This diagnostic protocol was adopted by the Standards Committee on behalf of the Commission on Phytosanitary Measures in January 2015.

The annex is a prescriptive part of ISPM 27.

ISPM 27
Diagnostic protocols for regulated pests

DP 7: *Potato spindle tuber viroid*

Adopted 2015; published 2016

CONTENTS

1. Pest Information

Viroids are unencapsidated, covalently closed circular single-stranded RNA molecules, 239–401 nucleotides in length that are replicated by host enzymes (Hammond & Owens, 2006). *Potato spindle tuber viroid* (PSTVd; genus *Pospiviroid*) is commonly 359 nucleotides in length but PSTVd isolates consisting of 341–364 nucleotides have been reported (Wassenegger *et al*., 1994; Shamloul *et al*., 1997; Jeffries, 1998). Mild and severe strains have been described based on symptoms produced in sensitive tomato cultivars; for example, *Solanum lycopersicum* L. (tomato) cv. *Rutgers* (Fernow, 1967).

The natural host range of PSTVd is relatively narrow. The primary natural hosts are stolon- and tuber-forming *Solanum* spp.; for example, *Solanum tuberosum* L. (potato) and *S. lycopersicum* (tomato). PSTVd has been found also in *Capsicum annuum*, *Persea americana* and *S. muricatum*. PSTVd has been detected in mainly vegetatively propagated ornamental plant species in the family Solanaceae – namely, *Brugmansia* spp., *Cestrum* spp., *Datura* sp., *Lycianthes rantonnetii*, *Petunia* spp., *Physalis peruviana*, *Solanum* spp. and *Streptosolen jamesonii* – but also in *Chrysanthemum* sp. and *Dahlia* × *hybrida* in the family Asteraceae (for natural host details, see CABI (n.d.)). The experimental host range of PSTVd is wide and includes 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)

PSTVd has been found infecting *S. tuberosum* in some countries or states in Africa, Asia, Eastern Europe, North America (EPPO/CABI, 1997), Central America (Badilla *et al*., 1999), South America and the Middle East (Hadidi *et al*., 2003) However, it has a wider geographical distribution in ornamental plant species and other hosts (see CABI (n.d.) for geographical distribution).

In *Solanum tuberosum* the main means of spread of PSTVd is 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 the aphids *Myzus persicae* or *Aulacorthum solani*. However, experimental acquisition and transmission of PSTVd by *M. persicae* from plants co-infected with PSTVd and *Potato leafroll virus* (PLRV) have been reported (Salazar *et al*., 1995; Singh & Kurz, 1997). PSTVd was subsequently shown to be heterologously encapsidated within particles of PLRV (Querci *et al*., 1997), a phenomenon that may have important implications for the epidemiology and spread of PSTVd under field conditions.

In *Solanum lycopersicum* , PSTVd is easily spread by contact and has been shown to be transmitted by pollen and seed (Kryczynski *et al*., 1988; Singh, 1970). Transmission via tomato seeds has been shown to contribute to the international spread of PSTVd (van Brunschot *et al*., 2014). It is possible that PSTVd is also spread in infected capsicum seeds (Lebas *et al*., 2005).

Infected ornamental plant species may act as an inoculum source if they are handled before touching other susceptible plants, and they have been shown to be a pathway for the international spread of PSTVd (Navarro *et al*., 2009; Verhoeven *et al.,* 2010). No transmission of PSTVd was shown with *Apis mellifera*, *Bombus terrestris*, *Frankliniella occidentalis* or *Thrips tabaci* (Nielsen *et al*., 2012).

PSTVd is the only viroid known to naturally infect cultivated species *Solanum*. However, *Mexican papita viroid* (MPVd) infects the wild species *S. 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 pospiviroids 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), *Pepper chat fruit viroid* (PCFVd; Reanwarakorn *et al*., 2011) *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).

2. Taxonomic Information

Name: *Potato spindle tuber viroid* (acronym PSTVd)

Synonyms: potato spindle tuber virus, potato gothic virus, tomato bunchy top virus

Taxonomic position: Pospiviroidae, *Pospiviroid*

Common names: potato spindle tuber

3. Detection

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 uprightness 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 (Mackie *et al*., 2002; Hailstones *et al*., 2003; Lebas *et al*., 2005). In *C. annuum*, symptoms are subtle, with leaves near the top of the plant showing a wavy-edged margin (Lebas *et al*., 2005). All ornamental plant species investigated to date do not show symptoms (Verhoeven, 2010).

Because PSTVd infections may be asymptomatic, tests are required for detection and identification of the viroid. 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 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 have the diagnosis confirmed by another laboratory.

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

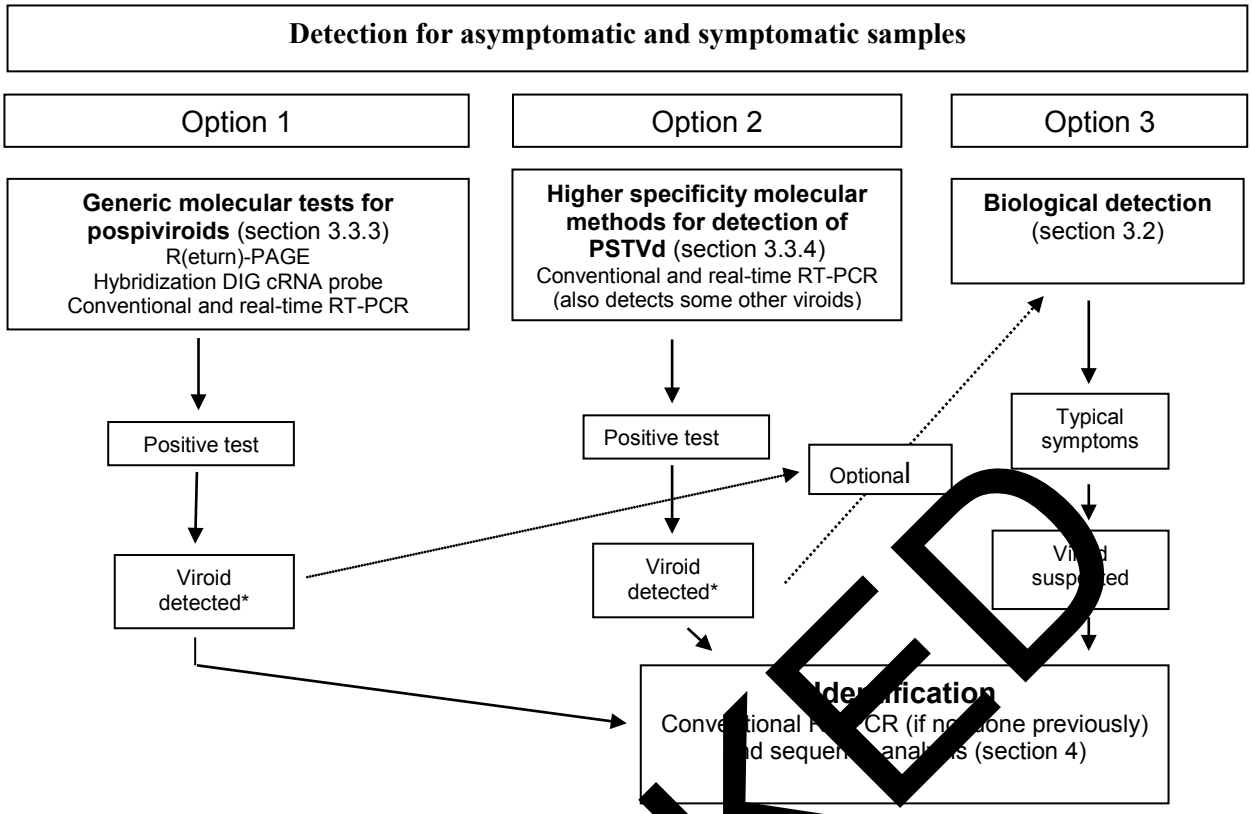

Figure 1. Minimum requirements for the detection and identification of *Potato spindle tuber viroid (PSTVd)*

* 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.

This annex is for the detection of PSTVd; it has not been developed for the detection and identification of other pospiviroid species. However, the possible presence of other viroids needs to be considered when choosing a detection and an identification method. Therefore, this annex describes non-specific detection methods that will detect all known viroids; including pospiviroids such as PSTVd. For identification, the PCR product will need to be sequenced.

Protocols for the detection of PSTVd in leaf, tuber and botanical (true) seed tissue are described, however reliable detection in seed tissue is particularly challenging.

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. Use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated. Recommendations on method validation in phytodiagnostics are provided by EPPO (2014).

The performance of a molecular test is determined by both the matrix to be tested and the choice of subsequent sample preparation, nucleic acid extraction, and detection and identification methods. Table 1 provides an overview of validation data that are available for different matrices and combinations of methods. Details of these methods are described in the corresponding paragraphs or indicated references.

3.1 Sampling

General guidance on sampling methodologies is described in ISPM 31 (*Methodologies for sampling of consignments*).

***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 in length and with well-formed leaves. For glasshouse-grown plants a fully expanded leaflet from each plant should be used. Viroid concentration is lower at low temperature and low light levels, so plants should be grown at a temperature of at least 18 °C 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 and must be validated.

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 and must be validated.

***S. tuberosum* tubers** PSTVd is systemically distributed in infected *S. tuberosum* tubers (Shamloul *et al*., 1997). It also occurs in almost equal amounts in different parts of both primarily and secondarily infected tubers (Roenhorst *et al*., 2006). The highest concentration is found immediately after harvest. In tubers stored at 4 °C the concentration does not decrease significantly for up to three months but after six months of storage, it may decrease by more than 10^4 times. A single core from any part of the tuber can be used as a sample and may be bulked; the bulking rate will depend on the test method used and must be validated.

Leaves of other crops and ornamental plant species Fully expanded young leaves are used. Leaves may be bulked together for testing; the bulking rate will depend on the test method used and must be validated. Note that the viroid concentration is influenced by the age/maturity of the plants, and there are often seasonal fluctuations. In addition, some species contain biochemicals that may inhibit transmission to test plants (e.g. *Brugmansia* spp.) or RT-PCR (e.g. *Calibrachoa* spp., *Solanum jasminoides* and *S. jamesonii*).

Seed Viroid concentration may vary greatly between seeds and the level of infection may vary from less than 1 to 100%. This makes it very difficult to recommend a sample size and bulking rate (EUPHRESCO, 2010). For *S. lycopersicum*, bulk rates of 100–1 000 have been used for a single test. The bulking rate will depend on the test method used and must be validated.

Potato seeds may be sown in growing medium (e.g. compost) in trays and the seedlings/plants tested non-destructively using the same procedure described for glasshouse-grown plants (EPPO, 2006).

3.2 Biological detection

Inoculation of *S. lycopersicum* plants (cultivars Rutgers, Moneymaker or Sheyenne) will allow the detection of many but not all viroids (e.g. tomato is not a host of the pospiviroid *Iresine viroid 1* (IrVd-1; Spieker, 1996; Verhoeven *et al.*, 2010)) and will provide visual evidence of pathogenicity. However, mild isolates may not be detected because of the absence of symptoms. Moreover, symptoms may not be diagnostic for PSTVd. 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. If it is less sensitive than the molecular methods, it might be less suitable for testing seed. However, it is possible that the viroid may be amplified in biological indexing to a level that allows detection by other methods.

Approximately 200–500 mg leaf, root 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 (pH 7.4) 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.

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 with a diurnal temperature fluctuation of 24–39 °C under a photoperiod of 14 h supplemented with sodium vapour illumination of approximately 650 μE/m²/s (Grassmick & Slack, 1985). Lower temperatures and less illumination may reduce the sensitivity of the assay. 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.

A bioassay on tomato will allow detection of many pospiviroids (except IrVd-1, see above); 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.

3.3 Molecular detection

3.3.1 Sample preparation

Microplants, leaf material and roots Mortars and pestles or homogenizers (e.g. Homex 6 (Bioreba)) with extraction bags (Bioreba) have been used successfully to grind material. Adding a small quantity of water or lysis buffer (the composition of which depends on the method used for nucleic acid extraction) or freezing the sample (e.g. in liquid nitrogen) may facilitate homogenization.

The following procedure has been validated (see Table 1) in combination with nucleic acid extraction using the magnetic bead extraction method 2 and the real-time RT-PCR GenProbe assay described in this annex. About 1 g tissue is homogenized in an extraction bag using a Homex 6 or handheld homogenizer (Bioreba) with 3.5 ml (range 1:2–1:5 (w/v)) GH plus lysis buffer (6 M guanidine hydrochloride; 0.2 M sodium acetate, pH 5.2; 25 mM ethylenediaminetetraacetic acid (EDTA); 2.5% polyvinylpyrrolidone (PVP)-10). Samples are then incubated for 10 min at 65 °C at 850 r.p.m. in a thermomixer (or by shaking (invert the tube 3 times) and additional centrifugation for 2 min at 16 000 *g*) before nucleic acid extraction.

S. tuberosum **tubers** Tuber cores are thoroughly homogenized in water or lysis buffer (the composition of which depends on the method used for nucleic acid extraction; 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. at –20°C) before adding the water or lysis buffer facilitates homogenization.

Seeds For small numbers of seeds (<200), a tissue lyser (e.g. Retsch TissueLyser (Qiagen)) may be used. For larger numbers of seeds, a paddle blender (e.g. MiniMix (Interscience)) or homogenizer (e.g. Homex 6) with a maximum quantity of lysis buffer (the composition of which depends on the method used for nucleic acid extraction) may be used. Seeds may also be crushed with a hammer (Bertolini *et al*., 2014b) or using a mortar and pestle. The latter may not be practical for routine use as cross-contamination may be difficult to control. Alternatively, liquid nitrogen may be used to freeze the sample, after which it is ground in a cell mill (this method can also be used for other tissue types).

The following procedure has been validated (see Table 1) in combination with nucleic acid extraction using the magnetic bead extraction method 2 and the real-time RT-PCR assay of Boonham *et al*. (2004) described in this annex. Each of three subsamples of 1 000 seeds are soaked in 20 ml GH plus lysis buffer in a 100 ml BagPage (Interscience) for 30–60 min at room temperature, homogenized for 90 s using a BagMixer (Interscience) and incubated (or shaken and centrifuged as described for microplants, leaf material and roots) before nucleic acid extraction

Tissue print and/or squash Leaf pedicels or detached shoots are pressed onto nylon membranes. Several partially overlapping imprints or squashes from different leaves and/or detached shoots may be made on approximately 0.5 cm² nylon membrane according to Bertolini *et al*. (2008, 2014a). The membrane containing the immobilized sample is cut and inserted into a micro tube. The immobilized sample should be handled with clean tweezers. The tissue-printed or squashed samples can be stored at room temperature in a dark and dry environment for at least three months. For extraction of target RNA from the membranes, 100 μl glycine buffer is added to each micro tube containing an immobilized sample, which is then vortexed and placed on ice until PCR amplification.

3.3.2 Nucleic acid extraction

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 kits, buffers and procedures have been used successfully for the detection of PSTVd.

Commercial kits Commercial extraction kits such as RNeasy (Qiagen), MasterPure (Epicentre) and Sbeadex maxi plant kit (LGC Genomics) may be used according to the manufacturer's instructions. RNeasy was evaluated for the extraction of PSTVd RNA from different matrices as part of the EUPHRESCO Detection and Epidemiology of Pospiviroids (DEP) project (EUPHRESCO, 2010).

Method described by Mackenzie *et al.* (1997) Plant tissue is homogenized (1:10 (w/v)) in lysis buffer (4 M guanidine isothiocyanate, 0.2 M sodium acetate, 25 mM EDTA, 2.5% PVP-40 (w/v, and 1% 2-mercaptoethanol (v/v) added just before use). One millilitre of homogenate is then mixed with 100 µl of 20% sarkosyl (w/v) and incubated at 70 °C for 10 min in a thermomixer, with agitation at 1 200 r.p.m.. This method can be used to extract quality RNA from a wide range of plant species.

Method using EDTA buffer Plant tissue may be homogenized (1:4 (w/v)) in a simple lysis buffer (50 mM NaOH, 2.5 mM 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, dependent on the level of sensitivity required, either be 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.

Phenol–chloroform and two-step PEG extraction Plant tissue is homogenized and nucleic acid extracted as described by EPPO (2004). This method has been used in combination with return (R)-polyacrylamide gel electrophoresis (RPAGE), DIG-RNA probe and the conventional RT-PCR methods described in this diagnostic protocol for a wide range of plant species and tissue types (e.g. leaves and potato tubers).

CTAB extraction Plant tissue is homogenized and nucleic acid extracted as described in EPPO (2004). The cetyl trimethylammonium bromide (CTAB) method has been used with real-time RT-PCR for a wide range of plant species and tissue types (e.g. leaves and tomato seeds; EUPHRESCO, 2010).

Magnetic bead extraction method 1 The following automated procedure is based on use of the KingFisher mL Magnetic Particle Processor (Thermo Scientific). With appropriate adjustment of volumes, other KingFisher models may be used.

For each sample, at least 200 mg leaf or tuber tissue or up to 100 seeds are macerated, and then extraction buffer is added immediately at a ratio of 1g leaf or tuber tissue to 10 ml buffer and 1 g seed to 20 ml buffer. Maceration is continued until a clear cell lysate with minimal intact tissue debris is obtained. Extraction buffer consists of 200 µl of 8.39% (w/v) tetrasodium pyrophosphate (TNaPP) solution (pH 10.0–10.9) and 100 µl Antifoam B Emulsion (Sigma) added to 9.8 ml guanidine lysis buffer (GLB). GLB consists of: 764.2 g guanidine hydrochloride, 7.4 g disodium EDTA dehydrate, 30.0 g PVP-10, 5.25 g citric acid monohydrate, 0.3 g tri-sodium citrate, 5 ml Triton X-100, 250 ml absolute ethanol and 750 ml water.

Approximately 2 ml lysate is decanted into a fresh microcentrifuge tube, which is centrifuged at approximately 5 000 *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 vortexed MAP Solution A magnetic beads (Invitek) 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 1× Tris-EDTA buffer.

The tube strip is placed in the KingFisher mL and the programme (see Figure 2) 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. On completion, the eluted nucleic acids are transferred to a new microcentrifuge tube.

This method has been used for a wide range of plant species as well as for potato tubers and tomato seeds. The method has been used with two of the real-time RT-PCR assays described in this annex (see sections 3.3.3.4 and 3.3.4.2). Cycle threshold (Ct) values several cycles higher than those for the other extraction methods described in this annex may be expected using the magnetic bead extraction method 1, but the increased throughput of samples that is achievable makes it a valuable extraction method (Roenhorst *et al.,* 2005).

Plate layout Default: Plate type = KingFisher tubestrip 1000 µl; Plate change messag… Chan… Default
A: volume = 1000, name = Cell lysate or tissue homogenate; volume = 50, name = …agnetic par…es;
B: volume = 1000, name = Washing buffer 1 (Various); **C**: volume = 1000, name = …ashing buffer… (Various);
D: volume = 1000, name = Washing buffer 3 (Various); **E**: volume = 200, nam… = Elu… buffer (Va…ous)
STEPS COLLECT BEADS Step parameters: Name = Collect Beads; W… = A, Defa… Begi…ing of step: Premix = No; Collect parameters: Collect count = 1. BIND Step parame…rs: Nam… Lysin… = A, Default; Beginning of step: Release = Yes, time = 1min 0s, speed = Fast dua…ix; Bind…arameters: Bind time = 4min 0s, speed = Slow; End of step: Collect beads = No. BIND Step pa…amet… …me = Lys…g, Well = A, Default; Beginning of step: Release = Yes, time = 1min 0s, speed = Fast…ual mix…d; Bind…rameters: Bind time = 4min 0s, speed = Slow; End of step: Collect beads = No. BIND…tep param…rs: …ame = Lysing, Well = A, Default; Beginning of step: Release = Yes, time = 1min 0s, spee… = Fast dual m… Bind parameters: Bind time = 4min 0s, speed = Slow; End of step: Collect beads = …s, c… 4. WASH Step parameters: Name = Washing, Well = B, Default; Beginning of step: Release = …me = 0s, speed = Fast; Wash parameters: Wash time = 3min 0s, speed = Fast dual mix; …tep: …llect beads = Yes, count = 3. WASH Step parameters: Name = Washing, Well = C, Defau… Beginni…f ste… Release = Yes, time = 0s, speed = Fast; Wash parameters: Wash time = 3min 0s, spe… = Fast dual …x; End of step: Collect beads = Yes, count = 3. WASH Step parameters; Name = Washing, …ell = D, Defau… Beginning of step: Release = Yes, time = 0s, speed = Fast; Wash parameters: Wash…me = …min 0s, spee… = Fast dual mix; End of step: Collect beads = Yes, count = 3. ELUTION Step parame…s; Na… = Elution…Well = E, Default; Beginning of step: Release = Yes, time = 10s, speed = Fast; Elution …rameter…on time = 20s, speed = Bottom very fast; Pause parameters: Pause for manual handling = Y…s, message = Heating, Post mix time = 30s, speed = Bottom very fast; Remove beads: Remove be… = Yes, …llect count = 4, disposal well = D

Figure 2. Programme for t… KingFisher mL M…etic Particle Processor (Thermo Scientific)

Magnetic bead extra…n metho… 2 This automated procedure uses the Sbeadex maxi plant kit (LGC Geno…es) …ith the …ingF…her 96 system (Thermo Scientific). The manufacturer's instructions should be …llowed …xcept th… GH plus lysis buffer is used instead of lysis buffer PN that is part of the kit.

3.3.3 Gener… molecular methods for pospiviroid detection

3.3.3.1 R-PAGE

R-PAGE has been recommended as a detection method for PSTVd infecting *S. tuberosum* leaves (EPPO, 2004), but it was less sensitive (limit of detection (LOD) 87 893 pg PSTVd) than the other molecular methods evaluated (LOD at least 17 pg PSTVd) in a ring test with DIG-labelled cRNA probe, two-step conventional RT-PCR using the primers of Shamloul *et al*. (1997) and the real-time method of Boonham *et al*. (2004) (Jeffries & James, 2005; see also Table 1).

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.

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.

3.3.3.2 Hybridization with a DIG-labelled cRNA probe

This method has been recommended for detection of PSTVd infecting *S. tuberosum* leaves (EPPO, 2004). Sensitivity for the detection of PSTVd in *S. tuberosum* leaves was at least 17 pg PSTVd (Jeffries & James, 2005). Other hosts have been tested successfully, including *Petunia* spp., *S. jasminoides, S. lycopersicum* and *S. tuberosum* (tubers).

The probe used is based on a full-length monomer of PSTVd produced by Agdia, Inc.[9] (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), polyethylene glycol (PEG) and other extraction buffers may be used for nucleic acid extraction.

This DIG-labelled cRNA probe method 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.

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

The primers used in this assay are the Pospi1 and Vid primers of Verhoeven *et al.* (2004). The Pospi1 primers will detect CEVd, *Chrysanthemum stunt viroid* (CSVd), IrVd-1, MPVd, PSTVd, TASVd, TCDVd and TPMVd. The Vid primers will detect PSTVd, TCDVd and, additionally, CLVd. Using the Pospi1 and Vid primers in two separate reactions will allow detection of all pospiviroids. However, sequence mismatch at critical positions of the primer target site may prevent the detection of some pospiviroid isolates (e.g. an isolate of CLVd was not detected using these primers; Steyer *et al*., 2010) and additional primers to detect these isolates will be required. *In silico* studies have shown that the following PSTVd isolates may not be detected because of primer–sequence mismatch at critical positions: Pospi1 primers: EU879925, EU2[illegible]604, EF45[illegible]97, AJ007489, AY372398, AY372394, FM998551, DQ308555, E00278; Vid primers: EU2[illegible]604. The Pospi1 primers are much more sensitive than the Vid primers for the detection of PSTVd.

Primers

Pospi1-FW: 5′-GGG ATC CCC GGG C[illegible]A AC-3′ (nucleotide (nt) 86–102)

Pospi1-RE: 5′-AGC TTC AG[illegible]TGT (T/A)TC CAC CGG GT-3′ (nt 283–261)

Vid-FW: 5′-TTC CTC G[illegible]A ACT A[illegible]C[illegible]G GTG-3′ (nt 355–16)

Vid-RE: 5′-CCA AC[illegible]GCG GTT CCA AGG G-3′ (nt 354–336)

Reaction conditions

The One-Step RT-PCR kit (Qiagen) has been shown to be reliable when used for the detection of PSTVd, CEVd, CLVd, CSVd, TASVd and TCDVd in individual samples (EUPHRESCO, 2010) and for other pospiviroids listed at the start of this section. It is not necessary to use the Q-solution described by EUPHRESCO (2010). 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 detected.

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× One-Step RT-PCR buffer, 1.0 µl One-Step RT-PCR enzyme mix, 1.0 µl dNTPs (10 mM each dNTP) and 14 µl water. The thermocyling 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.

Gel electrophoresis

After RT-PCR, the PCR products (approximately 197 bp and 359 bp for the Pospi1 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.

3.3.3.4 Real-time RT-PCR using the GenPospi assay (Botermans *et al.*, 2013)

The GenPospi 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 (see Table 1) on tomato leaves showed that the GenPospi assay detected isolates from all the known pospiviroid species up to a relative infection rate of 0.13% (which equals a 1:770 dilution). The assay was specific as no cross-reactivity was observed with other viroids, viruses or nucleic acid from host plants. Repeatability and reproducibility were 100% and the assay appeared robust in an inter-laboratory comparison. The GenPospi assay has been shown to be a suitable tool for large-scale screening for pospiviroid species. The assay will need to be validated for matrices other than tomato leaves.

Primers

TCR-F 1-1: 5′-TTC CTG TGG TTC ACA CCT GAC C-3′ (Botermans *et al.*, 2013)
TCR-F 1-3: 5′-CCT GTG GTG CTC ACC TGA CC-3′ (Botermans *et al.*, 2013)
TCR-F 1-4: 5′-CCT GTG GTG CAC TCC TGA CC-3′ (Botermans *et al.*, 2013)
TCR-F PCFVd: 5′-TGG TGC CTC CCC CGA A-3′ (Botermans *et al.*, 2013)
TCR-F IrVd: 5′-AAT GGT TGC ACC CCT GAC C-3′ (Botermans *et al.*, 2013)
TR-R1: 5′-GGA AGG GTG AAA ACC CTG TTT-3′ (Botermans *et al.*, 2013)
TR-R CEVd: 5′-AGG AAG GAG ACG AGC TCC TG[illegible]-3′ (Botermans *et al.*, 2013)
TR-R6: 5′-GAA AGG AAG GAT GAA AAT [illegible] TT T[illegible]′ (Botermans *et al.*, 2013)

CLVd-F: 5′-GGT TCA CAC CTG ACC C[illegible]G CAG-3′ (Monger *et al.*, 2010)
CLVd-F2: 5′-AAA CTC GTG GTT G[illegible]T C[illegible]G GTT-3′ (Monger *et al.*, 2010)
CLVd-R: 5′-CGC TCG GTC TGA GT[illegible]GCC[illegible]′ (Monger *et al.*, 2010)
nad5-F: 5′-GAT GCT TCT TGG GGC [illegible]C TTG TT-3′ (Menzel *et al.*, 2002)
nad5-R: 5′-CTC CAG TCA CC[illegible]CA T[illegible]G GCA TAA-3′ (Menzel *et al.*, 2002)

Probes

pUCCR: 6FAM-5′-[illegible]G GC[illegible]AAA CCT GGA-3′-MGB (Botermans *et al.*, 2013)
CLVd-P: 6FAM-5′-AC[illegible]GT CT[illegible]AGG AGC CCC GG-3′-BHQ1 (Monger *et al.*, 2010)
nad5-P: VI[illegible]-5′-A[illegible]GG A[illegible] C[illegible] ATA GCC CTC GAT TTA TGT G-3′-BHQ1 (Botermans *et al.*, 2013)

The two reaction mixes are based on the TaqMan RNA to Ct 1-Step Kit (Applied Biosystems).

Reaction mix 1 (all pospiviroids except CLVd + nad5)

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 make a final volume of 25 µl.

Reaction mix 2 (CLVd + nad5)

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 (CLVd-F, CLVd-F2 and *nad5*-F) and reverse primers (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 make a final volume of 25 µl.

Thermocycling conditions for both reaction mixes are 48 ºC for 15 min, 95 ºC for 10 min, followed by 40 cycles of (95 ºC for 15 s and 60 ºC for 1 min).

For this method, Botermans *et al*. (2013) interpreted Ct values <32 as positive; those between 32 and 37 as inconclusive, requiring confirmation; and those ≥37 as negative. However, these values may exclude low levels of infection in some tissues, and will need to be defined in each laboratory.

3.3.4 Higher specificity molecular methods for the detection of PSTVd

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

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. If RNA was not amplified using these primers, the Vid primers may be used.

Primers

3H1-F: 5′-ATC CCC GGG GAA ACC TGG AGC GAA C-3′ (nt 89–113)

2H1-R: 5′-CCC TGA AGC GCT CCT CCG AG-3′ (nt 88–69)

Method 1 *(SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen))*

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 2× 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, then 10 cycles of 94 °C for 30 s, 68 °C for 90 s and 72 °C for 45 s, followed by 20 cycles of 94 °C for 30 s, 64 °C for 90 s and 72 °C for 45 s, with a final extension of 72 °C for 10 min and 20 °C for 1 min.

Method 2 *(two-step RT-PCR)*

Using the two-step RT-PCR, the sensitivity for the detection of PSTVd in *S. tuberosum* is at least 17 pg PSTVd – the lowest concentration tested, but the sensitivity achieved varies between laboratories, with most laboratories detecting at least 89 pg PSTVd (Jeffries & James, 2005). See EPPO (2004) for a description of method 2.

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.

An internal control assay using *nad5* primers (Menzel *et al*., 2002) has been used with this method 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.

nad5 sense: 5′-GATGCTTCTTGGGGCTTCTTGTT-3′ (nt 968–987 and 1836–1838)

nad5 antisense: 5′-CTCCAGTCACCAACATTGGCATAA-3′ (nt 1973–1995)

3.3.4.2 Real-time RT-PCR using the primers of Boonham *et al.* (2004)

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 RT-PCR, the identity of the viroid will need to be determined using conventional RT-PCR and sequencing.

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 17 pg PSTVd, the lowest concentration tested (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 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, personal communication, 2011)[1].

Primers

PSTV-231-F: 5′-GCC CCC TTT GCGCTG T-3′ (nt 232–247)

PSTV-296-R: 5′-AAG CGG TTC TCG GGA GCT T-3′ (nt 297–279)

PSTV-251T: FAM-5′-CAG TTG TTT CCA CCG GGT AGTAGC CGA-3′ TAMRA (nt 278–252)

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

COX-F: 5′-CGT GCG ATT CCA GAT TAT CCA-3′

COX-R: 5′-CAA CTA CGG ATA TAT AAG RRC CRR ACC TG-3′

COXsol-1511T: VIC-5′-AGG GCA TTC CAT CCA GCG TAA GCA-3′ TAMRA

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 PSTVd and COX and a simplex reaction for detection of PSTVD (Roenhorst *et al.*, 2005).

The reaction mix consists of 13.75 µl water, 25 µl of 2× Master Mix (Applied Biosystems), 1.25 µl of 40× MultiScribe Reverse Transcriptase (Applied Biosystems), 1.5 µl of each primer PSTV-231-F and PSTV-296-R (10 µM) and 1.0 µl probe PSTV-251T (5 µM). This reaction mix is divided equally into two volumes of 22 µl, A and B. Two microlitres of water is added to A and to B is added 0.75 µl of each COX primer (10 µM) and 0.5 µl of the probe COXsol-1511T (5 µM). One microlitre of RNA target is added to each of A and B to make a final reaction mix of 25 µl for each well of the reaction plate. With reaction mix A, PSTVd will be detected and with reaction mix B, PSTVd and COX will be detected in a duplex reaction.

Thermocycling conditions are 48 °C for 30 min, 95 °C for 2 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

3.3.4.3 Real-time RT-PCR (Plant Print Diagnòstics kit)

The primers and probe used in this assay are those described by Bertolini *et al*. (2010) and they are available as a kit from Plant Print Diagnòstics (Ref. PSTVd/100). The assay will detect CLVd, PSTVd and TCDVd. All 37 PSTVd isolates present in GenBank should be detected because *in silico* studies showed that all primer–sequence mismatches were in non-critical positions (N. Duran-Vila, personal communication, 2014).

Validation data are provided in Table 1.

Primers

PSTVd-F: 5’-CCT TGG AAC CGC AGT TGG T-3’ (nt 339–357)

PSTVd-R: 5’-TTT CCC CGG GGA TCC C-3’ (nt 87–102)

PSTVdP: FAM-5’-TCCTGTGGTTCACACCTGACCTCCTGA-3’ TAMRA (nt 19–45)

The PCR cocktail contains lyophilized primers and probe (provided in the kit) to which any commercial RT-PCR master mix can be added. For each reaction, 3 µl template RNA is added to 9 µl PCR cocktail consisting of 6 µl commercial 2× RT-PCR buffer, 0.6 µl of each of forward and reverse primer (10 µM), 0.36 µl TaqMan probe (5 µM), 0.5 µl of 25× RT-PCR enzyme mix and 0.94 µl water to make a final reaction volume of 12 µl.

[1] As of 1 March 2010 (W. Monger, personal communication, 2011)

Thermocycling conditions are 45 °C for 10 min, 95 °C for 10 min and 40 cycles of (95 °C for 15 s and 60 °C for 1 min).

For this method a sample is considered positive when it produces a Ct value of <40 and negative controls are negative (no amplification). A sample is considered negative when it produces a Ct value of ≥40 and the positive controls show amplification.

3.4 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – 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) are the minimum controls that should be used.

Positive nucleic acid control This control is used to monitor the efficiency of the assay (apart from the extraction). 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. A limit of detection control (not mandatory) may also be used.

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 nucleic acid extraction failure or degradation or the presence of PCR inhibitors. Preferably, the internal control primers should be used in a multiplex 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.

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. sisymbrifolium*), *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.

Although COX has been used as an internal control in this protocol, COX primers will amplify RNA and DNA and therefore provide only an indication of the quality of amplifiable DNA rather than RNA alone and does not control the RT step.

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.

Negative amplification control (no template control) This control is necessary for conventional and real-time RT-PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

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 target viroid is detectable. Viroid nucleic acid is extracted from infected host tissue or healthy plant tissue that has been spiked with the viroid.

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.

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 with 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 with PCR amplicons of the correct size.

Negative extraction control This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified. Multiple controls are recommended to be included when large numbers of positive samples are expected.

3.5 Interpretation of results from conventional and real-time RT-PCR

3.5.1 Conventional RT-PCR

The viroid-specific PCR will be considered valid only if:

- the positive nucleic acid control produces the correct size product for the viroid and
- no amplicons of the correct size for the viroid are produced in the negative extraction control and the negative amplification control.

If the COX and/or *nad5* internal control primers are also used, then the negative (healthy plant tissue) control (if used), positive nucleic acid 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 nucleic acid 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 nucleic acid extract, or the nucleic acid has degraded.

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.

3.5.2 Real-time RT-PCR

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

- the positive nucleic acid control produces an amplification curve with the viroid-specific primers; and
- no amplification curve is seen (i.e. Ct value is 40 or other Ct value defined by the laboratory after validation) with the negative extraction control and the negative amplification control.

If the COX and/or *nad5* internal control primers are also used, then the negative control (if used), positive nucleic acid 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.

A sample will be considered positive if it produces a typical amplification curve. Specific information on the Ct cut-off value for two methods is provided in sections 3.3.3.4 and 3.3.4.3.

4. Identification

PSTVd should be identified by sequencing the product obtained from the conventional RT-PCR methods using the Shamloul or Vid primers described in sections 3.3.4.1 and 3.3.3.3, respectively, and by searching for a sequence match on the public genetic sequence databases. Sequence analysis specialists may be needed to assist in identification. If the PCR product is weakly amplified or if the

sample is infected by more than one pospiviroid, cloning the PCR product may be effective in enabling a sequence to be obtained.

A positive sample detected by real-time RT-PCR, should, if required for confirmation, be retested using conventional RT-PCR to enable the product to be sequenced and identified. Sequencing the real-time PCR product directly will give sequence information that does not allow reliable identification. It will allow the PCR product to be identified as a viroid but will not allow species identification or discrimination from the positive control used. However, because of the increased sensitivity of the real-time RT-PCR, a product may not be obtained with conventional RT-PCR. In the case of bulked samples, retesting smaller subsamples might increase the reliability of amplification by conventional RT-PCR. Alternatively, samples may be inoculated in tomato plants to increase the concentration of the viroid to levels that may be detectable by conventional RT-PCR. However, this approach has not been evaluated and if results are inconclusive then resampling and testing may be required.

4.1 Sequencing and sequence analysis

Sequence analysis should only be done by an experienced person. If facilities are not available for sequencing to be done in-house, a commercial company should be used. The company will specify their requirements for the sequencing of PCR products. The purified product (and forward and reverse primers if requested) is sent to the company to carry out the sequencing. Some companies may also purify the product if required.

If sequencing is done in-house, the methods should be established and followed. Each strand of the PCR product should be sequenced, using the PCR primers as the sequencing primers. The two independently sequenced DNA strands (from using forward and reverse primers) should be assembled into a single contig, confirming the base call (identity) of each nucleotide site. It is preferable to use assemblers (e.g. Geneious, CLC Genomics Workbench or Lasergene software) that use electropherograms (trace files) for the analysis. Disagreements between the two strands should be coded as ambiguous bases in the edited sequence. The edited consensus sequence (determined by comparing the two strands) can then be compared with pospiviroid sequences in a relevant database. In the case of a mixed infection, the chromatogram may not be readable and the PCR product should be cloned and sequenced.

Careful alignment is required for pospiviroids where a few nucleotide differences may be critical in identifying the viroid as a regulated or a non-regulated pest. For initial identification of PSTVd, the primer sequences (Shamloul PSTVd primers) in the consensus sequence may be kept because these primers are located in the most conserved regions of the viroid genome and are not likely to influence identification. Overhangs built on by the polymerase during elongation have to be removed if observed. For identification it is advisable to use an edited consensus sequence starting at position 1 of the viroid genome for comparison with one of the comprehensive nucleotide databases. The search should be done in the GenBank non-redundant nucleotide database at the website of the National Centre for Biotechnology Information (NCBI) or the European Nucleotide Archive at the website of the European Molecular Biology Laboratory (EMBL) by using the Basic Local Alignment Search Tool (BLAST). In addition, identification should be based on specific clustering of BLAST hit results in (neighbour joining) tree view.

According to the International Committee on Taxonomy of Viruses (ICTV) the main criterion for species identification is more than 90% sequence identity (Owens *et al*., 2011). However, if the sequence obtained shows identity close to 90%, additional parameters should be included, such as biological properties. The ICTV Viroid Study Group is currently discussing the viroid classification and the criteria for species demarcation.

When 100% sequence accuracy is required, for example when a sequence is to be submitted to a database or when a new viroid species is suspected, it is necessary to perform a second PCR. This PCR will cover the region of the primer sequences used for the first PCR as well as any ambiguous

bases from the first PCR. Design of a new set of primers from the initial sequence may be required for this purpose, but the use of the Shamloul and Vid primer-pairs may be sufficient.

5. Records

Records and evidence should be retained as described in ISPM 27 (*Diagnostic protocols for regulated pests*).

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:

- the original sample (if still available) should be kept frozen at −80ºC or freeze-dried and kept at room temperature
- if relevant, RNA extractions should be kept at −80ºC
- if relevant, RT-PCR amplification products should be kept at −20ºC to −80ºC
- the DNA sequence trace files used to generate the consensus sequence for identification of samples.

If the isolate is shown to have different molecular or biological characteristics to previously recorded isolates, it should be offered to a recognized plant pest collection/archive (e.g. Q-bank (Comprehensive Database on Quarantine Plant Pests and Diseases), DSMZ (Leibniz Institute-German Collection of Microorganisms and Cell Cultures).

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.

6. Contact Points for Further Information

Further information on this protocol can be obtained from

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

National Plant Protection Organization, PO Box 9102, 6700 HC Wageningen, The Netherlands (Dr J.W. Roenhorst, e-mail: jan.roenhorst@nvwa.nl; Dr J.Th.J. Verhoeven, e-mail: j.th.j.verhoeven@nvwa.nl).

Department of Environment and Primary Industries, Biosciences Research Division, AgriBio, 5 Ring Road, La Trobe University, Bundoora, Victoria 3083, Australia (Dr B. Rodoni, e-mail: brendan.rodoni@depi.vic.gov.au).

Canadian Food Inspection Agency (CFIA), Charlottetown Laboratory, 93 Mt Edward Road, Charlottetown, PE C1A 5T1, Canada (Dr H. Xu, e-mail: huimin.xu@inspection.gc.ca).

Conselleria d'Agricultura de la Generalitat Valenciana, Centro de Proteccion Vegetal y Biotecnologia (IVIA), 46113 Moncada (Valencia), Spain (Dr N. Duran-Vila, e-mail: duran_nur@gva.es).

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).

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).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will be forward it to the Technical Panel on Diagnostic Protocols (TPDP).

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8. References

The present standard refers to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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Table 1. Overview of and validation data for protocols used to detect *Potato spindle tuber viroid* in different types of host material

Matrix	Sample size	Sample preparation	Nucleic acid extraction	Detection method	Remarks on validation
Tomato leaves	1 g	3.5 ml (1:2–1:5 (w/v)) GH plus lysis buffer with Homex 6 (Bioreba)	RNeasy Plant Mini Kit (Qiagen) or Sbeadex maxi plant kit (LGC Genomics) on KingFisher 96 system (Thermo Scientific)	Real-time reverse transcription-polymerase chain reaction (RT-PCR): GenPospi assay, Botermans *et al*. (2013)	***Limit of detection***: detection of all pospiviroid species up to a relative infection rate[1] of 0.13% (equals 770 times dilution) with 99.7% certainty for dilution of infected tomato leaves in healthy tomato ***Analytical specificity***: highly specific for pospiviroid species ***Selectivity***: no influence of tomato leaves ***Repeatability and reproducibility***: 100% (Naktuinbouw, 2012a; Botermans *et al*., 2013; NPPO-NL, 2013d)
Tomato leaves	1 g	3.5 ml (1:2–1:5 (w/v)) GH plus lysis buffer with Homex 6	RNeasy Plant Mini Kit	Real-time RT-PCR: Boonham *et al*. (2004)	***Limit of detection***: detection up to 10 000 times dilution of infected tomato leaves in healthy tomato ***Analytical specificity***: detection of *Mexican papita viroid* (MPVd), *Potato spindle tuber viroid* (PSTVd), *Tomato chlorotic dwarf viroid* (TCDVd), *Tomato planta macho viroid* (TPMVd) (some isolates) ***Selectivity***: no influence of tomato leaves ***Repeatability and reproducibility***: 100% (Naktuinbouw, 2012b)
Tomato leaves	1 g	3.5 ml (1:2–1:5 (w/v)) GH plus lysis buffer with Homex 6	RNeasy Plant Mini Kit	RT-PCR: Pospi1-FW/Pospi1-RE primers, Verhoeven *et al*. (2004)	***Limit of detection***: detection of all pospiviroid species (except *Columnea latent viroid* (CLVd)) up to at least a relative infection rate of 2.5% for dilution of infected tomato leaves in healthy tomato ***Analytical specificity***: detection of *Hop latent viroid* (HpLVd, genus *Cocadviroid*) and PSTVd ***Selectivity***: no influence of tomato leaves ***Repeatability and reproducibility***: 100% (NPPO-NL, 2013a)
Tomato leaves	1 g	3.5 ml (1:2–1:5 (w/v)) GH plus lysis buffer with Homex 6	RNeasy Plant Mini Kit	RT-PCR: Vid-FW/Vid-RE primers, Verhoeven *et al*. (2004)	***Limit of detection***: detection of CLVd, *Potato spindle tuber viroid* (PSTVd) and TCDVd up to at least a relative infection rate of 100% (10% for CLVd*) for dilution of infected tomato leaves in healthy tomato * Primers originally designed to detect CLVd complementary to the Pospi1-FW/Pospi1-RE RT-PCR (Verhoeven *et al*., 2004) ***Analytical specificity***: detection of CLVd, PSTVd and TCDVd ***Selectivity***: no influence of tomato leaves ***Repeatability and reproducibility***: 100% (NPPO-NL, 2013b)
Tomato leaves	1 g	3.5 ml (1:2–1:5 (w/v)) GH plus lysis buffer with Homex 6	RNeasy Plant Mini Kit	RT-PCR: Shamloul *et al*. (1997)	***Limit of detection***: detection up to at least a relative infection rate of 10% for dilution of infected tomato leaves in healthy tomato ***Analytical specificity***: detection of MPVd, PSTVd, TCDVd, TPMVd (some isolates) ***Selectivity***: no influence of tomato leaves ***Repeatability and reproducibility***: 100% (NPPO-NL, 2013c)

Matrix	Sample size	Sample preparation	Nucleic acid extraction	Detection method	Remarks on validation
Tomato seeds	3 000 seeds (tested as three times 1 000)	20 ml (1:2–1:5 (w/v))GH plus lysis buffer with BagMixer (Interscience)	Sbeadex maxi plant kit on KingFisher 96 system	Real-time RT-PCR: Boonham *et al.* (2004)	Performance characteristics assay as for tomato leaves Probability of detection of one infected seed in a sample of 1 000 is >95% when testing three subsamples each of 1 000 seeds. Owing to rapid cross-contamination of PSTVd from infected fruits to healthy seeds during processing (using fermentation and pectinase treatment) of the seeds there is a high probability that more contaminated seeds will be present in a sample (Naktuinbouw, 2012c).
Potato leaves (growth room grown) and *in vitro* potato plants	200 mg	20 µL of 10% sodium dodecyl sulphate (SDS), 180 µL LiCl extraction buffer, 400 µL phenol–chloroform with mortar and pestle	Phenol–chloroform and two-step polyethylene glycol (PEG) extraction	Return (R)-polyacrylamide gel electrophoresis (PAGE)[2]	***Limit of detection:*** 2 365 pg PSTVd; this was the least sensitive of the molecular methods in an international ring test ***Analytical specificity*:** detection of all known pospiviroids ***Selectivity*:** no influence of potato variety, potato leaves or *in vitro* plants ***Repeatability and reproducibility*:** reproducibility 51% at 87 893 pg PSTVd (the highest concentration of PSTVd tested) and 42% at the limit of detection
Potato leaves (growth room grown) and *in vitro* potato plants	200 mg	1:1.5 (w/v) Ames buffer (EPPO, 2004) with mortar and pestle	Immobilization on membrane (Agdia, Inc.) phenol–chloroform and two-step PEG extraction	Digoxigenin (DIG) probe[2]	***Limit of detection:*** at least 17 pg PSTVd (the lowest concentration tested) ***Analytical specificity:*** detection of all known pospiviroids ***Selectivity*:** no influence of potato variety, potato leaves or *in vitro* plants ***Repeatability and reproducibility*:** reproducibility 100% at 87 893 pg PSTVd and 23% at 17 pg PSTVd
Potato leaves (growth room grown) and *in vitro* potato plants	50–500 mg	1:9 (w/v) RH buffer (Qiagen) with microcentrifuge tube and micropestle or Homex 6	RNeasy Plant Mini Kit	Two-step[2] conventional RT-PCR using the primers of Shamloul *et al.* (1997)	***Limit of detection:*** at least 17 pg PSTVd ***Analytical specificity*:** detection of MPVd, PSTVd, TCDVd and TPMVd ***Selectivity*:** no influence of potato variety, potato leaves or *in vitro* plants ***Repeatability and reproducibility*:** reproducibility 78% at 87 893 pg PSTVd (the highest concentration of PSTVd tested) and 44% at 17 pg PSTVd
Potato leaves (growth room grown) and *in vitro* potato plants	1 g	3.5 ml (1:2–1:5 (w/v)) GH plus lysis buffer with Homex 6	Sbeadex maxi plant kit on KingFisher 96 system	Real-time RT-PCR: GenPospi assay, Botermans *et al.* (2013)	Performance characteristics assay as for tomato leaves ***Analytical specificity*:** no cross-reaction with viruses commonly occurring in potato ***Selectivity*:** no influence of potato leaves and *in vitro* plants Validated for bulking rates up to 100 (100% detection in sample composed of 1 infected and 99 healthy leaves; NAK, 2011)
Potato leaves, (growth room grown) *in vitro* potato plants and tubers	1.5 g leaves or 5 g tubers	Approximately 6 ml buffer for leaves or approximately 3 ml buffer for tubers (buffer choice depending on method used for extraction)	RNeasy Plant Mini Kit, cetyl trimethylammonium bromide (CTAB) extraction or Purescript RNA isolation kit (Gentra Systems; note that this kit is not available anymore)	Real-time RT-PCR: Boonham *et al.* (2004)	***Limit of detection:*** detection up to 10 000 times dilution of infected tissue in healthy tissue ***Analytical specificity*:** detection of MPVd, PSTVd, TCDVd, TPMVd (some isolates); no cross-reaction with viruses commonly occurring in potato ***Selectivity*:** no influence of potato leaves, *in vitro* plants or tubers ***Repeatability and reproducibility*:** 100% (ring test of four laboratories) Validated for bulking rates up to 100 (100% detection in sample composed of 1 infected and 99 healthy leaves; Roenhorst *et al*., 2005, 2006)

Matrix	Sample size	Sample preparation	Nucleic acid extraction	Detection method	Remarks on validation
Ornamental plant species (leaves)	1 g	3.5 ml (1:2–1:5 (w/v)) GH plus lysis buffer with Homex 6	RNeasy Plant Mini Kit or Sbeadex maxi plant kit on KingFisher 96 system	Real-time RT-PCR: GenPospi assay, Botermans *et al*. (2013)	Performance characteristics assay as for tomato leaves ***Analytical sensitivity*:** concentration of pospiviroids and selectivity (inhibitory components) in leaf sap dependent on plant species Validated for bulking rates up to 25 for *Brugmansia*, *Calibrachoa*, *Cestrum*, *Dahlia*, *Nematanthus*, *Petunia*, *Solanum jasminoides* and *Streptosolen jamesonii*. Note that for *Calibrachoa*, *S. jasminoides* and *S. jamesonii* matrix effects have been observed at dilutions of more than 100. For some crops, such as *Dahlia*, only the summer period seems suitable for (reliable) testing (Naktuinbouw, 2012a).
Ornamental plant species (leaves)	1 g	3.5 ml (1:2–1:5 (w/v)) GH plus lysis buffer with Homex 6	RNeasy Plant Mini Kit or Sbeadex maxi plant kit on KingFisher 96 system	Real-time RT-PCR: Boonham *et al*. (2004)	Performance characteristics assay as for tomato leaves ***Analytical sensitivity*:** concentration of pospiviroids and selectivity (inhibitory components) in leaf sap dependent on plant species Validated for bulking rates up to 25 for *Brugmansia*, *Calibrachoa*, *Dahlia*, *Petunia, S. jasminoides* and *S. jamesonii*. Note that for *Calibrachoa*, *S. jasminoides* and *S. jamesonii* matrix effects have been observed at dilutions of more than 100. For some crops, such as *Dahlia*, only the summer period seems suitable for (reliable) testing (Naktuinbouw, 2012b).
Tomato leaves, potato leaves, tubers and seeds, and ornamental plant species (leaves)	1 g leaves or potato tubers or leaf prints on nylon membranes	10 ml (1:10 (w/v)) phosphate-buffered saline (PBS) with Homex 6	Direct methods (tissue print), RNeasy Plant Mini Kit or PowerPlant RNA Isolation Kit (Mo Bio)	Real-time RT-PCR Bertolini *et al*. (2010)	***Limit of detection:*** detection up to 10 000 times dilution of infected *S. jasminoides* leaves in healthy leaves of *S. jasminoides* and tomato ***Analytical specificity*:** detection of CLVd, PSTVd and TCDVd ***Selectivity*:** no influence of potato leaves, tubers or tomato seeds ***Repeatability and reproducibility*:** 100% (ring test of three laboratories) The diagnostic sensitivity was 100%, the diagnostic specificity was 100% and the relative accuracy compared with a molecular hybridization method (Murcia *et al*., 2009) was 100%. Validation of the test was performed with 208 field samples of *S. jasminoides*, *Brugmansia* spp., *Datura* spp., *Petunia* spp., *Dendrathema* spp., potato and tomato. Of the 208 samples, 43 were true positive and 150 true negative by both techniques. Fifteen samples were false positive by hybridization in which *Tomato apical stunt viroid* (TASVd) and *Citrus exocortis viroid* (CEVd) were detected. No samples were false negative.

[1] Because viroid concentration in the original test material is not known, for some of the assays the limit of detection (sensitivity) is expressed as a relative value. Undiluted infected leaf sap is considered 100% infected (at a ratio of 1 g leaf material : 3 ml buffer). The relative limit of detection was determined by testing eight serial dilutions of infected leaf sap in healthy leaf sap. The relative limit of detection is defined as the average of the lowest relative infection rate of each isolate that could still be detected (cycle threshold (Ct) <32), and three standard deviations were added to give a conservative measure with 99.7% certainty (Botermans *et al*., 2013).

[2] The three methods, R-PAGE, DIG probe and two-step conventional RT-PCR using the primers of Shamloul *et al*. (1997), were compared in an international ring test (Jeffries and James, 2005).

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This is not an official part of the standard

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ISPM 27. **Annex 7**. *Potato spindle tuber viroid* (2015). Rome, IPPC, FAO.

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