5. 1% Agarose gel with ethidium bromide

Use a 1% DNA grade agarose (w/v) gel made with 0.5x TBE solution, and stained with 0.03 μg/ml Ethidium bromide.

6. 1x TE Buffer

	Per 100 ml
1 M Tris-HCl (pH 8.0)	1 ml
0.5 M EDTA	200 µl

Adjust pH to 8.0± 0.2. Store at room temperature.

7. 6x loading dye

	Per 100 ml
1 x TE	10 ml
Glycerol	50 ml
Bromophenol blue	0.2%

Store at room temperature.

4.1.2.3 Method

This method is to be repeated for each set of the three primer pairs listed in Table 1. Use one-step RT-PCR reagents as specified by the manufacturer. Some volumes outlined below may vary depending on the buffer and enzyme concentrations specified by the manufacturer. Ensure that the final volume of the RT-PCR reaction is 25 µl by altering the volume of nuclease-free water accordingly.

1. Label sterile PCR tubes
2. Prepare "Master Mix" on ice in a sterile microcentrifuge tube.

   The “Master Mix” usually contains buffer, forward and reverse primers, RT/Taq and nuclease-free water.

   Prepare the “Master Mix” according to the RT/Taq manufacturer’s recommendations.

   Ensure that the final volume for each reaction is 24 µl.

   Add 24 µl of Master Mix to each PCR tube.
3. Add 1µl of template (total RNA extract) to the corresponding labelled 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 60 sec], 1 cycle [72°C for 5 mins] and 15°C hold.

   Please note that the PCR conditions have been adapted from Mayo *et al.* (1996) (25 cycles, annealing temperature of 50°C), Sokmen *et al.* (1998) (annealing temperature of 50°C), and Nassuth *et al*. (2000) (annealing temperature of 54°C for 45 seconds).

5. At the completion of the RT-PCR, mix 10 µl each PCR sample with 2 µl of 6x gel loading dye, and load samples onto a 1% agarose gel with ethidium bromide.
6. Electrophorese in 0.5X TBE at 100V for 45 minutes or until the bromophenol blue front has migrated half way down the length of the gel.
7. Visualise and photograph gel on UV transilluminator.
8. Criteria for determination of a valid assay: 196 bp product from samples containing plant extracts, no product in no template and negative plant extract controls, product of the expected size in positive control.

   Criterion for positive sample result: Product of the expected size in test sample, obtained from a valid assay.

4.1.3 DNA Sequencing of PCR Products

Sequencing is outsourced and the technique not described here. The following is the method used to prepare the sample for DNA sequencing.

4.1.3.1 Equipment required

1. 0-2 µL, 2-20 µl, 20-200 µl, and 200-1000 µl micropipettes and tips
2. 0.2 or 0.5 ml PCR tubes
3. 1.5 or 2 ml centrifuge tubes to store reagents
4. Bulb spinner or centrifuge
5. Freezer
6. Crushed ice
7. Latex gloves
8. PC with Internet access
9. Thermocycler
10. UV illuminator

4.1.3.2 Reagents

1. QIAQuick PCR Purification Kit, or gel purification kit if multiple bands are present. Available from Qiagen, Catalogue Number 28104
2. ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits

   Available from Applied Biosystems, www.appliedbiosystems.com
3. Forward primer (As per section 4.4.2.2).
4. Sterile dH_2O.

4.1.3.3 Method

PCR products of correct size are cleaned using the QIAquick Spin kit (Qiagen) as per manufacturer's instructions. The purified PCR products are prepared for sequencing with ABI Big Dye (Roche), as per the manufacturer's instructions. Sequencing is out-sourced and the DNA sequences are compared against sequences on the GenBank database (http://www.ncbi.nlm.nih.gov)using the program BlastN (Altschul *et al*., 1997), to confirm if the positive PCR product sequence is from PMTV (PMTV RNA2 Genbank accession No. D30753; PMTV RNA 3 accession No. D16193).

5 Contact points for further information

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6 Acknowledgements

This protocol was written by Jo Eyre, Bonny Rowles-van Rijswijk and Dr Brendan Rodoni from the Department of Primary Industries, Knoxfield, Private Mail Bag 15, Ferntree Gully Delivery Centre, Victoria 3156, Australia.

Dr John Thomas, AgriScience Queensland, peer reviewed and verified the protocol.

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