The International Diagnostic Protocol for PPV (ISPM-27 DP02) was released March 2012

https://www.ippc.int/publications/dp-2-2012-plum-pox-virus

<table>
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<th>PROTOCOL NUMBER</th>
<th>NDP 2</th>
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<tbody>
<tr>
<td>VERSION NUMBER</td>
<td>V3.1</td>
</tr>
<tr>
<td>STATUS</td>
<td>Endorsed</td>
</tr>
<tr>
<td>ISSUE DATE</td>
<td>January 2014</td>
</tr>
<tr>
<td>REVIEW DATE</td>
<td>January 2019</td>
</tr>
<tr>
<td>ISSUED BY</td>
<td>SPHDS</td>
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Prepared for the Subcommittee on Plant Health Diagnostic Standards (SPHDS)

This version of the National Diagnostic Protocol (NDP) for Plum Pox Virus (PPV) 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/

Where an IPPC diagnostic protocol exists it should be used in preference to the NDP. 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
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1 INTRODUCTION

This diagnostic protocol provides technical information for the identification of Plum pox virus (PPV). PPV causes fruit deformity of stonefruit, with rings on fruit and leaves.

1.1 Host range

The major hosts of PPV are in the genus Prunus, and include apricots (P. armeniaca), peaches (P. persica) and plums (P. domestica and P. salicina). Almond (P. dulcis) can express mild symptoms (Festic, 1978). PPV has been mechanically transmitted to Prunus species in the cherry group, and early research showed that infection remained localized and the virus did not translocate (Dosba et al., 1987). Cherries were considered immune to PPV infection but in 1994 sour cherry (P. cerasus) was reported being naturally infected with PPV (Kalashyan et al., 1994). Recently PPV has found to naturally infect sweet cherry (P. avium) (Crescenzi et al., 1996; James & Glasa, 2006).

Important wild or ornamental Prunus species that can naturally host PPV are P. besseyi, P. cerasifera, P. insititia, P. tomentosa, and P. spinosa. It is also accepted that many other cultivated or weedy annual plant species can potentially carry PPV inoculum, but natural transmission between such herbaceous plants and Prunus has not been demonstrated. Wild woody and herbaceous hosts are also widespread and are potential reservoirs of the disease.

Table 1 provides a list of common and botanic names of alternate hosts of PPV often used in Australia and New Zealand as street trees, or grown in domestic gardens.

1.2 Vectors

The virus is transmitted either by grafting or non-persistently by two main aphid vectors Aphis spiraecola and Myzus persicae. Other aphids able to transmit but at lower efficiency are: Aphis craccivora, A. fabae, Brachycerus cardui, B. helichrysi, B. persicae, Hyalopterus pruni, Myzus varians, Phorodon humuli (Kunze & Krczal, 1971; Leclant, 1973). Aphis gossypii, Rhopalosiphum padi and A. hederae have been recently reported as vectors (Avinent et al., 1994; Labonne et al., 1994).

Table 1. Alternate Rosaceae hosts of PPV often used in Australia and New Zealand as ornamental plants*.

<table>
<thead>
<tr>
<th>Botanic Name</th>
<th>Common Name</th>
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<tbody>
<tr>
<td>Prunus cerasifera</td>
<td>Cherry Plum, Myrobalan, Purple-leafed plum</td>
</tr>
<tr>
<td>Prunus glandulosa</td>
<td>Dwarf flowering almond, Almond cherry</td>
</tr>
<tr>
<td>Prunus insititia</td>
<td>Bully-bloom, Bullies, Bolas, Bullace, Wild Damson</td>
</tr>
<tr>
<td>Prunus japonica</td>
<td>Japanese single bush cherry</td>
</tr>
<tr>
<td>Prunus mahaleb</td>
<td>Mahaleb cherry</td>
</tr>
<tr>
<td>Prunus maritima</td>
<td>Beach plum</td>
</tr>
<tr>
<td>Prunus salicina</td>
<td>Japanese plum</td>
</tr>
<tr>
<td>Prunus sibirica</td>
<td>Siberian apricot</td>
</tr>
<tr>
<td>Prunus spinosa</td>
<td>Blackthorn, Sloe</td>
</tr>
<tr>
<td>Prunus tormentosa</td>
<td>Nanking cherry, Hansen bush cherry</td>
</tr>
</tbody>
</table>

*All listed Prunus species and their seed for sowing and/or nursery stock import specifications for New Zealand are available at the Plants Biosecurity Index (http://www1.maf.govt.nz/cgi-bin/bioindex/bioindex.pl).
2 TAXONOMIC INFORMATION

Genus Potyvirus
Family Potyviridae

Common names: Sharka, plum pox (English); variole duprunier, sharka (French); Scharka-Krankheit (German); and vaiolatura delle drupacee (Italian).

2.1 Virus strains

Six strains of PPV have been distinguished on the basis of symptoms on inoculated herbaceous indicator plants, and according to their serological and molecular properties (Kerlan & Dunez, 1979; James & Varga, 2005; Candresse & Cambra, 2006; James & Glasa, 2006). Differentiation of strains can be determined by antigenic virus properties, electrophoretic mobility of the coat protein, antigenic properties of the N and C regions of coat protein, divergence in the RNA sequence and the presence or absence of a specific restriction site in the C-terminal region of the coat protein. The six currently recognized subgroups, strains or serotypes of PPV have been identified as Dideron (PPV-D), Markus (PPV-M), El Amar (PPV-EA) (Wetzel et al., 1991a), Cherry (PPV-C), Wimona or W3174 (PPV-W) (James & Varga, 2005), as well as a widespread group of recombinants (of PPV-M and PPV-D) known as PPV-Rec. A "necrogenic" isolate of PPV-M which was previously referred to as PPV-SP, and further characterised by Adamolle et al. (1994), is not currently recognized as a separate strain from PPV-M. More recently a new recombinant group of PPV isolates have been characterised from Turkey and it has been proposed to be a novel strain referred to as PPV-T (Serçe et al., 2009). In addition, newly characterised sour cherry isolates of PPV from the Volga basin in Russia, have also recently been proposed as a novel cherry strain of PPV referred to as PPV-CR (Cherry Russia) (Glasa et al., 2013). The protocols described in this document will detect all currently known strains of PPV, including PPV-Rec, PPV-T and PPV-CR.

3 DETECTION

3.1 Symptoms

Symptom expression of PPV can depend on the strain involved, Prunus cultivar infected and weather conditions. Symptoms vary between different Prunus species. For example, in peach, symptoms are best observed on the flower petals, and depending on the conditions, may be evident for only short periods of the growing season. In plums, symptoms occur on the leaves and are more persistent. Some Prunus cultivars may remain symptomless. A useful website for images is http://extension.psu.edu/pests/ipm/biosecurity/plum-pox-virus-sharka (PennState College of Agricultural Sciences).

Other good websites for descriptions of symptoms and photos are:
http://www.caf.wvu.edu/kearneysville/disease_descriptions/ppvresources.html

2
3.1.1 Apricot (*Prunus armeniaca*) symptoms

**Fig 1:** Mild to moderately PPV infected apricot ‘Castle Brite’ fruit show spots which are slightly darker than the surrounding skin colour (left) in Chile. These darker spots may also be raised in severely infected fruit (right) (© M. Cambra)

**Fig 2 (Left) and 3 (Right):** Light rings on yellow fruits may be apparent on apricots near maturity. Valencia, Spain (© J.W. Travis).

**Fig 4:** Apricot fruit infected with PPV showing a misshapen bumpy appearance. Montpellier, INRA, France (© J.W. Travis)

**Fig 5:** Light ring spots on pit and deformities in the flesh of an apricot fruit ‘Castle Brite’ in Chile (© M. Cambra).
Fig 6: Misshapen and deformed apricot fruits infected with PPV (© M. Nemeth, Hungary).

Fig 7: Ring spots on apricot fruits’ pit infected with PPV (© J. Dunez, France).

Fig 8: Apricot deformities caused by PPV extending into the flesh and light ring spots which can occur on the pit (© M. Barba, Italy).

Fig 9: Ring spots on a pit of an apricot. Europe (© R. Scorza).

Fig 10: Light green ring spots on apricot leaf. Southern France © (J.W. Travis).

Fig 11: Light green ring spots on an apricot leaf. Murcia, Spain (© M. Cambra).
3.1.2 Plum (*Prunus domestica*) symptoms

![Fig 12: Apricot leaves showing a netting discolouration associated with the veins. France (© P. Gentit)](image1)

![Fig 13: One or several ring spots may appear on an apricot leaf. Valencia, Spain (© J.W. Travis)](image2)

**Fig 12:** Apricot leaves showing a netting discolouration associated with the veins. France (© P. Gentit)

**Fig 13:** One or several ring spots may appear on an apricot leaf. Valencia, Spain (© J.W. Travis)

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**Fig 14:** Yellow ring spot symptoms on a red plum variety infected with PPV. Southern France (© P. Gentit).

**Fig 15:** Speckling pattern that may be evident on some varieties of red plums. Pictured ‘Arm King’ plum. INRA, Montpellier, France (© J. Quiot).

**Fig 16:** Infected Japanese plum fruits ‘Red Beaut’ showing severe deformity. Sevilla, Spain (© M. Cambra; Cambra et al., 2008).

**Fig 17:** Immature plums showing irregular surface and red ring spots. Valencia, Spain (© F.E. Gildow).
Fig 18: Sunken lesions on plums infected with PPV (© M. Nemeth, Hungary)

Fig 19: Yellow plums showing red ring spots near harvest. Valencia, Spain (© F.E. Gildow)

Fig 20: Severely infected plums showing necrotic areas and a bumpy appearance. Valencia, Spain (© J.W. Travis)

Fig 21: Premature fruit fall on sensitive plum cultivars in Central and Eastern Europe (© M.Nemeth, PHSCS, Hungary). Levy et al. (2001): http://www.apsnet.org/online/feature/PlumPox/Top.html

Fig 22: Blotches of light green to yellow on plum leaves. Valencia, Spain (© F.E. Gildow).

Fig 23: Some plum varieties may have very large ring spots on their leaves. Quetsche, Southern France (© P. Gentit)
3.1.3 Peach (*Prunus persica*) symptoms.

![Fig 24: Speckling appearance on leaves is another symptom of a PPV infection. Montpellier, INRA, France (© P. Quiot).](image1)

![Fig 25: Light green ring spots on plum leaves. Europe (© R. Scorza)](image2)

![Fig 26: Necrotic areas on plum leaves often fall out giving a shot hole appearance. Valencia, Spain (© F.E. Gildow).](image3)

![Fig 27: Light green ring spots on plum leaves (© M. Nemeth, Hungary)](image4)

![Fig 28: Early symptom of the virus on peach flowers. Note the dark pink streaks. This is the most reliable symptom to look for on peaches. This picture shows infected “Babygold 5” flowers. Southern France (© P. Gentit)](image5)

![Fig 29: PPV ring spots. Pictured PPV-D infecting ‘Encore’ peach fruits from Adams County, Pennsylvania, USA (© F.E. Gildow)](image6)
Fig 30: ‘Encore’ peaches express PPV symptoms more clearly than other varieties. Pennsylvania, USA (© R. Welliver).

Fig 31: Obvious yellow rings on a red-skinned ‘Encore’ peach. Pennsylvania, USA (© K.D. Hickey).

Fig 32: Light green veins of a peach leaf. Montpellier, INRA, France (© J.W. Travis).

Fig 33: Peach leaves showing necrotic areas and yellow blotching patterns. Pennsylvania, USA (© J.W. Travis).

Fig 34: Veinal yellowing and deformity of peach leaves caused by PPV. Symptoms are similar to those caused by insect damage. Pennsylvania, USA (© J.W. Travis).
3.1.4 Cherry (*Prunus avium*) symptoms

![Cherry leaves showing mottling and necrotic spots](image)

**Fig 35:** Cherry leaves showing mottling and necrotic spots (© P. Gentit, France).

### 3.2 Sampling method

The distribution of the virus in a tree can be irregular. However, the detection from flowers, young leaves, old leaves, fruit, dormant wood, and roots is possible. The time of year when the sample is taken is critical and can greatly affect the test results. Optimal sampling time is during spring, or early summer (18-28 °C) but can vary from season to season depending on the weather conditions.

#### 3.2.1 Sampling the correct tissue from a tree

When sampling leaf material for the presence of PPV, leaves from the middle of the branch should be sampled from various points around the tree. Optimal tissue for peach samples is petals if possible.

![Leaf tip, Mid-branch leaves (Optimal), Oldest leaves](image)

**Fig 36:** Best section for sampling (mid branch) is shown in a characteristic *Prunus* scaffold branch.
3.3 Direct observation of Plum pox virus by electron microscopy

Transmission electron microscope (TEM) examination of grids prepared from small sections of homogenised leaf tissue can be negatively stained with 1% uranyl acetate (UA) for rapid detection of PPV. The grids are examined at 40,000 x magnification for flexuous filamentous potyvirus particles of 660-770 nm in length. Two detailed protocols are provided for crude sap preparation (Section 3.3.1) and crude sap preparation using immunosorbent electron microscopy (Section 3.3.2).

3.3.1 Crude sap preparation - TEM

Preparation for transmission electron microscope examination using uranyl acetate stain. Method adapted from Milne et al. (1996).

**Equipment:**
- 1.5 ml microcentrifuge tubes
- Sterile micropesle for 1.5 ml microcentrifuge tubes (e.g. Eppendorf part: 0030 120.937)
- Pipettes and tips
- Formvar-carbon reinforced copper grids, square 400 mesh (e.g. SPI supplies catalogue number 3440C-CF), store at room temperature
- Forceps (very fine tips)
- Filter paper (cut in small triangular pieces)
- Small containers (e.g. weight boat)
- Beakers
- Sharp blade or razor blade (one per sample)

**Reagents:**
- Deionised water
- Polyvinylpyrrolidone (PVP; MW 24,000-45,000)
- 0.5 M Phosphate Buffer pH 7 (stock solution, store at room temperature)
  - Prepare two stock solutions: 100 ml deionized water
  - 0.5 M KH₂PO₄ 6.8 g
  - 0.5 M K₂HPO₄ 8.71 g
  - Mix 39 ml of 0.5M KH₂PO₄ with 61 ml of 0.5 M K₂HPO₄
  - Working solution: 0.1 M phosphate buffer pH 7, prepare fresh
- 1% (w/v) aqueous uranyl acetate 50 ml
  - Uranyl acetate 0.5 g
  - Dissolve in deionised water
  - Store in the dark at room temperature

**HAZARD:** Uranyl acetate has a slight radioactivity emission.

A 0.5% (w/v) uranyl acetate stain may also be used if needing to clarify the background.

**Method:**
1. Prepare 0.1 M phosphate buffer (about 100 μl per sample) containing 2% (w/v) PVP [TEM extraction buffer]. The buffer may be kept up to one month in the fridge. Phosphate buffer may be modified depending on the tissue used.
2. Add 100 μl of TEM extraction buffer in a 1.5 ml microcentrifuge tube (1 tube
3. Cut a small piece of plant material (~5 mm²), preferably from the edge between symptomatic and asymptomatic tissue, using a clean blade and immediately transfer it into the 1.5 ml microcentrifuge tube containing TEM extraction buffer. Use a clean blade for each sample. If touching plant material with gloves, change gloves immediately.

NB: if using dry tissue, leave to soak in TEM extraction buffer for 5-10 min on ice before grinding.

4. Grind using the sterile micropestle. The crude sap should be a clear green colour. If dark green (too concentrated), add more TEM extraction buffer to dilute the sap.
5. Incubate crude sap preparation on ice for about 5 min, then briefly centrifuge at maximum speed for one min.
6. Hold a grid (bright side upward) by the edge using forceps.
7. Place a drop (~4 µl) of crude sap preparation on the grid and leave for about 5 min. The grid may also be incubated at room temperature for up to one hour in a sealable box containing damp paper.
8. Rinse with ~30 drops of deionised water by holding the grid slightly tilted on the side.
9. Add ~5 drops of 1% (w/v) uranyl acetate stain while holding the grid slightly tilted on the side. If touching uranyl acetate stain with gloves, change gloves immediately.
10. Leave the grid to dry on the bench for 10-15 min, alternatively it can be put in a 37°C incubator for 5 min.
11. Grid observation
   • Use liquid nitrogen in the EM cold finger all the time (where applicable)
   • Usually start observation at 25-30K
   • Need to see at least 3-4 particles before being confident that there is a virus present

3.3.2 Crude sap preparation – ISEM

For immunosorbent electron microscopy technique and uranyl acetate stain for transmission electron microscope examination. Method adapted from Matthews (1993).

Immunosorbent electron microscopy (ISEM) is used when particles in the crude sap preparation are low in concentration. In ISEM, the virus is first trapped with an antibody on the grid with the purpose of increasing the number of virus particles. This is followed by a second application of the same antibody.

Alternatively, if the virus particles in a crude sap preparation are known to be in high concentration, a decoration technique may be sufficient. Decoration of a crude sap preparation is used to confirm and/or identify the virus presence.

Equipment:
• 1.5 ml microcentrifuge tubes
• Sterile micropestle for 1.5 ml microcentrifuge tubes (e.g. Eppendorf part: 0030 120.937)
• Pipettes and tips
• Formvar-carbon reinforced copper grids, square 400 mesh (e.g. SPI supplies
catalogue number 3440C-CF), store at room temperature
• Forceps (very fine tips)
• Filter paper (cut in small pieces)
• Small containers (e.g. weight boat)
• Beakers
• Sharp blade or razor blade (one per sample)

Reagents:
• Deionised water
• Polyvinylpyrrolidone (PVP; MW 24,000-45,000)
• 0.5 M Phosphate Buffer pH 7 (stock solution, store at room temperature)
  • Prepare two stock solutions: 100 ml deionized water
  • 0.5 M KH2PO4 6.8 g
  • 0.5 M K2HPO4 8.71 g
  • Mix 39 ml of 0.5M KH2PO4 with 61 ml of 0.5 M K2HPO4
  • Working solution: 0.1 M phosphate buffer pH 7, prepare fresh
• 1% (w/v) aqueous uranyl acetate 50 ml
  • Uranyl acetate 0.5 g
  • Dissolve in deionised water
  • Store in the dark at room temperature

HAZARD: Uranyl acetate has a slight radioactivity emission.

A 0.5% (w/v) uranyl acetate stain may also be used if needing to clarify the background.
• Antisera specific to the targeted viruses

Method:
1. Dilute the antisera in 0.1 M phosphate buffer (without PVP).
2. Hold a grid (bright side upward) by the edge using forceps.
3. Add a drop (~4 µl) of the diluted antisera on a grid and incubate in a closed humid box at room temperature for 5 min.

NB: If using an antisera for the first time for decoration technique, several dilutions may be used starting 1/1, 1/10, 1/50 (v/v).

4. Wash with ~15 drops of 0.1 M phosphate buffer (without PVP) by holding the grid slightly tilted on the side.
5. Transfer 100 µl of 0.1 M phosphate buffer containing 2% (w/v) PVP [TEM extraction buffer] per 1.5 ml microcentrifuge tube (1 tube per sample).
6. Cut a small piece of plant material (~5 mm²), preferably from the edge between symptomatic and asymptomatic tissue, using a clean blade for each sample and immediately transfer it into the 1.5 ml microcentrifuge tube containing TEM extraction buffer. Change gloves immediately if they contact the plant material.

NB: if using dry tissue, leave to soak in TEM extraction buffer for 5-10 min on ice before grinding.

7. Grind using the sterile micropestle. The crude sap should be of a clear green colour. If dark green (too concentrated), add more TEM extraction buffer to dilute the sap.
8. Incubate crude sap preparation on ice for about 5 min, then briefly centrifuge at maximum speed for one min.
9. Place a drop (~4 µl) of crude sap preparation on a grid and incubate about 5 min in a closed humid box.
10. Wash with ~15 drops of 0.1 M phosphate buffer (without PVP) by holding the grid slightly tilted on the side.
11. Place a drop of the antisera and incubate in a closed humid box at room temperature for 15 min.
12. Rinse with ~30 drops of deionised water by holding the grid slightly tilted on the side.
13. Add ~5 drops of 1% (w/v) uranyl acetate stain while holding the grid slightly tilted on the side. If touching uranyl acetate stain with gloves, change gloves immediately.
14. Leave it to dry on the bench for 10-15 min alternatively it can be put in a 37°C incubator for 5 min.
15. Grid observation
   - Use liquid nitrogen in the EM cold finger all the time
   - Usually start observation at 25-30K
   - Need to see at least 3-4 particles before being confident that there is a virus in a sample

3.3.3 Tips for Grid preparation

For woody species (e.g. plum, apricot etc.), it is recommended to add 2% (w/v) polyvinylpyrrolidone (PVP; MW 24,000-45,000) or polyethylene glycol (PEG; MW 6,000). The 0.1 M phosphate buffer with 2% PVP may be stored in the fridge for up to one month.

<table>
<thead>
<tr>
<th>Problem when observing the grid under the microscope</th>
<th>Suggestions</th>
</tr>
</thead>
</table>
| Too dark                                             | 1. Too much plant material on grid  
|                                                      | 2. Too much stain |
| Dark lumps                                           | 3. Too much stain |
| Crystalline structures                               | 1. Stain precipitation or phosphate buffer  
|                                                      | 2. Grid was not rinsed enough with deionised water |

3.4 Direct DAS-ELISA for PPV.

This protocol (double antibody sandwich-enzyme linked immunosorbent assay) enables the serological detection and quantification of PPV capsid protein by using polyclonal antibodies. The selected kit of antibodies is the PPV Reagent Set ELISA for the detection of PPV-C, D, EA, M, and W. These polyclonal antibodies will also pick PPV-CR and will pick PPV-Rec and PPV-T as both are recombinants of PPV-M and PPV-D. This DAS-ELISA is an alkaline phosphatase label system distributed by Agdia Inc. USA, catalog number SRA 31505. The protocol is performed according to the manufacturer’s instructions. It is recommended to read and understand Section 3.4.1 before starting the test procedure.
3.4.1 ELISA – general considerations

Equipment and key consumables

- Measuring cylinders and beakers
- Bench centrifuge (refrigerated)
- Centrifuge tubes (Eppendorf 1.5 ml)
- Dispensing troughs
- Precision pipettes (Single channel & multichannel, calibrated)
- Disposable pipette tips
- Incubator (calibrated)
- Humidified chamber
- Refrigerator (or cool store for sample storage) at 4°C (calibrated)
- ELISA plate reader (calibrated)
- ELISA microplates and adhesive plate covers
- ELISA microplate washer
- Balances (calibrated)
- Homogeniser or alternative hand grinder
- Homogenisation bags
- PPV kit commercially available from: Agdia Incorporated.

Sample extraction

Samples should be tested as soon as possible after removal from the host plant and should be kept in a cold store or on crushed ice during the whole extraction procedure.

1. Transfer 5 ml of ELISA extraction buffer into a labelled extraction bag and store on crushed ice.

2. Remove the leaves from each sample bag and neatly stack on top of each other in the same orientation. From this stack hand tear or cut out the tissue for testing (if cutting, rinse the blade in water then wipe between each sample). Select the tissue from the petiole end of the leaf, while excluding the petiole itself. Weigh out 0.5 ± 0.04 g of tissue using an analytical balance. Make sure that each leaf is represented in the 0.5 g sample and quickly transfer into the prepared extraction bag. This gives a sample to buffer ratio of 1:10 (w/v).

3. Homogenise the plant tissue in the buffer, e.g. using a homogeniser or hand grinder. Homogenised samples should be stored on crushed ice until they are ready to be transferred into microcentrifuge tubes.

4. Pour about 1ml of homogenised plant extract into a labelled 1.5ml micro-centrifuge tube and centrifuge at full speed (16,000g) for 5 minutes at 4°C. The tubes containing the clarified samples should be stored on crushed ice until they are ready to be loaded into the ELISA microplate.

5. At least one (preferably several) healthy plant controls and one positive control must be included in each ELISA test performed. The positive control used is as supplied by Agdia (or equivalent), which consists of recombinant PPV coat protein prepared according to the manufacturer’s recommendations.

6. It is recommended that a “weak positive” should also be used. This consists of a one eighth dilution of the eluted Agdia PPV positive in Phosphate Buffer Saline (PBS; Sigma catalog P3813) (one part eluted positive control plus seven parts of PBS, by volume). Healthy controls are supplied by the manufacturer of the antiserum (usually dried extract), but wherever possible a control consisting of known healthy plant tissue of the same species and physiological condition as the sample should also be included.
7. All processed samples should be loaded within 1-2 hours of grinding. If the test does not work, samples may be re-loaded onto a new ELISA microplate from the original grinding bags stored in a monitored fridge or cold store at 4°C for up to 2 days. If possible, positive and negative controls must also be the originals used.

**ELISA procedure**

1. Unless otherwise indicated, all procedures are carried out at room temperature, i.e. 18-23°C, and away from direct sunlight.

2. As manufacturers change the procedures from time to time, it is important to follow the protocol on the brochure provided with the specific reagent lot. The procedure followed and the results obtained must be recorded.

3. Make-up the required ELISA reagents/buffers as necessary. Record batch numbers and expiry dates on the bottles.

4. Design a microplate layout or loading diagram.

5. Calculate the correct concentration and volume of reagents and antisera required at each ELISA step. Record the volumes and concentrations of reagents and antisera. Dispense the correct volumes of buffer or test sample in each ELISA well as per manufacturer’s recommendations of the kit in use.

6. The ELISA microplate is first coated with anti-PPV polyclonal immunoglobulins. Calculate the volume of concentrated coating antiserum required and dilute in coating buffer. Dispense coating antibody solution into the ELISA microplate (100μl per well for Agdia kit) and cover the microplate with an adhesive plate cover. Incubate the microplate according to the manufacturer’s recommendations.

7. Wash the microplate as specified by the manufacturer’s recommendations, or if not specified use 3 washes with a 30 second soak between washes, then aspirate and drain by inverting and tapping on a clean paper towel.

8. Add the clarified test sample extracts and controls into the ELISA microplate (100μl per well for Agdia kit) and cover the microplate with an adhesive plate cover and place immediately in a 4°C fridge. Incubate in the fridge for 16-18 hours.

9. Wash the sample extract from the microplate, as specified in the technical brochure; use additional washes if required to remove any remaining extract residue. Note number of additional washes used. If washing cycle is not specified, use 4 washes with a 1 minute soak between washes, plus additional washes required to remove visible residue. If there is more than one microplate, remove microplates from the fridge and wash at sufficient intervals to allow for successive microplate readings.

10. Calculate the volume of concentrated conjugated antibody required and dilute in the appropriate conjugate buffer. Dispense the conjugate antibody solution into the ELISA microplate (100 μl per well for Agdia kit) and cover the microplate with an adhesive plate cover. Incubate the microplate according to the manufacturer’s recommendations.

11. Wash the microplate as specified by the manufacturer, or if not specified, 3 washes with a 30 second soak between washes plus a final wash using an additional 50 μl per well. Aspirate and drain by inverting and tapping on a clean paper towel. If using an automatic plate washer with shaking function set to 12-15 cycles per second. If the automatic plate washer is unavailable, another alternative is manual washing using a wash bottle to fill the wells, but care should be taken to avoid cross-contamination, and soak times should be increased to 5 minutes.

12. Prepare the substrate whilst the microplate is washing at the end of conjugated
antibody incubation. Prepare 1 mg/ml p-nitrophenylphosphate (PNP) in substrate buffer. Note that PNP is supplied as 5 mg tablets, so the minimum volume of buffer that can be made is 5 ml. Ensure the buffer volume matches the number of tablets used e.g. 3 tablets = 15 mg in 15 ml buffer. Dissolve substrate tablets in buffer and use immediately. Return the PNP to the monitored -20ºC freezer immediately after use.

**Note:** To avoid contamination, do not touch the PNP tablets or solution, or put fingers inside the reagent reservoirs (wear gloves). Do not expose substrate solution to strong light.

13. Prepare the ELISA reader. Allow a warm-up period according to equipment specifications.

14. Quickly, dispense the freshly prepared substrate into the ELISA microplate (100 µl for Agdia kit) and cover the microplate with an adhesive plate cover. Incubate the microplate according to the technical brochure.

15. Read the microplate at 405nm wavelength and record the absorbance 60 minutes after addition of substrate. If at this point any sample absorbance readings exceed twice the healthy absorbance (average of healthy absorbance minus blank) then a further, final reading is made at 2.25 hours after addition of substrate.

**Interpretation of results**

The test results are interpreted as positive if the mean absorbance of the test sample is equal to or greater than 3 times the mean absorbance of the healthy controls (after blank subtraction). Samples having a mean absorbance (after blank subtraction) greater than 2.5 but less than 3 are considered to be uncertain and must be retested.

If the absorbance values for the positive and negative controls are outside the specifications, the test is invalid and must be repeated. Note if the control validation limits are not specified in the manufacturer’s recommendations, the maximum limit for healthy control minus blank is 0.2 absorbance units and the minimum for the positive control minus blank is 0.9 absorbance units, both 60 min after addition of substrate.

Re-testing may include some or all of the following:

1. Repeating the ELISA, using the homogenate of the original suspect sample provided it has not been stored for more than 2 days in a monitored 4°C fridge or cold store, or using freshly collected sample (if the initial sample was bulked from multiple trees, then retest samples individually) using several replicates for each sample.
2. Use of an alternative test method, such as RT-PCR.

**3.4.2 ELISA procedure for Agdia kit SRA 31505**

The following buffers are required but are not provided in the Agdia kit SRA 31505:

1. Carbonate coating buffer
2. Phosphate Buffer Saline Tween-20 (PBST) wash buffer
3. PNP substrate buffer (formulation below)
4. PNP Substrate tablets (Agdia catalog ACC 00404)

Buffer packs containing the above items can be purchased from Agdia or made from standard recipes provided with the SRA 31505 kit.
3.4.2.1 Coating of ELISA microplates

1. Prepare coating antibody

The coating antibody is provided in a concentrated solution and must be diluted with coating buffer before use. The recommended antibody to buffer ratio is given on the label. Prepare the volume of coating buffer needed for the test as indicated above. 100 µl of coating antibody solution is required for each well in use; therefore a 96 well microplate requires about 10 ml of coating buffer. After the coating buffer is measured, add the appropriate volume of concentrated coating antibody to the coating buffer at the dilution given on the label. Use a new, sterile pipette tip to prevent contamination of the concentrated coating antibody.

Example: If the dilution given on the bottle of concentrated coating antibody is 1:200, and 10 ml of coating antibody solution is required, mix 50 µl of the concentrated coating antibody with 10 ml of coating buffer.

Mix the prepared coating antibody thoroughly and use immediately.

2. Coat the microplate by pipetting 100 µl of the prepared coating antibody solution into each well.

3. Incubate the microplate for 4 hours at room temperature or overnight at 4°C.

3.4.2.2 Wash the ELISA microplates

Empty the wells into a waste container. Fill the wells to overflowing with 1X PBST, then quickly empty them again. Repeat 2 more times. Hold the microplate upside down and tap firmly on a folded paper towel to remove excess liquid.

Note: Use freshly coated microplates only. To store the microplates for future use it is necessary to apply Postcoat 10 which is available separately from Agdia (catalog number ACC 00650).

3.4.2.3 Prepare general extraction buffer

Preparation of the General Extract Buffer 4 (GEB4) GEB4 is used to dilute and extract samples.

- Buffer powder (provided in kit) 33 g
- Distilled water 1000 ml (1 litre) Tween 20 20 ml or 20 g

To make 1L of GEB4, add 50 ml of water to the powder and mix into a smooth slurry. While mixing, slowly add the remaining volume of water. Add Tween-20 to the solution. Stir for 30 minutes.

It is recommended preparing only as much buffer as is needed for one day. When storing buffers at room temperature add sodium azide (Sigma S-2002) to 1X liquid buffers at a rate of 0.2 g per litre 0.02%.

3.4.2.4 Sample extraction

Samples must be ground and diluted with General Extract Buffer 4 (GEB4). Samples can be ground in sample mesh bags with a tissue homogenizer. This method is especially useful for processing large numbers of samples. Use no more than eight Prunus leaves per
sample. Stack the leaves with the petioles at one end. Remove the petioles and discard them. Use 0.5 g of the basal portion of the stacked leaves. Grind plant tissue in GEB4 at a sample to buffer ratio of 1:10 (tissue weight in g: buffer volume in ml).

**Example:** A sample weighing 0.5 grams requires 5 ml of GEB4.

100 µl of the ground and diluted sample will be added to the test well.

### 3.4.2.5 Preparation of positive controls

Before opening the container of control strips, let the container warm at room temperature for 15 minutes. This maintains the shelf life of the strips. Do not allow the container to remain opened, keep tightly sealed between uses. Each control strip requires 500 µl of GEB4. Dispense the required amount of GEB4 into a tube or similar container. Dip the pad end of the strip into the buffer and let it sit for 5 minutes. Use the strips to stir the buffer before using. The control strip can only be used once, after which it must be discarded. Do not store the positive control solution. It should be discarded after one day.

### 3.4.2.6 Test Procedure

**Method:**

1. **Dispense** 100 µl of prepared sample into each sample well. Dispense 100 µl of positive control into the positive control wells, 100 µl of extracted negative tissue into the negative control wells, and 100 µl of GEB4 (the extraction buffer) into the buffer wells.

2. **Incubate** microplate inside a humid box for 2 hours at room temperature or overnight in the refrigerator (4°C) for 14-16 hours.

3. **Prepare the enzyme conjugate.** Always prepare enzyme conjugate within 10 minutes of use. The alkaline phosphatase enzyme conjugate is concentrated and must be diluted with RUB3 enzyme conjugate diluent before use. The recommended conjugate to diluent ratio is given on the label. Dispense the appropriate volume of RUB3 enzyme conjugate diluent into a dedicated container, 100 µl of diluent is required for each test well. Then, add the alkaline phosphatase enzyme conjugate according to the dilution given on the label. Use a new, sterile pipette tip to prevent contamination of the enzyme conjugate.

   **Example:** If the dilution given on the bottle of concentrated alkaline phosphatase conjugate is 1:200, you should first dispense 10 ml of RUB3 enzyme conjugate diluent. Then add 50 µl of the alkaline phosphatase enzyme conjugate. Mix the enzyme conjugate thoroughly.

4. **Wash microplate.** When the first incubation is complete empty the test wells into a sink or waste container in a manner that does not allow the content of one test well from going into another test well. Then, fill all the test wells to overflowing with 1X PBST, and quickly empty. Repeat this PBST wash step 6 to 8 times. It is very important that all test wells are thoroughly washed. After washing, hold the microplate upside down and tap firmly on a paper towel to remove excess liquid. Quickly inspect the test wells. All wells should be free of plant tissue and air bubbles. If tissue or bubbles are present repeat the washing and tap firmly on a paper towel.

5. **Add enzyme conjugate.** Dispense 100 µl of prepared enzyme conjugate to each test well.

6. **Incubate microplate.** Incubate the microplate in a humid box for 2 hours at room
temperature.

7. **Wash microplate.** As before, wash the microplate 6 to 8 times with 1X PBST.
   
   1. Quickly inspect the wells looking for the presence of air bubbles.

8. **Add PNP substrate solution.** Add 100 µl of PNP substrate solution to each test well.

9. **Incubate microplate.** Incubate the microplate for 60 minutes. Keep test wells away from strong light.

10. **Measure of optical density.** Measure the optical density of the test wells on a plate reader at 405 nm. Wells in which colour develops indicate positive results. Wells in which there is no significant colour development indicate negative results. Test results are valid only if positive control wells give a positive result and blank and negative control wells remain colourless. Calculate the mean absorbance of the healthy samples (Abs_{neg av}) and multiply it by 2.5 and by 3 to obtain the sample positive-negative cut-off points. Record all results.
3.5 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a rapid, specific and sensitive test that can be used to detect and diagnose PPV from extracted nucleic acids. The primers recommended are those described by Wetzel et al. (1991b), which have become standard primers used worldwide for the detection of PPV. These primers detect all strains of PPV and a restriction digestion test can be used to differentiate PPV strains.

3.5.1 RNA extraction from plant tissue using QIAGEN RNeasy™ Plant Mini Kit

**Equipment**

- Autoclave
- Sterile 1.5 ml microcentrifuge tubes
- 0-2 μl, 2-20 μl, 20-200 μl, and 200-1000 μl Pipettes and sterile tips
- Sterile micropesle (e.g. Eppendorf part: 0030 120.937) (if necessary)
- Balance
- Microcentrifuge (at room temperature)
- Freezer/Refrigerator
- Sterile scalpel blades (if necessary)
- Vortex
- Variable speed electric drill (optional)
- MagNa Lyser machine (optional)
- Dewar liquid nitrogen dispenser and liquid nitrogen (if appropriate for protocol)
- Water bath or Dry heat block at 56°C ± 2.5°C or 70°C ± 2.5°C (depending on the protocol used)

**Reagents**

- Ethanol 100% (room temperature)
- QIAGEN RNeasy™ Plant Mini Kit reagents
- BioRad Quantum Prep® Freeze ‘N Squeeze DNA gel Extraction Spin Columns.
- PVP-40 (if using the modified protocol)
- 20% (w/v) Sarkosyl (if using the modified protocol by MacKenzie et al., 1997)
- 2 M sodium acetate, pH 5 (if using the modified protocol by MacKenzie et al., 1997) For 100 ml: Sodium acetate 16.406 g
- Adjust pH to 5 using acetic acid

**Method**

The kit provides two extraction buffers, the RLT and the RLC, which contain guanidine isothiocyanate (GITC) and guanidine hydrochloride, respectively. In most cases, the buffer RLT is the lysis buffer of choice due to the greater cell disruption and denaturation properties of GITC. For woody plants samples and/or Prunus suspected to be PPV infected it is recommended to use the RLT buffer.

1. Before starting add 10 μl of β-Mercaptoethanol to 1 ml extraction RLT buffer.

Maintaining this proportion prepare the required volume according to the number of samples to be processed. Record samples and kit details in an appropriate nucleic acid extraction form.

2. Add 450μl of the prepared RLT buffer to a maximum of 0.1 g of fresh or 0.020 g
of frozen tissue per sample.

3. Grind the sample using a grinding machine or directly in 1.5 ml microcentrifuge tube or in a mortar and pestle with or without liquid nitrogen. It is recommended to incubate the lysate at 56°C ± 2.5°C for 1-3 minutes in a water bath or dry heat block to help to disrupt the tissue. However, for samples with high starch content, incubation at elevated temperatures should be omitted in order to prevent swelling of the starting material.

4. Pipette the lysate directly onto a LILAC QIAshredder spin column placed into a 2 ml collection tube.

5. Centrifuge for 2 min at 13,000 rpm.

6. Transfer the flow-through fraction to a new 1.5 ml microcentrifuge tube (not supplied)

7. without disturbing the cell-debris pellet in the collection tube.

8. Apply all samples including any precipitate that may have formed, to a PINK RNeasy Mini column placed in a 2 ml collection tube (supplied). Close the tube gently.

9. Centrifuge for 15 sec at 10,000 rpm.

10. Add 700 μl buffer RW1 to the RNeasy pink column. Close the tube gently.

11. Centrifuge for 15 sec at 10,000 rpm.

12. Discard the flow-through.

13. Transfer the RNeasy column into a new 2 ml-collection tube (supplied).

14. Pipette 500 μl of buffer RPE into the RNeasy column. Close the tube gently. Note: RPE Buffer is supplied as a concentrate. Ensure that ethanol is added to the RPE Buffer before use.

15. Centrifuge for 15 sec at 10,000 rpm.

16. Discard the flow-through.

17. Add another 500 μl of RPE Buffer to the RNeasy column. Close the tube gently.

18. Centrifuge for 15 sec at 10,000 rpm.

19. Discard the flow-through.

20. Re-centrifuge for 2 min at 10,000 rpm to dry the RNeasy silica-gel membrane.

21. Remove the RNeasy mini column carefully from the collection tube, so the column does not touch the inside of the collection tube, to a new 1.5 ml microcentrifuge tube.

22. Pipette 30 μl RNase-free water directly onto the RNeasy silica-gel membrane. Close the tube gently.

23. Centrifuge for 1 min at 10,000 rpm.

24. Optional: Repeat steps 22 and 23.

25. Label the tube and store at -80°C ± 5°C.
3.5.2 RT-PCR procedures

3.5.2.1 Method

Reverse Transcription-polymerase chain reaction can be performed either in a one or two step reaction. This document describes a two step RT-PCR reaction. This approach allows storage of the obtained cDNA template for future RT-PCR testing, and/or subsequent cloning. In this case, the reverse primer for the target region to be amplified is required. A cDNA synthesis reaction of 10 - 20 µl is normally made but a 50 µl reaction is recommended when the cDNA product will be used in several different PCR testing or cloned to guarantee enough cDNA provision.

Reverse Transcription

As mentioned above, cDNA synthesis can be performed separately from the PCR amplification.

<table>
<thead>
<tr>
<th>Primer (Choose one)</th>
<th>Name</th>
<th>Sequence (5'-&gt;3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse</td>
<td>P1 (Wetzel)</td>
<td>ACC GAG ACC ACT ACA CTC CC</td>
</tr>
<tr>
<td>Random</td>
<td>Random hexamers</td>
<td>(Invitrogen)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reagent master mix A</th>
<th>Volume reaction (8 µl)</th>
<th>Incubation (Time-temperature)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile distilled H₂O</td>
<td>XX* µl</td>
<td>Incubate at 70°C +/-3°C for 10 min.</td>
</tr>
<tr>
<td>5X first strand buffer</td>
<td>2.0 µl</td>
<td>Centrifuge up to 10,000 rpm for 30 sec.</td>
</tr>
<tr>
<td>40 U/µl RNASin Plus</td>
<td>1.0 µl</td>
<td>Leave at room temperature for 15 min.</td>
</tr>
<tr>
<td>Primer*</td>
<td>XX* µl</td>
<td></td>
</tr>
<tr>
<td>RNA template</td>
<td>2.0 µl</td>
<td></td>
</tr>
</tbody>
</table>

* Add 2.0 µl of 5 µM of P1 PPV reverse primer or 0.25 µl of 0.5 ng/µl of random hexamers. Adjust the volume of water so the reaction volume of mix A equals 8 µl.

<table>
<thead>
<tr>
<th>Reagent master mix</th>
<th>Volume reaction (2 µl)</th>
<th>Incubation (Time-temperature)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M DTT</td>
<td>1.0 µl</td>
<td>Mix 2 µl of master mix B with master mix A for a total volume of 10 µl.</td>
</tr>
<tr>
<td>40 U/µl RNASin Plus</td>
<td>0.25 µl</td>
<td>Incubate for 1 hour at 42°C to 50°C +/-2.5°C depending on the reverse transcriptase^ used.</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>0.5 µl</td>
<td>Store cDNAs at -20°C +/-3°C.</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>0.25 µl</td>
<td></td>
</tr>
</tbody>
</table>

^ 42°C +/-2.5°C for Superscript II and 50°C +/-2.5°C for Superscript III.
Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>Primer</th>
<th>Name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse</td>
<td>P1 (Wetzel)</td>
<td>ACC GAG ACC ACT ACA CTC CC</td>
</tr>
<tr>
<td>Forward</td>
<td>P2 (Wetzel)</td>
<td>CAG ACT ACA GCC TCG CCA GA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCR Reagent mix</th>
<th>Volume (20µl)</th>
<th>Reaction</th>
<th>Cycling parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile distilled H2O</td>
<td>12.2 µl</td>
<td></td>
<td>94°C 2 min x 1</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>2.0 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mM Mg Cl2</td>
<td>1.2 µl</td>
<td></td>
<td>92°C 30 sec x 40</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>0.4 µl</td>
<td></td>
<td>60°C 30 sec</td>
</tr>
<tr>
<td>5 µM P1 (Wetzel) primer</td>
<td>1.0 µl</td>
<td></td>
<td>72°C 1 min</td>
</tr>
<tr>
<td>5 µM P2 (Wetzel) primer</td>
<td>1.0 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.2 µl</td>
<td></td>
<td>72°C 30 sec</td>
</tr>
<tr>
<td>cDNA template</td>
<td>2.0 µl</td>
<td></td>
<td>4°C 1 min x 1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCR controls</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Known PPV-infected sample (a positive control may be purchased from Loewe, Germany; DSMZ, Germany; Bioreba, Switzerland; or alternatively use the target product cloned into a plasmid).</td>
</tr>
<tr>
<td>Negative</td>
<td>Healthy plant tissue (a negative control may be purchased from Loewe, Germany; DSMZ, Germany; Bioreba, Switzerland).</td>
</tr>
<tr>
<td>No template control</td>
<td>Water</td>
</tr>
</tbody>
</table>

Electrophoresis

<table>
<thead>
<tr>
<th>Electrophoresis</th>
<th>Description</th>
<th>Buffer</th>
<th>Predicted size amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose gel</td>
<td>2%</td>
<td>1X TAE</td>
<td>243 bp for primers P1 &amp; P2</td>
</tr>
</tbody>
</table>

For better resolution of the PCR products it is recommended to perform a 2% agarose-TAE gel electrophoresis at 90 volts for 40-90 min (depending on gel size) and using a 100 bp marker. It is also recommended to load 10 µl or the complete volume of the RT-PCR reaction in each electrophoretic cell if further testing such as restriction enzyme is planned. Stain the gel either with SYBR safe or Ethidium bromide. Visualise the RT-PCR products using an UV trans-illuminator, photograph the gel and record the results.

Band excision and further testing

Excise the band of the expected PCR product with a sterile blade and place it into a Quantum Prep BioRad gel extraction column to purify the amplicon. Freeze for 5 min and centrifuge full speed at room temperature for 3 min. The amplicon will be ready for immediate cloning (see Sambrook et al., 1989 for standard cloning procedures), direct sequencing, and/or restriction digestion. Freeze the remaining product at -20°C for up to 6 weeks. The DNA will progressively degrade.
3.6 Herbaceous Indexing

**Equipment**

- mortar and pestle
- wash bottle
- tags
- pen

**Reagents**

- 0.5X PBS
- 0.68 M sodium chloride
- 40 mM sodium phosphate dibasic anhydrous
- 7 mM potassium phosphate
- 3 mM potassium chloride
- Carborundum powder (Saint-Gobain Industrial Ceramics Pty)

**Indicator plants (x2)**

- *Chenopodium foetidum*
- *Nicotiana benthamiana*

**Method**

1. Choose two leaves from each indicator and punch a small hole in the end of the leaf.
2. Lightly cover both leaves with fine carborundum powder.
3. Grind leaf sample in mortar and pestle with 0.5X PBS buffer
4. Dip fingers in homogenised sample and gently rub liquid 8 times onto leaves sprinkled with carborundum powder.
5. Wash off all traces of carborundum powder as it burns the leaves.
6. Check indicators every week for 6 weeks for symptoms such as:
   - *Chenopodium foetidum* – local lesions
   - *Nicotiana benthamiana* – mosaic symptoms (PPV-M), delayed mosaic (PPV-D).

3.7 Woody Indexing

The recommended woody indicator plant is *Prunus tomentose* or Peach GF 305. An indexing test requires at least 5 replicates per sample. This is not a rapid test to be implemented as a routine diagnostic test, but can be used to maintain the virus in an alternate host for further characterisation. The woody indexing can be used to assist with strain differentiation. The PPV strains are capable of inducing differential leaf symptoms on these indicators. PPV-D produces chlorotic, vein-associated patterns and after several weeks some of the chlorotic areas become necrotic. PPV-EA produces mild chlorotic patterns with some necrotic flecking. PPV-M produces strong chlorotic, vein-associated patterns followed quickly by necrotic flecking and vein-associated necrotic patterns. See Nemeth (1986) for the method of indexing and Damsteegt *et al.* (1997) for images of symptoms.
4 IDENTIFICATION

The diagnostic procedure described in this chapter follows the diagnostic scheme depicted in Figure 37. In general, when pursuing a high standard of unequivocal diagnostics, the diagnostician must consider four core methods: electron microscopy, mechanical transmission, serological and molecular tests. Some variation to this scheme can be given depending on the scope of the screening, whether it is for quarantine purposes only, for general surveillance or during incursion responses. However, its application can be also extended to quality control in nursery production industries or production of virus-free plants under a national certification programme.

Symptom expression in the field and the results obtained from mechanical inoculation assays on herbaceous indicators or woody indexing indicators, gives an indication of the infectivity and transmissibility through sap of the virus under investigation. However, this information is not sufficient for a definitive diagnosis. Mixture of viruses with similar morphology and symptomatology (Pseudopox) may be a cause of confusion. For example, the pseudopox caused by the mixed infection of *Prunus necrotic ringspot virus* and *Apple chlorotic leaf spot virus* (Lebas et al., 2004).

Therefore, positive or negative samples derived from mechanical inoculation assays require additional serological or molecular validation. Another useful and rapid method for the identification of PPV-like particles is the observation of leaf-dip preparations by electron microscopy. However, observing the virus structures under the electron microscope can only be used as a general PPV detection method. The observation of crude sap preparation using Immunosorbent Electron Microscopy Technique and decoration may lead to a more definitive diagnostic result, but still requires validation by ELISA and/or RT-PCR.

In general, the specificity and high throughput of the serological methods such as ELISA can be exploited to specifically target PPV during surveys in production areas, commercial nurseries or for quarantine purposes, at the border and post entry quarantine. On the other hand, the RT-PCR technique is very sensitive and specific and allows the detection of minimal amounts of target RNA, which facilitates the validation of results from all other previously applied techniques. These results require further validation by sequencing the DNA amplicon. The obtained sequence is required to be aligned with sequences held in the NCBI-GenBank database using the Basic Local Alignment Search Tool (BLAST). Note that RT-PCR is not a high throughput method when compared with ELISA. Moreover, the overall cost of RT-PCR is higher. Therefore, RT-PCR is not recommended for the screening of large numbers of routine samples. This is a process that can be better achieved using ELISA, especially when conducted during optimum time of year for virus detection.

If the identification of virus strains is the objective, then the use of restriction enzymes on the RT-PCR product is the option proposed by this diagnostic standard.

It is also important to note that there are other recently developed techniques and diagnostic strategies which can also be used as alternative methods. For example, the dot blot hybridization using specific PPV probes, Western blot, Real-time RT-PCR (e.g. Schneider et al., 2004; Mavrodieva et al., 2005), and LAMP (e.g. Varga & James, 2006; Hadersdorfer et al., 2011) to name a few. These methods and strategies are not considered in this diagnostic standard.
Fig 37: Diagnostic flow chart for detection and identification of PPV.
5. CONTACT POINTS

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6. ACKNOWLEDGEMENTS

Brendon Rodoni, Renae Sarec, Ross Mann, Jane Moran, Peter Merriman (Australia) and Francisco Ochoa Corona (New Zealand) for providing first drafts of their respective PPV protocols for Australia and New Zealand.

The document and procedures were reviewed by Francisco Ochoa-Corona & Gerard Clover, NZ MPI. The NDP was reviewed by Dr Catia Delmiglio, NZ MPI.

The Pennsylvania State College of Agricultural Sciences, Department of Plant Pathology and Cooperative Extension and the authors and contributors of Plum Pox Virus and Other Diseases of Stone Fruits Symptoms Booklet (AGRS-81) from which most of the pictures for this protocol were taken.

7. REFERENCES


### 7.1. Additional useful references


QIAGEN RNeasy™ Plant Mini Kit for RNA isolation from plant tissue Catalogue # 74904.
