



[1] **DRAFT ANNEX to ISPM 27:2006 – Potato spindle tuber viroid (2006-022)**

[2] **Development history**

[3]

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<b>Discipline leads history</b>	2008-04 SC Gerard CLOVER (NZ) 2010-11 SC Delano JAMES (CA)
<b>Consultation on technical level</b>	The first draft of this protocol was written by (lead author and editorial team) <ul style="list-style-type: none"> <li>• Colin JEFFRIES (Science and Advice for Scottish Agriculture, Edinburgh, UK);</li> <li>• Jorge ABAD (USDA-APHIS, Plant Germplasm Quarantine Program, Beltsville, USA);</li> <li>• Nuria DURAN-VILA (Conselleria de Agricultura de la Generalitat Valenciana, IVIA, Moncada, Spain);</li> <li>• Ana ETCHERVERS (Dirección General de Servicios Agrícolas, Min. de Ganadería Agricultura y Pesca, Montevideo, Uruguay);</li> <li>• Brendan RODONI (Dept of Primary Industries, Victoria, Australia);</li> <li>• Johanna ROENHORST (National Reference Centre, National Plant Protection Organization, Wageningen, the Netherlands);</li> <li>• Huimin XU (Canadian Food Inspection Agency, Charlottetown, Canada).</li> </ul> <p>In addition, JThJ VERHOEVEN (National Reference Centre, National Plant Protection Organization, The Netherlands) was significantly involved in the</p>

	<p>development of this protocol.</p> <p>The draft, in whole or part, has also been commented upon by:</p> <ul style="list-style-type: none"> <li>• SL NIELSEN (Denmark);</li> <li>• L. SEIGNER, S. WINTER, M. WASSENEGGER (Germany);</li> <li>• H. KOENRAADT (The Netherlands);</li> <li>• A. FOX, T. JAMES, W. MONGER, V. MULHOLLAND (UK).</li> </ul> <p>In addition, the draft has also been subject to expert review and the following international experts submitted comments:</p> <ul style="list-style-type: none"> <li>• Neil BOONHAM (The Food and Environment Research Agency, York, UK);</li> <li>• Gerard CLOVER (Plant Health and Environment laboratory, Min. of Agriculture and Forestry, Auckland, New Zealand);</li> <li>• Ricardo FLORES (UPV-CSIS, Universidad Politecnica, Valencia, Spain);</li> <li>• Ahmed HADIDI (USDA, ARA, Beltsville, USA);</li> <li>• Rick MUMFORD (The Food and Environment Research Agency, York, UK);</li> <li>• Teruo SANO (Plant Pathology laboratory, Hirosaki University, Japan);</li> <li>• Mathuresh SINGH (Agricultural Certification Services Inc., New Brunswick, Canada);</li> <li>• Rudra SINGH (Potato Research Centre, AAFC, Fredericton, Canada).</li> </ul>
<p><b>Main discussion points during development of the diagnostic protocol</b> (to be updated during development as needed)</p>	<ul style="list-style-type: none"> <li>• Reducing the text for 1. Pest information and the length of introduction and references</li> <li>• Whether generic tests should be included in preference to more specific tests</li> <li>• Whether bioassay, R-PAGE and dig probe should be included (but particularly bioassay, R-PAGE)</li> <li>• Whether more extraction sets published primer sets and reaction conditions should be included</li> <li>• Test validation data (or more specifically the lack of it)</li> <li>• Which controls to include</li> <li>• Interpretation of results for RT-PCR methods</li> <li>• Whether to leave a section for inclusion of a generic real time detection method that is to be published soon.</li> </ul>
<p><b>Notes</b></p>	<p>2013-05-07 edited</p>

[4] **1. Pest Information**

[5] Viroids are unencapsidated, small (239–401 nucleotides), covalently closed circular single-stranded RNA molecules capable of autonomous replication in infected hosts (Hammond & Owens, 2006). *Potato spindle tuber viroid* (PSTVd; genus *Pospiviroid*) is commonly 359 nucleotides in length but nucleotide lengths of 341–364 have been reported (Jeffries, 1998; Shamloul *et al.*, 1997; Wassenegger *et al.*, 1994). Mild and severe strains have been described based on symptoms produced in sensitive tomato cultivars; for example, *Solanum lycopersicum* (tomato) cv. Rutgers (Fernow, 1967).

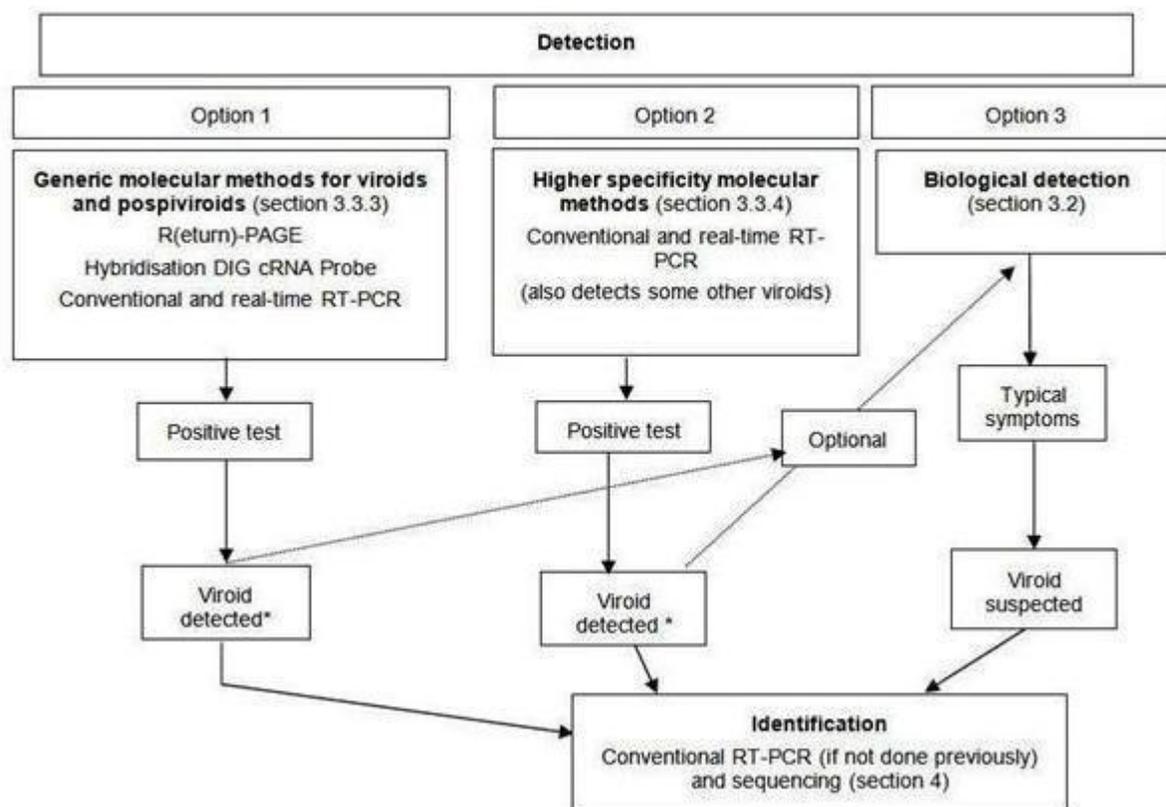
- [6] The natural host range of PSTVd is relatively narrow. The primary hosts are *Solanum tuberosum* (potato) and stolon- and tuber-forming *Solanum* spp. and *S. lycopersicum*. PSTVd has been found also in *Capsicum annuum* (pepper), *Persea americana* and *S. muricatum*. Recently, PSTVd has been detected in mainly vegetatively propagated ornamental plant species in the family Solanaceae – namely, *Brugmansia* spp., *Cestrum* spp., *Datura* sp., *Lycianthes rantonetti*, *Petunia* spp., *Physalis peruviana*, *Solanum* spp. and *Streptosolen jamesonii* – but also *Dahlia × hybrida* in the family Asteraceae (for natural host details, download the European and Mediterranean Plant Protection Organization (EPPO) Plant Quarantine Data Retrieval System (PQR) database available at <http://www.eppo.int/DATABASES/databases.htm>). The experimental host range of PSTVd is wide. It primarily infects species in the family Solanaceae but also some species in at least nine other families. Most hosts express few or no disease symptoms (Singh, 1973; Singh *et al.*, 2003).
- [7] PSTVd has been found infecting *S. tuberosum* in Africa (Nigeria), Asia (Afghanistan, China, India), parts of Eastern Europe, North America (EPPO/CABI, 1997), Central America (Badilla *et al.*, 1999), the Middle East (Hadidi *et al.*, 2003) and Argentina (Bartolini & Salazar, 2003). However, it has a wider geographical distribution in ornamentals and other hosts.
- [8] In potato, the main means of spread of PSTVd is by vegetative propagation. It is also spread by contact, mainly by machinery in the field and by cutting seed potato tubers (Hammond & Owens, 2006). PSTVd is transmitted in true potato seed – up to 100% of the seed may be infected (Fernow *et al.*, 1970; Singh, 1970) – and also in pollen (Grasmick & Slack, 1985; Singh *et al.*, 1992). De Bokx and Pirone (1981) reported a low rate of transmission of PSTVd by the aphid *Macrosiphum euphorbiae* but not by *Myzus persicae* or *Aulacorthum solani*. However, experimental acquisition and transmission of PSTVd by *Myzus persicae* from plants co-infected by *Potato leafroll virus* (PLRV) have been reported (Salazar *et al.*, 1995, 1996; Singh & Kurz, 1997). PSTVd was subsequently shown to be heterologously encapsidated within particles of PLRV (Querici *et al.*, 1997), a phenomenon that may have important implications for the epidemiology and spread of PSTVd under field conditions. In tomato, PSTVd is easily spread by contact and has been shown to be transmitted by pollen and seed (Kryczynski *et al.*, 1988; Singh, 1970). It is also possible that infected ornamental species may act as an inoculum source if handled before touching other susceptible plants (Verhoeven *et al.*, 2010). No transmission of PSTVd was shown with *Apis mellifera*, *Bombus terrestris*, *Frankliniella occidentalis* or *Thrips tabaci* (Nielsen *et al.*, 2012).
- [9] PSTVd is the only viroid known to infect cultivated species of *S. tuberosum* naturally. However, *Mexican papita viroid* infects the wild species *Solanum cardiophyllum* (Martinez-Soriano *et al.*, 1996). Experimentally, other viroid species in the genus *Pospiviroid* infect *S. tuberosum* (Verhoeven *et al.*, 2004). In addition to PSTVd, other viroids have been found infecting *S. lycopersicum* naturally, including *Citrus exocortis viroid* (CEVd; Mishra *et al.*, 1991), *Columnea latent viroid* (CLVd; Verhoeven *et al.*, 2004), *Mexican papita viroid* (MPVd; Ling & Bledsoe, 2009), *Tomato apical stunt viroid* (TASVd; Walter, 1987), *Tomato chlorotic dwarf viroid* (TCDVd; Singh *et al.*, 1999) and *Tomato planta macho viroid* (TPMVd; Galindo *et al.*, 1982).
- [10] **2. Taxonomic Information**
- [11] **Name:** *Potato spindle tuber viroid* (acronym PSTVd)
- [12] **Synonyms:** potato spindle tuber virus, potato gothic virus
- [13] **Taxonomic position:** Pospiviroidae, *Pospiviroid*
- [14] **Common names:** potato spindle tuber
- [15] **3. Detection**
- [16] Symptom appearance and severity depend on PSTVd strain, cultivar and environment. In *S. tuberosum*, infection may be symptomless or produce symptoms ranging from mild to severe (reduction in plant size and uprightiness and clockwise phyllotaxy of the foliage when the plants are viewed from above; dark green and rugose leaves). Tubers may be reduced in size, misshapen, spindle or dumbbell shaped, with conspicuous

prominent eyes that are evenly distributed (EPPO, 2004). In *S. lycopersicum*, symptoms include stunting, epinasty, rugosity and lateral twisting of new leaflets, leaf chlorosis, reddening, brittleness, necrosis, reduction in fruit size, and fruit not fully ripening (Hailstones *et al.*, 2003; Lebas *et al.*, 2005; Mackie *et al.*, 2002). In *C. annuum*, symptoms are subtle, with leaves near the top of the plant showing a wavy-edged margin (Lebas *et al.*, 2005). In ornamental plant species symptoms are absent (Verhoeven, 2010).

[17] Because PSTVd may be asymptomatic, tests are required for its detection and identification. Detection of PSTVd can be achieved using the biological and molecular tests shown as options in Figure 1, but for identification, the polymerase chain reaction (PCR) product/amplicon must be sequenced as the tests are not specific for PSTVd and will detect other viroids. Sequencing will also contribute to preventing the reporting of false positives. If pathogenicity is considered to be important, biological indexing may be done. If the identification of PSTVd represents the first finding for a country, the laboratory may wish to have the diagnosis confirmed by another laboratory.

[18] Appropriate controls should be included in all tests to minimize the risk of false positive or false negative results.

[19]



\* Identification may not be needed for every viroid positive sample in certain situations; for example, when dealing with a PSTVd outbreak.

Note: If a viroid is suspected in a sample (i.e. typical symptoms are present) but a test gives a negative result, another of the tests should be carried out for confirmation of the result.

**Figure 1.** Minimum requirements for the detection and identification of *Potato spindle tuber viroid*

[20] The presence of other viroids needs to be considered when choosing a detection and identification method. This protocol describes non-specific detection methods that will detect all known viroids, pospiviroids and PSTVd (as well as some other closely related viroids). Identification is achieved by sequencing the PCR

product.

[21] In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The protocols described in this standard do not imply that other protocols used by a laboratory are unsuitable, provided that they have been adequately validated. Recommendations on method validation in phytodiagnostics are provided by EPPO (2010).

### [22] 3.1 Sampling

[23] General guidance on sampling methodologies is described in ISPM 31:2008, *Methodologies for sampling of consignments*.

[24] **S. tuberosum microplants and glasshouse grown S. tuberosum plants** For microplants the whole plant should be used as the sample or the top two-thirds of the plant should be sampled under aseptic conditions so as to enable the rest of the plant to continue growing. Microplants should be four to six weeks old with stems of about 5 cm length and with well-formed leaves. For glasshouse grown plants a fully expanded leaflet from each plant should be used. Viroid concentration is affected by temperature and light levels, so plants should be grown preferably at a temperature of 18 °C or higher and with a photoperiod of at least 14 h. Microplants or leaves may be bulked; the bulking rate will depend on the test method used. The bulking rate must be validated.

[25] **Field grown S. tuberosum plants** A fully expanded non senescing terminal leaflet from the top of each plant should be used. Leaves may be bulked together for testing; the bulking rate will depend on the test method used. The bulking rate must be validated.

[26] **S. tuberosum tubers** PSTVd is systemically distributed in infected *S. tuberosum* tubers, that is, in the “eye”, periderm, cortical zone containing cortical parenchyma and external phloem tissue, xylem ring, perimedullary zone containing internal phloem and phloem parenchyma strands tissue and perimedullary starch-storage parenchyma, and pith (Shamloul *et al.*, 1997). It also occurs in almost equal amounts in different parts of both primarily and secondarily infected tubers (Roehorst *et al.*, 2006), that is, in the top and other eyes, heel ends, peel fragments and flesh cores throughout the whole tuber. The highest concentration is found immediately after harvest and hardly decreases during storage at 4 °C for up to three months. Six months after harvest and storage at 4 °C, concentrations may decrease by more than 10<sup>4</sup> times. A core from any part of the tuber can be used as a sample. Up to 100 small cores weighing about 50 mg each may be bulked together for extraction if using real-time reverse transcription (RT)-PCR. Bulking for other methods should be validated.

[27] **Leaves of other crops and ornamental plant species** Samples from fully expanded young leaves are used. For *Brugmansia* spp., leaf sap is not suitable for inoculation of test plants; root material should be used instead.

[28] **Seed** Viroid concentration may vary greatly between seeds and the level of infection may vary from 100% to less than 5%. This makes it very difficult to recommend a maximum bulking rate. For *S. lycopersicum*, bulking rates of 100–1 000 have been used (EUPHRESKO, 2010) for testing samples of 1 000–3 000 seeds. In some countries bulking rates of 400 seeds are being used for testing samples of 20 000 seeds (H. Koenraad, Naktuinbouw, the Netherlands, personal communication, 2012).

[29] Seeds may also be sown in compost in trays and the seedlings tested destructively or non-destructively.

### [30] 3.2 Biological detection

[31] Inoculation of *S. lycopersicum* plants (cvs Rutgers, Moneymaker or Sheyenne) will allow the detection of many viroids, but will not detect certain viroids such as the pospiviroid *Iresine viroid 1* (IrVd-1). The method is sensitive, results are repeatable and reproducible, and visual evidence of pathogenicity may be observed. However, some isolates may not be detected because of the absence of symptoms, and if symptoms are produced, they may not be diagnostic for PSTVd. Moreover, biological indexing may require a great deal of greenhouse space, it is labour intensive, and several weeks or more may be needed before the test is completed. No work has been done to compare the sensitivity of this method with other methods described in

this protocol.

- [32] Approximately 200–500 mg leaf or tuber tissue is ground in a small quantity of 0.1 M phosphate inoculation buffer (a 1:1 dilution is adequate) containing carborundum (400 mesh). Phosphate buffer is made by combining 80.2 ml of 1 M  $K_2HPO_4$  with 19.8 ml of 1 M  $KH_2PO_4$  and adjusting the volume to 1 litre with distilled water.
- [33] Young tomato plants with one or two fully expanded leaves are inoculated. Using a gloved finger, a cotton bud, or a cotton swab dipped into the inoculum, the leaf surface is gently rubbed with the inoculum and then the leaves are immediately rinsed with water until the carborundum has been removed. The plants are grown at 25–39° C under a photoperiod of 14 h. If necessary, supplemental illumination is provided (approximately 650  $\mu E/m^2/s$ ; Grassmick & Slack, 1985). The plants are inspected weekly for symptoms for up to six weeks after inoculation. Symptoms of PSTVd infection include stunting, epinasty, rugosity and lateral twisting of new leaflets, leaf chlorosis, reddening, brittleness and necrosis.
- [34] A bioassay on tomato will allow detection of many pospiviroids; therefore, RT-PCR should be carried out on the nucleic acid extracted from symptomatic indicator plants and the PCR product should be sequenced for identification.
- [35] **3.3 Molecular detection**
- [36] **3.3.1 Tissue maceration**
- [37] **Microplants, leaf material and roots** Mortars and pestles or homogenizers (e.g. Homex 6, Bioreba)<sup>1</sup> with extraction bags have been used successfully to grind material. Adding a small quantity of water or lysis extraction buffer or freezing the sample (e.g. in liquid nitrogen) may facilitate homogenization.
- [38] ***S. tuberosum* tubers** Tuber cores are thoroughly homogenized in water or lysis buffer (1 ml per g tuber core). A grinder such as the Homex 6 with extraction bags has been used successfully. Freezing the cores (e.g. –20°C) before adding the water or lysis buffer facilitates homogenization.
- [39] **Seeds** For small numbers of seeds (<100) a tissue lyser (e.g. Retsch TissueLyser, Qiagen<sup>2</sup>) may be used. Although mortars and pestles may be used they are probably not practical for routine use, and cross-contamination may be more difficult to control. For larger numbers of seeds, a paddle blender (e.g. MiniMix®, Interscience)<sup>3</sup> or homogenizer (e.g. Homex 6) with a minimum quantity of extraction buffer for the initial homogenization may be used. Alternatively, use liquid nitrogen to freeze the sample, and grind it in a cell mill (this method can also be used for other tissue types).
- [40] **3.3.2 Nucleic acid extraction**
- [41] A wide range of nucleic acid extraction methods may be used, from commercial kits to methods published in scientific journals. The following nucleic acid extraction methods have been used successfully for the detection of PSTVd, as indicated for individual methods.
- [42] **Commercial kits** Commercial extraction kits such as RNeasy® (Qiagen)<sup>4</sup> and MasterPure™ (Epicentre Biotechnologies)<sup>5</sup> may be used according to the manufacturer's instructions. RNeasy® was evaluated for the extraction of PSTVd RNA from *S. lycopersicum* seed as part of the EUPHRESKO DEP project (EUPHRESKO, 2010).
- [43] **Lysis buffer** A modified extraction lysis buffer described by Mackenzie *et al.* (1997) can be used. It extracts quality RNA from a wide range of plant species.
- [44] **EDTA buffer** Plant tissue may be homogenized in a simple extraction buffer (50 mM NaOH, 2.5 mM ethylenediaminetetraacetic acid (EDTA)) and then incubated (at approximately 25° C for 15 min) or centrifuged (at 12 000 g at 4 °C for 15 min). The supernatant can then, depending on the level of sensitivity required, be either used directly for RT-PCR (less sensitive) or spotted onto a nitrocellulose membrane and eluted using sterile distilled water (more sensitive) (Singh *et al.*, 2006). Although the concentration of viroid is lower for the EDTA method than for the other extraction methods described, this should not be a limiting factor when the method is used with RT-PCR or the digoxigenin (DIG) probe. The method has been used

with *S. lycopersicum* and *S. tuberosum* and a range of ornamental plant species.

- [45] **Two-step PEG extraction** This extraction method using polyethylene glycol (PEG) (EPPO, 2004) has been used in combination with return (R)-polyacrylamide gel electrophoresis (PAGE), DIG-RNA probe and conventional RT-PCR methods described in this protocol for a wide range of plant species and tissue types (e.g. leaves and potato tubers).
- [46] **CTAB** This extraction method using cetyl trimethylammonium bromide (CTAB) (EPPO, 2004) has been used on leaves of a wide range of plant species and tomato seed with real-time RT-PCR.
- [47] **KingFisher (Thermo Scientific<sup>6</sup>)** The following automated procedure is based on use of the KingFisher mL Magnetic Particle Processor. With appropriate adjustment of volumes, other KingFisher models may be used. The extraction method has been used on leaves of a wide range of plant species, *S. tuberosum* tubers and *S. lycopersicum* seed. The method has been used with the real-time RT-PCR methods described in this standard. Cycle threshold (Ct) values several cycles higher may be expected using the KingFisher compared with the other extraction methods described in this protocol, but the increased throughput of samples that is achievable makes this a valuable extraction method. To make up the extraction buffer (EB), 200 µl of 8.39% (w/v) tetrasodium pyrophosphate (TNaPP) solution (pH 10.0–10.9) and 100 µl Antifoam B Emulsion (AB) (Sigma)<sup>7</sup> are added to 9.8 ml guanidine lysis buffer (GLB). GLB comprises water, 750 ml; absolute ethanol, 250 ml; guanidine-HCl, 764.2 g; disodium EDTA dehydrate, 7.4 g; polyvinylpyrrolidone (PVP), 30.0 g; citric acid monohydrate, 5.25 g; tri-sodium citrate, 0.3 g; and Triton™ X-100, 5 ml. GLB may be stored indefinitely. Store EB at 4° C and discard at the end of the day any that has not been used.
- [48] For each sample, at least 200 mg leaf or tuber tissue or up to 100 seeds are macerated, and then EB is added immediately at a ratio of 10 ml buffer per 1 g plant tissue or 20 ml buffer per 1 g seed. Maceration is continued until a clear cell lysate with minimal intact tissue debris is obtained.
- [49] Approximately 2 ml lysate is decanted into a fresh microcentrifuge tube, which is centrifuged at approximately 5000 g for 1 min. One millilitre of supernatant is removed and placed in the first tube (A) of the KingFisher mL rack, to which 50 µl of vortexed MAP Solution A magnetic beads (Invitex/Thistle Scientific)<sup>8</sup> are added. Tube B has 1 ml GLB added to it; tubes C and D 1 ml of 70% ethanol; and tube E 200 µl water or 1x Tris-EDTA (TE) buffer.
- [50] The tube-strip is placed in the KingFisher mL and the programme (see Table 1) is run. After 20 min, the machine will pause to allow a heating step. The tube-strip is placed in an oven at 65–70 °C for 5 min and then returned to the KingFisher mL, and the programme is resumed. Other models may have a heating or holding evaporation step built in.
- [51] On completion, the eluted nucleic acids are transferred to a new microcentrifuge tube and stored between –20 °C and –80 °C.
- [52] **Table 1.** Programme for the KingFisher Magnetic Particle Processor

[53]

*Plate layout* Default: Plate type = KingFisher tubestrip 1000 µl; Plate change message = Change Default  
**A:** volume = 1000, name = Cell lysate or tissue homogenate; volume = 50, name = Magnetic particles;  
**B:** volume = 1000, name = Washing buffer 1 (Various); **C:** volume = 1000, name = Washing buffer 2 (Various); **D:** volume = 1000, name = Washing buffer 3 (Various); **E:** volume = 200, name = Elution buffer (Various)  
**STEPS COLLECT BEADS** Step parameters: Name = Collect Beads; Well = A, Default; Beginning of step: Premix = No; Collect parameters: Collect count = 1. **BIND** Step parameters: Name = Lysing, Well = A, Default; Beginning of step: Release = Yes, time = 1min 0s, speed = Fast dual mix; Bind parameters: Bind time = 4min 0s, speed = Slow; End of step: Collect beads = No. **BIND** Step parameters: Name = Lysing, Well = A, Default; Beginning of step: Release = Yes, time = 1min 0s, speed = Fast dual mix Bind; Bind parameters: Bind time = 4min 0s, speed = Slow; End of step: Collect beads = No. **BIND** Step parameters: Name = Lysing, Well = A, Default; Beginning of step: Release = Yes, time = 1min 0s, speed = Fast dual mix; Bind parameters: Bind time = 4min 0s, speed = Slow; End of step: Collect beads = Yes, count = 4.  
**WASH** Step parameters: Name = Washing, Well = B, Default; Beginning of step: Release = Yes, time = 0s, speed = Fast; Wash parameters: Wash time = 3min 0s, speed = Fast dual mix; End of step: Collect beads = Yes, count = 3. **WASH** Step parameters: Name = Washing, Well = C, Default; Beginning of step:

Release = Yes, time = 0s, speed = Fast; Wash parameters: Wash time = 3min 0s, speed = Fast dual mix; End of step: Collect beads = Yes, count = 3. WASH Step parameters; Name = Washing, Well = D, Default; Beginning of step: Release = Yes, time = 0s, speed = Fast; Wash parameters: Wash time = 3min 0s, speed = Fast dual mix; End of step: Collect beads = Yes, count = 3. ELUTION Step parameters; Name = Elution, Well = E, Default; Beginning of step: Release = Yes, time = 10s, speed = Fast; Elution parameters: Elution time = 20s, speed = Bottom very fast; Pause parameters: Pause for manual handling = Yes, message = Heating, Post mix time = 30s, speed = Bottom very fast; Remove beads: Remove beads = Yes, collect count = 4, disposal well = D

[54] **3.3.3 Generic molecular methods for pospiviroid detection**

[55] **3.3.3.1 R-PAGE (EPPO, 2004)**

[56] R-PAGE has been recommended as a detection method for PSTVd infecting *S. tuberosum* leaves (EPPO, 2004), but it is less sensitive than the other molecular methods evaluated. It detects the equivalent of 5–20 mg PSTVd-infected leaf tissue (when mixed with a standard amount of healthy leaf tissue) depending on the laboratory, whereas other methods detect as little as 15.5 µg infected leaf tissue (the lowest weight tested). Results are repeatable and mostly reproducible, with three out of four laboratories detecting PSTVd (Jeffries & James, 2005).

[57] This method has also been used successfully with other host plants; for example, *C. annuum*, *S. tuberosum* (tubers) and *S. lycopersicum*. Because of its low sensitivity, bulking of samples would need to be validated.

[58] R-PAGE will detect all known pospiviroids; therefore, for identification of PSTVd, RT-PCR on the nucleic acid followed by sequencing of the PCR product must be carried out.

[59] **3.3.3.2 Hybridization with a DIG-labelled cRNA probe**

[60] This method has been recommended for detection of PSTVd infecting *S. tuberosum* leaves (EPPO, 2004). Sensitivity for the detection of PSTVd in *S. tuberosum* is at least 15.5 µg PSTVd-infected leaf tissue (the lowest weight tested), which is equivalent to 17 pg PSTVd (Jeffries & James, 2005). Results are repeatable and mostly reproducible, but although the nine laboratories that evaluated this method all detected PSTVd, the level of sensitivity achieved varied between them, with most laboratories detecting 0.25–0.5 mg infected leaf tissue. Other hosts have been tested successfully, including *Petunia* spp., *Solanum jasminoides*, *S. lycopersicum* and *S. tuberosum* (tubers).

[61] This DIG-labelled cRNA probe method will detect the following viroids: *Chrysanthemum stunt viroid* (CSVd), CEVd, CLVd, IrVd-1, MPVd, PSTVd, TASVd, TCDVd and TPMVd (T. James, Science and Advice for Scottish Agriculture (SASA), UK, personal communication, 2010). *Pepper chat fruit viroid* (PCFVd), a proposed new species in the genus *Pospiviroid* (Verhoeven *et al.*, 2009), is also detected by this method (T. James, SASA, UK, personal communication, 2010).

[62] The probe used is based on a full-length monomer of PSTVd produced by Agdia, Inc.<sup>9</sup> (Cat. No. DLP 08000/0001). This probe should be used according to the manufacturer's instructions, or refer to EPPO (2004) for details of the method. In addition to the Ames buffer (EPPO 2004), PEG and other extraction buffers may be used for nucleic acid extraction.

[63] To identify PSTVd, conventional RT-PCR should be carried out, followed by sequencing of the PCR product.

[64] **3.3.3.3 Conventional RT-PCR using the primers of Verhoeven *et al.* (2004)**

[65] The primers used in this assay are the Posp1 and Vid primers of Verhoeven *et al.* (2004). The Posp1 primers will detect CEVd, CSVd, IrVd-1, MPVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd. The Vid primers will detect PSTVd, TCDVd, and, additionally CLVd. Using the Posp1 and Vid primers in two separate reactions will allow detection of all pospiviroids. Sequence mismatch at critical positions of the primer target

site may prevent the detection of some isolates (e.g. an isolate of CLVd was not detected using these primers; Steyer *et al.*, 2010). *In silico* studies have shown that the following PSTVd isolates may not be detected because of primer/sequence mismatch at critical positions: Posp1 primers: EU879925, EU273604, EF459697, AJ007489, AY372398, AY372394, FM998551, DQ308555, E00278; Vid primers: EU273604<sup>10</sup>. The Posp1 primers are much more sensitive than the Vid primers for the detection of PSTVd.

[66] **Primers**

[67] Posp1-forward (F): 5'-GGG ATC CCC GGG GAA AC-3' (nucleotide (nt) 86–102)

[68] Posp1-reverse (R): 5'-AGC TTC AGT TGT (T/A)TC CAC CGG GT-3' (nt 283–261)

[69] Vid-F: 5'-TTC CTC GGA ACT AAA CTC GTG-3' (nt 355–16)

[70] Vid-R: 5'-CCA ACT GCG GTT CCA AGG G-3' (nt 354–336)

[71] Although various RT-PCR kits and reaction conditions may be used, they should be validated to check that they are fit for the purpose intended, with all relevant pospiviroids able to be detected.

[72] **Reaction conditions**

[73] The Qiagen<sup>11</sup> OneStep RT-PCR Kit has been shown to be reliable when used for the detection of PSTVd, CEVd, CLVd, CSVd TASVd and TCDVd (EUPHRESCO, 2010) and for other pospiviroids listed at the start of this section (T. James, SASA, UK, personal communication, 2010). It is not necessary to use the Q-solution described by EUPHRESCO (2010).

[74] Two microlitres of template is added to 23 µl master mix comprising 1.0 µl each of forward and reverse primer (10 µM), 5 µl of 5× Qiagen OneStep RT-PCR buffer, 1.0 µl Qiagen OneStep RT-PCR enzyme mix, 1.0 µl dNTPs (10 mM each dNTP), and 14 µl water. The thermocycling programme is as follows: 50 °C for 30 min; 95 °C for 15 min; 35 cycles of 94 °C for 30 s, 62 °C for 60 s and 72 °C for 60 s; and a final extension step of 72 °C for 7 min.

[75] **Gel electrophoresis**

[76] After RT-PCR, the PCR products (approximately 197 bp and 359 bp for the Posp1 and Vid primers, respectively) should be analysed by gel electrophoresis (2% agarose gel) and the PCR amplicons of the correct size sequenced to identify the viroid species. In practice, sequencing the 197 bp product has always resulted in the same identification as sequencing the complete viroid genome.

[77] **3.3.3.4 Real-time RT-PCR using the GenPosp1 assay (Botermans *et al.*, 2013)**

[78] The GenPosp1 assay uses TaqMan® real-time RT-PCR to detect all known species of the genus *Pospiviroid*. It consists of two reactions running in parallel: the first (reaction mix 1) targets all pospiviroids except CLVd (Botermans *et al.*, 2013); the second (reaction mix 2) specifically targets CLVd (Monger *et al.*, 2010). To monitor the RNA extraction a *nad5* internal control based on primers developed by Menzel *et al.* (2002) to amplify mRNA from plant mitochondria (the mitochondrial NADH dehydrogenase gene) is included. Method validation on tomato leaves showed that the GenPosp1 assay detects all pospiviroids up to a relative infection rate of 0.13% (which equals 770× dilution). The assay was specific as no cross-reactivity was observed with other viroids, viruses or nucleic acid from plant hosts. Repeatability and reproducibility were 100% and the assay appeared robust in an inter-laboratory comparison. The GenPosp1 assay has been shown to be a suitable tool for large-scale screening for all known pospiviroids. Although it has been validated for tomato leaves it can potentially be used for any crop.

**[79] Primers**

- [80] TCR-F 1-1: 5'-TTC CTG TGG TTC ACA CCT GAC C-3' (Botermans *et al.*, 2013)
- [81] TCR-F 1-3: 5'-CCT GTG GTG CTC ACC TGA CC-3' (Botermans *et al.*, 2013)
- [82] TCR-F 1-4: 5'-CCT GTG GTG CAC TCC TGA CC-3' (Botermans *et al.*, 2013)
- [83] TCR-F PCFVd: 5'-TGG TGC CTC CCC CGA A-3' (Botermans *et al.*, 2013)
- [84] TCR-F IrVd: 5'-AAT GGT TGC ACC CCT GAC C-3' (Botermans *et al.*, 2013)
- [85] TR-R1: 5'-GGA AGG GTG AAA ACC CTG TTT-3' (Botermans *et al.*, 2013)
- [86] TR-R CEVd: 5'-AGG AAG GAG ACG AGC TCC TGT T-3' (Botermans *et al.*, 2013)
- [87] TR-R6: 5'-GAA AGG AAG GAT GAA AAT CCT GTT TC-3' (Botermans *et al.*, 2013)
- [88] CLVd-F: 5'-GGT TCA CAC CTG ACC CTG CAG-3' (Monger *et al.*, 2010)
- [89] CLVd-F2: 5'-AAA CTC GTG GTT CCT GTG GTT-3' (Monger *et al.*, 2010)
- [90] CLVd-R: 5'-CGC TCG GTC TGA GTT GCC-3' (Monger *et al.*, 2010)
- [91] *nad5*-F: 5'-GAT GCT TCT TGG GGC TTC TTG TT-3' (Menzel *et al.*, 2002)
- [92] *nad5*-R: 5'-CTC CAG TCA CCA ACA TTG GCA TAA-3' (Menzel *et al.*, 2002)

**[93] Probes**

- [94] pUCCR: 6FAM-5'-CCG GGG AAA CCT GGA-3'-MGB (Botermans *et al.*, 2013)
- [95] CLVd-P: 6FAM-AGC GGT CTC AGG AGC CCC GG-3'-BHQ1 (Monger *et al.*, 2010)
- [96] *nad5*-P: VICr-AGG ATC CGC ATA GCC CTC GAT TTA TGT G-3'-BHQ1 (Botermans *et al.*, 2013)
- [97] The two reaction mixes are based on the TaqMan® RNA-to-CT™ 1-Step Kit (Applied Biosystems)<sup>12</sup>.

**[98] Reaction mix 1 (all pospiviroids except CLVd + nad5)**

- [99] The reaction mix consists of 12.5 µl of 2× TaqMan® RT-PCR mix, 0.6 µl of 1× TaqMan® RT enzyme mix, 0.75 µl (10 µM) forward primers (TCR-F 1-1, TCR-F 1-3, TCR-F 1-4, TCR-F IrVd, TCR-F PCFVd and *nad5*-F) and reverse primers (TR-R1, TR-R CEVd, TR-R6 and *nad5*-R) (final concentration 0.3 µM each), 0.25 µl (10

µM) TaqMan® probe pUCCR (final concentration 0.1 µM) and 0.5 µl (10 µM) TaqMan® probe *nad5*-P (final concentration 0.2 µM). Molecular grade water and 2 µl RNA template are added to a final volume of 25 µl.

**[100] Reaction mix 2 (CLVd + nad5)**

**[101]** The reaction mix consists of 12.5 µl of 2x TaqMan® RT-PCR mix, 0.6 µl of 1x TaqMan® RT enzyme mix, 0.75 µl (10 µM) forward primers (CLVd-F, CLVd-F2 and *nad5*-F) and reverse primer (CLVd-R and *nad5*-R) (final concentration 0.3 µM each), 0.25 µl (10 µM) TaqMan® probe CLVd-P (final concentration 0.1 µM) and 0.5 µl (10 µM) TaqMan® probe *nad5*-P (final concentration 0.2 µM). Molecular grade water and 2 µl RNA template are added to a final volume of 25 µl.

**[102]** Thermocycling conditions for both reaction mixes are 48 °C for 15 min, 95 °C for 10 min, followed by 40 cycles of (95 °C for 15 s and 60 °C for 1 min).

**[103]** For this method, Botermans *et al.* (2013) interpreted cycle threshold (Ct) values <32 as positive; those between 32 and <37 as doubtful, requiring confirmation; and those ≥37 as negative. However, these values need to be defined in each laboratory.

**[104] 3.3.4 Higher specificity molecular methods for the detection of PSTVd**

**[105] 3.3.4.1 Conventional RT-PCR using the primers of Shamloul *et al.* (1997)**

**[106]** The RT-PCR primers used in this assay are those of Shamloul *et al.* (1997), which are also described by Weidemann and Buchta (1998). The primers will detect MPVd, PSTVd, TCDVd and TPMVd. *In silico* studies have shown that the following PSTVd isolates may not be detected because of primer/sequence mismatch at critical positions: AY372394, DQ308555, EF459698 for the reverse primer<sup>2</sup>.

**[107] Primers**

**[108]** 3H1-F: 5'-ATC CCC GGG GAA ACC TGG AGC GAA C-3' (nt 89–113)

**[109]** 2H1-R: 5'-CCC TGA AGC GCT CCT CCG AG-3' (nt 88–69)

**[110] Method 1 (Invitrogen<sup>13</sup> SuperScript® One-Step RT-PCR with Platinum® Taq)**

**[111]** For each reaction, 1 µl template RNA is added to 24 µl master mix consisting of 1.7 µl each of forward and reverse primer (15 µM), 12.5 µl of 2x Reaction Buffer, 0.5 µl RT/Platinum® Taq and 7.6 µl water. The thermocycling programme is as follows: 43 °C for 30 min, 94 °C for 2 min, 10x (94 °C for 30 s, 68 °C for 90 s, 72 °C for 45 s), 20x (94 °C for 30 s, 64 °C for 90 s, 72 °C for 45 s), 72 °C for 10 min and 20 °C for 1 min.

**[112] Method 2 (Two-step RT-PCR)**

**[113]** Using the two-step RT-PCR, the sensitivity for the detection of PSTVd in *S. tuberosum* is at least 15.5 µg PSTVd-infected leaf tissue, but the sensitivity achieved varies between laboratories, with most laboratories detecting at least 62 µg infected leaf tissue (Jeffries & James, 2005). See EPPO (2004) for the description of Method 2.

**[114]** After RT-PCR, the PCR products (approximately 360 bp) are analysed by gel electrophoresis as described and PCR amplicons of the correct size are sequenced to identify the viroid species.

**[115]** An internal control assay using *nad5* primers (Menzel *et al.*, 2002) has been used with this assay in a simplex (separate) reaction (Seigner *et al.*, 2008). Primers are used at a final concentration of 0.2 µM. The amplicon is 181 bp.

[116] *nad5* sense: 5'-GATGCTTCTTGGGGCTTCTTGTT-3' (nt 968–987 and 1836–1838)

[117] *nad5* antisense: 5'-CTCCAGTCACCAACATTGGCATAA-3' (nt 1973–1995)

[118] **3.3.4.2 Real-time RT-PCR**

[119] The primers and probe used for this assay are those described by Boonham *et al.* (2004). However, neither this assay nor any of the published real-time assays will specifically identify PSTVd. If a positive is obtained by real-time PCR, the identity of the viroid will need to be determined using conventional RT-PCR and sequencing.

[120] The assay will detect PSTVd, MPVd, TCDVd and TPMVd. Sensitivity for the detection of PSTVd in *S. tuberosum* using the CTAB extraction method was at least 15.5 µg PSTVd-infected leaf tissue (the lowest weight tested), which is equivalent to 17 pg PSTVd (Jeffries & James, 2005). By testing variants of PSTVd and synthetic oligonucleotides it has been shown that this assay detects all known sequence variants. These were identified from *in silico* studies as primer/sequence mismatches with the potential for the failure of detection (Boonham *et al.*, 2005). However, the divergent isolates VIR-06/7L and VIR-06/10L described recently by Owens *et al.* (2009) may not be detected because of the insertion of (an) additional base(s) at the probe binding site (W. Monger, SASA, UK, personal communication, 2011).

[121] **Primers**

[122] PSTV-231-F: 5'-GCC CCC TTT GCGCTG T-3' (nt 232–247)

[123] PSTV-296-R: 5'-AAG CGG TTC TCG GGA GCT T-3' (nt 297–279)

[124] PSTV-251T: FAM-5'-CAG TTG TTT CCA CCG GGT AGTAGC CGA-3' TAMRA (nt 278–252)

[125] The internal control COX primers amplify the *cytochrome oxidase 1* gene found in plant mitochondria (Weller *et al.*, 2000).

[126] COX-F: 5'-CGT GCG ATT CCA GAT TAT CCA-3'

[127] COX-R: 5'-CAA CTA CGG ATA TAT AAG RRC CRR ACC TG-3'

[128] COXsol-1511T: VIC-5'-AGG GCA TTC CAT CCA GCG TAA GCA –3'TAMRA

[129] The reaction mix is for a 96-well plate and is a modification of the EPPO method (EPPO, 2004) as it incorporates a duplex reaction for detection of viroid and COX and a simplex reaction for detection of viroid (Roehorst *et al.*, 2005).

[130] The reaction mixture consists of 13.75 µl water, 25 µl of 2x Master Mix (Applied Biosystems)<sup>14</sup>, 1.25 µl of 40x MultiScribe™ (Applied Biosystems), 1.5 µl of each primer PSTV231F and PSTV296R (10 µM) and 1.0 µl probe PSTV251T (5 µM). This reaction mixture is divided equally into two volumes of 22 µl, part A and part B. Two microlitres of water is added to A and to B 0.75 µl of each COX primer (10 µM) and 0.5 µl of the probe COXsol1511T (5 µM). One microlitre of RNA target is added to each of A and B to make a final reaction mixture of 25 µl for each well of the reaction plate. With reaction mixture A, PSTVd will be detected and with reaction mixture B, PSTVd and COX will be detected in a duplex reaction.

[131] Thermocycling conditions are 48 °C for 30 min, 95 °C for 2 min and 40x (95 °C for 15 s, 60 °C for 1 min).

**[132] 3.4 Controls for molecular tests**

- [133] For a reliable test result to be obtained, appropriate controls – which will depend on the type of test used and the level of certainty required – are essential. The following controls should be considered for each series of nucleic acid isolation and amplification of the target pest or target nucleic acid. For RT-PCR, a positive nucleic acid control, an internal control and a negative amplification control (no template control) should be used as a minimum.
- [134] **Positive nucleic acid control** Pre-prepared (stored) viroid nucleic acid, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product) generated using the same primer pair as used for detection may be used as a control to monitor the efficiency of the test method (apart from the extraction) and with RT-PCR, the amplification.
- [135] **Internal control** For conventional and real-time RT-PCR, a plant housekeeping gene (HKG) such as COX or NAD should be incorporated into the RT-PCR protocol to eliminate the possibility of false negatives due to extraction failure, nucleic acid degradation or the presence of PCR inhibitors. Preferably, the internal control primers should be used in a duplex reaction with the pospiviroid/PSTVd primers. However, as this may be difficult to achieve without reducing the sensitivity of the test for the viroid, it is recommended where practical to run a duplex reaction of the pospiviroid/ PSTVd primers with the HKG primers and also a simplex reaction with only pospiviroid/PSTVd primers.
- [136] The *nad5* mitochondrial *NADH dehydrogenase 5* gene fragment has been shown to be a reliable indicator of the performance of the extraction procedure and RT step for conventional RT-PCR (Menzel *et al.*, 2002). It has been tested against many plant species, including *S. tuberosum* and other *Solanum* species (*S. bonariensis*, *S. dulcamara*, *S. jasminoides*, *S. nigrum*, *S. pseudocapsicum*, *S. rantonnetii* and *S. sisymbriifolium*), *Acnistus arborescens*, *Atropa belladonna*, *Brugmansia* spp., *Capsicum* spp., *Cestrum* spp., *Lochroma cyanea*, *Nicotiana* spp. and *Physalis* spp. (Seigner *et al.*, 2008). The *nad5* primers span an intron and will therefore not amplify from DNA. RNA is amplified after the intron is removed.
- [137] Although COX has been used as an internal control in this protocol, COX primers will amplify RNA and DNA. It therefore provides only an indication of the quality of amplifiable DNA rather than RNA alone and does not control the RT step.
- [138] When the internal control COX or *nad5* is not mentioned in the description of a PCR method, the laboratory should choose an internal control and validate it.
- [139] **Negative amplification control (no template control)** For conventional and real-time RT-PCR, PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage to rule out false positives due to contamination during preparation of the reaction mixture.
- [140] **Positive extraction control** This control is used to ensure that target viroid nucleic acid extracted is of sufficient quantity and quality for RT-PCR and that the viroid is detected. Viroid nucleic acid is extracted from infected host tissue or healthy plant tissue that has been spiked with the viroid.
- [141] The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the RNA extraction. If bulking of samples is done then the quantity of positive control should be adjusted accordingly (e.g. 10 lots of 20 mg sample bulked for RNA extraction, 2 mg infected leaf + 198 mg healthy potato tissue). If this is not detected then the test should be repeated or the bulking rate reduced until reliable detection is achieved.
- [142] For RT-PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples. The positive control used in the laboratory should be sequenced so that this sequence can be readily compared to the sequence obtained from PCR amplicons of the correct size. Alternatively, synthetic positive controls can be made with a known sequence that, again, can be compared to PCR amplicons of the correct size.
- [143] **Negative extraction control** This control is used to monitor contamination during nucleic acid extraction and cross-reaction with the host tissue, and it requires nucleic acid extraction and subsequent amplification of uninfected host tissue. Multiple controls are recommended when large numbers of positive samples are expected.

[144] **3.5 Interpretation of results from conventional and real-time RT-PCR**

[145] **Conventional RT-PCR**

[146] The pathogen-specific PCR will be considered valid only if:

- [147] • the positive control produces the correct size product for the viroid; and
- [148] • no bands of the correct size for the viroid are produced in the negative extraction control and the negative amplification control.

[149] If the *nad5* internal control primers are also used, then the negative (healthy plant tissue) control (if used), positive control, and each of the test samples must produce a 181 bp band (*nad5*). Failure of the samples to amplify with the internal control primers suggests, for example, that the RNA extraction has failed, the nucleic acid has not been included in the reaction mixture, the RT step has failed, compounds inhibitory to PCR are present in the RNA extract, or the RNA or DNA has degraded.

[150] A sample will be considered positive if it produces an amplicon of the correct size. For identification of the viroid species the PCR product must be sequenced.

[151] **Real-time PCR**

[152] The real-time RT-PCR will be considered valid only if:

- [153] • the positive control produces an amplification curve with the pathogen-specific primers; and
- [154] • no amplification curve is seen (i.e. cycle threshold (Ct) value is 40) with the negative extraction control and the negative amplification control or other Ct value defined by the laboratory after validation.

[155] If the COX internal control primers are also used, then the negative control (if used), positive control, and each of the test samples must produce an amplification curve. Failure of the samples to produce an amplification curve with the internal control primers suggests, for example, that the nucleic acid extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the nucleic acid extract, or the nucleic acid has degraded.

[156] A sample will be considered positive if it produces a typical amplification curve. For one method, specific information on the Ct cut-off value is provided (section 3.3.3.4).

[157] **4. Identification**

[158] Identification should be done by sequencing the product obtained from any of the conventional RT-PCR methods described in section 3 (3.3.3.3 and 3.3.4.1). If the PCR product is weak, cloning the PCR product may be effective in allowing sequence to be obtained.

[159] For identification of a positive sample detected by real-time PCR, the sample should be retested using conventional RT-PCR to enable the product to be sequenced. However, because of the increased sensitivity of the real-time assay, a product may not be obtained with conventional RT-PCR. Sequencing the real-time amplicon directly will give sequence information that does not allow reliable identification. It will allow the amplicon to be identified as a viroid but will not allow species identification or discrimination from the positive control used.

[160] **4.1 Sequencing and sequence analysis**

- [161] If facilities are not available for sequencing to be done in-house or by known commercial companies, consult the Web for companies offering this service. The company will specify their requirements for the sequencing of PCR products. Send the purified product (and forward and reverse primers if requested) to the company to carry out the sequencing.
- [162] If sequencing is done in-house, the methods should be established and followed. Sequence the PCR product on each strand using the PCR primers as the sequencing primers.
- [163] Export the sequence data output files for the two strands and observe the base calls (A, C, G and Ts) generated by the sequencing instrument's software to detect errors. Sequences extending into a PCR primer site should be truncated to exclude the primer sequence as mismatches between the primer sequence and the binding site will be missed because the primer sequence will be amplified, not the binding site in the viroid genome. Including the primer site may skew the comparison of results. The two independently sequenced DNA strands (forward and reverse primers) should be assembled into a single contig, confirming the base call (identity) of each nucleotide site. Disagreements between the two strands should be coded as ambiguous bases (N) in the edited sequence. The edited consensus sequence (determined by comparing the two strands) can then be compared to a database of pospiviroid sequences.
- [164] Consensus nucleotide sequences should be searched against a nucleotide database (e.g. GenBank searched by the Basic Local Alignment Search Tool (BLAST)) to identify the most similar sequences. It may be necessary to use an alignment program (e.g. Clustal or MEGA) to obtain full length sequence alignments, as BLAST may not give uninterrupted alignments of whole genomes.
- [165] Careful alignment is required for pospiviroids where a few base pair differences may be the difference between classifying the viroid as a regulated or a non-regulated pest.
- [166] For viroid species identification, the demarcation criteria of the International Committee on Taxonomy of Viruses should be followed (Flores *et al.*, 1998, 2005; Owens *et al.*, 2011). In most cases the arbitrary level of 90% sequence identity establishes a clear border that separates species from variants. Consequently, a sample is identified as the species with which it shares the greatest similarity if (1) that similarity is >90% and (2) the sample is also <90% similar to other species in the database. For characterization of a species, however, Flores *et al.* (1998, 2005) also mentions the evaluation of biological properties.
- [167] **5. Records**
- [168] Records and evidence should be retained as described in section 2.5 of ISPM 27:2006.
- [169] In instances where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance and where PSTVd is found in an area for the first time, the following additional material should be kept in a manner that ensures complete traceability:
- [170] - the original sample (if still available) should be kept frozen at –80°C or freeze-dried and kept at room temperature
- [171] - if relevant, RNA extractions should be kept at –80°C
- [172] - if relevant, RT-PCR amplification products should be kept at –20°C to –80°C
- [173] - the DNA sequence trace files used to generate the consensus sequence for identification of samples.
- [174] If the isolate is shown to have different molecular or biological characteristics to previously recorded isolates, it should be offered to a national plant pest herbarium (e.g. Q-bank, DSMZ).
- [175] If there is evidence of any of the tests described failing to detect an isolate of PSTVd, isolate details (preferably the GenBank accession number) should be sent to the IPPC Secretariat.

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**[176] 6. Contact Points for Further Information**

**[177]** Further information on this protocol can be obtained from:

**[178]** Science and Advice for Scottish Agriculture (SASA), Roddinglaw Road, Edinburgh EH12 9FJ, Scotland (Dr C.J. Jeffries, e-mail: colin.jeffries@sasa.gsi.gov.uk).

**[179]** National Plant Protection Organization, PO Box 9102, 6700 HC Wageningen, the Netherlands (Dr J.W. Roenhorst, e-mail: j.w.roenhorst@minlnv.nl; Dr J.Th.J. Verhoeven, e-mail: j.th.j.verhoeven@minlnv.nl).

**[180]** Department of Primary Industries, Knoxfield Centre, Private Bag 15, Ferntree Gully Delivery Centre, Victoria, Australia (e-mail: Dr B. Rodoni, e-mail: brendan.rodoni@dpi.vic.gov.au).

**[181]** CFIA, Charlottetown Laboratory, 93 Mt Edward Road, Charlottetown, PE, C1A 5T1, Canada (Dr H. Xu, e-mail: huimin.xu@inspection.gc.ca).

**[182]** Conselleria de Agricultura de la Generalitat Valenciana, Centro de Proteccion Vegetal y Biotecnologia, IVIA, 46113 Moncada (Valencia), Spain (Dr N. Duran-Vila, e-mail: nduran@ivia.gva.es).

**[183]** USDA-APHIS, Plant Germplasm Quarantine Program BARC-E, BLD 580, Powder Mill Road, Beltsville, MD 20705, USA (Dr J.A. Abad, e-mail: jorge.a.abad@aphis.usda.gov).

**[184]** Laboratorios Biológicos, Dirección General de Servicios Agrícolas, Ministerio de Ganadería, Agricultura y Pesca, Millán 4703, Montevideo, Uruguay (Dr A. Etchevers, e-mail: anitaetchevers@hotmail.com).

**[185] 7. Acknowledgements**

**[186]** The first draft of this protocol was written by C.J. Jeffries (SASA, UK), J.W. Roenhorst (National Plant Protection Organization, the Netherlands), B. Rodoni (Department of Primary Industries, Australia), H. Xu (CFIA, Canada), N. Duran-Vila (INIA, Spain), A. Etchevers (Laboratorios Biológicos, Uruguay) and J.A. Abad (USDA-APHIS, USA) (see section 6 for contact details). In addition, J.Th.J. Verhoeven (National Plant Protection Organization, the Netherlands) was significantly involved in the development of this protocol.

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- [244] **Footnote 1.** The use of products of the brand Bioreba in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.
- [245] **Footnote 2.** The use of products of the brand Retsch, Qiagen and Interscience in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[246] **Footnote 3.** See Footnote 2.

[247] **Footnote 4.** See Footnote 2.

[248] **Footnote 5.** The use of products of the brand Epicentre Biotechnologies in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[249] **Footnote 6.** The use of products of the brand ThermoScientific in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[250] **Footnote 7.** The use of products of the brand Sigma in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[251] **Footnote 8.** The use of products of the brands Invitek and Thistle Scientific in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[252] **Footnote 9.** The use of products of the brand Agdia, Inc. in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[253] **Footnote 10.** As of 1 March 2010.

[254] **Footnote 11.** See Footnote 2.

[255] **Footnote 12.** The use of products of the brand Applied Biosystems in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[256] **Footnote 13.** The use of products of the brand Invitrogen in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[257] **Footnote 14.** See Footnote 12.