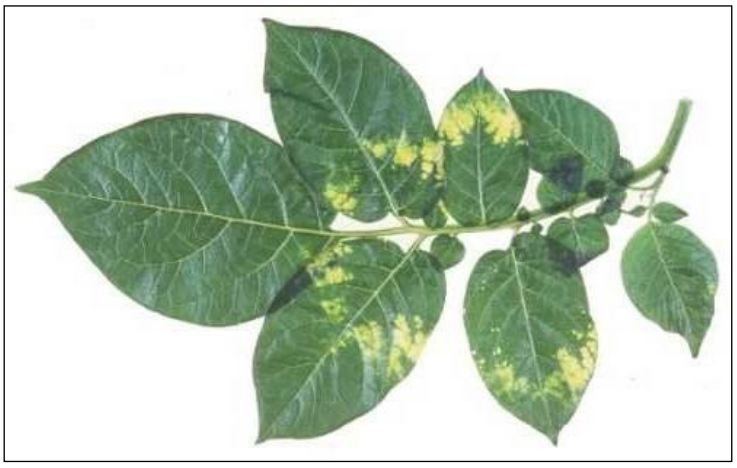
National Diagnostic Protocol

Potato Mop-Top Virus (PMTV)



NDP 15 V2

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Department of Agriculture and Water Resources

Street Address 1: 18 Marcus Clarke Street, Canberra City ACT 2601
Street Address 2: 7 London Circuit, Canberra City ACT 2601
Postal Address: GPO Box 858, Canberra City ACT 2601
Switchboard Phone: 02 6272 3933
Web: http://www.agriculture.gov.au

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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 although 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 http://plantbiosecuritydiagnostics.net.au/sphd/sphd-reference-standards/

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 Mop-Top Virus (PMTV) 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 project should be sent to: sphds@agriculture.gov.au

Contents

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; Yellareddygari *et al.,* 2018).

1.1 Host range

PMTV affects potato (*Solanum tuberosum*).

Weeds from the Chenopodiaceae and Solanaceae are susceptible (https://link.springer.com/article/10.1007/BF02732217, Anderson *et al* 2002)., 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; Santal *et al.,* 2010), 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*. More recently, Tsror et al., (2020) observed *S. subterranean* successfully infecting a wider weed host range including species within the families of *Malvaceae* and *Zygophyllaceae*.

2 TAXONOMIC INFORMATION

Species *Potato mop top virus*
Genus *Pomovirus*
Family *Virgaviridae*
Order *Martellivirales*
Class *Alsuviricetes*
Phylum *Kitrinoviricota*
Kingdom *Orthornavirae*
Realm *Riboviria*

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) (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).

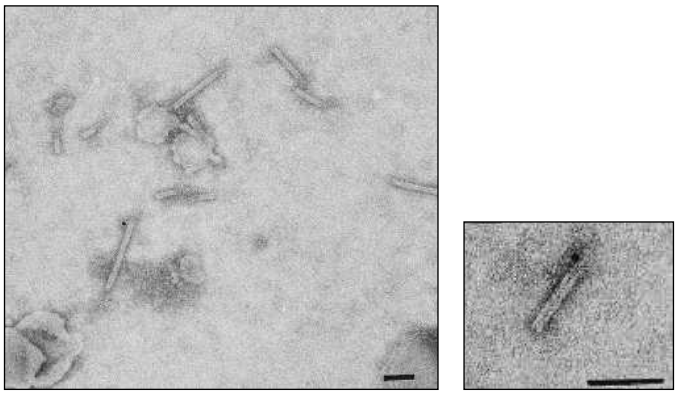


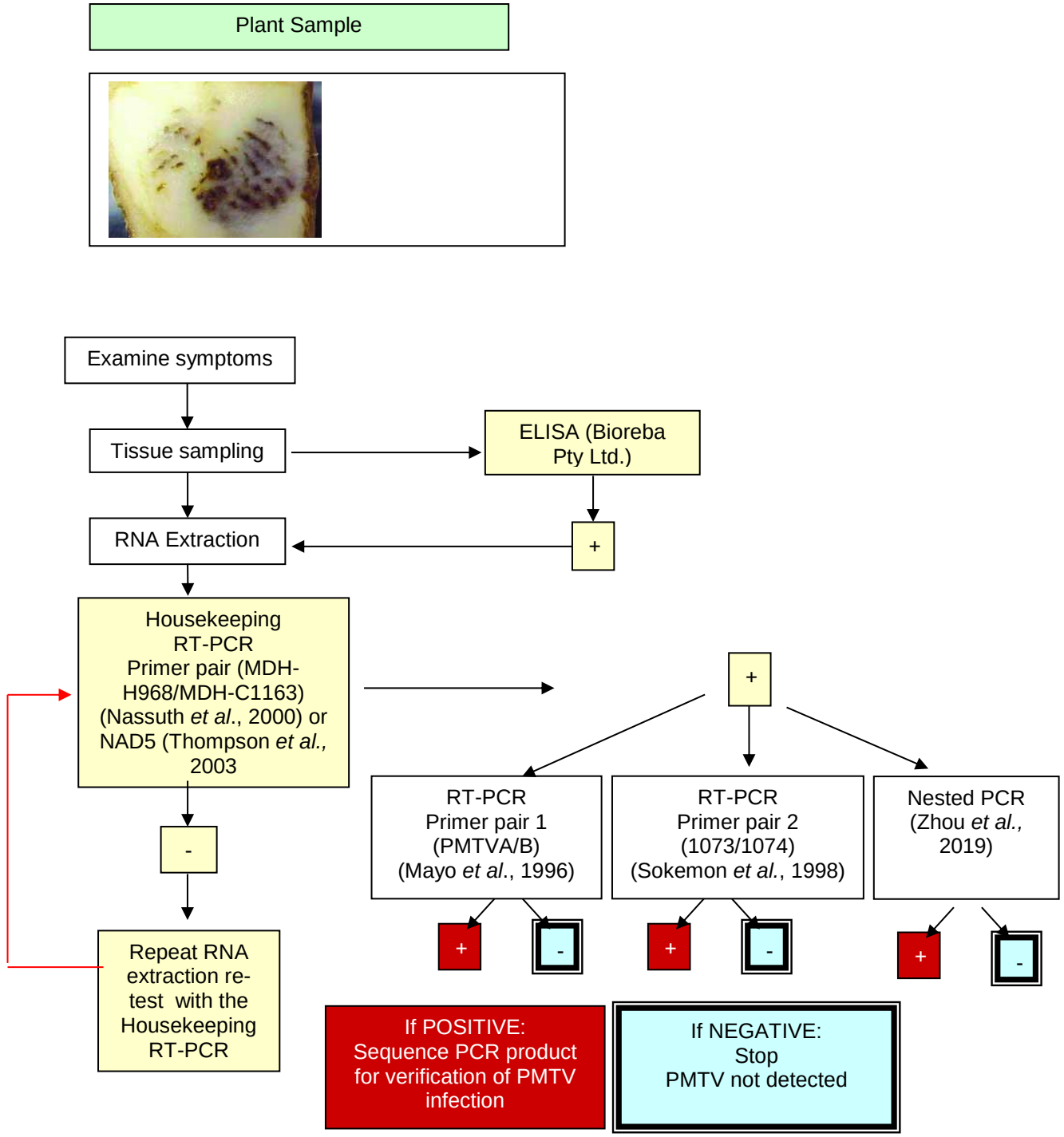
Figure 1. PMTV particles (left), uncoiling of the PMTV particle (right). In each electron micrograph, the bar represents 100 nm (Cowan *et al*., 1997)**.**

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). The 3'- and 5'-terminal sequences of PMTV are highly conserved and could prove useful in the design of specific primers (Kalyandurg *et al.,* 2017; Gil *et al.,* 2016).

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). An additional nested PCR (Zhou *et al.,* 2019) can also be used.

3.1 Diagnostic flow chart



3.2 Symptom description

PMTV symptoms on potato include 'mop-top' stunting of foliage (Figure), yellow blotching (Figure) and yellow V-shaped markings (**Error! Reference source not found.**) 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; Santala *et al.,* 2010; Gil *et al.,* 2016).

Tubers infected with PMTV often display rust coloured, necrotic arcs, flecks, rings or lines through the tuber flesh (Figure , 11, 12, 13). The brown necroses often penetrate into the tuber's flesh almost without interruption (**Error! Reference source not found.**). 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°C) (Sokmen *et al.*, 1998). External symptoms include cracking (Figure), raised necrotic rings (Figure) and raised necrotic lines (**Error! Reference source not found.**). Tuber symptoms may be absent at harvest, but can develop during storage. Visual diagnosis based on spraing 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).

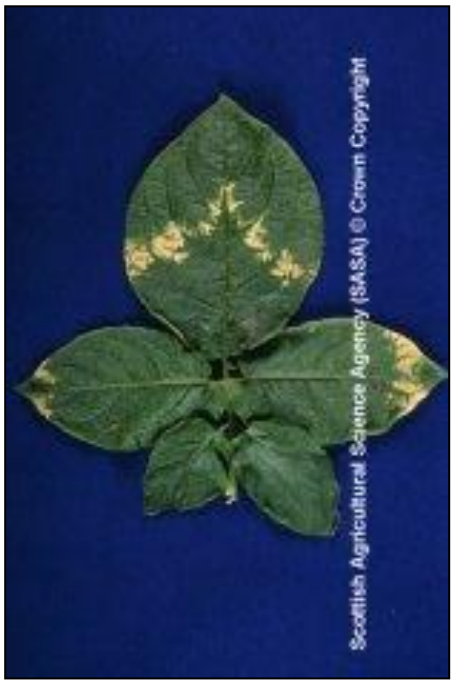


Figure 4. Chlorotic V-shaped markings in the leaflets (© Scottish Agricultural Science Agency)

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

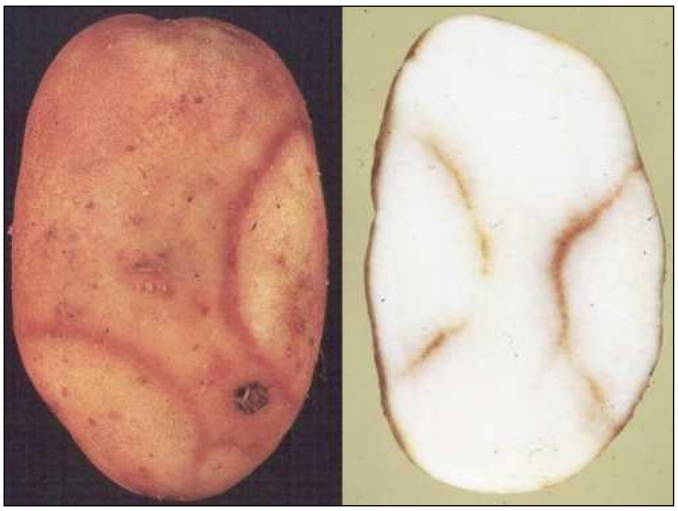


Figure 6. External (left) and internal (right) PMTV spraing symptoms in naturally infected potato tubers cv. Arran Pilot (© Scottish Agricultural Science Agency).

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).

3.3 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 () and necrotic arcs in tubers () similar to PMTV. The tuber necrosis strain of *Potato virus Y* (PVY^{NTN}) causes necrotic rings to appear on potato tubers (**Error! Reference source not found.**). The physiological disorder, "internal rust spot" can also cause tuber symptoms (**Error! Reference source not found.**) easily confused with PMTV.

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

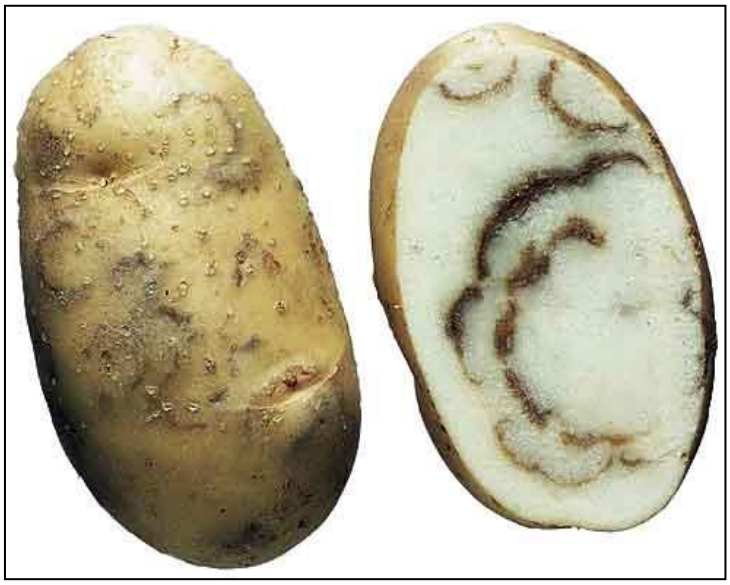


Figure 1. Potato tuber showing necrotic arcs in the flesh caused by infection by TRV (Source: British potato council, http://www.potato.org.uk/department/knowledge_transfer/pests_and_diseases/ref.html?item=27.)

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).

3.4 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.

3.4.1 Leaf sampling

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

3.4.2 Tuber sampling

Tubers removed from cold storage should be incubated at 20°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) 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).

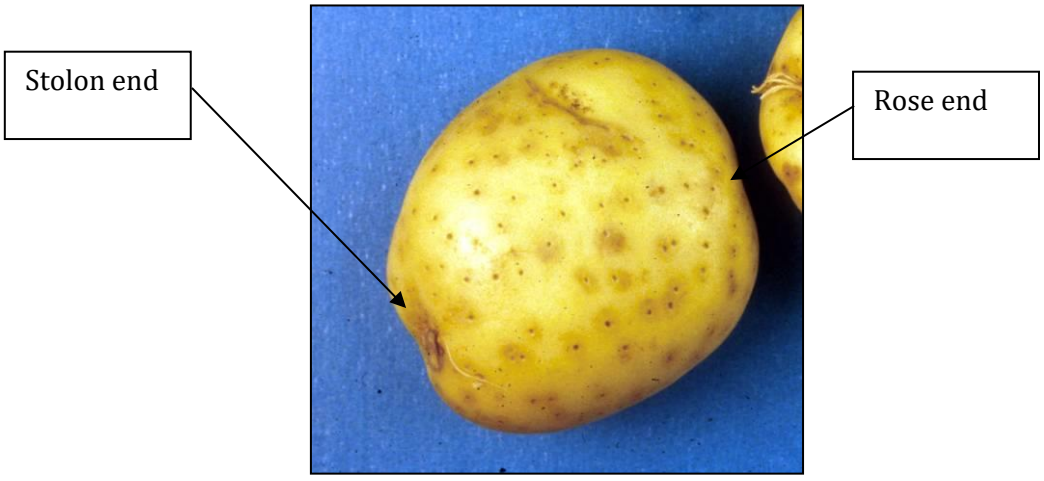


Figure 183. Stolon and rose end of tuber (Source: B Hall, SARDI)

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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.

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™) (Or similar RNA extraction Kit)
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

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

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 four RT-PCR tests, as outlined below, with primer sequences and annealing temperatures listed in Table 1 and 2.

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. **Nested PCR:** 1st Round Primer pair H360 (F) / C819 (R) and 2nd Round Primer pair PMTV-171 (F) / PMTV-433 (R). These primer pairs anneal to the PMTV coat protein, resulting in amplicons at sizes of 460bp and 263bp respectively (Zhou *et al.,* 2019)
4. **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. Alternatively, the Primer pair of **NAD2.1a / Nad 2.2b** can be used to detect mRNA of the NADH dehydrogenase ND2 subunit; the amplicon size is approximately 188bp (Thompson *et al.*, 2003).

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

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			
Nad2.1a[4]	GGA CTC CTG ACG TAT ACG AAG GAT C	NADH dehydrogenase ND2 subunit	50˚C	188bp
Nad2.2b[4]	AGC AAT GAG ATT CCC CAA TAT CAT			

[1](Mayo *et al.,* 1996), [2](Sokmen *et al.,* 1998), [3](Nassuth *et al*., 2000) [4](Thompson *et al.,* 2003).

Table 2 Primers required for the detection of PMTV using a nested-PCR

Primer	Sequence (5'-3')	Target	Anneal temp.	PCR product size
H360 (F)	CAT GAA GGC TGC CGT GAG GAA GT	PMTV Coat Protein (1st Round)	58˚C	460bp
C819 (R)	CTA TGC ACC AGC CCA GCG TAA CC			
PMTV-171 (F)	TAG CGT GTT GAG CCA GGT GT	PMTV Coat Protein (2nd Round)	58˚C	263bp
PMTV-433 (R)	CCT CTG CGA GTT GAT GTG CC			

(Zhou *et al.,* 2019)

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 DSMZ or contact author for further information).
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 or Nad2.1a/Nad2.2b, 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 (196/188bp bp respectively) 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/PCR Kit (Kits validated within laboratories can also be used):
   - Invitrogen® SuperScript™ III One-Step RT-PCR with Platinum® *Taq* DNA Polymerase, Catalogue No. 12574026, is recommended.
   - Qiagen One step RT –PCR kit (Cat. No. 210212) was successfully used in verification of protocol while following the manufacturer's instructions.
2. For the nested PCR Invitrogen® *Taq* DNA Polymerase Catalogue No. 109660108 is recommended for the second PCR.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. SYBR safe (Invitrogen)

Use a 1% DNA grade agarose (w/v) gel made with 0.5x TBE solution, and stained with 10% (v/v) SYBR safe.

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.

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).

   Cycling conditions for the nested-PCR round 1 are as follows: 1 cycle [48°C for 45 min], 1 cycle [94°C for 2 min], 40 cycles [92°C for 30 secs, annealing temperature as in 2 for 30 secs, 72°C for 60 sec], 1 cycle [72°C for 10 mins] and 15°C hold. Following completion of 1st round, follow manufacturer's instructions for DNA polymerase 2nd round PCR.
5. Cycling conditions for the nested-PCR round 2 are as follows: 1 cycle [94°C for 2 min], 40 cycles [92°C for 30 secs, annealing temperature as in 2 for 30 secs, 72°C for 60 sec], 1 cycle [72°C for 10 mins] and 15°C hold (Fig 19).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 SYBR™ DNA Gel Stain (Invitrogen).

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.

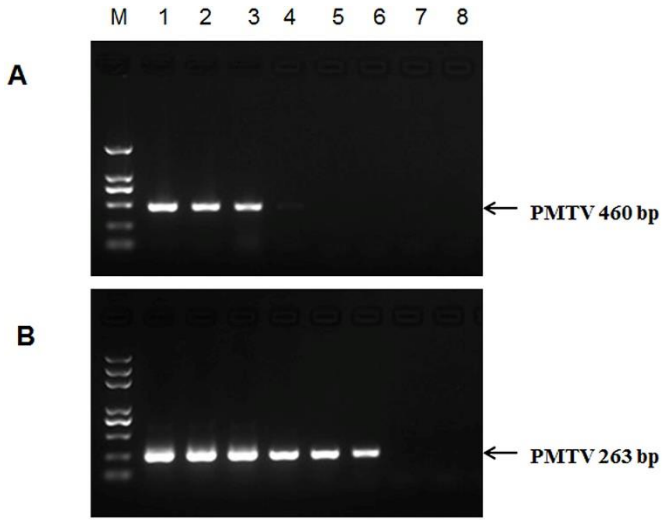

Fig. 19. The results of conventional RT-PCR and nested RT-PCR with serial RNA dilutions. Lane M, DNA marker; lanes 1–8 correspond to RNA dilutions of 100, 10−1, 10−2, 10−3, 10−4, 10−5, 10−6 and 10−7, respectively. A. Conventional RT-PCR. B. Nested RT-PCR using 0.5 µL of the external PCR products as the template (Source: Zhou *et al.,* 2019).

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.

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

Reagents

1. QIAQuick PCR Purification Kit, or gel purification kit if multiple bands are present. Available from Qiagen, Catalogue Number 28104 (Alternatively any validated kit may be used).Forward and Reverse primer (As per section 0).
2. Sterile dH_2O.

Method

PCR products of correct size are purified using the QIAquick PCR Purification Kit (Qiagen) as per manufacturer's instructions. Sequencing can be out-sourced to a commercia provider (e.g. Micromon, Monash University; AGRF etc) 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).

4.2 Serological Tests – ELISA

The ELISA protocol for PMTV (Bioreba is the preferred manufacturer) detects all known strains of PMTV and does not cross react with other viruses.

For PMTV detection using double-antibody sandwich (DAS)-ELISA using the BIOREBA (Reinach, Switzerland) including interpretation of results, follow the manufacturer's instructions. For Further information: http://www.bioreba.ch/saas/web/bioreba/web.aspx?PageID=58&search=PMTV

The buffer recipes are include in Appendix 8.1.

4.2.1 Equipment

- 20, 100 and 1000ul pipettes and tips
- Microcentrifuge and microcentrifuge tubes (2.0mL)
- Container with ice
- Mortar and pestle (or similar macerating device)
- ELISA plates
- ELISA plate reader.

4.2.2 Reagents

- Coating Antibody (provided by supplier)
- Detecting Conjugate (provided by supplier)
- Controls: Positive and negative (provided by supplier – Permit required).
- 5X PBS Buffer
- PBS-Tween-Buffer
- Coating Buffer
- ELISA extraction Buffer
- Conjugation Buffer
- Substrate Buffer

5 CONTACTS FOR FURTHER INFORMATION

AUSTRALIA	UNITED STATES OF AMERICA
David Lovelock	**Steven B. Johnson**
Department of Jobs, Precincts and Regions	Extension crops specialist
Agribio	Extension Professor
5 Ring Road, La Trobe University	University of Maine Cooperative Extension
Bundoora, Victoria 3083	Aroostook City Ces
Phone: 03 9032 7170	Aroostook County
david.lovelock@agriculture.vic.gov.au	Phone: 764-3361
	stevenj@maine.edu

CANADA	SCOTLAND
Will Proctor	**Lesley Torrance**
Agriculture Information Officer	Scottish Crop Research Institute
440 University Avenue	Invergowrie
Charlottetown	Dundee, DD2 5DA
Prince Edward Island C1A 4N6	Scotland, UK
Phone: (902) 620-3285	Tel +44(0)1382 5627331
waproctor@gov.pe.ca	l.torrance@scri.sari.ac.uk

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Dr John Thomas, AgriScience Queensland, peer reviewed and verified the protocol. It was reviewed and updated by Dr. David Lovelock at AgriBio.

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8 APPENDIX

8.1 ELISA Buffers

Coating buffer

Dissolve in distilled water to 1000 ml:

Sodium carbonate (anhydrous)	1.59 g
Sodium hydrogen carbonate	2.93 g

Adjust pH to 9.6 and store at 4° C.

PBS-Tween Buffer (Wash Buffer)

Dissolve in distilled water to 1000 ml:

Sodium chloride	8.0 g
Sodium phosphate, dibasic (anhydrous)	1.15 g
Potassium phosphate, monobasic (anhydrous)	0.2 g
Potassium chloride	0.2 g
Tween-20	0.5 g

Adjust pH to 7.4. Store at 4°C.

ECI Buffer (use as Sample Buffer and as Conjugate Buffer)

Add to 1000 ml 1X PBST:

Bovine serum albumin (BSA)	2.0 g
Polyvinylpyrrolidone (PVP) MW 24-40,000	20.0 g

Adjust pH to 7.4. Store at 4° C.

PNP Buffer

Dissolve in 800 ml distilled water:

Magnesium chloride hexahydrate	0.1 g
Sodium azide	0.2 g
Diethanolamine	97.0 ml

Adjust pH to 9.8 with 0.1 N hydrochloric acid. Adjust final volume to 1000 ml with distilled water. Store at 4° C.

9 DIAGNOSTICS PROCEDURES TO SUPPORT SURVEILLANCE

9.1 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; Yellareddygari *et al.,* 2018).

The current methods for detection include two RT-PCRs and ELISA and a newly added nested PCR. In situations where volumes of material/samples are very high (+300), it is recommended that the laboratory undertake ELISA initially, followed up with a more targeted approach using RT-PCR Table 1).

Table 1: Diagnostic methods for the detection of PMTV.

Method	**Identification level**	**Identification Confidence**	**Deployment (Field/Lab)**	**Required Time**	**Throughput (No. of samples)**
RT-PCR (4.1.2)	To Species/Strain	High (99%+)	Laboratory	<1 w	High (100s)
ELISA (4.2)	To Species	Medium (90-99%)	Laboratory	<1 w	Very High (1000s)

9.2 Sampling

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. Further information can be found in the NDP sections 3.4.1 and 3.4.2.

9.3 In Field Tests

There are currently no known in field tests for PMTV.

9.4 Laboratory Tests

The laboratory tests including RT-PCR and ELISA can be found in the NDP for PMTV in sections 4.1.2 and 4.2 respectively.

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9.6 References

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