Bulletin OEPP/EPPO Bulletin (2021) **51** (1), 144–177 ISSN 0250-8052. DOI: 10.1111/epp.12717

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/138 (1)

Diagnostics

PM 7/138 (1) Pospiviroids (genus *Pospiviroid*)

Specific scope

This Standard describes a generic diagnostic protocol for the detection of viroids belonging to the genus *Pospiviroid* and the identification of species within this genus, in particular the species recommended for regulation CSVd and PSTVd.

This protocol replaces the EPPO Standard PM 7/6 *Chrysanthemum stunt pospiviroid* (EPPO, 2002) and PM 7/33 *Potato spindle tuber pospiviroid* (EPPO, 2002).

This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment

Approved in 2020-10.

1. Introduction

Viroids are subviral agents with small genomes (239–401 nt) that infect plants. They consist of circular, single-stranded RNA molecules. Viroids do not code for any protein. A viroid replication mechanism uses RNA polymerase II, a host cell enzyme normally associated with synthesis of messenger RNA from DNA, which instead catalyses 'rolling circle' synthesis of new RNA using the viroid's RNA as a template. The taxon is unique among plant pathogens and consists of two families, the *Avsunviroidae* and the *Pospiviroidae*. The genus *Pospiviroid* is one of five genera in the family *Pospiviroidae* (Di Serio *et al.*, 2014).

The genus *Pospiviroid* comprises nine viroid species (Verhoeven *et al.*, 2011a; Owens *et al.*, 2012; ICTV online): Potato spindle tuber viroid (PSTVd; type species), Chrysanthemum stunt viroid (CSVd), Citrus exocortis viroid (CEVd), Columnea latent viroid (CLVd), Iresine viroid 1 (IrVd-1), Pepper chat fruit viroid (PCFVd), Tomato apical stunt viroid (TASVd), Tomato chlorotic dwarf viroid (TCDVd), Tomato planta macho viroid (TPMVd, including the former Mexican papita viroid) and one tentative species portulaca latent viroid (PoLVd; Verhoeven *et al.*, 2015a). Species demarcation is based on sequence similarity level (less than 90% sequence identity of the total viroid genome) and on distinctive biological properties, in particular host range and symptoms (Owens *et al.*, 2012; Di Serio *et al.*, 2014). Some pospiviroids represent clusters of very similar genome sequences (>90% sequence identity, e.g. PSTVd/TCDVd) but differ in host range and symptom expression (Martinez-Soriano *et al.*, 1996; Singh *et al.*, 1999; Matsushita *et al.*, 2009) and are therefore accepted as distinct species.

A summary of the host range of pospiviroids is presented in Table 1. While the natural host range of most pospiviroids seems limited, all pospiviroids (except IrVd-1 and the related PoLVd) could be transmitted to potato and tomato, and elicit similar symptoms under controlled conditions (Verhoeven *et al.*, 2004; EFSA, 2011). Pospiviroids can be mechanically transmitted (Verhoeven *et al.*, 2010a). Evidence of mechanical transmission of pospiviroids by crop handling within and between plant species is supported by the observation that in tomatoes PSTVd, CEVd, CLVd and TCDVd generally spread along rows in greenhouses (Verhoeven *et al.*, 2004; Matsushita *et al.*, 2008). In addition, several pospiviroids have been reported to be transmitted via pollen and seeds (e.g. Matsushita & Tsuda, 2016). The mechanism of seed transmission and the role in spreading, however, is not understood since studies with different pospiviroids, hosts and conditions gave contradictory results

(e.g. Matsushita & Tsuda, 2016; Yanagisawa & Matsushita, 2017).

A decision scheme for detection and identification and/or confirmation of the presence of a pospiviroid as well as for the detection and identification of PSTVd and CSVd is presented in Fig. 1. Positive results in a detection test should be confirmed by a different test (Table 2 and Appendix 8) or by sequencing and sequence analysis of the amplicon. Identification should be based on either a specific test or sequence analysis of the amplicon, preferably comprising the complete genome. However, when dealing with low levels of viroids, as often is the case in seed samples, this might not be feasible and other options should be considered.

2. Identity

2.1. Identity of viroid species covered

Taxonomic position (of the different species) Family *Pospiviroidae*, Genus *Pospiviroid*.

Name: Chrysanthemum stunt viroid.
Other scientific names: Chrysanthemum stunt mottle virus, Chrysanthemum stunt pospiviroid.
Acronym: CSVd.
EPPO Code: CSVD00.
Phytosanitary categorization: EPPO A2 n°92, EU RNQP Annex IV.

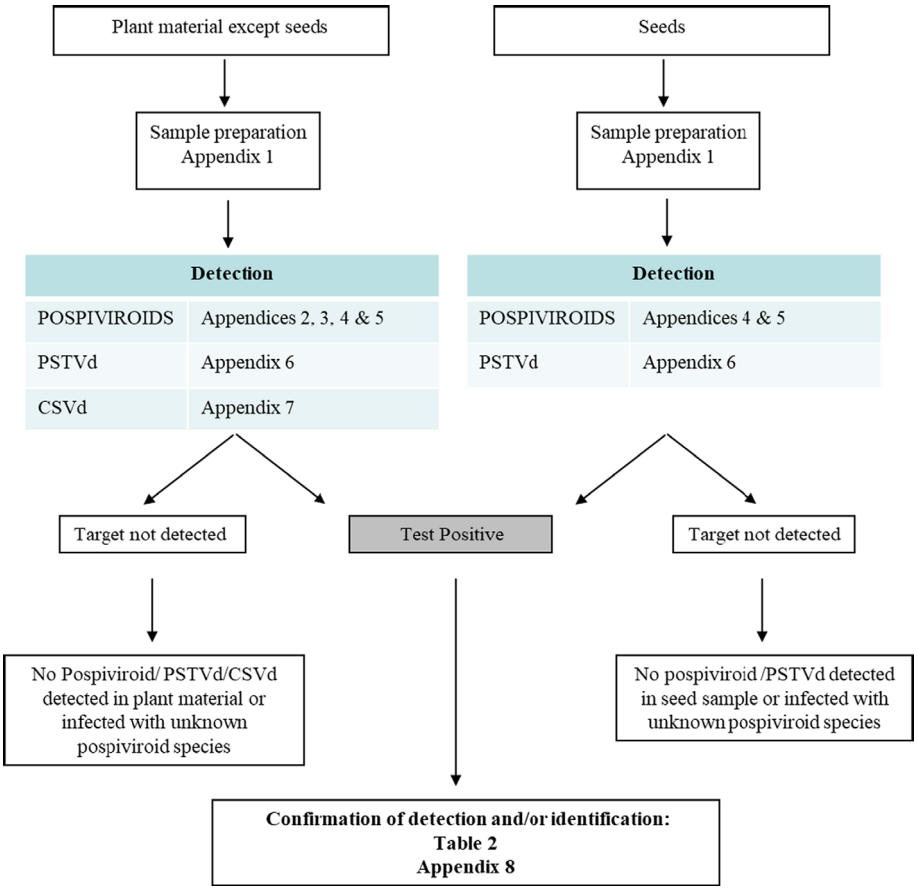

Fig 1 Decision scheme for testing plant samples for pospiviroids. Table 2 and Appendix 8 provide an overview of tests that can be used for detection, confirmation of detection and/or identification of pospiviroids.

Name Citrus exocortis viroid.
Other scientific names: Indian tomato bunchy top viroid, Citrus exocortis pospiviroid.
Acronym: CEVd.
EPPO Code: CEVD00.
Phytosanitary categorization: None.

Name: Columnea latent viroid.
Other scientific name: Columnea latent pospiviroid.
Acronym: CLVd.
EPPO Code: CLVD00.
Phytosanitary categorization: None.

Name: Iresine viroid 1.
Other scientific names: Iresine pospiviroid, Iresine viroid.
Acronym: IrVd-1.
EPPO Code: IRVD00.
Phytosanitary categorization: None.

Name: Pepper chat fruit viroid.
Other scientific name: Pepper chat fruit pospiviroid.
Acronym: PCFVd.
EPPO Code: PCFVD0.
Phytosanitary categorization: None.

Name Potato spindle tuber viroid.
Other scientific name: Potato spindle tuber pospiviroid,
Acronym: PSTVd.
EPPO Code: PSTVD0.
Phytosanitary categorization: EPPO A2 n°92; EU RNQP Annex IV.

Name: Tomato apical stunt viroid.
Other scientific name: Tomato apical stunt pospiviroid.
Acronym: TASVd.
EPPO Code: TASVD0.
Phytosanitary categorization: EPPO Alert List.

Name: Tomato chlorotic dwarf viroid.
Other scientific name: Tomato chlorotic dwarf pospiviroid.
Acronym: TCDVd.
EPPO Code: TCDVD0.
Phytosanitary categorization: None.

Name: Tomato planta macho viroid.
Other scientific name: Tomato planta macho pospiviroid.
Acronym: TPMVd.
EPPO Code: TPMVD0.
Phytosanitary categorization: None.

Portulaca latent viroid (PoLVd) Verhoeven *et al.* (2015a)

PoLVd should be considered as a new species within the pospiviroid genus based on its molecular characteristics. However, since no biological differences have yet been found with its closest relative IrVd-1, PoLVd does not fulfil all ICTV criteria for species demarcation and is not included in this Standard.

Note Virus nomenclature in Diagnostic protocols is based on the latest release of the official classification by the International Committee on Taxonomy of Viruses (ICTV, Release 2019, https://talk.ictvonline.org/taxonomy/). Accepted species names are italicized when used in their taxonomic context, whereas virus names are not, corresponding to ICTV instructions. The integration of the genus name within the name of the species is currently not consistently adopted by ICTV working groups and therefore species names in diagnostic protocols do not include genus names. Names of viruses not included in the official ICTV classification are based on first reports.

3. Detection

3.1. Symptoms

Pospiviroids are generally distributed in most parts of the plants, including seeds. Their propensity to develop symptoms largely depends on the viroid species, strain, host species, cultivar and environmental conditions. Infected ornamental species are often symptomless. Although pospiviroids are mainly found in solanaceous species, they have also been reported infecting other plant species (see Table 1, for review see EFSA, 2011). On their main hosts, the following symptoms have been observed:

Table 1. Species in the genus *Pospiviroid*: natural host range and symptomatology*

Name and acronym	Hosts	Symptoms
Chrysanthemum stunt viroid (CSVd)	*Argyranthemum frutescens* *Dendranthema* × *grandiflorum* *Gerbera* spp. *Petunia* spp. *Solanum* spp. *Verbena* spp.	 Courtesy J. Dunez (France).  Courtesy J. Dunez (France).  Courtesy M Visage (LSV-ANSES)
Citrus exocortis viroid (CEVd)	*Cestrum* spp. *Citrus* spp. *Impatiens* spp. *Solanum* spp. *Verbena* spp.	 Courtesy A. Olmos (Spain)  Courtesy J. Dunez (France).
Columnea latent viroid (CLVd)	*Brunfelsia* spp. *Columnea* spp. *Gloxinia* spp. *Nematanthus wettsteinii* *Solanum* spp.	 Courtesy Defra (UK, Crown Copyright).  Courtesy M Visage (LSV-ANSES)
Iresine viroid 1 (IrVd-1)	*Alternanthera* spp. *Celosia* spp. *Iresine* spp. *Portulacaceae* spp. *Solanum* spp. *Verbenaceae* spp. *Vinca major*	Information on symptoms not available.

(continued)

Table 1 *(continued)*

Name and acronym	Hosts	Symptoms
Pepper chat fruit viroid (PCFVd)	*Capsicum* spp. *Solanum* spp.	 (EPPO Global Database)  (EPPO Global Database)  Ref.: Verhoeven *et al*. (2009a)
Potato spindle tuber viroid (PSTVd)	*Capsicum* sp. *Dahlia* spp. *Datura* spp. *Ipomoea* spp. *Nicandra* spp. *Nicotiana* spp. *Persea* spp. *Petunia* spp. *Physalis* spp. *Solanum* spp.	 Courtesy Defra (UK, Crown Copyright).  Courtesy SA Slack.
Tomato apical stunt viroid (TASVd)	*Brugmansia* spp. *Capsicum annuum* (seed) *Cestrum* spp. *Solanum* spp. *Streptosolen jamesonii*	 Courtesy NPPO (The Netherlands).

(continued)

Table 1 *(continued)*

Name and acronym	Hosts	Symptoms
Tomato chlorotic dwarf viroid (TCDVd)	*Brugmansia* spp. *Petunia* spp. *Pittosporum* spp. *Solanum* spp. *Verbena* spp. *Vinca* spp.	 Courtesy R. Singh (Canada)  Courtesy R. Singh (Canada)  Courtesy LSV-ANSES
Tomato planta macho viroid (TPMVd)	*Solanum lycopersicum*	 http://vegetablemdonline.ppath.cornell.edu/DiagnosticKeys/TomLeaf/PlantaMacho_Tom.htm)

*Note that *Pospiviroid* infections may be asymptomatic in many hosts. In *S. lycopersicum* symptoms are not specific for the viroid species, i.e. variation in symptoms within a species is similar to variation between species.

- *Capsicum annuum*: In pepper natural infections have been recorded for only two pospiviroids, i.e. PCFVd and PSTVd. In the case of PCFVd infection, plant growth is slightly reduced, leaves appear slightly pale and fruit size is reduced, down to 50%. In some instances, vein necrosis has been observed (Verhoeven *et al.*, 2009, 2011b). Symptoms of PSTVd in pepper plants were very mild, showing only a wavy margin on the leaves near the top of the plant (Lebas *et al.*, 2005) and symptomless infections also occur. In addition to PCFVd and PSTVd, TASVd has been detected in an old pepper seed lot (Verhoeven *et al.*, 2017), suggesting that pepper is a natural host of TASVd as well (Verhoeven *et al.*, 2017).
- *Chrysanthemum* spp.: The main symptom of CSVd in chrysanthemum is stunting (Hollings & Stone, 1973; Diener & Lawson, 1973). Stems might become brittle, readily breaking at the branch point. Other common symptoms are reduced flower size and premature flowering. In certain cultivars, especially red-pigmented ones, symptoms can also include flower break or bleaching. Foliar symptoms are less common, and the presence of pale, upright young leaves is often the only indication of infection. Sometimes leaf spots or flecks are observed, which might be associated with leaf distortions (crinkling). However, many chrysanthemum cultivars are symptomless. When symptoms are seen, they are often variable and dependent on environmental conditions, especially temperature and light.
- *Citrus* spp.: CEVd is the causal agent of exocortis disease (also reported as scaly butt disease and Rangpur lime disease). CEVd may cause scaling, shelling and splitting of the bark of citrus trees and stunting (Semancik & Weathers, 1972a,b; EFSA., 2008).
- *Solanum lycopersicum*: In the early stages of infection, a growth reduction and chlorosis in the upper leaves and

reduced fruit size are generally observed (Verhoeven *et al.*, 2004). In addition, other types of symptoms such as rugosity and irregular ripening might occur. Growth reduction may develop into stunting and bunchy growth, and the chlorosis may become more severe, turning into reddening, purpling and/or necrosis. At this stage, leaves may become deformed and brittle. As stunting begins, flower and fruit initiation stop. Generally, this stunting is permanent; occasionally, plants may either die or partially recover (EFSA, 2011). Isolates from different tomato-infecting pospiviroids may cause a similar diversity of symptoms irrespective of the species.

- *Solanum tuberosum*: PSTVd is the only viroid known to infect cultivated species of potato naturally. In potato, PSTVd may cause severe to mild symptoms and also symptomless infections occur. Severe symptoms might include reduction in plant size, uprightness and clockwise phyllotaxy of the foliage if viewed from above, and dark green and rugose leaves (Pfannenstiel & Slack, 1980). Tubers may be reduced in size, misshapen, spindle or dumbbell-shaped, with conspicuous prominent eyes which are evenly distributed. Under experimental conditions, all pospiviroids (except IrVd-1) could cause tuber symptoms similar to PSTVd (Verhoeven *et al.*, 2004, 2010b). So far, there is one report of CSVd isolated from potato, hence CSVd could potentially infect potato naturally (Matsushita *et al.*, 2019).

3.2. Test sample requirements

Pospiviroids can infect a wide range of plant species, including both herbaceous and woody species. The viroid concentration in different hosts and tissue types might vary significantly. Sampling is described for the main hosts and/or matrices. Plant material may be bulked to specific rates depending on the test, tissue and purpose of testing. In all cases bulking rates must be validated.

3.2.1. Bark and woody tissues

Bark tissue from citrus species (e.g. *Citrus medica* L., *Poncirus trifoliata*) can be sampled from symptomatic or asymptomatic seedlings and young shoots (Rizza *et al.*, 2009). In the case of trees, which might display scaling symptoms on the rootstock, green bark tissues can be collected during the period of growth, i.e. summertime (Ragozzino *et al.*, 2005). Since viroid concentrations are expected to be low, bulking should be kept to a minimum and validated.

3.2.2. Leaves

In general top and fully expanded young leaves, i.e. growing tissue, are most suitable for testing. Since viroid concentrations might differ considerably and are dependent on environmental conditions (temperature and photoperiod); the bulk size should be adapted to the host plant and test characteristics (analytical sensitivity). For leaves of potato and tomato, bulking rates up to 100 have been used for real-time reverse transcriptase-polymerase chain reaction (RT-PCR) tests, whereas for pepper and ornamentals, such as *Brugmansia* spp., *Chrysanthemum* spp., *Dahlia* spp. and *S. jasminoides*, bulking rates of up to 25 were found adequate (E. Meekes, pers. comm.; Verhoeven *et al.*, 2008, 2016).

3.2.3. Microplants

For *Solanum tuberosum* (potato), whole microplants should be tested or the top two-thirds when at least 4–6 weeks old and with stems of at least 5 cm in length with well-developed leaves. Microplants may be bulked. The bulking rate will depend on the test method used and should be validated (IPPC, 2015).

3.2.4. Seeds

For seed testing it is difficult to recommend sample sizes and bulking rate because the level of pospiviroids can differ considerably between individual seeds. For seed lots of pepper and tomato, protocols using weighed samples of approximately 3000 seeds, tested in three subsamples of 1000 seeds, has been validated for real-time RT-PCR (Appendices 4 and 5). However, both sample and subsample size might have to be adapted due to technical restrictions or to meet specific import requirements. Furthermore, it should be noted that the likelihood of viroid detection in a seed lot might be higher than expected depending on the ratio of infected fruits/plants, since seeds from healthy tomato fruits might become contaminated when extracted together with seeds from viroid-infected fruits (Verhoeven *et al.*, 2015b).

3.2.5. Tubers

For *Solanum tuberosum* (potato), samples can be taken from tuber eyes, heel end, peel fragments and flesh cores throughout the whole tuber, since PSTVd has been found to be present in almost equal amounts in different parts of both primarily and secondarily infected tubers (Shamloul *et al.*, 1997; Roenhorst *et al.*, 2006). The highest concentration is found immediately after harvest and hardly decreases during storage at 4°C for at least up to 3 months. After that period, concentrations may decrease in time. Up to 100 small cores weighing about 50 mg each may be bulked together for RNA extraction if using real-time RT-PCR (Boonham *et al.*, 2004; IPPC, 2015).

3.3. Screening tests

For the detection of pospiviroids different tests are available, depending on the type, source of the material and purpose of testing. Appendix 1 describes sample preparation and RNA extraction methods for different host plants and tissue types. The currently most widely used molecular tests are described and recommended:

- Conventional RT-PCR (Appendix 2)
- Real-time RT-PCR (Appendix 3)
- Real-time RT-PCR for seed testing (Appendices 4 and 5).

It should be noted that for all molecular tests sequence mismatch may hamper detection of specific isolates.

3.3.1. Conventional one-step RT-PCR

RT-PCR using so-called 'generic' pospiviroid primers is an efficient and sensitive method to detect and identify most pospiviroids. There are several RT-PCR tests suitable for the detection of pospiviroids by using combinations of different primer sets (Verhoeven *et al.*, 2004; Luigi *et al.*, 2014; Olivier *et al.*, 2014; ANSES, 2017). Some of these tests were evaluated in an interlaboratory comparison (Olivier *et al.*, 2016). The test described by Verhoeven *et al.* (2004) using primer sets Pospi1 and Vid allows detection of all known pospiviroids with the exception of one CLVd isolate so far (GenBank acc. no. FM995506, Steyer *et al.*, 2010). The CLV4-primer set described by Spieker (1996a) additionally detects this CLVd variant. A multiplex test combining modified versions of the Pospi1 and CLV4 primers detects all nine pospiviroids in one reaction (Olivier *et al.*, 2014). Although this test is less sensitive than the original tests, it allowed the detection of all pospiviroids up to a relative infection rate of at least 1%. The most widely used RT-PCR tests are described in Appendix 2, i.e. the tests using Pospi1 primers (Verhoeven *et al.*, 2004) and CLV4 primers (Spieker, 1996a). Further details on the characteristics of these tests can be found in Table 2. Figure 2 gives a schematic overview of the positions of the respective amplicons.

3.3.2. Real-time RT-PCR

Real-time RT-PCR is the preferred method for large-scale screening of plant material and seeds. Several real-time RT-PCR tests have been developed to detect a subset of pospiviroids. The GenPospi test (Botermans *et al.*, 2013) detects all known pospiviroids and is described in Appendix 3. Figure 2 gives a schematic representation of the positions of the amplicons. For testing seeds of pepper, tomato and potentially other solanaceous species, more sensitive tests have been designed. Appendix 4 describes a test consisting of four parallel reactions designed by Naktuinbouw (unpublished) which has been validated in the framework of the EU project TESTA (Testa, 2015). Appendix 5 describes the PospiSense test consisting of two parallel reactions designed by Botermans *et al.* (2020). The two latter tests only target pospiviroid species relevant for pepper and tomato and have not been designed to detect CSVd and IrVd-1. Appendix 6 (Boonham *et al.*, 2004) describes a real-time RT-PCR test for the detection of PSTVd and is included in ISPM 27 DP 7: *Potato spindle tuber viroid* (IPPC, 2015). Appendix 7 (Mumford *et al.*, 2000) describes a real-time RT-PCR test for the detection of CSVd.

Further details on the specificity of the different real-time RT-PCR tests can be found in Table 2.

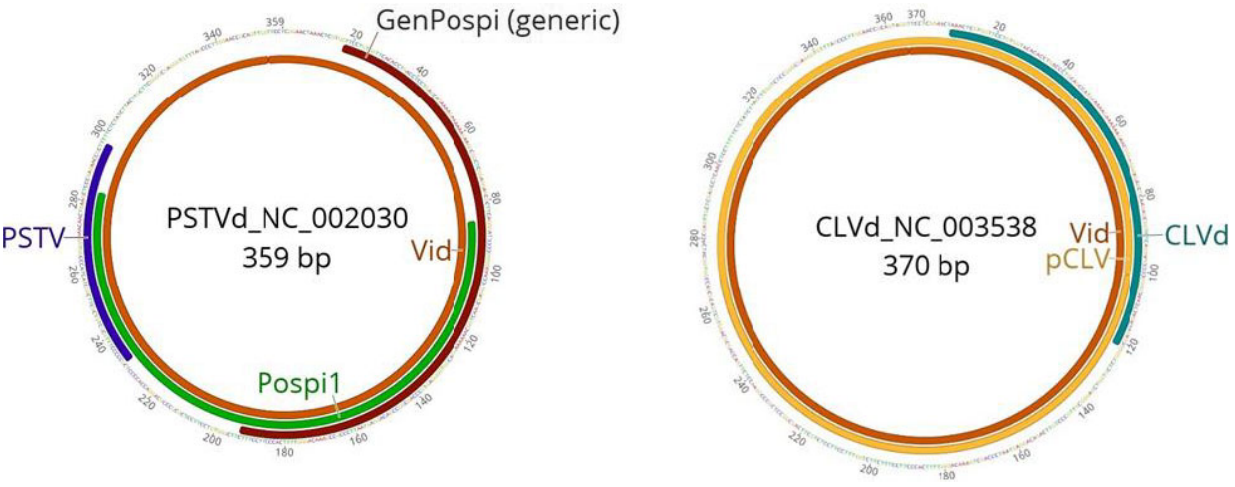

Fig 2 Schematic representation of a PSTVd and CLVd genome based on their reference sequences. Amplicons of recommended tests are colour-coded and indicated by names of the primers: PSTV (Boonham *et al.*, 2004), Pospi1 (Verhoeven *et al.*, 2004), Vid (Verhoeven *et al.*, 2004), GenPospi (Botermans *et al.*, 2013), pCLV (Spieker, 1996a) and CLVd (Monger *et al.*, 2010).

Table 2. Overview of recommended tests for all listed pospiviroid species

	Primers or mix	CSVd	CEVd	CLVd	IrVd-1	PCFVd	PSTVd	TASVd	TCDVd	TPMVd	Position of amplicon*	Fragment size (if relevant)	Remarks on validation	References
Appendix 2	Pospi1[†]	+	+	–	+	+	+	+	+	+	86–283 in PSTVd NC_002030	198 nt	EPPO validation data NPPO-NL (2013a)	Verhoeven *et al.* (2004)
	pCLV	nt	–	+	–	nt	nt	–	nt	nt	102–101 in CLVd NC_003538	370 nt	Olivier *et al.*, 2016; NPPO-NL (unpublished)	Spieker (1996a)
Appendix 3	GenPospi Mix	+	+	–	+	+	+	+	+	+	18–193 in PSTVd NC_002030	–	EPPO validation data NPPO-NL (2013c)	Botermans *et al.* (2013)
	CLVd Mix	–	–	+	–	–	–	–	–	–	8–121 in CLVd NC_003538	–	EPPO validation data NPPO-NL (2013c)	Monger *et al.* (2010)
Appendix 4	Mix A	nt	–	–	–	+	+	–	+	+[††]	232–297 in PSTVd NC_002030 7–72 in PCFVd NC_011590	–	Testa (2015); EPPO validation data (Naktuinbouw, 2017)	Boonham *et al.* (2004); Naktuinbouw (2017)
	Mix B	–	+	+	nt	–	–	+[‡‡]	–	–	206–299 in CEVd NC_001464I8-121 in CLVd NC_003538	–	Testa (2015); EPPO validation data (Naktuinbouw, 2017)	Monger *et al.* (2010); Naktuinbouw (2017)
	Mix C	nt	–	–	nt	–	–	–	–	+	57–130 in TPMVd NC_001558	–	Testa (2015); EPPO validation data (Naktuinbouw, 2017)	Botermans *et al.* (2013); Naktuinbouw (2017)
	Mix D	nt	–	–	nt	–	–	+	–	–	197-263 in TASVd NC_001553	–	Testa (2015); EPPO validation data (Naktuinbouw, 2017)	Monger *et al.* (2010)
Appendix 5	PospiSense1	nt	–	+	nt	+	+	–	+	+				Botermans *et al.* (2020)
	PospiSense2	nt	+	–	nt	–	–	+	–	–				Botermans *et al.* (2020)
Appendix 6	PSTVd	–	–	–	–	–	+	–	+	+*	232-297 in PSTVd NC_002030	–	Naktuinbouw (2012a), NAK (2015)	Boonham *et al.* (2004)
Appendix 7	CSVd	+	nt	nt	nt	nt	–	nt	nt	nt	222-299 in CSVd NC_002015	–	Fera	Mumford *et al.* (2000)

+, detected; –, not detected; nt, not tested.

*Position of amplicon in reference sequence of indicated species in NCBI GenBank.

[†]Sequence of PCR product can be used for (preliminary) identification.

[††]It is known that at least one isolate of TPMVd (GenBank acc. no. K00817.1) will not or only be poorly detected (Testa, 2015; EPPO validation data (Naktuinbouw, 2017).

[‡‡]May cross-react with some isolates (Monger *et al.*, 2010).

3.3.3. Other methods

- *Biological tests*: Inoculation of suitable indicator plants (e.g. *S. lycopersicum*) and monitoring symptoms development. Propagation on indicator plants can be used for maintenance or production of material for further testing and identification. Inoculation of *S. lycopersicum* plants (cultivars Rutgers, Moneymaker or Sheyenne) might allow the detection of all viroids, except IrVd-1 and an isolate of TASVd (Spieker, 1996b; Verhoeven *et al.*, 2010a, 2015a, 2017), and might provide visual evidence of pathogenicity. While not generally suitable for detection and identification of pospiviroids, this method might be useful in detecting infections of yet uncharacterized viroid species. Further details on mechanical inoculation are described in Appendix 9.
- *Hybridization with DIG-labelled probe*: Nucleic-acid hybridization using species-specific probes that cross-hybridize with other pospiviroids at low stringency conditions (Owens & Diener, 1981; Singh, 1999; IPPC, 2015). Nucleic acid-hybridization using a Digoxigenin (DIG)-labelled PSTVd cRNA probe (Agdia Inc., USA) is a sensitive detection method but less amenable to high-throughput diagnosis in comparison to molecular tests. A full-length monomer PSTVd DIG-labelled cRNA probe will detect all known pospiviroids from a range of hosts including *Petunia* spp., *S. jasminoides*, *S. lycopersicum* and *S. tuberosum* (Torchetti *et al.*, 2012; Monger & Jeffries, 2015). Sensitivity of detection was at least 17 pg PSTVd (Jeffries & James, 2005). Probe preparation, sample and test membrane preparation, and hybridization conditions are as described in Appendix 10.
- *Return polyacrylamide gel electrophoresis (R-PAGE)*: Generic method allowing the detection (not the identification) of all known and unknown viroids. This method has been used successfully with various host plants including *C. annuum*, *Dendranthema grandiflorum*, *Petunia hybrida*, *S. lycopersicum* and *S. tuberosum*, and often provided the initial step in discovering yet undescribed species. R-PAGE is about 100-fold less sensitive than other molecular methods evaluated (Jeffries & James, 2005). R-PAGE is described by Roenhorst *et al.* (2000). Because of its low sensitivity and practicality this method is not recommended for high-throughput application.

4. Identification

For the identification of pospiviroid species, the following tests can be used:

- Conventional RT-PCR using 'generic' primers as described in Appendix 2 or other primers provided in Appendix 8, followed by sequencing and sequence analysis of the amplicon (see section 4.1).
- Conventional and real-time RT-PCR tests using specific primers (and probes) for individual pospiviroid species as described in Appendix 8 and Table 2.

4.1. Conventional RT-PCR followed by sequencing and sequence analysis

Appendix 7 of EPPO PM 7/129 *DNA barcoding as identification tool for a number of regulated pests* (EPPO, 2016 and new version in press) provides general guidance on sequencing and sequence analysis. For the identification of pospiviroids, preferably the sequence of the complete genome should be used for further analysis. 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.*, 2012). 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.

The test using the Pospi1 primers (Verhoeven *et al.*, 2004) has been found to be the most sensitive conventional RT-PCR test, in some cases comparable to real-time RT-PCR. This test is not the best choice for identification, as the amplicon only covers about half of the pospiviroid genome. Nevertheless, thus far this partial sequence appeared suitable for a correct identification of the species (NPPO-NL, 2013a). The Pospi2 primers (Verhoeven *et al.*, 2017) which have the opposite orientation can be used to sequence the complete genome, although this test is less sensitive than the Pospi1 test. The Vid (Verhoeven *et al.*, 2004) and pCLV (Spieker, 1996a) primers produce amplicons spanning the whole genome and therefore are suitable for identification (Figure 2).

It should be noted that in some cases where it is not feasible to obtain the complete genome sequence, species identification can only be concluded based on a partial sequence in combination with the results of another test. Especially in the case of seed testing, where viroid concentrations might be low, conventional RT-PCR tests might lack the sensitivity to produce an amplicon. Choices for further testing therefore have to be adjusted depending on the initial test. Examples of test combinations suitable to substantiate a diagnostic result are described in Roenhorst *et al.* (2018).

Furthermore, when 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 test for confirmation.

4.2. Conventional and/or real-time RT-PCR using specific primers (and probes)

Table 2 provides an overview of conventional and real-time RT-PCR tests. Specific tests can be used for identification provided that these are adequately validated on analytical specificity. In other cases, subsequent sequencing and sequence analysis of the amplicons is required as well (see section 4.1).

4.3. Other tests

The Panel on Diagnostics in Virology and Phytoplasmology noted that high throughput sequencing (HTS) is a technology that may be used for obtaining (almost) complete genome sequences, from which analysis can be used for identification of a virus isolate. An EPPO Standard on the use of HTS for plant pest diagnostics is in preparation.

5. Reference material

Pospiviroid isolates for reference are available from:

Leibniz-Institut DSMZ-Deutsche Sammlung vorn Mikroorganismen und Zellkulturen GmbH, Inhoffenstrasse 7B, 38124, Braunschweig, Germany (https://www.dsmz.de/catalogues/catalogue-plant-viruses-and-antisera.html and https://qbank.eppo.int/virus/).

National Plant Protection Organization, National Reference Centre, PO Box 9102, 6700 HC Wageningen, the Netherlands. (see https://qbank.eppo.int/virus/).

6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM 7/77 *Documentation and reporting on a diagnosis*.

7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8. Further information

Further information on these organisms can be obtained from:

National Plant Protection Organization, National Reference Centre, PO Box 9102, 6700 HC Wageningen, the Netherlands (for PCR, in particular tests described in Appendices 2, 3 and 5).

Naktuinbouw Laboratories, Sotaweg 25, PO Box 135, 2370 AC Roelofarendsveen, the Netherlands (for PCR, in particular tests described in Appendices 3 and 4).

SASA, Roddinglaw Road, Edinburgh, EH12 9FJ, United Kingdom.

9. Feedback on this Diagnostic Protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website. When errata and corrigenda are in press, this will also be marked on the website.

Acknowledgements

This protocol was originally drafted by: C. Lacomme, SASA, Roddinglaw Road, Edinburgh, EH12 9FJ, United Kingdom; M. Botermans and J.W. Roenhorst, National Plant Protection Organization, National Reference Centre, PO Box 9102, 6700 HC Wageningen, the Netherlands; F. Faggioli, CREA – Centro di Ricerca per la Patologia Vegetale, Via C.G. Bertero, 22 00156 Roma, Italy. The Panel on Diagnostics in Virology and Phytoplasmology reviewed this protocol.

References

ANSES (2017) *Détection des Pospiviroïdes par RT-PCR en point final sur feuilles de plantes hotes*. ANSES/LSV/MA 034, version 2.

Astruc N, Marcos JF, Macquaire G, Candresse T & Pallas V (1996) Studies of diagnosis of hop stunt viroid in fruit trees: detection by molecular hybridisation and relationship with specific maladies affecting peach and pear trees. *European Journal of Plant Pathology* **102**, 837–46.

Bernad L & Duran-Vila N (2006) A novel RT-PCR approach for detection and characterization of citrus viroids. *Molecular and Cellular Probes* **20**, 105–113.

Boonham N, González L, Lilia Peralta E, Blockley A, Walsh K, Barker I & *et al*. (2004) Development of a real-time RT-PCR assay for the detection of Potato spindle tuber viroid (PSTVd). *Journal of Virological Methods* **116**, 139–146.

Botermans M, Roenhorst JW, Hooftman M, Verhoeven JThJ, Metz E, Van Veen EJ *et al*. (2020) *Development and validation of a real-time RT-PCR test for screening pepper and tomato seed lots on the presence of pospiviroids*. https://doi.org/10.1371/journal.pone.0232502

Botermans M, van de Vossenberg BTLH, Verhoeven JThJ, Roenhorst JW, Hooftman M, Dekter R & *et al*. (2013) Development and validation of a real-time RT-PCR test for generic detection of pospiviroids. *Journal of Virological Methods* **187**, 43–50.

Chomczynski P & Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156–159.

Di Serio F, Flores R, Verhoeven JThJ, Li FS, Pallás V, Randles JW *et al*. (2014) Current status of viroid taxonomy. *Archives of Virology* **159**, 3467–3478.

Diener TO & Lawson RH (1973) Chrysanthemum stunt: a viroid disease. *Virology* **51**, 94–101.

EFSA (2008) Pest risk assessment made by France on Citrus exocortis viroid (CEVd) considered by France as harmful in French overseas department of Réunion. *EFSA Journal* **685**, 1–17.

EFSA (2011) Scientific Opinion on the assessment of the risk of solanaceous pospiviroids for the EU territory and the identification

and evaluation of risk management options. *EFSA Journal* **9**, 2330. [133 pp.] https://doi.org/10.2903/j.efsa.2011.

EPPO (2002) PM 7/6. Diagnostic protocols for regulated pests. Chrysanthemum stunt pospiviroid. *EPPO Bulletin* **32**, 245–253.

EPPO (2004) PM 7/33. Diagnostic protocols for regulated pests. *Potato spindle tuber viroid. EPPO Bulletin* **34**, 155–157.

EPPO (2016 and new version in press) PM 7/129. DNA barcoding as an identification tool for a number of regulated pests. *EPPO Bulletin* **46**, 501–537.

Hollings M & Stone OM (1973) Some properties of chrysanthemum stunt, a virus with the characteristics of an uncoated ribonucleic acid. *Annals of Applied Biology* **74**, 333–348.

Hooftman R, Arts MJ, Shamloul AM, Van Zaayen A & Hadidi A (1996) Detection of chrysanthemum stunt viroid by revers transcription-polymerase chain reaction and by tissue blot hybridization. *Acta Horticulturae* **432**, 88–94.

IPPC (2015) *ISPM 27, Diagnostic protocols for regulated pests, DP7 2015. Potato spindle tuber viroid*. https://www.ippc.int/static/media/files/publications/en/2015/02/18/dp_07_2015_2006-022_draftdp_pstvd_2015-02-06.pdf.

Jeffries C & James C (2005) Development of an EU protocol for the detection and diagnosis of Potato spindle tuber pospiviroid. *EPPO Bulletin* **35**, 125–132.

Kiefer MC, Owens RA & Diener TO (1983) Structural similarities between viroids and transposable genetic elements. *Proceedings National Academy of Sciences USA* **80**, 6234–6238.

Lebas BSM, Clover GRG, Ochoa-Corona FM, Elliott DR, Tang Z & Alexander BJR (2005) Distribution of Potato spindle tuber viroid in New Zealand glasshouse crops of capsicum and tomato. *Australasian Plant Pathology* **34**, 129–133.

Luigi M, Costantini E, Luison D, Mangiaracina P, Tomassoli L & Faggioli F (2014) A diagnostic method for the simultaneous detection and identification of pospiviroids. *Journal of Plant Pathology* **96**, 151–158.

Martinez-Soriano JP, Galindo-Alonso J, Maroon CJM, Yucel I, Smith DR & Diener TO (1996) Mexican papita viroid: Putative ancestor of crop viroids. *Proceedings of the National Academy of Sciences USA* **93**, 9397–9401.

Matsushita Y, Kanda A, Usugi T & Tsuda S (2008) First report of a Tomato chlorotic dwarf viroid disease on tomato plants in Japan. *Journal of General Plant Pathology* **74**, 182–184.

Matsushita Y & Tsuda S (2016) Seed transmission of potato spindle tuber viroid, tomato chlorotic dwarf viroid, tomato apical stunt viroid, and Columnea latent viroid in horticultural plants. *European Journal of Plant Pathology* **145**, 1007–1011.

Matsushita Y, Usugi T & Tsuda S (2009) Host range and properties of tomato chlorotic dwarf viroid. *European Journal of Plant Pathology* **124**, 349–352.

Matsushita Y, Yanagisawa H, Khiutti A, Mironenko N, Ohto Y & Afanasenko O (2019) First report of chrysanthemum stunt viroid isolated from potato (*Solanum tuberosum*) plants in Russia. *Journal of General Plant Pathology* **85**, 311–313.

Mehle N, Kogovsek P, Racki N, Jakomin T, Gutierrez-Aguirre I, Kramberger P & *et al*. (2017) Filling the gaps in diagnostics of pepino mosaic virus and potato spindle tuber viroid in water and tomato seeds and leaves. *Plant Pathology* **66**, 1191 https://doi.org/10.1111/ppa.12710.

Menzel W, Jelkmann W & Maiss E (2002) Detection of four apple viruses by multiplex RT-PCR tests with co-amplification of plant mRNA as internal control. *Journal of Virological Methods* **99**, 81–92.

Monger W & Jeffries C (2015) Detection of Potato spindle tuber viroid and other related viroids by a DIG labelled RNA probe. In *Methods in Molecular Biology – Plant Pathology: Techniques and Protocols* (ed. Lacomme C), pp. 259–271. USA: Springer.

Monger W, Tomlinson J, Boonham N, Virscek Marn M, Mavric Plesko I, Molinero-Demilly V *et al*. (2010) Development and inter-laboratory evaluation of real-time PCR tests for the detection of pospiviroids. *Journal of Virological Methods* **169**, 207–210.

Mumford RA, Walsh K & Boonham N (2000) A comparison of molecular methods for the routine detection of viroids. *EPPO Bulletin* **30**, 341–346.

Naktuinbouw. (2017) *Reference protocols Naktuibouw. Real-Time RT-PCR (RT Taqman PCR) for pospiviroids (CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd) on seeds of tomato (Solanum lycopersicum) and pepper Capsicum annuum) and validation data available in the EPPO Database on Diagnostic expertise: section validation data for diagnostic tests* https://dc.eppo.int/validation_data/validationlist. Pospiviroids: validation sheet for 'Detection of pospiviroids by real-time RT-PCR on tomato and pepper seeds, i.e. CEVd, CLVd, PCFVd, TASVd, TCDVd, TPMVd').

NPPO-NL (2013a) *Pospiviroid: Validation of a conventional RT-PCR assay for detection and preliminary identification of pospiviroids (except CLVd) by Pospi1-FW/Pospi1-RE. EPPO Database on Diagnostic Expertise*. https://dc.eppo.int/validation_data/validationlist [accessed 2020-10-14].

NPPO-NL (2013b) *Potato spindle tuber viroid: Validation of a conventional RT-PCR assay for detection and identification of CLVd, PSTVd and TCDVd using primers Vid-FW/RE. EPPO Database on Diagnostic Expertise*. https://dc.eppo.int/validation_data/validationlist [accessed 2020-10-14].

NPPO-NL (2013c) *Pospiviroid: Development and validation of a real-time RT-PCR assay for generic detection of Pospiviroids. EPPO Database on Diagnostic Expertise*. https://dc.eppo.int/validation_data/validationlist [accessed 2020-10-14].

NPPO-NL (2013d) *Potato spindle tuber viroid: Validation of a conventional RT-PCR test for detection and identification of PSTVd TCDVd MPVd and TPMVd using primers 2H1/3H1 described by Shamoul et al. (1997)* https://dc.eppo.int/validation_data/validationlist [accessed 2020-10-14].

Olivier T, Demonty E, Frauche F & Steyer S (2014) Generic detection and identification of pospiviroids. *Archives of Virology* **159**, 2097–2102.

Olivier T, Šveikauskas V, Demonty E, De Jonghe K, Gentit P, Virščeк-Marn M *et al*. (2016) Inter-laboratory comparison of four RT-PCR based methods for the generic detection of pospiviroids in tomato leaves and seeds. *European Journal of Plant Pathology* **144**, 645–654.

Önelge N (1997) Direct nucleotide sequencing of citrus exocortis viroid (CEV). *Turkish Journal of Agriculture and Forestry* **21**, 419–422.

Owens RA & Diener TO (1981) Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic-acid hybridization. *Phytopathology* **71**, 770.

Owens RA, Flores R, Di Serio F, Li SF, Pallas V, Randles JW*et al*. (2012) Viroids. In King AMQ, Adams MJ, Carstens EB & Lefkowitz EJ eds. *Virus Taxonomy, Classification and Nomenclature of Viruses, Ninth Report of the International Committee on Taxonomy of Viruses*, pp. 1221–1234. London, Elsevier Academic Press. 1340 pp.

Pfannenstiel MA & Slack SA (1980) Response of potato cultivars to infection by the potato spindle tuber viroid. *Phytopathology* **70**, 922–926.

Ragozzino E, Faggioli F & Barba M (2005) Distribution of citrus exocortis viroid and hop stunt viroid in citrus orchards of central Italy as revealed by one-tube one-step RT-PCR. *Phytopathologia Mediterranea* **44**, 322–326.

Rizza S, Nobile G, Tessitori M, Catara A & Conte E (2009) Real time RT-PCR assay for quantitative detection of Citrus viroid III in plant tissues. *Plant Pathology* **58**, 181–185.

Roenhorst JW, Butot RPT, Van der Heijden KA, Hooftman M & Van Zaayen A (2000) Detection of chrysanthemum stunt viroid and potato spindle tuber viroid by return-polyacrylamide gel electrophoresis. *EPPO Bulletin* **30**, 453–456.

Roenhorst JW, De Krom C, Fox A, Mehle N, Ravnikar M & Werkman AW (2018) Ensuring validation in diagnostic testing is fit for purpose: a view from the plant virology laboratory. *EPPO Bulletin* **48**, 105–115.

Roenhorst JW, Jansen CCC, Kox LFF, De Haan EG & Van den Bovenkamp GW (2006) Real-time RT-PCR voor grootschalige toetsing van aardappel op het aardappelspindelknolviroïde. *Gewasbescherming* **37**, 198–203.

Semancik JS & Weathers LG (1972a) Exocortis disease: Evidence for a new species of 'infectious' low molecular weight RNA in plants. *Nature New Biology* **237**, 242–244.

Semancik JS & Weathers LG (1972b) Exocortis virus: An infectious free-nucleic acid plant virus with unusual properties. *Virology* **46**, 456–466.

Shamloul AM, Hadidi A, Zhu SF, Singh RP & Sagredo B (1997) Sensitive detection of potato spindle tuber viroid using RT-PCR and identification of a viroid variant naturally infecting pepino plants. *Canadian Journal of Plant Pathology* **19**, 89–96.

Singh RP (1999) Development of the molecular methods for potato virus and viroid detection and prevention. *Genome* **42**, 592–604.

Singh RP, Nie XZ & Singh M (1999) Tomato chlorotic dwarf viroid: an evolutionary link in the origin of pospiviroids. *Journal of General Virology* **80**, 2823–2828.

Spieker RL (1996a) A viroid from *Brunfelsia undulata* closely related to the Columnea latent viroid. *Archives of Virology* **141**, 1823–32.

Spieker RL (1996b) The molecular structure of Iresine viroid, a new viroid species from *Iresine herbstii* ('beefsteak plant'). *Journal of General Virology* **77**, 2631–2635.

Steyer S, Oliviera T, Skelton A, Nixon T & Hobden E (2010) Columnea latent viroid (CLVd): first report in tomato in France. *Plant Pathology* **59**, 794.

Testa. (2015) *TESTA: Development of seed testing methods for pests and pathogens of plant health concern, Work package 5. Validation of detection methods, Deliverable: D5.3 Validated methods for viruses and viroids*. See EPPO Database on Diagnostic Expertise, Validation data for diagnostic tests, Pospiviroids: Detection of pospiviroids by PCR in seeds (Naktuinbouw). https://dc.eppo.int/validation_data/validationlist [accessed 2020-10-14].

Tiberini A & Barba M (2012) Optimization and improvement of oligonucleotide microarray-based detection of tomato viruses and pospiviroids. *Journal of Virological Methods* **185**, 43–51.

Torchetti EZ, Navarro B & Di Serio F (2012) A single polyprobe for detecting simultaneously eight pospiviroids infecting ornamentals and vegetables. *Journal of Virological Methods* **186**, 141–146.

Verhoeven JThJ, Botermans M, Jansen CCC & Roenhorst JW (2011b) First report of Pepper chat fruit viroid in capsicum pepper in Canada. *New Disease Reports* **23**, 15. https://doi.org/10.5197/j.2044-0588.2011.023.015.

Verhoeven JThJ, Hüner L, Virscek Marn M, Mavric Plesko I & Roenhorst JW (2010a) Mechanical transmission of Potato spindle tuber viroid between plants of *Brugmansia suaveoles*, *Solanum jasminoides* and potatoes and tomatoes. *European Journal of Plant Pathology* **128**, 417–421.

Verhoeven JThJ, Jansen CCC, Botermans M & Roenhorst JW (2010b) First Report of Iresine viroid 1 in *Celosia plumosa* in the Netherlands. *Plant Disease* **94**, 920.

Verhoeven JThJ, Jansen CCC & Roenhorst JW (2008) First report of pospiviroids infecting ornamentals in the Netherlands: *Citrus exocortis* viroid in Verbena sp., Potato spindle tuber viroid in *Brugmansia suaveolens* and *Solanum jasminoides*, and Tomato apical stunt viroid in Cestrum sp. *Plant Pathology* **57**, 399.

Verhoeven JTJ, Jansen CCC, Roenhorst JW, Flores R & De la Peña M (2009) Pepper chat fruit viroid: biological and molecular properties of a proposed new species of the genus Pospiviroid. *Virus Research* **144**, 209–214.

Verhoeven JTJ, Jansen CCC, Willemen TM, Kox LFF, Owens RA & Roenhorst JW (2004) Natural infections of tomato by *Citrus exocortis* viroid, *Columnea latent* viroid, Potato spindle tuber viroid and Tomato chlorotic dwarf viroid. *European Journal of Plant Pathology* **110**, 823–831.

Verhoeven JThJ, Koenraadt HMS, Westenberg M & Roenhorst JW (2017) Characterization of tomato apical stunt viroid isolated from a 24-years old seed lot of Capsicum annuum. *Archives of Virology* **162**, 1741–1744.

Verhoeven JThJ, Roenhorst JW, Hooftman M, Meekes ETM, Flores R & Serra P (2015a) A pospiviroid from symptomless portulaca plants closely related to iresine viroid 1. *Virus Research* **205**, 22–26.

Verhoeven JThJ, Roenhorst JW & Owens RA (2011a) *Mexican papita* viroid and Tomato planta macho viroid belong to a single species in the genus Pospiviroid. *Archives of Virology* **156**, 1433–1437.

Verhoeven JThJ, Roenhorst JW, Van Vliet ACA, Ebskamp MJM & Koenraadt HMS (2015b) A potato spindle tuber viroid-positive seed lot of tomato may produce a viroid-free crop. *Program and abstract book of the 5th Conference of the International Working Group on Legume and Vegetable Viruses, Haarlem, the Netherlands*, p 95.

Verhoeven JTJ, Westenberg M, Van Ede EPM, Visser K & Roenhorst JW (2016) Identification and eradication of potato spindle tuber viroid in dahlia in the Netherlands. *European Journal of Plant Pathology* **146**, 443–447. https://doi.org/10.1007/s10658-016-0911-0.

Weller SA, Elphinstone JG, Smith NC, Boonham N & Stead DE (2000) Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan). *Assay. Applied and Environmental Microbiology* **66**(7), 2853–2858. https://doi.org/10.1128/AEM.66.7.2853-2858.2000.

Yanagisawa H & Matsushita Y (2017) Host ranges and seed transmission of Tomato planta macho viroid and Pepper chat fruit viroid. *European Journal of Plant Pathology* **149**, 211–217.

Appendix 1 – Sample preparation and RNA extraction for different matrices

This appendix describes sample preparation and RNA extraction methods for different hosts and types of plant material. These initial steps are critical for the result of a PCR test and are more dependent on the matrix than the following amplification steps, therefore they are described in this separate appendix.

A wide range of RNA extraction methods may be used, from commercial kits to methods published in scientific journals. The RNeasy Plant Mini kit (Qiagen), or the Sbeadex® maxi plant kit (LGC Genomics) can be used following the manufacturers' instructions or the instructions in this appendix. The Sbeadex® maxi plant kit can be used in combination with a KingFisher KF96 system for large-scale throughput. RNA extraction using CTAB (Gambino *et al.*, 2008) is robust and is considered by the Panel on Diagnostics in Virology and Phytoplasmology as an appropriate method for RNA extraction for all types of tissues which will not affect the performance of the molecular tests.

Other extraction methods may be used if they are verified.

Extracted RNA should be stored refrigerated for short-term storage (<8 h), at −20°C (<1 month) or at −80°C for longer periods.

1. Bark and woody tissues

RNA extraction can be performed using 100–500 mg of tissue depending on the method. The following extraction methods are used in different laboratories in the EPPO region, but validation data are lacking.

1.1. RNA extraction using guanidine lysis buffer and the RNeasy Plant Mini Kit (Qiagen) (Bernad & Duran-Vila, 2006)

Approximately 100 mg of tissue is homogenized in RNA lysis buffer (Table A1). The soluble fraction is concentrated by isopropyl alcohol precipitation and resuspended in TE buffer (Table A2). Subsequently, the RNA is purified using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions for RNA cleanup and resuspended in 50 µL of water.

Table A1. RNA lysis buffer

	Amount	Final concentration
Guanidine thiocyanate	472.64	4 M
Tris-HCl (1 M, pH 8.0)	100 mL	100 mM
$MgCl_2$	2.3 g	25 mM
EDTA (0.5 M, pH 7.5)	50 mL	25 mM
Distilled water to	1.0 L	

Table A2. TE buffer

	Amount	Final concentration
Tris-HCl (1 M, pH 8.0)	20 mL	20 mM
EDTA (0.5 M, pH 8.0)	2 mL	1 mM
Distilled water to	1.0 L	

1.2. RNA extraction using the SDS/potassium acetate method (Astruc *et al.*, 1996; Bernad & Duran-Vila, 2006)

Approximately 500 mg of tissue is homogenized in extraction buffer (Table A3) in sealed plastic bags. The homogenate is treated with SDS (65°C for 20 min) and potassium acetate (for 20 min on ice). The soluble fraction is concentrated by ethanol precipitation and resuspended in 40 µL of water.

Table A3. Extraction buffer

	Amount	Final concentration
Tris-HCl (1 M, pH 8.0)	100 mL	100 mM
EDTA (0.5 M, pH 8.0)	100 mL	50 mM
NaCl (5 M)	100 mL	0.5 M
2-mercaptoethanol (14.3 M)	699 µL	10 mM
Distilled water to	1.0 L	

1.3. RNA extraction using the phenol/guanidine isothiocyanate method (Chomczynski & Sacchi, 1987; Bernad & Duran-Vila, 2006)

This method can be used for tissues rich in polysaccharides, phenolic compounds or other secondary metabolites. Approximately 100 mg of tissue is homogenized in TRIzol reagent (Invitrogen). The nucleic acids in the aqueous phase obtained after chloroform separation are precipitated with isopropyl alcohol and resuspended in 50 µL of water.

2. Leaves (including *in vitro* grown microplants)

This extraction method was validated in combination with RT-PCR and real-time RT-PCR (see Appendices 2 and 3).

2.1. Individual plants and/or small samples

For testing individual plants and/or small samples the RNeasy Plant Mini kit (Qiagen) can be used according to the manufacturer's instructions.

2.2. Pooled samples

Pooled leaf samples should consist of equal amounts of each plant. For leaf material, this can be achieved by stacking leaves and preparing leaf discs using a disposable 4 mm leaf punch or by cutting or tearing the top parts. For pooled samples, however, larger amounts of plant material are involved. In this case other buffers can be used for homogenization, such as GH + buffer as specified in Table A4.

Approximately 1 g of plant tissue is put in an extraction bag and homogenized in 3.5 mL (range 1:2–1:5, w/v) of

GH + buffer. Samples are incubated for 10 min at 65°C. After centrifugation at 12 000*g* for 2 min, 500 µL of supernatant is loaded on the QIAshredder spin column and centrifuged. Thereafter the manufacturer's instructions in the RNeasy Plant Mini kit (Qiagen) should be followed.

For high throughput RNA extraction, the Sbeadex® maxi plant kit can be used in combination with a Kingfisher KF96 system. In this system 250 µL of the supernatant is transferred to a binding plate containing 450 µL of binding buffer and 50 µL of particle suspension (both included in the kit) and RNA is extracted following the manufacturer's instructions.

Table A4. GH + buffer

	Amount	Final concentration
Guanidine hydrochloride	573.18 g	6 M
Sodium acetate (4 M, pH 5.2)	50 mL	0.2 M
EDTA-Na_2 $2H_2O$	9.3 g	25 mM
PVP-10	25.0 g	2.5% w/v
Distilled water to	1.0 L	

3. Seeds

Sample preparation, including grinding, and RNA extraction from seeds can be challenging. Therefore, different equipment and reagents (kits) have been compared, resulting in the recommendation of the following procedures (these two procedures performed equally well). RNA extraction was validated in combination with real-time RT-PCR (see Appendices 4 and 5).

3.1 Homogenization using GH + buffer

For both tomato and pepper, three subsamples of 1000 seeds are transferred to a grinding bag (e.g. Interscience BagPage 100 mL) and 20 mL GH + buffer (Table A4) is added. The seeds are soaked at room temperature for 30–60 min before homogenization (e.g. with an Interscience BagMixer on position 4) for 90 s (tomato) or 4 min (pepper).

Alternatively, dry seeds are ground with a Genogrinder. Three subsamples of 1000 tomato or six subsamples of 500 pepper seeds are transferred to a 50-mL tube and a steel ball (14 mm) is added. Seeds are ground, tubes upside down, at 1700 rpm for 4 min for tomato and 7 min for pepper seeds. After grinding GH + buffer is added, 20 mL for tomato or 10 mL for pepper samples. Tubes are shaken by hand to obtain homogenous solutions. Duplicate pepper homogenates are combined and mixed before further processing to make three subsamples.

One millilitre of the seed homogenate is transferred into a 1.5-mL tube and 30 µL of dithiothreitol (DTT, 5 M) is added, followed by incubation in a thermoshaker at 850 rpm and 65°C for 15 min. After centrifugation at 16 000*g* for 10 min, 750 µL of supernatant is loaded on the QIAshredder spin column and centrifuged. Thereafter the manufacturer's instructions forf the RNeasy Plant Mini Kit (Qiagen) are followed.

For high throughput RNA extractions, the Sbeadex® maxi plant kit can be used in combination with a Kingfisher KF96 system. In this system 250 µL of the supernatant is transferred to a binding plate containing 600 µL of binding buffer and 50 µL of particle suspension (both included in the kit) and RNA is extracted following the manufacturer's instructions.

Note that when dahlia latent viroid (DLVd) is used as internal control, this virus is added to GH + buffer (DLVd infected leaf material homogenized in a dilution of approximately 10^4-fold aiming for a Ct value of 28–32; Naktuinbouw, 2017).

3.2 Homogenization in phosphate buffer

For both tomato and pepper, 12 subsamples of 250 seeds can be immersed in 10 mL of 0.1 M phosphate buffer (Na_2HPO_4/KH_2PO_4, pH 7.2), incubated at 4 ± 2°C overnight, and then ground with, for example, a FastPrep homogenizer at 5 m/s for 40 s. After centrifugation at 10 000*g* at 4°C for 10 min, the supernatant can be used for RNA extraction using the RNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions with some minor modifications. The homogenates can be processed separately or combined to three subsamples of four homogenates.

Briefly, 600 µL of the supernatant is added to 600 µL of RLT buffer (without β-mercaptoethanol). Two aliquots of 600 µL of this mix are loaded one after the other on the same QIAshredder spin column and centrifuged. Subsequently, the manufacturers protocol is followed until the elution step. RNA is eluted from the RNeasy Mini Spin columns by applying 50 µL of RNase-free warm water (65°C) followed by centrifugation. To maximize RNA recovery, an additional elution step is performed using the same procedure.

In critical cases, increasing the pospiviroid RNA concentration might be desirable (Mehle *et al.*, 2017). This can be achieved by transferring 4.5 mL of the supernatant to a 5-mL tube containing 0.5 g Amberlite IRA-900 anion-exchange resin (Polysciences, Warrington, PA, USA). In the next step, RNA is bound to the resin by continuous stirring (about 27 rpm) at RT for 3 h, followed by centrifugation at 5000*g* for 1 min and removal of the supernatant. The resin-absorbed RNA is eluted by adding 560 µL of AVL buffer (QIAamp Viral RNA Mini kit; Qiagen, Hilden, Germany) to the pelleted Amberlite beads, followed by incubation and occasional agitation at RT for 10 min. After centrifugation at 5000*g* for 1 min, the supernatant (containing the nucleic acids) is transferred to a 1.5 mL tube and applied to the QIAamp column, washed and processed according to the manufacturer's instructions. Finally, the RNA is eluted from the QIAamp column in 45 µL of RNase-free water pre-warmed to 65°C. Note that the QIAamp Viral RNA Mini Kit can be also used for RNA extraction from non-concentrated seed samples.

4. Tubers

Tuber cores are homogenized in water or lysis buffer (1 mL per gram of tuber core) in extraction bags by a homogenizer. Freezing the cores before adding the water or lysis buffer facilitates homogenization (IPPC, 2015). Either the RNeasy Plant Mini kit or Sbeadex® maxi plant kit can be used for RNA extraction.

Extraction was validated in combination with real-time RT-PCR (Boonham *et al.*, 2004), see Appendix 6.

Appendix 2 – Conventional one-step RT-PCR (Spieker, 1996a; Verhoeven *et al.*, 2004)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1 This one-step RT-PCR uses Pospi1 (Verhoeven *et al.*, 2004) and pCLV4 primer sets (Spieker, 1996a) for generic detection of pospiviroids.
1.2 The test has been successfully used on a wide range of plant species and matrices.
1.3 Oligonucleotides

Primer	Sequence (5′-3′)	Primer location	Viroids detected	Amplicon size (bp)
Pospi1-FW	GGGATCCCCG GGGAAAC	86–102*	CEVd CSVd	197
Pospi1-RE	AGCTTCAGTT GT(T/A)TCC ACCGGGT	283–261*	IrVd-1 PCFVd PSTVd TASVd TCDVd TPMVd	
pCLVR4	GGGGCAACT CAGACC GAG C	102–120†	CLVd	370
pCLV4	GGGGCTCCT GAGACCG CTCTTG-3’	101–80†		

*Location in PSTVd NC_002030.
†Location in CLVd NC_003538.

1.4 The test has been successfully performed on different PCR systems, including C1000 (Bio-Rad Laboratories).

2. Methods

2.1 RNA extraction
See Appendix 1.
2.2 Reaction mix for RT-PCR

Pospi1 primers: CEVd, CSVd, IrVd-1, PCFVd, PSTVd, TASVd, TCDVd, TPMVd

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	–	15.0	–
One-Step RT-PCR buffer (Qiagen)	5×	5.0	1×
dNTP mix (Qiagen)	10 mM	1.0	0.4 mM
Pospi1-FW	10 µM	1.0	0.4 µM
Pospi1-RE	10 µM	1.0	0.4 µM
One-Step RT-PCR Enzyme Mix (Qiagen)	–	1.0	–
Subtotal		24.0	
RNA template		1.0	
Total		25.0	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

pCLV primers: CLVd

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	–	15.0	–
One-Step RT-PCR buffer (Qiagen)	5×	5.0	1×
dNTP mix (Qiagen)	10 mM	1.0	0.4 mM
pCLVR4	10 µM	0.5	0.2 µM
pCLV4	10 µM	0.5	0.2 µM
One-Step RT-PCR Enzyme Mix (Qiagen)	–	1.0	–
Subtotal		23.0	
RNA template		2.0	
Total		25.0	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

2.3 RT-PCR cycling parameters

Pospi primers

Reverse transcription at 50°C for 30 min; denaturation at 95°C for 15 min; 14 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 90 s and elongation at 72°C for 45 s, followed by 29 cycles of denaturation at 94°C for 30 s,

annealing at 60°C for 90 s and elongation at 72°C for 45 s; terminal elongation at 72°C for 10 min and kept at 20°C.

pCLV primers

Reverse transcription at 50°C for 30 min; denaturation at 95°C for 15 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 90 s and elongation at 72°C for 45 s; terminal elongation at 72°C for 10 min; and kept at 20°C.

2.4 Gel electrophoresis

After RT-PCR, the PCR products (approximately 197 and 370 bp for the Pospi1 and pCLV primers, respectively) should be analysed by gel electrophoresis (2% agarose gel).

Note that if other RT-PCR reagents, such as Invitrogen RT/Platinum Taq Mix, are used, the conditions during the RT step might be different, i.e. 43°C for 30 min, 94°C for 2 min.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, appropriate controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid. The inclusion of suitable controls should be in accordance with the type of matrix used.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: naturally infected host tissue or host tissue spiked with one of the target pospiviroids. Preferably, a different species or 'uncommon' or 'deviating' genotype is used as positive control, since this allows exclusion of false positives due to cross-contamination by the positive control based on the nucleotide sequences.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection. For the choice of the PAC, see also PIC.

In addition to the external positive controls (PIC and/or PAC), an internal positive control (IPC) can be used to monitor each individual sample separately. The IPC can include an endogenous nucleic acid of the matrix using conserved primers, preferably amplifying RNA targets, such as nad5 (Menzel *et al.*, 2002). The use of the IPC is optional but is recommended for detection tests.

3.2. Interpretation of results for conventional PCR tests

Verification of the controls:

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of approximately 197 bp for the Pospi primers or 370 bp for the pCLV primers.
- IPC if used should produce amplicons of expected size (~181 bp for nad5).

When these conditions are met:

- A test will be considered positive if amplicons are produced of approximately 197 bp for the Pospi primers or 370 bp for the pCLV primers, respectively.
- A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

It should be noted that for viruses and viroids bands of different sizes may correspond to different strains of the target organism and care should be taken when interpreting the results of conventional PCR, in particular the sizes of bands.

4. Performance criteria available

Validation data (EPPO database) were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*. Pospi1 primers were validated at the National Plant Protection Organization of the Netherlands (NPPO-NL, 2013a,b), pCLV4 primers were validated at Laboratoire de la Sante des Vegetaux (LSV) ANSES (France).

Pospi1 primers

4.1. Analytical sensitivity:

Pospi1 primers detected all pospiviroid species (except CLVd) up to at least a relative infection rate of 2.5% for dilution of infected tomato leaves in healthy tomato. Amplicons could be successfully sequenced up to a relative infection rate of 1%.

4.2. Analytical specificity

Pospi1 primers have been found to detect all pospiviroid isolates (except CLVd) encountered at the NPPO-NL thus far. No reactions were obtained for isolates of the following viroid species in the family *Avsunviroidae*, i.e. Avocado sunblotch viroid (*Avsunviroid*), Chrysanthemum chlorotic mottle viroid (*Pelamoviroid*), Eggplant latent viroid (*Elaviroid*), and family *Pospiviroidae*, i.e. Apple scar skin viroid (*Apscaviroid*), Coleus blumei viroid 1 (*Coleviroid*) and Hop stunt viroid (*Hostuviroid*). A cross-reaction was observed for an isolate of Hop latent viroid (*Cocadviroid*). *In silico* analysis did not reveal cross-reactions with other tomato-infecting viruses and host plant sequences.

4.3. Selectivity

No apparent matrix effects have been observed in a broad variety of host plants, in particular in the families *Apocynaceae*, *Gesneriaceae* and *Solanaceae*.

4.4. Repeatability and reproducibility

100%.

pCLV4 primers

This test has been initially validated with the following kit: SuperScript® One-Step RT-PCR system with Platinum® Taq DNA polymerase (Reference: Thermo Fisher Scientific™, August 2016, no. 10928042). The reaction mix includes dNTP and $MgCl_2$.

4.5. Analytical sensitivity

pCLV4 primers detected all tested CLVd isolates up to at least a relative infection rate of 1% for dilution of infected tomato leaves in healthy tomato leaves (1×10^{-2}, six replicates for each sample).

4.6. Analytical specificity

So far, pCLV4 primers have been found to detect all CLVd isolates encountered at the LSV-ANSES. No cross-reactions were obtained for isolates of other viroid species in the genus *Pospiviroid*. *In silico* analysis did not reveal cross-reactions with other tomato-infecting viruses and host plant sequences (six replicates for each sample).

4.7. Selectivity

No apparent matrix effects have been observed in a broad variety of host plants, in particular in the families *Asteraceae*, *Chenopodiaceae* and *Solanaceae* (six replicates for each sample).

4.8. Repeatability and reproducibility

The test was validated in both intra- and interlaboratory comparison. Repeatability and reproducibility were shown to be 100% (six replicates for each sample).

Furthermore, these tests have been compared for detection of *Pospiviroid* in tomato leaves and seeds by interlaboratory comparison (Olivier *et al.*, 2016).

Appendix 3 – Real-time RT-PCR (Botermans *et al.*, 2013)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The GenPospi test (Botermans *et al.*, 2013) uses real-time RT-PCR to detect all known pospiviroids in leaves, tubers and fruits. The test is not recommended for testing seeds because the sensitivity might be too low for this matrix. Other more sensitive tests (Appendix 4) will detect the most relevant pospiviroid species in seeds of *C. annuum* and *S. lycopersicum*.

1.2. The GenPospi test consists of two reactions running in parallel: the first (reaction mix 1) targets all pospiviroids except CLVd; the second (reaction mix 2) specifically targets CLVd. In both reactions nad5 is included as an internal (isolation) control.

Reaction mix 1: CEVd, CSVd, IrVd-1, PCFVd, PSTVd, TASVd, TCDVd, TPMVd

Reaction mix 2: CLVd

1.3. Oligonucleotides

Primers and probes list for real-time RT-PCR identification of all known pospiviroids.

Primers and probes	Sequence (5′-3′)	Reference
Reaction mix 1		
TCR-F 1-1	TTCCTGTGGTTCACACCTGACC	1
TCR-F 1-3	CCTGTGGTGCTCACCTGACC	1
TCR-F 1-4	CCTGTGGTGCACTCCTGACC	1
TCR-F PCFVd	TGGTGCCTCCCCCGAA	1
TCR-F IrVd	AATGGTTGCACCCCTGACC	1
TR-R1	GGAAGGGTGAAAACCCTGTTT	1
TR-R CEVd	AGGAAGGAGACGAGCTCCTGTT	1
TR-R6	GAAAGGAAGGATGAAAATCCT GTTTC	1
pUCCR	*FAM*-CCGGGGAAACCTGGA-*MGB*	1
TCR-F 1-1	TTCCTGTGGTTCACACCTGACC	1
TCR-F 1-3	CCTGTGGTGCTCACCTGACC	1
TCR-F 1-4	CCTGTGGTGCACTCCTGACC	1
TCR-F PCFVd	TGGTGCCTCCCCCGAA	1
TCR-F IrVd	AATGGTTGCACCCCTGACC	1
TR-R1	GGAAGGGTGAAAACCCTGTTT	1
TR-R CEVd	AGGAAGGAGACGAGCTCCTGTT	1
TR-R6	GAAAGGAAGGATGAAAATCCT GTTTC	1
Reaction mix 2		
CLVd-F	GGTTCACACCTGACCCTGCAG	2
CLVd-F2	AAACTCGTGGTTCCTGTGGTT	2
CLVd-R	CGCTCGGTCTGAGTTGCC	2
CLVd-P	*FAM*-AGCGGTCTCAGGAGCCC CGG-*BHQ1*	2
Internal control		
nad5-F	GATGCTTCTTGGGGCTTCTTGTT	3
nad5-R	CTCCAGTCACCAACATTGGCATAA	3
nad5-P	*VIC*-AGGATCCGCATAGCCCTCG ATTTATGTG-*BHQ1*	1

References: [1]Botermans *et al.*, 2013; [2]Monger *et al.*, 2010; [3]Menzel *et al.*, 2002.

1.4. Test has been successfully performed on different real-time PCR systems including ABI 7900 HT (Applied Biosystems) and CFX96 (Bio-Rad Laboratories).

1.5. Data were analysed with CFX Manager software 2.0 (Bio-Rad Laboratories).

2. Methods

2.1. RNA extraction

See Appendix 1.

2.2. Reaction mixes for real-time RT-PCR

2.2.1. Preparation of GenPospi primer mix

GenPospi primer mix	Working concentration (µM)	Volume (µL)	Final concentration (µM)
Molecular-grade water*	–	720	–
TCR-F 1-1	100	10	1.25
TCR-F 1-3	100	10	1.25
TCR-F 1-4	100	10	1.25
TCR-F IrVd	100	10	1.25
TCR-F PCFVd	100	10	1.25
TR-R1	100	10	1.25
TR-R CEVd	100	10	1.25
TR-R6	100	10	1.25
Total		800	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

2.2.2. Reaction mixtures

The use of reagent TaqMan RNA-to-CT 1-Step Kit (Applied Biosystems, ABI) is critical as Ct values have been found to increase by 8–10 when using other kits (Botermans *et al.*, 2013).

GenPospi Reaction Mix: CEVd, CLVd, IrVd, PCFVd, PSTVd, TASVd, TCDVd, TPMVd + nad5

Reagent	Working concentration (µM)	Volume per reaction (µL)	Final concentration (µM)
Molecular-grade water*	–	1.65	–
TaqMan® RT-PCR mix (ABI)**	2×	12.5	1×
TaqMan® RT enzyme mix (ABI)**	40×	0.6	~1×
GenPospi- primer mix	**10 (1.25 each)**	**6.0**	0.3 (each)
Primer nad5-F	10	0.75	0.3
Primer nad5-R	10	0.75	0.3
TaqMan® probe pUCCR	10	0.25	0.1
TaqMan® probe nad5-P	10	0.5	0.2
Subtotal		23.0	
RNA		2.0	
Total		25.0	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

CLVd Reaction Mix 2: CLVd + nad5

Reagent	Working concentration (µM)	Volume per reaction (µL)	Final concentration (µM)
Molecular-grade water*	–	5.4	–
TaqMan® RT-PCR mix (ABI)**	2×	12.5	1×
TaqMan® RT enzyme mix (ABI)**	40×	0.6	~1×
Primer CLVd-F	10	0.75	0.3
Primer CLVd-F2	10	0.75	0.3
Primer nad5-F	10	0.75	0.3
Primer CLVd-R	10	0.75	0.3
Primer nad5-R	10	0.75	0.3
TaqMan® probe CLVd-P	10	0.25	0.1
TaqMan® probe nad5-P	10	0.5	0.2
Subtotal		23.0	
RNA		2.0	
Total		25.0	

*Molecular-grade water should be used preferably or prepared purified (deionized or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

**TaqMan RNA-to-CT 1 Step Kit, ABI. Note that the use of this reagent can be critical as Ct values have been found to increase by 810 when using another kit (Botermans *et al.*, 2013).

2.3. RT-PCR cycling conditions

Both reaction mixes: reverse transcription at 48°C for 15 min; denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s; annealing and elongation at 60°C for 60 s.

3. Essential Procedural Information

3.1 Controls

For a reliable test result to be obtained, appropriate controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid. The inclusion of suitable controls should be in accordance with the type of matrix used.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: naturally infected host tissue or host tissue spiked with one of the target pospiviroids. Preferably, a different species or 'uncommon' or 'deviating' genotype is used as positive control, since this allows exclusion of false positives due to cross-contamination by the positive control based on the nucleotide sequences.

- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

In addition to the external positive controls (PIC and/or PAC), an internal positive control (IPC) is used to monitor each individual sample separately. The IPC includes the endogenous nucleic acid of the matrix using conserved primers amplifying the RNA target nad5 (Menzel *et al.*, 2002; Botermans *et al.*, 2013). The use of the IPC is optional but is recommended for detection tests.

3.2 Interpretation of results

Verification of the controls:

- The PIC and PAC (as well as IC and IPC if applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Validation data were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* at the NPPO of the Netherlands (NPPO-NL, 2013c; Botermans *et al.*, 2013).

4.1. Analytical sensitivity

The GenPospi test was found to detect isolates from all the known pospiviroid species up to a relative infection rate of 0.13% in tomato leaf material (which equals a 770-fold dilution).

4.2. Analytical specificity

The GenPospi test was found to detect all 33 tested isolates of the targeted pospiviroids, i.e. CEVd (3), CLVd (3), CSVd (4), IrVd-1 (2), PCFVd (1), PSTVd (10), TASVd (3), TCDVd (5) and TPMVd (2). No reactions were obtained for isolates of the following viroid species in the family *Avsunviridae*, Avocado sunblotch viroid (*Avsunviroid*), Chrysanthemum chlorotic mottle viroid (*Pelamoviroid*), Eggplant latent viroid (*Elaviroid*), and family *Pospiviroidae*, Apple scar skin viroid (*Apscaviroid*), Coleus blumei viroid 1 (*Coleviroid*), Hop latent viroid (*Cocadviroid*), Hop stunt viroid (2) (*Hostuviroid*) and (tomato)viruses: Alfalfa mosaic virus (AMV), Cucumber mosaic virus (CMV), Pepino mosaic virus (PepMV), Potato virus Y (PVY), Tomato mosaic virus (ToMV), Tobacco mosaic virus (TMV), Tomato chlorosis virus (ToCV) and Tomato yellow leaf curl virus (TYLCV).

4.3. Selectivity

No apparent matrix effects were observed in a wide range of host plants, including a range of tomato cultivars.

4.4. Repeatability and reproducibility

The test was validated in both an intra- and interlaboratory comparison for IrVd-1, PSTVd, TASVd and TCDVd and repeatability and reproducibility were shown to be 100%.

Appendix 4 – Real-time RT-PCR for seed testing (Naktuinbouw, 2017)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. This test was specifically designed for testing seeds because available tests at the time lacked the sensitivity to detect the (usually) low amounts of pospiviroid present in contaminated seed lots of *C. annuum* (pepper) and *S. lycopersicum* (tomato). The test is described for standard samples of approximately 3000 seeds, tested in three subsamples of 1000 seeds.

1.2. The test consists of four reactions running in parallel, which each target one or several Pospiviroid species. The test will detect all Pospiviroid species with the exception of CSVd and IrVd-1. Subsequent reaction mixtures will detect the following species:

Reaction mix A: PCFVd, PSTVd, TCDVd and TPMVd (not all isolates)

Reaction mix B: CEVd and CLVd

Reaction mix C: TPMVd (genotype not detected by Mix A; GenBank acc. no. NC_001558; Kiefer *et al.*, 1983)

Reaction mix D: TASVd

In Reaction mixes A and B, Dahlia latent viroid (DLVd; genus *Hostuviroid*) is included as (exogenous) internal control. In reaction mix C nad5 is included as internal control.

1.3. Oligonucleotides

Primers and probes list for real-time RT-PCR for seed testing

Primers and probes	Sequence (5′-3′)	Ref.
Mix A		
PSTV-231F	GCCCCCTTTGCGCTGT	1
PSTV-296R	AAGCGGTTCTCGGGAGCTT	1
PSTV-251T	*FAM*-CAGTTGTTTCCACCGGG TAGTAGCCGA-*BHQ1*	1
PCFVd-F	TCTTCTAAGGGTGCCTGTGG	2
PCFVd-R	GCTTGCTTCCCCTTTCTTTT	2
PCFVd-P	*VIC*-CTCCCCCGAAGCCCGCT TAG-*BHQ1*	2
Mix B		
CLVd-F	GGTTCACACCTGACCCTGCAG	3
CLVd-F2	AAACTCGTGGTTCCTGTGGTT	3
CLVd-R	CGCTCGGTCTGAGTTGCC	3
CLVd-P	*FAM*-AGCGGTCTCAGGAGCCC CGG-*BHQ1*	3
CEVd-F2-304	CTCCACATCCGRTCGTCGCTGA	3
CEVd-R2-399	TGGGGTTGAAGCTTCAGTTGT	3
CEVd-P2-337	*FAM*-CCCTCGCCCGGAGCTTC TCTCTG-*BHQ1*	3
Mix C		
TPMVd-F1	AAAAAAGAATTGCGGCCAAA	2
TPMVd-R	GCGACTCCTTCGCCAGTTC	2
pUCCR	*FAM*-CCGGGGAAACCTGGA-*MGB*	4
Mix D		
TASVd-F2-200	CKGGTTTCCWTCCTCTCGC	3
TASVd-R2-269	CGGGTAGTCTCCAGAGAGAAG	3
TASVd-P2-228	*FAM*-TCTTCGGCCCTCGCCCGR-*BHQ1*	3
Internal controls		
DaVd1-FT	GCTCCGCTCCTTGTAGCTTT	2
DaVd1-RT	AGGAGGTGGAGACCTCTTGG	2
DaVd1-P	*Texas red*-CTGACTCGAGGACGC GACCG-*BHQ2*	2
nad5-F	GATGCTTCTTGGGGCTTCTTGTT	5
nad5-R	CTCCAGTCACCAACATTGGCATAA	5
nad5-P	*VIC*-AGGATCCGCATAGCCCTCGA TTTATGTG-*BHQ1*	4

References: [1]Boonham *et al.* (2004); [2]Naktuinbouw (2017); [3]Monger *et al.* (2010); [4]Botermans *et al.* (2013); [5]Menzel *et al.* (2002).

1.4. The test has been successfully performed on different real-time PCR systems, including CFX96 (Bio-Rad Laboratories).
1.5. Data were analysed with Bio-Rad CFX Manager software 2.0 (Bio-Rad Laboratories).
1.6. Further details can be found at the website of Naktuinbouw (Naktuinbouw, 2017).

2. Methods

2.1. RNA extraction
See Appendix 1.
2.2. Real-time RT-PCR
2.2.1 Preparation of primer and probe mixtures

Reaction mix A primers

Primers RMA	Working concentration (µM)	Volume (µL)	Final concentration (µM)
Molecular-grade water*	–	400	–
PSTV-231F	100	100	10
PSTV-296R	100	100	10
PCFVd-F	100	100	10
PCFVd-R	100	100	10
DaVd1-FT	100	100	10
DaVd1-RT	100	100	10
Total		1000	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

Reaction mix A probes

Probes RMA	Working concentration (µM)	Volume (µL)	Final concentration (µM)
Molecular-grade water*	–	350	–
PSTV-251T	100	50	10
PCFVd-P	100	50	10
DaVd1-P	100	50	10
Total		500	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

Reaction mix B primers

Primers RMB	Working concentration (µM)	Volume (µL)	Final concentration (µM)
Molecular-grade water*	–	300	–
CLVd-F	100	100	10
CLVd-F2	100	100	10
CLVd-R	100	100	10
CEVd-F2-304	100	100	10
CEVd-R2-399	100	100	10
DaVd1-FT	100	100	10
DaVd1-RT	100	100	10
Total		1000	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

Reaction mix B probes

Probes RMB	Working concentration (µM)	Volume (µL)	Final concentration (µM)
Molecular-grade water*	–	350	–
CLVd-P	100	50	10
CEVd-P2	100	50	10
DaVd1-P	100	50	10
Total		500	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

Reaction mix C primers

Primers RMC	Working concentration (µM)	Volume (µL)	Final concentration (µM)
Molecular-grade water*	–	600	–
TPMVd-F1	100	100	10
TPMVd-R	100	100	10
nad5-F	100	100	10
nad5-R	100	100	10
Total		1000	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

Reaction mix C probes

Probes RMC	Working concentration (µM)	Volume (µL)	Final concentration (µM)
Molecular-grade water*	–	400	–
pUCCR	100	50	10
nad5-P	100	50	10
Total		500	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

2.2.2. Reaction mixtures

All reactions can be performed with the UltraPlex 1-Step ToughMix 4x (Quanta Biosciences) and AgPath-IDTM One-step RT-PCR mix (Ambion; P/N: 4387424). These mixes have been shown to improve performance in comparison with the qScript XLT Multiplex One-Step RT qPCR Tough Mix 2x (Quanta Biosciences), which has been used for validation of the original protocol (TESTA, 2015). Pipetting schemes are provided for the UltraPlex 1-Step ToughMix 4x (Quanta Biosciences).

Reaction mix A: PCFVd, PSTVd, TCDVd, TPMVd + DLVd

Reagent	Working concentration (µM)	Volume per reaction (µL)	Final concentration (µM)
Molecular-grade water*	–	11.5	–
RT-PCR mix (Quanta Biosciences)	4×	6.25	1×
RMA primers mix PSTV-231F/ PSTV-296R PCFVd-F/ PCFVd-R DaVd1-FT/ DaVd1-RT	10 (each)	0.75	0.3 (each)
RMA probes mix PSTV-251T PCFVd-P DaVd1-P	10 (each)	0.5	0.2 (each)
Subtotal		19.0	
RNA		6.0	
Total		25.0	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

Reaction mix B: CEVd, CLVd + DLVd

Reagent	Working concentration (µM)	Volume per reaction (µL)	Final concentration (µM)
Molecular-grade water*	–	11.5	–
RT-PCR mix (Quanta Biosciences)	4×	6.25	1×
RMB primers mix CLVd-F, CLVd-F2/ CLVd-R CEVd-F2-304/ CEVd-R2-399 DaVd1-FT/ DaVd1-RT	**10 (each)**	**0.75**	**0.3 (each)**
RM2 Probes mix CLVd-P CEVd-P2-337 DaVd1-P	**10 each**	**0.5**	**0.2 (each)**
Subtotal		19.0	
RNA		6.0	
Total		25.0	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

Reaction mix C: TPMVd + nad5

Reagent	Working concentration (µM)	Volume per reaction (µL)	Final concentration (µM)
Molecular-grade water*	–	11.5	–
RT-PCR mix (Quanta Biosciences)	4×	6.25	1×
RMC primers mix	**10 (each)**	**0.75**	**0.3 (each)**
TPMVd-F1/ TPMVd-R			
nad5-F/ nad5-R			
RMC Probes mix	**10 (each)**	**0.5**	**0.2 (each)**
pUCCR			
nad5-P			
Subtotal		19.0	
RNA		6.0	
Total		25.0	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

Reaction mix 4: TASVd

Reagent	Working concentration (µM)	Volume per reaction (µL)	Final concentration (µM)
Molecular-grade water*	–	10.75	–
RT-PCR mix (Quanta Biosciences)	4×	6.25	1×
TASVd-F2-200	10	0.75	0.3
TASVd-R2-269	10	0.75	0.3
TASVd-P2-228	10	0.5	0.2
Subtotal		19.0	
RNA		6.0	
Total		25.0	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

2.3. Real-time RT-PCR cycling conditions (UltraPlex 1-Step ToughMix 4x (Quanta Biosciences))

All reaction mixtures reverse transcription at 50°C for 15 min; denaturation at 95°C for 3 min; 40 cycles of denaturation at 95°C for 10 s, annealing and elongation at 60°C for 60 s.

3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid. The inclusion of suitable controls should be in accordance with the type of matrix used.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of healthy seeds or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: contaminated seeds or seeds spiked with one of the targets pospiviroids. Preferably, a different species or 'uncommon' or 'deviating' genotype is used as positive control, since this allows exclusion of false positives due to cross contamination by the positive control based on the nucleotide sequences.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection. For the choice of the PAC, see also PIC.

In addition to the external positive controls (PIC and/or PAC), internal positive controls (IPCs) are used to monitor each individual sample separately. The IPCs include:

- Endogenous nucleic acid of the matrix using conserved primers preferably amplifying the RNA target *nad5* (Menzel *et al.*, 2002; Botermans *et al.*, 2013).
- Exogenous nucleic acid that has no relation with the target nucleic acid, i.e. DLVd.

The use of the IPCs is optional but is recommended for detection tests.

3.2 Interpretation of results

Verification of the controls:

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

As a Ct cut-off value is equipment, material and chemistry dependent, it needs to be verified in each laboratory when implementing the test.

4. Performance criteria available

Validation data were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* at Naktuinbouw, NL (Testa, 2015; Naktuinbouw, 2017).

4.1. Analytical sensitivity

For both pepper and tomato seeds one contaminated seed in a sample of 1000 seeds could be detected for CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and one contaminated in samples of 100 seeds for TPMVd.

4.2. Analytical specificity

All primers/probe combinations appeared specific to the tested targets (18 isolates of seven species) except for the CEVd primers/probe, which were found to cross-react with TASVd. No cross-reactions have been observed for the following viroids: Chrysanthemum chlorotic mottle viroid (*Pelamoviroid*), CSVd, IrVd-1, Hop stunt viroid (*Hostuviroid*) and viruses Alfalfa mosaic virus, Arabis mosaic virus, Beet ringspot virus, Cucumber mosaic virus, Pepper mild mottle virus, Pepino mosaic virus, Potato virus Y, Tobacco mosaic virus, Tobacco rattle virus, Tobacco ringspot virus, Tobacco streak virus, Tomato mosaic virus, Tomato black ring virus, Tomato spotted wilt virus and Tomato yellow leaf curl virus.

4.3. Selectivity

No apparent matrix effects were observed in pepper and tomato seeds or in leaf material of pepper, potato, tomato and various ornamental crops.

4.4. Repeatability and reproducibility

For both pepper and tomato seeds 100% for all target species.

Performance characteristics of the original version of this test have been described in the report of the EU-project TESTA (Testa, 2015). In addition, Mix A of the tomato seed test performed well for PSTVd in a proficiency test with naturally contaminated seeds, where it produced consistent positive results for samples consisting of 10 contaminated in samples of 1000 seeds (Naktuinbouw, unpublished).

Appendix 5 – PospiSense: Real-time RT-PCR for seed testing (Botermans *et al.*, 2020)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. The PospiSense test (Botermans *et al.*, 2020) provides an alternative for seed testing, allowing sensitive detection of all pospiviroids known to infect pepper and tomato naturally. The test makes use of a single fluorophore, which implies that it does not discriminate between species. The test is described for samples of app. 3000 seeds, tested in three subsamples of 1000 seeds.

1.2. The test consists of two reactions running in parallel: PospiSense 1 and PospiSense 2, together targeting CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd.

Reaction mix 1: CLVd, PCFVd, PSTVd, TCDVd, TPMVd

Reaction mix 2: CEVd, TASVd

In both reactions Dahlia latent viroid (DLVd; genus *Hostuviroid*) is used as an internal (isolation) control. At high concentrations individual pospiviroids might produce a signal in both reactions.

1.3. Oligonucleotides

Primers and probes list for real-time RT-PCR for seed testing

Primers and probes	Sequence (5′-3′)	Ref.
PospiSense 1		
PospiFW1	TGCGCTGTCGCTTCG	1
PospiFW5a	CCTTCCTTTCTTCGGGTTTC	1
PospiRV1	AGAAAAAGCGGCGCTTG	1
PospiRV2	TAGAGAAAAAGCGGTTCTCGG	1
PospiRV5a	GAAAAAGCACCTCTGTCAGTTGTA	1
CLVd-F	GGTTCACACCTGACCCTGCAG	2
CLVd-F2	AAACTCGTGGTTCCTGTGGTT	2
CLVd-R	CGCTCGGTCTGAGTTGCC	2
PospiP1a	*FAM*-CGGTGGAAACAACTG-*MGB*	1
PospiP3a	*FAM*-CGGCCTTCTCGCGCA-*MGB*	1
CLVd-P	*FAM*-AGCGGTCTCAGGAGCCCCGG-*BHQ1*	2
PospiSense 2		
PospiFW6a	GGATCTTTCTTGAGGTTCCTGT	1
PospiFW6b	GGAACTTTCTTGAGGTTCCTGT	1
PospiFW6c	TCTTTCCTTGTGGTTCCTGTG	1
PospiRV6a	CGACTTCCTCCAGGTTTCC	1
PopspiP5	*FAM*-CTGCAGGGTCAGGTG-*MGB*	1
Internal control		
DaVd1-FT	GCTCCGCTCCTTGTAGCTTT	3
DaVd1-RT	AGGAGGTGGAGACCTCTTGG	3
DaVd1-P	*Texas Red*-CTGACTCGAGGACGC GACCG-*BHQ2*	3

References: [1]Botermans *et al.* (2020), [2]Monger *et al.* (2010); [3]Naktuinbouw (unpublished).

1.4. The test has been successfully performed on different real-time PCR systems including CFX96 (Bio-Rad Laboratories) and QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific).

1.5. Data were analysed with a CFX Manager software 2.0 (Bio-Rad Laboratories).

2. Methods

2.1 RNA extraction

See Appendix 1.

2.2 Reaction mixes for real-time RT-PCR

PospiSense 1 primers mix

	Working concentration (µM)	Volume (µL)	Final concentration (µM)
Molecular-grade water*	–	20	–
PospiFW1	100	10	10
PospiFW5a	100	10	10
PospiRV1	100	10	10
PospiRV2	100	10	10
PospiRV5a	100	10	10
CLVd-F	100	10	10
CLVd-F2	100	10	10
CLVd-R	100	10	10
Total		100	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

PospiSense 1 probes mix

	Working concentration (µM)	Volume (µL)	Final concentration (µM)
Molecular-grade water*	–	70	–
PospiP1a	100	10	10
PospiP3a	100	10	10
CLVd-P	100	10	10
Total		100	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

PospiSense 2 primers mix

	Working concentration (µM)	Volume (µL)	Final concentration (µM)
Molecular-grade water*	–	60	–
PospiFW6a	100	10	10
PospiFW6b	100	10	10
PospiFW6c	100	10	10
PospiRV6a	100	10	10
Total		100	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

DLVd primers mix (internal control)

	Working concentration (µM)	Volume (µL)	Final concentration (µM)
Molecular-grade water*	–	80	–
DaVd1-FT	100	10	10
DaVd1-RT	100	10	10
Total		100	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

2.2.2 Reaction mixtures

PospiSense Reaction mix 1: CLVd, PCFVd, PSTVd, TCDVd, TPMVd + DLVd

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	–	11.2	–
RT-PCR mix (Quanta Biosciences)	4×	5.0 µL	1×
PospiSense 1 primer mix	10 µM each	0.6	0.3 µM (each)
PospiSense 1 probe	10 µM each	0.2	0.1 µM (each)
DLVd primer mix	10 µM each	0.6	0.3 µM (each)
DLVd probe	10 µM	0.4	0.2 µM (each)
Subtotal		18	
RNA		2.0	
Total		20	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

PospiSense Reaction mix 2: CEVd, TASVd + DLVd

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	–	11.2	–
RT-PCR mix (Quanta Biosciences)	4×	5.0	1×
PospiSense 2-primer mix	10 µM each	0.6	0.3 µM
PospiP5 - probe	10 µM	0.2	0.1 µM
DLVd - primer mix	10 µM each	0.6	0.3 µM
DLVd - probe	10 µM	0.4	0.2 µM
Subtotal		18	
RNA		2.0	
Total		20	

*Molecular-grade water, purified (deionised or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

2.3 RT-PCR cycling conditions (UltraPlex 1-Step ToughMix 4x (Quanta Biosciences))

Both reaction mixes: reverse transcription at 50°C for 10 min; denaturation at 95°C for 3 min; 40 cycles of denaturation at 95°C for 10 s; annealing and elongation at 60°C for 60 s.

3. Essential Procedural Information

3.1 Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid. The inclusion of suitable controls should be in accordance with the type of matrix used.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of healthy seeds or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: contaminated seeds or seeds spiked with one of the target pospiviroids. Preferably, a different species or 'uncommon' or 'deviating' genotype is used as positive control, since this allows exclusion of false positives due to cross-contamination by the positive control based on the nucleotide sequences.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection. For the choice of the PAC, see also PIC.

In addition to the external positive controls (PIC and/or PAC), an internal positive control (IPC) is used to monitor each individual sample separately. The IPC includes an exogenous nucleic acid that has no relation with the target nucleic acid, i.e. DLVd (Appendix 4, Naktuinbouw, 2017).

The use of the IPC is optional but is recommended for detection tests.

3.2 Interpretation of results

Verification of the controls:

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Validation data were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* at the NPPO of the Netherlands (Botermans *et al.*, 2020).

4.1 Analytical sensitivity

For both tomato and pepper seeds one contaminated seed in a sample of 1000 seeds could be detected for CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd.

4.2 Analytical specificity

The PospiSense test was found to detect all 40 tested isolates of the seven target pospiviroid species, i.e. CEVd (5), CLVd (5), PCFVd (3), PSTVd (12), TASVd (6), TCDVd (6) and TPMVd (3).

Cross-reactions have been found to occur with CSVd, Eggplant latent viroid (*Elaviroid*) and IrVd-1, when present in high concentrations. Of these viroid species, however, no natural infections in pepper and tomato have been reported. Also one out of two isolates of Tomato infectious chlorosis virus produced a cross-reaction at high concentration.

No cross-reactions were observed for the *hostuviroid* Hop stunt viroid, and the following pepper and tomato viruses: Alfalfa mosaic virus, Cucumber mosaic virus, Pepper mild mottle virus, Pepino mosaic virus, Potato virus Y, Tobacco mosaic virus, Tomato chlorosis virus, Tomato mosaic virus, Tomato spotted wilt virus and Tomato yellow leaf curl virus. Furthermore, no cross-reactions have been observed for *Clavibacter michiganensis* subsp. *michiganensis*.

4.3 Selectivity

No apparent matrix effects were observed for pepper and tomato seeds.

4.4 Repeatability and reproducibility

The test was validated in both an intra- and interlaboratory comparison. For pepper seeds contaminated with PSTVd, TASVd and both PCFVd and CLVd, as well as tomato seeds contaminated with TASVd, TCDVd and TPMVd, both repeatability and reproducibility were 100%.

4.5. Diagnostic sensitivity and diagnostic specificity

Comparison of the PospiSense with the real-time RT-PCR for seed testing (Appendix 4) by testing 40 pospiviroid-infected samples and four healthy samples showed 100% concordance. It should be noted, however, that the PospiSense test appeared less sensitive for CEVd and TASVd,

Appendix 6 – PSTVd real-time RT-PCR test (Boonham 2004)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. This real-time RT-PCR test uses primers and a probe described by Boonham *et al.* (2004). The test will detect PSTVd, TCDVd and TPMVd (not all isolates, see Table A2).

This test uses COX primers and a probe which amplify the *cytochrome oxidase 1* as an internal positive control (IPC). Consequently, this test does not allow the reverse-transcription step to be monitored (contrary to the *nad5* primers and probes as described in other reported tests). However, this test has been reported to perform in a comparable fashion using either COX or *nad5* as IPC (Appendix 3, Botermans *et al.*, 2013).

1.2. The test consists of two reactions running in parallel, with reaction mix A targeting PSTVd, TCDVd and TPMVd, and reaction mix B targeting PSTVd, TCDVd TPMVd and COX in a duplex reaction.

This test has been successfully used on a wide range of plant species and matrices.

1.3. Oligonucleotides

Primers and probes	Sequence (5′-3′)	Primer location	Viroids detected
PSTV-231-F	GGGCCCTTTG CGCTGT	232–247	PSTVd, TCDVd, TPMVd
PSTV-296-R	AAGCGGTTCT CGGGAGCTT	297–279	
PSTV-251T	CAGTTGTTTC CACCGGGTA GTAGCCGA	278–252	
COX-F	CGTGCGATTC CAGATTATCCA		
COX-R	CAACTACGGA TATATAAGRR CCRRACCTG		
COXsol-1511T	AGGGCATTCCA TCCAGCGTAAGCA		

1.4. The test has been successfully performed on different real-time PCR systems including ABI 7900 HT (Applied Biosystems).

2. Methods

2.1. RNA extraction

See Appendix 1.

2.2. Real-time RT-PCR

Reagent	Working concentration (µM)	Volume per reaction (µL)	Final concentration (µM)
Pre-mix			
Molecular-grade water*	–	13.75	–
Master Mix (Applied Biosystems)	2×	25.0	1×
MultiScribe Reverse Transcriptase (ABI)	40×	1.25	1×
PSTV-231-F	10	1.5	0.34
PSTV-296-R	10	1.5	0.34
PSTV-251T	5	1.0	0.114
Subtotal		44.0	
Reaction mix A			
Pre-mix		22.0	
Molecular-grade water*		2.0	
RNA		1.0	
Reaction mix B			
Pre-mix		22.0	
COX-F	10	0.75	0.3
COX-R	10	0.75	0.3
COXsol-1511T	5	0.5	0.1
RNA		1	
Total		25	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

2.3 Real-time RT-PCR cycling conditions

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. Essential Procedural Information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid. The inclusion of suitable controls should be in accordance with the type of matrix used.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of healthy plant material or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: plant material or contaminated seeds or material spiked with one of the target pospiviroids. Preferably, a different species or 'uncommon' or 'deviating' genotype is used as positive control, since this allows exclusion of false positives due to cross-contamination by the positive control based on differences of the nucleotide sequences.

- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection. For the choice of the PAC, see also PIC.

In addition to the external positive controls (PIC and/or PAC), an internal positive control (IPC) is used to monitor each individual sample separately. The IPC includes:

- an endogenous nucleic acid of the matrix (COX, Weller *et al.*, 2000)
- an exogenous nucleic acid that has no relation with the target nucleic acid, e.g. DLVd (Appendix 4, Naktuinbouw, 2017).

The use of the IPC is optional but is recommended for detection tests.

3.2. Interpretation of results

Verification of the controls:

- The PIC and PAC (as well as IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Validation data were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*. Validation data available from EPPO website (Testa, 2015; Naktuinbouw, 2017).

4.1. Analytical sensitivity

Analytical sensitivity for the detection of PSTVd in *S. tuberosum* using the CTAB extraction method was found to be 17 pg PSTVd (this was the lowest concentration tested) (Jeffries & James, 2005).

Solanum lycopersicum: Probability of detection of one infested seed in a sample of 1000 is >95% when testing three subsamples of 1000 seeds each. A comparative study using two naturally contaminated seed lots showed that increasing the sample size to 20 000 seeds combined with decreasing the size of the subsamples to 400 did not influence the overall outcome of the test.

4.2. Analytical specificity

By testing variants of PSTVd and synthetic oligonucleotides it has been shown that this test detects all sequence variants that were known when the test was developed. These were identified from *in silico* studies as primer–sequence mismatches with the potential for failure of detection (Boonham *et al.*, 2004). However, the divergent isolates VIR-06/7L and VIR-06/10L described more 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, pers. comm., 2011). Five TCDVd isolates were detected including four isolates on seeds. TPMVd can be detected on plant material when its concentration is high, which is unlikely to occur in seed samples. This test does not detect CEVd, CSVd, IrVd-1, PCFVd and TASVd.

4.3. Selectivity

No apparent matrix effects were observed.

4.4. Repeatability and reproducibility

The test was validated in both an intra- and interlaboratory comparison. 100% repeatability and reproducibility: 100 infested seed in 1000 seeds, 10 infested seeds in 1000, 5 infested seeds in 1000 and 1 infested seed in 1000 tomato seeds.

Appendix 7 – CSVd Real-time RT-PCR test (Mumford *et al.*, 2000)

The test below is described as it was carried out to generate the validation data provided in Section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1 This real-time RT-PCR test uses primers and a probe described by Mumford *et al.* (2000) to detect CSVd. This test uses COX primers and probes which amplify the *cytochrome oxidase 1* as internal positive control (IPC). Consequently, this test does not allow the reverse-transcription step to be monitored of (contrary to using *nad5* primers and probes as described in other reported tests). However, this test has been reported to perform in a comparable fashion using either COX or *nad5* as IPC.

1.2 The described test uses two separate real-time reactions for CSVd and COX (Weller *et al.*, 2000). This test has been successfully used on a wide range of plant species and matrices.

1.3 Oligonucleotides

Primers and probe	Sequence (5′-3′)	Primer location*	Viroids detected
CSVd 220F	CTGCCCTAGCCCG GTCTT	222–239	CSVd
CSVd 297R	GGAAAAAAAGGC GTTGAAGCTT	278–289	
CSVd 249T	CAGTTGTTTCCAC CGGGTAGTAGCCAA	251–278	
COX-F	CGTGCGATTCCAG ATTATCCA		
COX-R	CAACTACGGATATA TAAGRRCCRRACCTG		
COXsol-1511T	AGGGCATTCCATCC AGCGTAAGCA		

*Location in CSVd NC_0020151 Location in CSVd NC_002015.

1.4 The test has been successfully performed on different real-time PCR systems including ABI 7900 HT (Applied Biosystems).

2. Methods

2.2. RNA extraction

See Appendix 1.

2.3. Real-time RT-PCR

Reagent	Working concentration (µM)	Volume per reaction (µL)	Final concentration (µM)
CSVd primers/probe mix			
CSVd 220F	7.5	1.0	0.375
CSVd 297R	7.5	1.0	0.375
CSVd 249T	5	0.5	0.125
Total		2.5	
CSVd reaction mix			
Molecular-grade water*	–	6.45	–
iTaq Master Mix (Bio-Rad)	2×	10.0	1×
iScript RNase H + Reverse Transcriptase (BioRad)	–	0.05	1×
CSVd Primers/ Probe mix		2.5	
RNA	5	1.0	
Total		20	
COX primers/probe mix			
COX F	7.5	1.0	0.375
COX R	7.5	1.0	0.375
COXsol-1511T	5	0.5	0.125
Total		2.5	

(continued)

Table *(continued)*

Reagent	Working concentration (µM)	Volume per reaction (µL)	Final concentration (µM)
COX reaction mix			
Molecular-grade water*	–	6.45	–
Taq Master Mix (BioRad)	2×	10	1×
iScript RNase H + Reverse Transcriptase (BioRad)		0.05	1×
COX Primers/ Probe mix		2.5	
RNA		1	
Total		20	

*Molecular-grade water, purified (deionized or distilled) nuclease-free water, sterile water (autoclaved or 0.22 µm filtered) should be used.

2.4. Real-time PCR cycling conditions

Thermocycling conditions are 50°C for 10 min, 95°C for 2 min and 40 cycles of 95°C for 15 s and 60°C for 1 min.

3. Essential PROCEDURAL information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid. The inclusion of suitable controls should be in accordance with the type of matrix used.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of healthy plant material or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: plant material infected by CSVd. Preferably, a different species or 'uncommon' or 'deviating' genotype is used as positive control, since this allows exclusion of false positives due to cross-contamination by the positive control based on differences in the nucleotide sequences.
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome

amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection. For the choice of the PAC, see also PIC.

In addition to the external positive controls (PIC and/or PAC), an internal positive control (IPC) is used to monitor each individual sample separately. The IPC includes:

- an endogenous nucleic acid of the matrix (COX, Weller *et al.*, 2000)
- an exogenous nucleic acid that has no relation with the target nucleic acid, e.g. DLVd (Appendix 4, Naktuinbouw, 2017).

The use of the IPC is optional but is recommended for detection tests.

3.2. Interpretation of results

Verification of the controls:

- The PIC and PAC (as well as IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

Validation data were generated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*. Validation data available from Fera (UK).

4.1. Analytical sensitivity

Analytical sensitivity for the detection of CSVd in chrysanthemum in a dilution of 1:100 000. There was no difference in the analytical sensitivity using different RNA extraction methods (CTAB, Promega SV and magnetic probe capture Kingfisher).

4.2. Analytical specificity

By testing variants of CSVd and synthetic oligonucleotides it has been shown that this test detects all known sequence variants. A survey done on 77 samples concluded that CSVd was detected in all CSVd-infected samples and no cross-reaction was observed on PSTVd-infected samples.

4.3. Selectivity

CSVd was detected in a wide range of chrysanthemum samples representing more than 750 samples from more than 20 varieties. No apparent matrix effects were observed.

4.4. Repeatability and reproducibility

The test was validated in both an intra- and interlaboratory comparison. The test displayed high repeatability and reproducibility (Ct values deviation between 0.2 and 2 with an average of 1 for 20 independent samples tested).

Appendix 8 – Overview of additional conventional RT-PCR and real-time RT-PCR tests suitable for pospiviroid detection and/or identification

This table presents individual tests whereas Table 2 presents combination of tests.

References	Primers/ probes	CSVd	CEVd	CLVd	IrVd-1	PCFVd	PSTVd	TASVd	TCDVd	TPMVd	Position of amplicon*	Fragment size (if relevant)	Remarks on validation
Boonham *et al.* (2004)	PSTV	–	–	nt	–	–	+	–	+	+[a]	232-297 in PSTVd NC_002030	–	Naktuinbouw (2012a), NAK (2015)
Hooftman *et al.* (1996)	CSVd h/c	+	nt	nt	nt	nt	nt	nt	nt	nt	62-112 in CSVd NC_002015	Complete genome[b]	see publication
Mumford *et al.* (2000)[c]	Vir2/1	+	+	nt	nt	nt	+	nt	nt	nt	17-280 in CSVd NC_002015	264 nt	see Appendix 7 (Fera, UK)
	CSVd	+	nt	nt	nt	nt	–	nt	nt	nt	222-299 in CSVd NC_002015	–	
Önelge (1997)	CEVd	nt	+	nt	nt	nt	nt	+[d]	nt	nt	80-117 in CEVd NC_002015	Complete genome[b]	see publication
Shamloul *et al.* (1997)	3H1/2H1[e]	nt	nt	nt	nt	nt	+	nt	+	+[a]	69-113 in PSTVd NC_002030	Complete genome[b]	EPPO validation data EPPO validation data NPPO-NL (2013d)
Spieker (1996a)	pCLVR4/ pCLV4	nt	–	+	–	nt	nt	–	nt	nt	102-101 in CLVd NC_003538	Complete genome[b]	see reference, NPPO-NL (unpublished)
Verhoeven *et al.* (2009)	AP-FW1/RE2	nt	nt	nt	nt	+	nt	nt	nt	nt	178-164 in PCFVd NC_011590	*ca.* complete genome (−13 nt)	see publication
Verhoeven *et al.* (2004)	Vid-FW/RW	–	–	+	–	–	+	–	+	–	355-354 in PSTVd NC_002030	Complete genome	EPPO validation data NPPO-NL (2013b)
Verhoeven *et al.* (2010b)	IrVd-1	nt	nt	nt	+	nt	nt	nt	nt	nt	168-167 in IrVd-1 NC_003613	Complete genome[b]	see publication
Verhoeven *et al.* (2017)	Pospi2[f]	+	+	–	+	+	+	+	+	+	261-103 in PSTVd NC_002030	*ca.* half genome	see publication
Monger *et al.* (2010); Naktuinbouw (unpublished)	Generic	+	+	+	nt	nt	+	+	+	nt	327-97 in PSTVd NC_002030	–	see publication Testa (2015)
	CEVd	–	+	–	nt	–	–	+[g]	–	–	206-299 in CEVd NC_001464I	–	
	CLVd	–	–	+	nt	–	–	–	–	–	8-121 in CLVd NC_003538	–	
	TASVd	–	–	–	nt	–	–	+	–	–	197-263 in TASVd NC_001553	–	
Naktuinbouw (unpublished)	PCFVd	nt	–	–	nt	+	–	–	–	–	7-72 in PCFVd NC_011590	–	Testa, 2015

*Position of amplicon in reference sequence of indicated species in NCBI GenBank; nt: not tested.

[a]It is known that at least one isolate of TPMVd (GenBank acc. no. K00817.1) will not or only be poorly detected (Testa, 2015; EPPO validation data; Naktuinbouw, 2017).

[b]Complete sequence includes primer sequences (because of the circular genome it might be advisable to include these sequences in BLAST searches).

[c]EPPO (2002) EPPO Bulletin 32, 245–253.

[d]All tested TASVd isolates tested at NPPO-NL were detected so far.

[e]Primer names used in IPPC protocol DP-07 PSTVd.

[f]Primers complementary to pospi1.

[g]CEVd primers and probe cross react with TASVd isolates.

Appendix 9 – Mechanical inoculation of test plants

Mechanical inoculation

Approximately 200–500 mg leaf, root or tuber tissue is ground in 0.1 M phosphate inoculation buffer (w/v 1:1 dilution is recommended) containing carborundum (400 mesh). 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.

It should be noted that total RNA extracts and (complete genome) PCR-products are also infectious and can be used for mechanical inoculation of test plants.

Growing conditions

Since viroid concentration is affected by temperature and light level, microplants and glasshouse plants for testing should be grown in controlled conditions, at least 24°C and photoperiod of 14 h (Grassmick & Slack, 1985, IPPC, 2015). Lower temperatures and less illumination may reduce the sensitivity of the test. The plants are inspected weekly for symptoms for up to 6 weeks after inoculation.

Symptomatology

Typical symptoms of pospiviroid infection are described in Section 3.1. It should be noted that variations in symptoms are frequently observed between and within species, therefore it is not possible to discriminate between pospiviroid species on that basis.

Appendix 10 – Hybridization tests using digoxigenin-labelled RNA probe

1. General information

Digoxigenin (DIG) is a system for nonradioactive labelling of nucleotides or nucleic acids (Monger & Jeffries, 2015). The probe is a full-length PSTVd monomer (Agdia, Inc., Cat. No. DLP 08000/0001) which will hybridize with other pospiviroids at the low stringency conditions described, the extent depending on sequence similarity. The labelled probes may then be hybridized with complementary nucleic acid spotted onto a membrane. An antidigoxigenin antibody conjugated to alkaline phosphatase is then added and this binds to the hybridized probe. The antibody-probe hybrids are then visualized by addition of a chemiluminescent substrate that produces light in the presence of alkaline phosphatase, and this is recorded on X-ray film or digitally.

2. Methods

2.1. Buffers and reagents

Ames buffer:

NaCl	12 g
$MgCl_2$	0.4 g
Sodium acetate	8.2 g
Ethanol	40 mL
Distilled water	160 mL
Adjust to pH 6 with HCl or NaOH	

20 × saline-sodium citrate buffer (SSC) (or use Sigma Aldrich cat no. S6639):

NaCl	173.5 g
Sodium citrate	88 g
Distilled water to	1000 mL
Adjust to pH 7 with NaOH	

10% SDS:

Sodium dodecyl sulphate (SDS)	50 g
Distilled water to	500 mL

Wash buffer 1:

20 × SSC	100 mL
10% SDS	10 mL
Distilled water to	1000 mL

Wash buffer 2:

20 × SSC	5 mL
10% SDS	10 mL
Distilled water to	1000 mL

Reagents:

Ribonuclease (RNase) A (Sigma Aldrich, UK)
DIG Easy Hyb solution (Sigma Aldrich, UK)
DIG Luminescent Detection Kit (Sigma Aldrich, UK)
Digoxigenin Labelled PSTVd Probe (Agdia, Germany)
Nylon membrane for hybridization (Agdia, Germany)
DIG Wash and Block Buffer Set (Sigma Aldrich, UK)
Hybridization bags (Sigma Aldrich, UK) only for use when using a shaking water bath. If using a hybridization oven, use hybridization tubes.

2.2. Sampling

For testing single plants, use a leaf tissue sample of 200 mg.

For the positive control, use not more than 2 mg of PSTVd-infected tissue added to 198 mg healthy tissue. The

test is sensitive enough always to detect this level. If it is not detected, the test should be repeated. The test is sensitive enough to allow bulking of 10 plants (at least 20 mg of tissue per plant).

One or more positive control(s) should be used for each batch of sample extractions and applied to each membrane. A healthy potato control should also be used. Process positive controls last to avoid possible splash contamination of individual tubes.

2.3. RNA extraction and spotting samples onto the membrane

Grind the tissue sample in a small amount of Ames buffer. Add the remaining buffer to a concentration ratio of 1:1.5 (sample weight: buffer volume), e.g. 200 mg of tissue per 300 µL of Ames buffer. Other extraction buffers may be used for nucleic acid extraction.

Transfer to a 1.5-mL microcentrifuge tube. Cover tubes and incubate at 37°C for 15 min. Add an equal volume of molecular biology grade chloroform to each tube and mix the contents thoroughly by vortexing or inverting until an emulsion has formed. Centrifuge the tubes briefly to separate the contents into aqueous (top) and chloroform (bottom) layers or place the tubes in a refrigerator (4°C) to separate overnight.

The layers should be clearly separated before proceeding since material from the interphase can cause false positives.

Spotting samples onto the membrane: Pipette 3 µL from the aqueous layer onto the membrane (Agdia) and air-dry the membrane at room temperature. Store the sample extracts at 4°C. If it is necessary to retest any samples, the stored extracts may be used. Chloroform-extracted samples can be retained for at least several months and a spotted, dried membrane may be retained in a dry location at room temperature for several years without affecting the results.

2.4. Preparation of probe

The probe should be used according to the manufacturer's instructions. Labelled probes can be stored for at least a year, and hybridization solutions can be reused several times.

If necessary, consult the Roche DIG online manual at http://biochem.roche.com/prodinfo_fst.htm?/prod_inf/manuals/dig_man/dig_toc.htm.

2.5. Hybridization

Having established an optimum exposure time for UV exposure, UV-crosslink the air-dried membranes on 20 × SSC wetted filter paper in a UV Crosslinker (or for a transilluminator type light box, place the dry membrane face down and expose to UV light, or bake the membrane at 80°C in an oven for 2 h). Briefly centrifuge the tube containing the lyophilized DIG-labelled PSTVd probe (Agdia, Germany) before opening. Avoid RNase contamination (wear gloves). Resuspend the lyophilized DIG labelled PSTVd probe in 100 µL DIG Easy-Hyb Buffer (Sigma Aldrich, UK). The ratio of probe to hybridization-buffer volume will be stated on the tubes of probe supplied. Place the membrane in a glass hybridization tube 4. Add 100 µL of resuspended DIG-labelled probe to 8 mL DIG Easy Hyb Buffer (Sigma Aldrich, UK) and pour over the membrane to cover it (about 4 mL is needed for 100 cm^2 of membrane). The remaining hybridization buffer with added probe can be stored. Hybridize in a hybridization incubator overnight at 55°C.

For the following procedures, through incubation in Detection Buffer, the membrane should never be allowed to dry. Washes can be done in a heat resistant dish. The next day carefully draw off the hybridization buffer and store in a sterile tube. The probe in hybridization buffer can be stored at −70°C and reused (defrost and denature at 65°C for 15 min).

Wash the membrane for 5 min at room temperature in 200 mL of wash buffer 1, then for 15 min at room temperature in 200 mL of wash buffer 1 containing 1 µg mL^{-1} RNase A. The addition of RNase A is essential to avoid false positives with healthy material. Wash the membrane twice, for 15 min per wash, at 65°C in 200 mL of preheated wash buffer 2. Rinse the membrane in 50–100 mL of maleic acid buffer (100 mm maleic acid, pH 7.5; 150 mm NaCl, supplied as 10 × maleic acid buffer in the DIG Wash and Block Buffer Set, Roche Diagnostics) for 1 min at room temperature. Pour off the solution. Then block for 1–2 h at room temperature in 25 mL of blocking solution per membrane (i.e. 2.5 mL of 10 × blocking solution + 22.5 mL of 10 × maleic acid buffer, supplied in the DIG Wash and Block Buffer Set, Roche Diagnostics) using an orbital shaker at 100–150 rpm. Do not pour off the solution. Centrifuge the anti-DIG-alkaline phosphatase solution (supplied in the DIG Luminescent Detection Kit, Sigma Aldrich, UK) at 10 000–12 000*g* for 5 min to remove small antibody aggregates that may be present and which can lead to speckling background. Add the anti-DIG alkaline phosphatase directly to the blocking solution used in the previous blocking step at a dilution of 1:10 000, taking care not to add the anti-DIG-alkaline phosphatase directly onto the membrane. Handling the stock anti-DIG-alkaline phosphatase and CSPD substrate solutions should be performed in sterile environment. Incubate the membrane for 30 min at room temperature on the shaker. Pour off the solution and wash the membrane twice, for 15 min, at room temperature in about 150 mL of maleic acid buffer. Use the orbital shaker at 50–80 rpm (or tilting table). Dilute CSPD 1:100 in 0.5 mL of detection buffer (supplied in the DIG Luminescent Detection Kit, Sigma Aldrich). Wash the membrane once for 5 min at room temperature in 50–100 mL of detection buffer. Place the wet membrane, sample side up, on an acetate sheet (or clingfilm) and pour diluted CSPD all over the membrane using a pipette (about 0.5 mL of CSPD per 100 cm^2 membrane). Carefully pick up the sheet and membrane and gently move around to disperse the substrate. Carefully place another acetate sheet (or clingfilm) over the membrane and gently remove air bubbles and further disperse the substrate. Place the membrane in an autoradiography cassette. Expose membranes to film for 2.5–3.0 h at room temperature or 1.0–1.5 h at 37°C and develop the film. The positive reaction is a very intense to easily visible spot. A barely visible spot or spot outline may be positive or negative.

2.6. Detection

Detection is performed using CSPD substrate (Tropix), following the protocol recommended by Roche Diagnostics for chemiluminescent detection of DIG-labelled RNA probes (http://www.roche-applied-science.com/wcsstore/RASCatalogAssetStore/Articles/05353149001_08.08.pdf). When using Kodak X-Omat film, an exposure of 1 h usually gives a clear result. However, a second exposure (longer or shorter) may be necessary depending on the strength of signal detected.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, appropriate controls should be included for each series of nucleic acid extraction and hybridization of the target organism and target nucleic acid. The inclusion of suitable controls should be in accordance with the type of matrix used.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: naturally infected host tissue or host tissue spiked with one of the target pospiviroids.
- Negative control (NC) to rule out false positives due to contamination during the preparation of the reaction mix: molecular-grade water that was used to prepare the reaction mix.
- Positive control (PC) to monitor the efficiency of the hybridization: nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole-genome amplified DNA or a synthetic control (e.g. cloned PCR product).
- 3.2. Interpretation of results

Verification of the controls:

- NIC and NAC should produce no hybridization signal.
- PIC and PAC should produce a hybridization signal.

When these conditions are met:

- A test will be considered positive if it produces an easily visible spot.
- Tests should be repeated if a barely visible spot is produced.

4. Performance criteria available

4.1. Analytical sensitivity

In a test performance study involving nine laboratories the DIG method was shown to be sensitive to at least 0.0155 mg (which is equivalent to 17 pg of PSTVd) of infected leaf tissue for the highest performing laboratories.

4.2. Analytical specificity

The probe is a full-length monomer and will detect PSTVd, CEVd, CSVd, CLVd, TASVd and TCDVd.

4.3. Reproducibility and repeatability

Reproducibility was 100% for all laboratories down to 10 mg of infected tissue. Reproducibility was better than or comparable to other diagnostic tests evaluated at the same time Jeffries & James (2005).

Results have been repeatable over a season.