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EPPO STANDARD ON DIAGNOSTICS

PM 7/32 (2) *Plum pox virus*

Specific scope: This Standard describes a diagnostic protocol for *Plum pox virus*.[1]

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

Specific approval and amendment: Approved in 2003-09 Revised in 2023-07.

Although this EPPO Diagnostic Standard differs in terms of format, it is in general consistent with the content of the IPPC Standard adopted in 2018 on *Plum pox virus* (Annex 2 to ISPM 27); however, tests evaluated in the framework of the EU funded project VALITEST have been added.

Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Sharka (plum pox) is one of the most serious viral diseases of stone fruit. The disease was first reported in *Prunus domestica* in Bulgaria in 1917–1918 and was described as a viral disease in 1932. Since then, the virus has spread progressively to a large part of Europe, around the Mediterranean basin and the Near East. It has a restricted distribution in the Americas and Asia (CABI, 2008; EPPO, 2006). The disease, caused by *Plum pox virus* (PPV), affects plants of the genus *Prunus*. It is particularly detrimental in *P. armeniaca*, *P. domestica*, *P. persica* and *P. salicina* because it reduces quality and causes premature fruit drop. It is estimated that the costs of managing sharka worldwide since the 1970s have exceeded 10 000 million EUR (Cambra et al., 2006).

Under natural conditions, PPV readily infects plants of the genus *Prunus* used as commercial varieties or rootstocks: *P. armeniaca* (apricot), *P. cerasifera* (Myrobalan flowering plum), *P. davidiana* (Chinese wild peach), *P. domestica* (European plum), *P. salicina* (Japanese plums), *P. mahaleb* (Mahaleb cherry), *P. mariana* (Marianna plum), *P. mume* (Japanese apricot), *P. persicae* (peach) and interspecific hybrids between these species. *Prunus avium* (sweet cherries), *P. cerasus* (sour cherries) and *P. dulcis* (almond) may be infected occasionally or only by specific PPV strains (García et al., 2014). The virus also infects many wild and ornamental *Prunus* species such as *P. besseyi* (western sand cherry), *P. cistena* (purple-leaved sand cherry), *P. glandulosa* (dwarf flowering almond), *P. insititia* (damson plum), *P. laurocerasus* (cherry laurel), *P. spinosa* (blackthorn), *P. tomentosa* (Nanking cherry) and *P. triloba* (flowering almond) (Damsteegt et al., 2007; James & Thompson, 2006).

Plum pox virus is a member of the genus *Potyvirus* in the family *Potyviridae*. The virus particles are flexuous rods of approximately 700 × 11 nm and are composed of a single-stranded RNA molecule consisting of almost 10 000 nucleotides coated by up to 2000 subunits of a single coat protein (García & Cambra, 2007). *Plum pox virus* is transmitted in the field by aphids in a non-persistent manner, but movement of infected propagative plant material is the main way in which PPV is spread over long distances. *Plum pox virus* isolates can currently be classified into 10 monophyletic strains, presented in Table 1.

The strains have specific genome sequences and may vary in their symptomatology, pathogenicity, host range, epidemiology and aphid transmissibility. Most PPV isolates belong to the strains D and M. PPV-D and -M can easily infect *P. armeniaca* and *P. domestica* but differ in their ability to infect *P. persica* cultivars. These two strains also differ in their pathogenicity, with M isolates generally causing more rapid epidemics and more severe symptoms than D isolates in *P. armeniaca*, *P. domestica*, *P. persica* and *P. salicina*. Limited information is available about the epidemiology and biological properties of the other PPV isolates.

Further information on the biology of PPV, including illustrations of disease symptoms, can be found in the EPPO datasheet (EPPO, 2023a) and in the EPPO Global Database (EPPO, 2023b).

A flow diagram describing the procedure for PPV detection and identification of strains is presented in Figure 1.

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

TABLE 1 Monophyletic strains of *Plum pox virus*.

D	Dideron	Widespread in Europe and the cause of most outbreaks in north and South America and Asia; found in all *Prunus* spp. except cherry and almond and less frequently associated with peaches
M	Marcus	Mainly reported in Central and Southern Europe; affects all *Prunus* spp. except cherry but causes rapid epidemics in different peach cultivars
C	Cherry	Relatively common in Moldova and occasionally reported in Belarus, Croatia, Hungary and Italy; restricted to *Prunus avium* and *Prunus cerasus*
EA	El Amar	Only reported in Egypt in several *Prunus* species except for cherry and almond
W	Winona	Reported in Eastern European countries and Canada; found in several *Prunus* species except for cherry and almond
Rec	Recombinant	Recombinant between D and M found in several European countries as well as outside Europe; epidemiologically similar to D
T	Turkish	Common in Turkey and exceptionally reported in Albania; found in several *Prunus* species except for cherry and almond (Ulubaş Serçe et al., 2009)
CR	Cherry Russian	Reported in Russia; cherry adapted strains infecting *Prunus avium* and *Prunus cerasus*
CV	Cherry Volga	Four unusual isolates were discovered on *Prunus cerasus* in Russia. The first two: Tat 2 and 4 have been sequenced and represent a new PPV cherry-adapted strain (CV) (Chirkov et al., 2016, 2018, Sheveleva et al., 2018). The second two: Tat 3 and 26 are different from CV, C and CR (Chirkov et al., 2018); so far, no full genome is available to characterize them
An	Ancestor Marcus	Found in Eastern Albania in several species except for cherry and almond (García et al., 2014; James et al., 2013); it has been proposed as a potential ancestor of PPV-M (Palmisano et al., 2012)

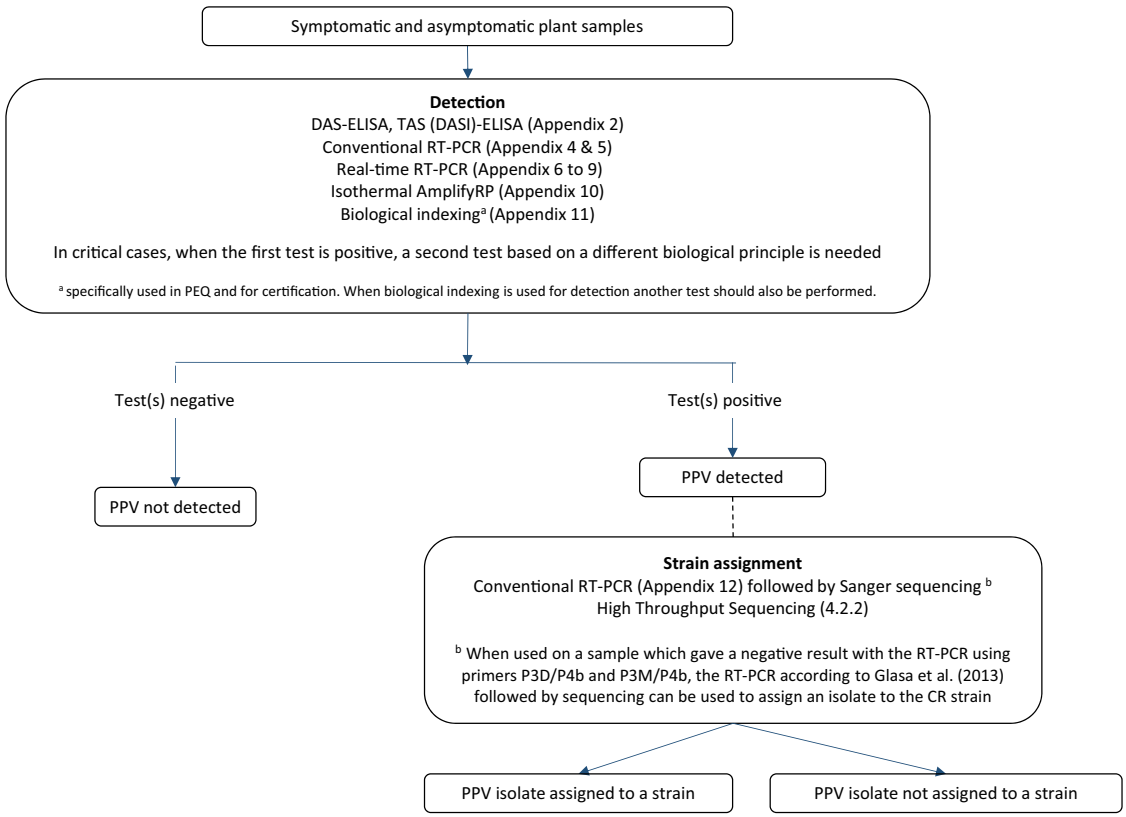

FIGURE 1 Flow diagram describing the diagnostic procedure for *Plum pox virus* in plant samples.

2 | IDENTITY

Name: *Plum pox virus*.
Other names: *Plum pox potyvirus*, *Sharka virus*.
Acronym: PPV.

Taxonomic position: viruses: *Potyviridae*: *Potyvirus*.
EPPO Code: PPV000.
Phytosanitary categorization: EPPO A2 list no. 96, EU RNQP (Annex IV).

Note: PPV has at least 10 monophyletic strains.

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 2021, 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 the genus name. Names of viruses not included in the official ICTV classification are based on first reports. Transfer to a binomial nomenclature is in progress (ICTV website) and will be implemented gradually in EPPO Diagnostic Protocols.

3 | DETECTION

3.1 | Symptoms

Plum pox virus symptoms on *Prunus* spp. may appear on leaves, bark, flowers and fruits (including stones). Symptom development and intensity depend strongly on the host plant and climatic conditions, for example the virus may be latent for several years in cold climates.

Plum pox virus can have an uneven distribution in infected trees and therefore symptoms may be overlooked or may be missing or difficult to detect late in the season. Up to 20% of infected trees in orchards may not be detected by visual inspections. In trees and nursery plants recent infections can be symptomless or produce insconspicuous symptoms that may be missed during inspection even by experienced persons. Infected tolerant cultivars or species can be symptomless.

3.1.1 | Symptoms on leaves and bark

Early in the growing season, symptoms on leaves are usually distinct and include mild light-green discoloration, chlorotic spots, bands or rings, vein clearing or yellowing and/or leaf deformation. Some of these leaf symptoms are similar to those caused by other viruses, such as American plum line pattern virus. *Prunus cerasifera* cv. GF 31 rootstock shows rusty-brown corking and cracking of the bark.

3.1.2 | Symptoms on flowers

Flower symptoms may include discoloration (pinkish streaks) on flower petals and flower-breaking symptoms (Barba et al., 2011).

3.1.3 | Symptoms on fruits

Infected fruits show chlorotic spots or lightly pigmented yellow rings or line patterns. Fruits may become deformed or irregular in shape and develop brown or necrotic areas under the discoloured rings. Some fruit deformations, especially in *P. armeniaca* and *P. domestica*, are similar to those caused by apple chlorotic leaf spot virus. Diseased fruits may show internal browning and gummosis of the flesh and reduced quality. In severe cases the diseased fruits drop prematurely from the tree. In general, the fruits of early maturing cultivars show more marked symptoms than those of late maturing cultivars.

Stones from diseased apricot fruits show typical pale rings or spots. Photos of typical symptoms are shown in Figure 2.

3.2 | Test sample requirements

Details of the plant parts to be sampled are described below. The preparation of samples is described in Appendix 1.

Appropriate sample selection is critical for PPV detection. When possible, it is recommended to collect plant material by hand and not by using scissors to avoid contamination between samples. Flowers, leaves, shoots and fruit skin can be stored at approximately 4°C for not more than 10 days before processing. Fruits can be stored for 1 month at approximately 4°C before processing.

Sampling should take into account virus biology and local climatic conditions, in particular the weather conditions during the growing season. Sampling should be avoided in periods where the temperature is above 25°C (i.e. when the virus titre decreases). It should be noted that the virus titre in samples collected in winter (dormant period) is lower and tests with the highest analytical sensitivity should be used during this period.

In some circumstances (e.g. during the routine diagnosis of a pest widely established in a country), multiple plants may be tested simultaneously using a bulked sample. The decision to test individual plants or composite plant samples by serological or molecular methods depends on the expected prevalence of the PPV in the area, the level of confidence required by the NPPO, the analytical sensitivity of the test and the type of tissue sampled, as described in the following sections. When not specified serological and molecular tests can be used.

Guidance for sampling in nurseries, included in this section, has been generated in the EU FP7 funded project SharCo (including pooling guidance).

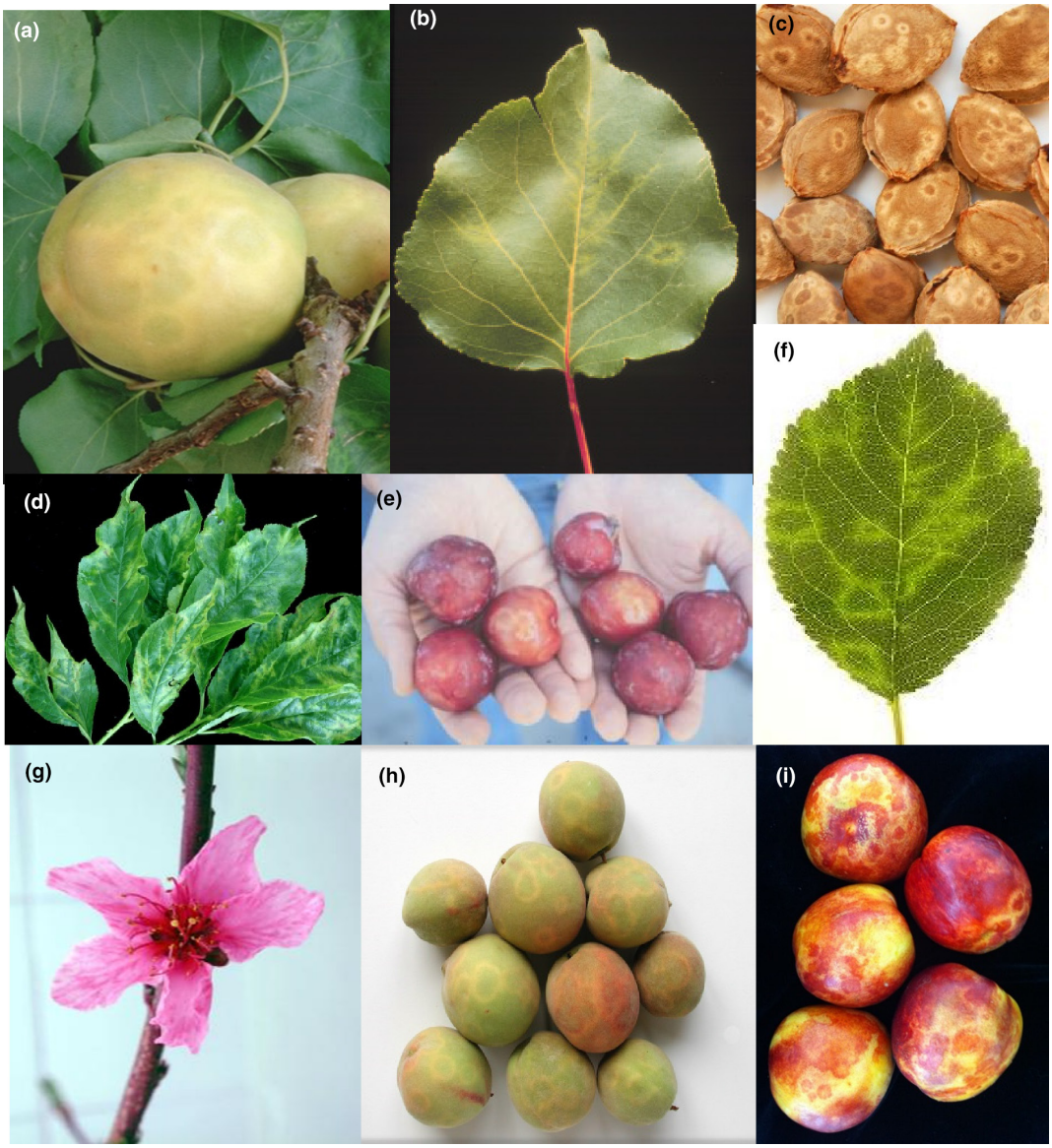

FIGURE 2 Typical symptoms induced by *Plum pox virus* on apricot fruit (a), apricot leaf (b), apricot stones (c), Japanese plum leaves (d), Japanese plum fruits (e), European plum leaf (f), peach flower (g) and peach fruits (h and i). Courtesy Dr M. A. Cambra, Centro de Protección Vegetal y Certificación, Diputación General de Aragón, Montañana-Zaragoza, Spain.

3.2.1 | Leaves and shoots

3.2.1.1 | *Symptomatic plants*

In symptomatic plants, leaves with typical symptoms should be collected.

3.2.1.2 | *Asymptomatic plants*

- Orchards

In asymptomatic plants, samples should be taken from at least 1-year-old shoots with mature or fully expanded leaves collected from the middle of each of the main branches (detection is not reliable in shoots <1 year old).

Plant material should preferably be collected from the internal parts of the tree canopy.

A standard sample consists of four or five shoots or eight to 10 fully expanded leaves collected around the canopy of each individual adult tree from the middle of each scaffold branch. If mature leaves are collected during periods when temperatures are above 25°C, molecular tests should be used.

- Nurseries

A standard sample should consist of three or four expanded leaves per nursery plant or 10 expanded leaves per mother plant. Leaves should preferably be collected from the internal structure of the nursery plant or collected around the canopy of each individual mother plant from the middle of each scaffold branch.

Composite samples: during the EU FP7 funded project SharCo it was shown that for serological tests, a bulked sample should not comprise leaves collected from more than four nursery plants.

3.2.2 | Buds

- Orchards

Dormant buds may also be collected (eight to 10 per tree) but should be tested using molecular tests.

- Nurseries

A standard sample should consist of three or four dormant buds per nursery plant or 15–20 per mother plant, collected from the apical, middle and basal part of shoots. These samples should be tested using molecular tests.

During the SharCo projects, it was shown that for molecular tests, a bulked sample can be composed of three buds per plant collected from 10 nursery plants.

3.2.3 | Flowers

Flowers are sampled when typical symptoms are present.

3.2.4 | Fruits

Skin from symptomatic fruits can be tested during all periods (including samples from packing houses).

3.2.5 | Bark

In winter, bark tissues from the basal part of twigs, shoots or branches, or complete spurs, can be sampled. Validation data for this matrix is lacking.

3.3 | Screening tests

Serological and molecular tests are commonly used for detection in plant material from nurseries and orchards.

Bioassays with indicator plants are widely used in certification schemes but do not detect all strains. Consequently, another test should also be performed. In addition bioassays are time consuming and require adequate facilities.

In critical cases when the first test is positive, another test based on different biological principle should also be performed.

3.3.1 | Sample preparation

The sample preparation for plant material is described in Appendix 1.

3.3.2 | Serological tests

Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) or triple antibody sandwich (TAS but also called DASI) ELISA are recommended for detection using polyclonal antibodies or a mix of monoclonal antibodies.

DAS- or TAS-ELISA is performed according to PM 7/125 *ELISA tests for viruses* with the antibodies recommended in Appendix 2.

Lateral flow devices and sticks for PPV detection in symptomatic samples are also available (Appendix 2). However, limited validation data is available.

3.3.3 | Molecular tests

Several molecular tests have been developed for the detection of PPV. Table 2 provides information regarding the strains covered by a selection of diagnostic tests.

The following molecular tests which have been evaluated in a Test Performance Study performed in the framework of the EU funded VALITEST project (https://www.valitest.eu/) are recommended to detect PPV. The tests were selected based on their ability to detect the following strains: An, C, CR, D, EA, M, Rec, T and W.

Nucleic acid extraction is described in Appendix 3.

- Conventional RT-PCR (Wetzel et al., 1991), described in Appendix 4.
- Conventional RT-PCR (Levy & Hadidi, 1994), described in Appendix 5.
- Real-time RT-PCR (Schneider et al., 2004 and FAO, 2018), described in Appendix 6.
- Real-time RT-PCR (Olmos et al., 2005), described in Appendix 7.
- Real-time RT-PCR kit from Qualiplante based on Olmos et al. (2005), described in Appendix 8.
- Real-time RT-PCR (Naktuinbouw, unpublished), described in Appendix 9.
- Isothermal AmplifyRP® Acceler8™ using reverse transcription-recombinase polymerase amplification for PPV detection is also available for the detection of PPV (Zhang et al., 2014) and is described in Appendix 10.

High-throughput sequencing (HTS) may also be used for screening of samples of *Prunus* species but is usually not used in the framework of PPV targeted surveys.

Information on molecular tests and strains detected is presented in Table 2. Data on strains generated in the framework of the EU funded Valitest project is indicated in red.

3.4 | Other test: Bioassay

Bioassays are commonly used in the framework of certification programmes for *Prunus* fruit trees. However, the bioassay for PPV has some disadvantages: it is time consuming (symptom development requires up

TABLE 2 Molecular tests and strains detected.

Test	Appendix	D	M	Rec	EA	T	W	C	CR	CV	An
Conventional RT-PCR (Wetzel et al., 1991)	Appendix 4	+	+	+	+	+	+	+	+	nt	+
Conventional RT-PCR (Levy & Hadidi, 1994)	Appendix 5	+	+	+	+	+	+	+	+	nt	+
Real-time RT-PCR (FAO, 2018; Schneider et al., 2004)	Appendix 6	+	+	+	+	+	+	+	+	nt	+
Real-time RT-PCR (Olmos et al., 2005)	Appendix 7	+	+	+	+	+	+	+	+	nt	+
Real-time RT-PCR kit Qualiplante based on Olmos et al. (2005)	Appendix 8	+	+	+	+	+	+	+	+	nt	+
Real-time RT-PCR (Naktuinbouw, unpublished)	Appendix 9	+	+	+	+	+	+	+	+	nt	+
Isothermal AmplifyRP® Acceler8™	Appendix 10	+	+	+	+	+	+	+	+	+	+
Conventional RT-PCR kit Qualiplante based on Wetzel et al. (1991)	–	+	+	+	+	+	+	+	+	nt	+
Real-time RT-PCR (Fotiou et al., 2019)	–	+	+	+	+	+	+	+	+	nt	+
Conventional RT-PCR (Olmos et al., 1997)	–	+	+	nt	nt	nt	nt	nt	nt	nt	nt
Real-time RT-PCR (Capote et al., 2006)	–	+	+	nt	nt	nt	nt	nt	nt	nt	nt
Conventional RT-PCR (Šubr et al., 2004)	–	+	+	+	nt	nt	nt	nt	nt	nt	nt
Real-time RT-PCR (Varga & James, 2006)	–	+	nt	nt	+	nt	+	+	nt	nt	nt
Real-time RT-PCR (Varga & James, 2005)	–	+	+	nt	nt	nt	nt	nt	nt	nt	nt
Conventional RT-PCR (Glasa et al., 2013)	–	nt	nt	nt	nt	nt	nt	nt	+	nt	nt

Note: +, strains detected.
Abbreviation: nt, not tested.

to 6 months post-inoculation); it requires dedicated containment facilities such as temperature-controlled insect-proof greenhouses; and it requires experienced staff who can accurately interpret disease symptoms that can be confused with symptoms of other graft-transmissible organisms. The bioassay is described in Appendix 11.

4 | IDENTIFICATION

4.1 | Identification of PPV

Serological and molecular tests described in Section 3 allow both the detection and identification of PPV. For strain assignment see Section 4.2.

4.2 | Assignment to a strain

4.2.1 | Molecular tests for strain assignment

Isolates of D, M, Rec, EA, T, W and An strains can be assigned with conventional RT-PCRs using primers P3D/P4b and P3M/P4b (Candresse et al., 1998, 2011), followed by Sanger sequencing of the amplicon(s). The test is described in Appendix 12 and is performed on nucleic acid extracts that tested positive with a detection test. Sequence analysis should follow the guidelines described in Appendices 7 and 8 of the EPPO Standard PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2021).

When used on a sample which gave a negative result with the RT-PCR using primers P3D/P4b and P3M/P4b, the RT-PCR according to Glasa et al. (2013) followed by sequencing can be used to assign an isolate to the CR strain.

4.2.2 | High-throughput sequencing

High-throughput sequencing technologies may be used to obtain (near) complete genome sequences, which can be used for assignment to a virus strain. An EPPO Standard PM 7/151 *Considerations for the use of high throughput sequencing in plant health diagnostics* has been approved (EPPO, 2022).

5 | REPORTING AND DOCUMENTATION

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

6 | PERFORMANCE CHARACTERISTICS

When performance characteristics 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.).

7 | REFERENCE MATERIAL

Lyophilized PPV-infected and healthy controls can be obtained through the SharCo collection of lyophilized PPV isolates maintained at Instituto Valenciano de Investigaciones Agrarias, Carretera Moncada-Náquera km 5. 46113 Moncada (Valencia), Spain (Antonio Olmos; olmos_antcas@gva.es).

An *in vivo* collection of key PPV isolates is maintained at Instituto di Virologia Vegetale del CNR, Sezione di Bari, via Amendola 165/A, I-70126 Bari, Italy (Dr Donato Boscia. E-mail: d.boscia@ba.ivv.cnr.it).

Alternatively, lyophilized PPV-infected controls are commercially available (Agritest, www.agritest.it; Agdia, www.agdia.com; Bioreba, www.bioreba.ch; DSMZ, https://www.dsmz.de/collection/catalogue/plant-viruses-and-antisera/catalogue; Loewe, www.loewe-info.com; Plant Print Diagnòstics, www.plantprint.net; Sediag, www.sediag.com).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

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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.fr.

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.

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This protocol was originally drafted by: M Cambra, E Bertolini, A Olmos & MT Gorris, Instituto Valenciano de Investigaciones Agrarias, Centre of Protección Vegetal y Biotecnología, Carretera de Moncada-Náquera km 5, 46113 Moncada (Valencia), Spain. This revision was prepared by A Olmos (lead author), F Faggioli (CREA, IT), A Fox (Fera, GB), C Lacomme (SASA, GB) and K De Jonghe (ILVO, BE). Appendix 12 was prepared with the contribution of JP Renvoisé (ANSES, FR). The protocol was reviewed by the Panel on Diagnostics in Virology and Phytoplasmology.

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APPENDIX 1 - SAMPLE PREPARATION FOR PLANT MATERIAL

For serological testing, 0.2–0.5 g fresh plant material is cut into small pieces with disposable razor blades or bleach-treated scissors and placed into a suitable tube or plastic bag. The sample is homogenized thoroughly in 2–10 mL (from 1:10 to 1:20 w/v) extraction buffer (as recommended in the kit) using an electrical tissue homogenizer, a manual roller, a hammer, or a similar tool.

For molecular testing, fresh plant material, 0.2 g for samples from individual trees up to 2 g for pooled samples (pooled samples consisting of equal amounts from each tree), is cut into small pieces as described above, placed into individual plastic bags and homogenized thoroughly in 1–20 mL (from 1:5 to 1:10 w/v) extraction buffer (PVP-PBS-DIECA or GH+ buffer, see below).

Extraction buffer

PVP-PBS-DIECA

PBS buffer (see below) supplemented with 2 g sodium diethyl dithiocarbamate (DIECA) and 20 g polyvinylpyrrolidone (PVP-10 average molecular weight 10 000 g/mol) per 1 L.

Phosphate-buffered saline (PBS)

NaCl	8 g
KCl	0.2 g
$Na_2HPO_4 \cdot 12H_2O$	2.9 g
KH_2PO_4	0.2 g
Distilled water	1 L

Adjust pH to 7.2–7.4.

GH+ buffer (Menzel et al., 2002)

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

APPENDIX 2 - SEROLOGICAL TESTS

DAS-ELISA or TAS (DASI)-ELISA tests are performed according to PM 7/125 *ELISA tests for viruses* (EPPO, 2015). The tests are performed on plant crude extracts.

1. Antibodies for DAS or TAS (DASI) ELISA

DAS-ELISA kits for universal PPV detection, based on polyclonal and/or monoclonal antibodies are commercially available from: Agdia, Agritest, Bioreba, Loewe, Neogen, Prime Diagnostics and SEDIAG.

Performance characteristics of selected antibodies

Data from the EU funded VALITEST project for DAS-ELISA. Details are available in the TPS report in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

During the TPS preliminary studies DAS-ELISA was performed according to PM 7/125 *ELISA tests for viruses* and consequently the buffers used during the evaluation were not those recommended by the companies.

The TPS was performed with the kits from Agdia, Bioreba and SEDIAG because these showed the highest analytical sensitivity during the TPS preliminary studies. The sample panel comprised 22 samples, including controls. Six laboratories from 6 countries participated.

- Analytical sensitivity

For the three selected kits (Agdia, Bioreba and SEDIAG), extracts prepared from *Nicotiana benthamiana* infected by PPV diluted up to at least 10× in PPV free *Prunus* sp. could be detected.

- Analytical specificity

The analytical specificity was evaluated during preliminary studies conducted in the framework of the project.

Inclusivity: inclusivity was evaluated for the following strains: An, C, CR, EA and Rec. The five PPV strains were detected in undiluted samples.

Exclusivity: exclusivity was evaluated with apple chlorotic leaf spot virus, cherry virus A, little cherry virus 1 and prunus necrotic ringspot virus. No cross reaction was noted.

- Diagnostic sensitivity

The diagnostic sensitivity was evaluated comparing the test result with an expected status of samples (for details see TPS report).

Agdia 80%
Bioreba 81.3%
SEDIAG 45%

- Diagnostic specificity

The diagnostic specificity was calculated comparing the test result with an expected status of samples (for details see TPS report).

Agdia 91.7%
Bioreba 93.8%
SEDIAG 98.3%

- Repeatability (evaluated on two replicates)

Agdia 83.33%
Bioreba 87.50%
SEDIAG 33.33%

- Reproducibility
  Agdia 85.83%
  Bioreba 87.50%
  SEDIAG 71.67%

2. Lateral flow devices

Lateral flow devices and sticks, based on polyclonal antibodies, for PPV detection are available from: Agdia, www.agdia.com and Bioreba, www.bioreba.ch.

PPV ImmunoStrip® Agdia: PPV strains An, C, CR, D, EA, M, Rec, T and W were detected. Performance characteristics are available from the Agdia website (https://orders.agdia.com/agdia-immunostrip-for-ppv-isk-31505) and from Zhang et al. (2014).

PPV AgriStrip® Bioreba: PPV strains C, D, EA, M and W are detected. Performance characteristics are available from the Bioreba website and Virscek Marn et al. (2014).

APPENDIX 3 - RNA EXTRACTION

This appendix describes RNA extraction methods for plant material. These initial steps are critical for the results of a test and are often more related to the matrix than the specific test. 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.

Care should be taken to prevent cross contamination when handling samples, especially when high concentrations of virus are expected.

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. Extraction with RNeasy Plant Mini kit (Qiagen): for leaves, flowers and buds (used in ANSES)

One gram of plant material (0.5 g for buds) is ground in 10 mL of PVP-PBS-DIECA buffer (Appendix 1) (or 5 mL for buds) and 200 µL is added to 350 µL of RLT buffer (without β-mercaptoethanol or dithiothreitol) and then purified with the RNeasy Plant Mini Kit (Qiagen).

2. Extraction with QuickPick™ SML Plant DNA kit[2] (Bio-Nobile, Pargas, Finland) and KingFisher™ mL System (Thermo Fisher Scientific, Vantaa, Finland) for leaves (used in ANSES)

The protocol uses specific KingFisher™ mL plastic consumables (five-well strips [one per sample] and tip combs). The KingFisher™ mL script was validated with the QuickPick™ SML Plant DNA as follows.

One gram of leaves is ground in 5 mL of PVP-PBS-DIECA buffer. A volume of 150 µL of extract is added to 150 µL of lysis buffer and 10 µL of proteinase K, vortexed, incubated at 65°C for 20 min and centrifuged for 5 min at 18 000*g* (at room temperature). The supernatant is transferred in the first well containing 250 µL of binding buffer and 10 µL of magnetic beads, then mixed for 10 min at medium speed. The beads are then collected using the magnetic rod inserted in a tip comb (five displacements ["counts"] from the top of the liquid to the bottom with a 10 s pause at the bottom ["collect time"]). The beads are washed three times with 500 µL of wash buffer in the next three wells (for each washing step: the beads are released for 10 s at medium speed, mixed for 20 s at medium speed and collected; five "counts" with a "collect time" of 10 s). The nucleic acids are eluted in 100 µL of elution buffer in the last well, "elution well" (the beads are released for 10 s at medium speed, mixed for 10 min at slow speed and collected: five "counts" with a "collect time" of 10 s). The beads are released for 5 s at fast speed in the last washing well. The nucleic acids are in the elution well.

Total KingFisher™ mL procedure duration is 32 min.

3. Procedure used in the VALITEST test performance study

"Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi" from the RNeasy® Mini Handbook using RNeasy Plant Mini kit (Qiagen) with a GH+ buffer (Appendix 1) in the grinding step. 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.

Important points:

GH+ buffer may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).
Unless specified otherwise perform all steps of the procedure at room temperature. It is important to work quickly during this procedure.
Perform all centrifugation steps at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

Other kits or extraction methods may be used if verified (see PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*) with the molecular test to be used.

[2]Although this is a DNA extraction kit it was evaluated and performed adequately.

APPENDIX 4 - RT-PCR WETZEL ET AL. (1991)

The test below differs from the one described in the original publication.

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

1. General information

1.1. RT-PCR for detection of PPV.
1.2. The test can be performed on any kind of plant material.
1.3. Amplicon sequence location: CP gene.
1.4. Oligonucleotides:

Primers	Sequence (5′–3′)	Amplicon size
P1	ACC GAG ACC ACT ACA CTC CC	243 bp
P2	CAG ACT ACA GCC TCG CCA GA	

2. Methods

2.1. Nucleic acid extraction
See Appendix 3.
2.2. Reverse transcription polymerase chain reaction—RT-PCR Master mix

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	–	15	–
OneStep RT-PCR buffer (Qiagen)	5×	5	1×
dNTP mix (10 mM each, Qiagen)	10 mM	1	0.4 mM
P1 primer	50 µM	0.5	1.0 µM
P2 primer	50 µM	0.5	1.0 µM
OneStep RT-PCR enzyme mix		1	
Subtotal	–	23.0	–
RNA		2.0	
Total PCR volume	–	25.0	–

2.3. RT-PCR cycling conditions: An initial step (50°C for 30 min and 95°C for 15 min) followed by 40 cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 60 s) and one final step at 72°C for 10 min before cooling at 4°C.

3. Essential procedural information

3.1. Controls:
For a reliable test result to be obtained, the following (external) controls should be included for each series of extraction phase and amplification of the target organism.

- 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 present: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives owing to contamination during the preparation of the reaction mix: application of the amplification procedure to 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). The PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the PIC, internal positive controls (IPC) can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene, nad5 or eukaryotic 18S rDNA).
- Specific amplification or co-amplification of nucleic acid from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

3.2. Interpretation of results: the following criteria should be followed:

Verification of the controls:
- NIC and NAC: no band is visualized.
- PIC, PAC a band of 243 bp is visualized. If relevant, a band of the expected size is visualized for the IPC.

When these conditions are met:

- A test will be considered positive if a band of 243 bp is visualized.
- 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.

4. Performance characteristics available

Data generated during the preliminary study and the TPS performed in the EU funded project VALITEST. Details are available in the TPS report in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).The TPS involved 12 laboratories from nine countries. The panel was composed of 22 samples (including controls).

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1. Analytical sensitivity

PPV-infected *Nicotiana benthamiana* extracts diluted up to at least 10^4 times in PPV free *Prunus* sp. extract.

4.2. Analytical specificity

Inclusivity 100% evaluated on PPV strains: An, C, CR, D, EA, M and Rec, T.

Exclusivity 100%. Exclusivity was evaluated during the preliminary studies with the following non-targets: apple chlorotic leaf spot virus, apple mosaic virus, Asian prunus virus 3, cherry associated luteovirus, cherry green ring mottle virus, cherry virus A, little cherry virus 1, nectarine stem pitting associated virus, plum bark necrosis stem pitting-associated virus, prune dwarf virus and prunus necrotic ringspot virus.

4.3. Diagnostic sensitivity

100% evaluated comparing the test result with an expected status of samples (for details see TPS report).

4.4. Diagnostic specificity

87.5% evaluated with samples of known status (verified using HTS).

4.5. Repeatability

100% evaluated with two replicate samples.

4.6. Reproducibility

98.75%.

APPENDIX 5 - CONVENTIONAL RT-PCR LEVY AND HADIDI (1994)

The test below differs from the one described in the original publication.

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

1. General information

1.1. RT-PCR for the detection of PPV.
1.2. The test can be performed on any kind of plant material.
1.3. Amplicon sequence location: CP gene.
1.4. Oligonucleotides:

Primers	Sequence (5′–3′)	Amplicon size
3′NCR sense	GTA GTG GTC TCG GTA TCT ATC ATA	220 bp
3′NCR antisense	GTC TCT TGC ACA AGA ACT ATA ACC	

2. Methods

2.1. Nucleic acid extraction

See Appendix 3.

2.2. Reverse transcription polymerase chain reaction—RT-PCR

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	–	15	–
OneStep RT-PCR buffer (Qiagen)	5×	5	1×
dNTP mix (10 mM each, Qiagen)	10 mM	1	0.4 mM
3′NCR sense primer	50 µM	0.5	1.0 µM
3′NCR antisense primer	50 µM	0.5	1.0 µM
OneStep RT-PCR enzyme mix (Qiagen)		1	
Subtotal	–	23.0	–
RNA		2.0	
Total PCR volume	–	25.0	–

2.3. RT-PCR cycling conditions: an initial step (50°C for 30 min and 95°C for 15 min) followed by 40 cycles (94°C for 30 s, 62°C for 30 s, and 72°C for 60 s) and one final step at 72°C for 10 min before cooling at 4°C.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of extraction phase and amplification of the target organism.

- 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.
- PIC to ensure that nucleic acid of sufficient quantity and quality is present: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- NAC to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- 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). The PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the PIC, IPC can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene, nad5 or eukaryotic 18S rDNA).
- Specific amplification or co-amplification of nucleic acid from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

3.2. Interpretation of results: the following criteria should be followed:

Verification of the controls:

- NIC and NAC no band is visualized.
- PIC, PAC a band of 220 bp is visualized. If relevant, a band of the expected size is visualized for the IPC.

When these conditions are met:

- A test will be considered positive if a band of 220 bp is visualized.
- 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.

4. Performance characteristics available

Data generated during the preliminary study and the TPS performed in the EU funded project VALITEST. Details are available in the TPS report in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).The TPS involved 12 laboratories from nine countries. The panel was composed of 22 samples (including controls).

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1. Analytical sensitivity

Plum pox virus-infected *Nicotiana benthamiana* extracts diluted up to at least 10^4 times in PPV free *Prunus* sp. extract.

4.2. Analytical specificity

Inclusivity 100% evaluated on PPV strains: An, C, CR, D, EA, M, Rec and T.

100%. Exclusivity was evaluated during the preliminary studies with the following non-targets: apple chlorotic leaf spot virus, apple mosaic virus, Asian prunus virus 3, cherry associated luteovirus, cherry green ring mottle virus, cherry virus A, little cherry virus 1, nectarine stem pitting associated virus, plum bark necrosis stem pitting-associated virus, prune dwarf virus and prunus necrotic ringspot virus.

4.3. Diagnostic sensitivity

100% evaluated comparing the test result with an expected status of samples (for details see TPS report).

4.4. Diagnostic specificity

90% evaluated with samples of known status (verified using HTS).

4.5. Repeatability

100% evaluated with two replicate samples.

4.6. Reproducibility

99%.

APPENDIX 6 - REAL-TIME RT-PCR SCHNEIDER ET AL. (2004) AND FAO (2018)

The test below differs from the one described in the original publication.

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

1. General information

1.1. Real-time PCR adapted from Schneider et al. (2004) and FAO (2018) using a TaqMan probe for the detection of PPV.
1.2. The test can be performed on any kind of plant material.
1.3. Amplicon sequence location: CP gene.
1.4. Oligonucleotides:

Primers and probes	Sequence (5′–3′)
PPV FWD primer	CCA ATA AAG CCA TTG TTG GAT C
PPV REV primer	TGA ATT CCA TAC CTT GGC ATG T
PPV TaqMan probe	FAM—CTT CAG CCA CGT TAC TGA AAT GTG CCA—TAMRA

2. Methods

2.1. Nucleic acid extraction:
See Appendix 3.
2.2. Real-time RT-PCR master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water		8.625	
TaqMan® RT-PCR Mix (Applied Biosystems)[a]	2×	12.5	1×
PPV FWD primer	10 µM	0.5	0.2 µM
PPV REV primer	10 µM	0.5	0.2 µM
PPV TaqMan probe	10 µM	0.25	0.1 µM
TaqMan® RT Enzyme Mix (Applied Biosystems)[a]	40×	0.625	1×
Subtotal		23.0	
RNA Extract		2.0	
Final volume		25.0	

[a]From the TaqMan® RNA-to-Ct™ 1-Step Kit (Applied Biosystems).

2.3. Real-time RT-PCR cycling conditions: an initial step (48°C for 15 min and 95°C for 10 min) followed by 40 cycles (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 (external) controls should be included for each series of extraction phase and amplification of the target organism.

- 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.
- PIC to ensure that nucleic acid of sufficient quantity and quality is present: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- NAC to rule out false positives owing to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- 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). The PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the PIC, IPC can be used to monitor each individual sample separately.
These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene, nad5 or eukaryotic 18S rDNA).
- Specific amplification or co-amplification of nucleic acid control that has been spiked to the sample and has no relation with the target nucleic acid (e.g. synthetic internal amplification control).

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

3.2. Interpretation of results:

The C_t cut-off value given below is as established in the framework of the VALITEST project. As a C_t cut-off value is equipment, material and chemistry dependent, it needs to be verified in each laboratory when implementing the test.

Verification of the controls:

- The PIC and PAC (and if relevant IC and IPC) 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 and a C_t value <35.
- A test will be considered negative, if it either does not produce an exponential amplification curve or produces an exponential curve with C_t value ≥35 or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

Data generated during the preliminary study and the TPS performed in the EU funded project VALITEST. Details are available in the TPS report in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php). The TPS involved 12 laboratories from nine countries. The panel was composed of 22 samples (including controls).

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1. Analytical sensitivity

PPV-infected *Nicotiana benthamiana* extracts diluted up to at least 10^4 times in PPV free *Prunus* sp. extract.

4.2. Analytical specificity

Inclusivity 100% evaluated on PPV strains: An, C, CR, D, EA, M, Rec and T.

Exclusivity 100%. Exclusivity was evaluated during the preliminary studies with the following non-targets: apple chlorotic leaf spot virus, apple mosaic virus, Asian prunus virus 3, cherry associated luteovirus, cherry green ring mottle virus, cherry virus A, little cherry virus 1, nectarine stem pitting associated virus, plum bark necrosis stem pitting-associated virus, prune dwarf virus and prunus necrotic ringspot virus.

4.3. Diagnostic sensitivity

98.6% evaluated comparing the test result with an expected status of samples (for details see TPS report).

4.4. Diagnostic specificity

87.5% evaluated with samples of known status (verified using HTS).

4.5. Repeatability

100% evaluated with two replicate samples.

4.6. Reproducibility

97.5%.

APPENDIX 7 - REAL-TIME RT-PCR OLMOS ET AL. (2005)

The test below differs from the one described in the original publication.

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

1. General information

1.1. Real-time RT-PCR using TaqMan probe for the detection of PPV.
1.2. The test can be performed on plant material (evaluated on leaves, buds and flowers).
1.3. Amplicon sequence location: CP gene.
1.4. Oligonucleotides:

Primers and probes	Sequence (5′–3′)	Amplicon size
P241	CGT TTA TTT GGC TTG GAT GGA A	76 bp
P316D	GAT TAA CAT CAC CAG CGG TGT G	
P316M	GAT TCA CGT CAC CAG CGG TGT G	
PPV-DM probe	FAM—CGT CGG AAC ACA AGA AGA GGA CAC AGA—TAMRA	

2. Methods

2.1. Nucleic acid extraction:

At ANSES, nucleic acid extraction described in Appendix 3 points 1 and 2 are used.

2.2. Real-time- RT-PCR

Reagents	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	–	5.26	–
TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems)	2×	15.00	1×
MultiScribeTM Reverse Transcriptase (Applied Biosystems)	50 U/µL	0.60	30 U
RNase Inhibitor (Applied Biosystems)	20 U/µL	1.50	30 U
P241 primer	25 µM	1.20	1.0 µM
P316D primer	25 µM	0.60	0.5 µM
P316M primer	25 µM	0.60	0.5 µM
PPV-DM probe	25 µM	0.24	0.2 µM
Subtotal	–	25.0	–
RNA extract	–	5.0	–
Total volume	–	30.0	–

2.3. RT-PCR cycling conditions: Initial step (48°C for 30 min and 95°C for 10 min) followed by 45 cycles (95°C for 15 s, and 60°C for 30 s).

3. Essential procedural information

3.1. Controls:

- 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.
- PIC to ensure that nucleic acid of sufficient quantity and quality is present: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue extract spiked with the target organism).
- NAC to rule out false positives owing to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- 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). The PAC should preferably be near to the limit of detection.

As alternative (or in addition) to the PIC, IPC can be used to monitor each individual sample separately.

These can include:

- Specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene, nad5 or eukaryotic 18S rDNA).
- Specific amplification or co-amplification of nucleic acid from a sample spiked with material (e.g. biological material, synthetic nucleic acids) that has no relation with the target nucleic acid.

IPC primers are not included in the Master Mix table (see point 2.2). Consequently, if the laboratory plans to use an IPC in multiplex reactions, it should demonstrate that this co-amplification does not negatively affect the performance of the test.

3.2. Interpretation of results:

The C_t cut-off value given below is as established at ANSES. As a C_t cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

Verification of the controls:

- The PIC and PAC (and if relevant IC and IPC) 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 and a C_t value <40.
- A test will be considered negative, if it either does not produce an exponential amplification curve or produces an exponential curve with a C_t value ≥40 or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance characteristics available

The test has been validated by ANSES on different matrices using the RNA extraction kits mentioned in Appendix 3 points 1 and 2.

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding

test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1. Analytical sensitivity data

Evaluated on dilution series made from five samples (two field samples of plum trees infested with PPV strain not known, one peach tree infested with M strain, one peach tree infested with D strain and one plum tree infested with Rec strain).

Analytical sensitivity:

Sample with low viral load 10^{-2}.
Sample with high viral load 10^{-4} (but see complementary study below).

Complementary study

Analytical sensitivity: 1/100 000, with the QuickPick SML Plant DNA and KingFisher™ ML and RNeasy Plant Mini Kit evaluated on dilution series of leaf extracts prepared from PPV-Rec infested plum tree leaves diluted in *Prunus persica*.

4.2. Analytical specificity data

Inclusivity 100%, evaluated with the following strains: D, M, Rec, C, EA, T, CR, W and An (confirmed with QuickPick SML Plant DNA and KingFisher™ ML or RNeasy Plant Mini Kit).

Exclusivity 100%, evaluated with the following non-targets: apple chlorotic leaf spot virus, Asian prunus virus, prune dwarf virus, prunus necrotic ringspot virus, tomato ringspot virus and potato virus Y.

4.3. Diagnostic sensitivity

Leaves 100%, evaluated on 10 samples: five field samples (from three plum trees and two peach trees infested with PPV; precise plant species and strain not known), two samples from peach trees infested with M strain, two samples from peach trees infested with D strain and one sample from a plum tree infested with PPV-Rec.

4.4. Diagnostic specificity

- Leaves

98.8%, using the RNeasy plant mini kit (Qiagen) evaluated on 10 samples (four field samples [two plum trees and two peach trees; precise species not known] free from PPV, six samples infected by non-target species). One well corresponding to a replicate of a peach tree free from PPV was positive.

100% using the extraction QuickPick SML Plant DNA and five prunus samples (*Prunus armeniaca*, *Prunus domestica*, *Prunus mahaleb* × *Prunus avium*, *Prunus persica* × *Prunus davidiana* and *Prunus salicina* × *Prunus spinosa*).

- Buds

100%, evaluated on five samples of *Prunus* species or hybrids free from PPV (*Prunus armeniaca*, *Prunus domestica*, *Prunus mahaleb* × *Prunus avium*, *Prunus persica* and *Prunus salicina* × *Prunus spinosa*).

- Flowers

100%, evaluated with three samples of *Prunus* species or hybrids free from PPV (one *Prunus domestica* × *Prunus davidiana*, two *Prunus persica*).

4.5. Repeatability

98.6% evaluated based on three replicates per sample and each sample tested twice.

4.6. Reproducibility

98.3% (involving two laboratories, three thermocyclers).

4.7. Selectivity

- Leaves

100%, evaluated with nucleic acid extracts of six *Prunus* species or hybrids (*Prunus persica*, *Prunus armeniaca*, *Prunus domestica*, *Prunus persica* × *Prunus davidiana*, *Prunus mahaleb* × *Prunus avium* and *Prunus salicina* × *Prunus spinosa*) spiked with nucleic acid extracts of PPV-D and PPV-M.

100% with leaf extracts of the same *Prunus* species (except *Prunus persica*) spiked with extract from leaf infested by PPV-Rec followed by an RNA extraction using QuickPick SML Plant DNA and KingFisher™ ML or RNeasy Plant Mini Kit.

- Buds

96.7%, evaluated with nucleic acid extracts of five *Prunus* species or hybrids (*Prunus persica*, *Prunus armeniaca*, *Prunus domestica*, *Prunus mahaleb* × *Prunus avium* and *Prunus salicina* × *Prunus spinosa*) spiked with PPV and tested in six replicates each.

Plum pox virus was detected in each replicate of the spiked extracts except in one replicate of the spiked extract of *Prunus domestica* (as spiked samples were close to the limit of detection it cannot be excluded that the lack of detection is linked to repeatability and not selectivity).

- Flowers

100%, evaluated with nucleic acid extracts of flowers from three *Prunus* species or hybrids (two *Prunus persica*, one *Prunus domestica* × *Prunus davidiana*) spiked with PPV.

4.8. Accuracy

99.4 %.

Data on leaves, using the RNeasy plant mini kit (Qiagen) and calculated with the results obtained from the

samples evaluated for the diagnostic sensitivity and diagnostic specificity.

APPENDIX 8 - REAL-TIME RT-PCR KIT QUALIPLANTE BASED ON OLMOS ET AL. (2005)

Validation data on this kit was obtained during the EU funded VALITEST project.

1. General information
   - 1.1. OneStep real-time RT-PCR (Qualiplante, cat. No. qPCR-PPV-100Liq) for the detection of PPV.
   - 1.2. The test can be performed on any kind of plant material.
   - 1.3. Amplicon sequence location: CP gene.

2. Method
   - 2.1. Nucleic acid extraction:
     See Appendix 3.
   - 2.2. Real-time RT-PCR
     Follow manufacturer's instructions.

3. Validation data

Data generated during the TPS performed in the EU funded project VALITEST. The TPS involved 12 laboratories from nine countries. The panel was composed of 22 samples (including controls). Details are available in the TPS report in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

3.1. Analytical sensitivity

PPV-infected *Nicotiana benthamiana* extracts diluted up to at least 10^4 times in PPV free *Prunus* sp. extract.

3.2. Analytical specificity

Inclusivity 100% evaluated on PPV strains: An, C, CR, D, EA, M, Rec and T.

Exclusivity 100%. Exclusivity was evaluated during the preliminary studies on the test described by Olmos et al. (2005) with the following non-targets: apple chlorotic leaf spot virus, apple mosaic virus, Asian prunus virus 3, cherry associated luteovirus, cherry green ring mottle virus, cherry virus A, little cherry virus 1, nectarine stem pitting associated virus, plum bark necrosis stem pitting-associated virus, prune dwarf virus and prunus necrotic ringspot virus.

3.3. Diagnostic sensitivity

100% evaluated with samples of known status (positive samples with known C_t values were diluted in PPV free *Prunus* extract).

3.4. Diagnostic specificity

89.5% evaluated with samples of known status (verified using HTS).

3.5. Reproducibility

98.5%.

3.6. Repeatability

100% evaluated with two replicate samples.

APPENDIX 9 - REAL-TIME RT-PCR (NAKTUINBOUW, UNPUBLISHED)

The test below differs from the one originally described by Naktuinbouw.

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

1. General information

- 1.1. Real-time RT-PCR (Naktuinbouw, unpublished) for the detection of PPV.
- 1.2. The test can be performed on leaves and shoots of *Prunus* spp.
- 1.3. The PCR targets the coat-protein gene. The primers and probe are designed on an alignment of approximately 200 PPV accessions from NCBI GenBank (date 13-3-2018). These accessions include all currently known PPV strains.
- 1.4. The test is described and validated in combination with an internal positive control.
- 1.5. Oligonucleotides

Primers and probes	Sequence (5′–3′)	Position[a]
PPV		
PPV-Fn	CAR AAT CGT TTA TTT GGC TTG GA	9450–9472
PPV-R2n	AGG AGG TTR TGC ATG TTG CG	9531–9550
PPV-Pn	FAM-AGA GGA CAC AGA GAG RCA CAC CGC TG-BHQ1	9494–9519
Internal control[b]		
nad5-F	GAT GCT TCT TGG GGC TTC TTG TT	
nad5-R	CTC CAG TCA CCA ACA TTG GCA TAA	
nad5-P	Texas Red-AGG ATC CGC ATA GCC CTC GAT TTA TGT G-BHQ1	

[a] Position in PPV-D, GenBank accession numbers LT600779–LT600782.

[b] Based on Botermans et al. (2013) and Menzel et al. (2002).

1.6. The test has been successfully performed using the TaqMan® RNA-to-Ct™ 1-Step Kit (Applied Biosystems, Valitest) and the UltraPlexTM 1-step ToughMix® (Quanta Biosciences, Naktuinbouw) on the real-time PCR system CFX96 (Bio-Rad Laboratories).

1.7. Data were analysed with CFX Manager software 3.1 (Bio-Rad Laboratories).

2. Methods

2.1. Nucleic acid extraction and purification

See Appendix 3.

2.2. One-step real-time RT-PCR

2.2.1. Master mix

Reagents	Working concentration (µM)	Volume per reaction (µL)	Final concentration (µM)
Molecular grade water	N.A.	7.575	N.A.
TaqMan® RT-PCR Mix (Applied Biosystems®)[a]	2×	12.5	1×
PPV-Fn primer	10 µM	0.75	0.3
PPV-R2n primer	10 µM	0.75	0.3
PPV-Pn probe	10 µM	0.25	0.1
nad5-F primer	10 µM	0.15	0.06
nad5-R primer	10 µM	0.15	0.06
nad5-P TaqMan® probe	10 µM	0.25	0.1
Taqman RT-Enzyme Mix (Applied Biosystems®)[a]	40×	0.625	1×
Subtotal		23.0	
RNA		2.0	
Total		25.0	

[a] From the TaqMan® RNA-to-Ct™ 1-Step Kit (Applied Biosystems).

2.3. RT-PCR cycling conditions

Initial step (48°C for 15 min and 95°C for 10 min) followed by 40 cycles (95°C for 15 s, 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.

- 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.
- PIC to ensure that nucleic acid of sufficient quantity and quality is isolated: naturally infected host tissue or host tissue spiked with preferably a known genotype of PPV.
- NAC to rule out false positives owing to contamination during the preparation of the reaction mix: molecular grade water that was used to prepare the reaction mix.
- 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). The PAC should preferably be near to the limit of detection.

In addition to the PIC, an IPC nad5 is used in this test to monitor each individual sample separately.

3.2. Interpretation of results

Verification of the controls:

- The PIC, PAC and IPC 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 characteristics available

Data generated during the preliminary study and the TPS performed in the EU funded project VALITEST. The TPS involved 12 laboratories from nine countries. The panel was composed of 22 samples (including controls). Details are available in the TPS report in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

The test may have been adapted further and validated or verified using other critical reagents, instruments and/or further modifications. If so, the corresponding test descriptions and validation data can be found in the EPPO database on diagnostic expertise (section validation data http://dc.eppo.int/validationlist.php).

4.1. Analytical sensitivity

Plum pox virus-infected *Nicotiana benthamiana* extracts diluted up to at least 10^4 times in PPV free *Prunus* sp. extract.

4.2. Analytical specificity

Inclusivity 100% evaluated on PPV strains: An, C, CR, D, EA, M, Rec and T.

Exclusivity 100%. Exclusivity was evaluated during the preliminary studies with the following non-targets: apple chlorotic leaf spot virus, apple mosaic virus, Asian prunus virus 3, cherry associated luteovirus, cherry green ring mottle virus, cherry virus A, little cherry virus 1, nectarine stem pitting associated virus, plum bark necrosis stem pitting-associated virus, prune dwarf virus and prunus necrotic ringspot virus.

4.3. Diagnostic sensitivity

98.6% evaluated with samples of known status (positive samples with known C_t values were diluted in PPV free *Prunus* extract).

4.4. Diagnostic specificity

87.5% evaluated with samples of known status (all samples were sequenced using HTS to verify viral content [PPV and other viruses]).

4.5. Reproducibility

97.5%.

4.6. Repeatability

100% evaluated with two replicate samples.

APPENDIX 10 - THE AGDIA AMPLIFYRP® ACCELER8® FOR PPV TEST (ZHANG ET AL., 2014)

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 provideded that verification (see PM 7/98) is carried out.

1. General information

1.1. This test is suitable for the detection of PPV in stone fruit crops.

1.2. This test is based on Zhang et al. (2014)

1.3. The probe and primer sequences are protected by IP.

2. Methods

2.1. Sample preparation using the kit

2.1.1. Place 300 mg of basal part of leaf in GEB4 buffer at a 1:10 weight to volume ratio and grind.

2.1.2. Rehydrate the reaction pellet with 10 µL of PD1 Pellet Diluent.

2.1.3. Transfer 1 µL of GEB4-extracted sample into the rehydrated reaction pellet mix immediately using a 1 µL transfer loop or pipette and mix via vortex or by flicking the bottom of the tube six to eight times.

2.2. Test procedure.

2.2.1. Agdia AmplifyRP® Acceler8® for PPV kits are commercially available to perform end-point tests (15 min) on a portable heat block (Agdia) at 39°C followed by placing the completed reaction inside an amplicon detection chamber (20 min) that houses a lateral flow strip.

3. Essential procedural information

3.1. Interpretation of results

3.1.1. Results are visible on the lateral flow device inside the amplicon detection chamber. After completion of the migration of the reaction mix along the lateral flow device, red/purple lines will be visible on the device.

A test will be considered positive if two lines (test line and control line) are present. A test will be considered negative if the test line does not appear and only the control line is visible.

The test is considered invalid if no lines are visible.

4. Performance characteristics available

A. Data from VALITEST

Agdia's AmplifyRP® Acceler8® for PPV was evaluated during a test performance study organized in on-site tests in 2020. Unlike the evaluation performed and reported in B, the TPS was performed on freeze-dried infected samples. In the VALITEST TPS, lyophilized tissue was used and an additional dilution step of 1:10 of the extracted samples in GEB4 buffer was introduced in order to counteract matrix inhibition.

4.1. Analytical sensitivity

1/16 dilution of the infected samples (isolate T+PPV#1-M strain). 1/16 was the highest dilution included in the panel of samples. The test may be able to detect an isolate at higher dilutions.

4.2. Diagnostic sensitivity:

95% (compared with an RT-real-time PCR adapted from Olmos et al., 2005).

4.3. Diagnostic specificity

93.8% (compared with an RT-real-time PCR adapted from Olmos et al., 2005).

4.4. Repeatability

90.9%, 11 samples tested in duplicates.

4.5. Reproducibility

90.9%, six laboratories participated.

B. Additional data from Zhang et al. (2014) and Agdia

Analytical specificity

Inclusivity: 100%, the test was evaluated with 14 isolates representing the following strains: An, C, CR, CV, D, EA, M, Rec, T and W.

Exclusivity: 100% no cross-reaction with the following non-target viruses: American plum line pattern virus, cherry leaf roll virus, peach rosette mosaic virus, pepper mottle virus, potato virus Y, prune dwarf virus, prunus necrotic ringspot virus and tomato ringspot virus.

Selectivity

No matrix effect observed with *Prunus armeniaca*, *P. avium*, *P. domestica*, *P. dulcis* and *P. persica* leaves.

APPENDIX 11 - BIOASSAY

1. Indicator plants

The main indicator plants used for PPV testing are seedlings of *P. cerasifera* cv. GF31, *P. persica* cv. GF305, *P. persica* × *P. davidiana* cv. Nemaguard, or *P. tomentosa*.

2. Preparation of the indicator plants

Indicator plants are raised from seed, planted in a well-drained soil mixture, and maintained in an insect-proof greenhouse between 18 and 25°C until they are big enough to graft (usually 25–30 cm high with a diameter of 3–4 mm). Alternatively, seedlings of other *Prunus* species may be grafted with indicator plant scions.

3. Inoculation

The indicator plants are inoculated using the chip budding technique (Desvignes, 1999) (with or without buds), using at least four replicates per indicator plant and negative and positive controls. Two 10–15 mm stem pieces originating from the plant to be tested are grafted on opposite sides of the main stem (one slightly above the other), 10–12 cm above the collar, just beneath an eye of the indicator plant. The inoculated plants are maintained in the conditions described in Section 2. After 1 week, they are pruned a few centimetres below the top (3–4 cm recommended) and pruned again 1 week later, just above the eye previously chosen (Gentit, 2006). After 2 weeks, the first leaves appear, and symptoms of *Plum pox virus* may be seen. One shoot, located just above the upper bark chip, is selected for observation of symptoms and the other buds are removed. For the detection of PPV, the grafted plants should be inspected for symptoms every week for at least 6 weeks.

Symptoms

Symptoms, in particular chlorotic banding and patterns are observed on the new growth after 3–4 weeks and should be compared with positive and healthy controls. Illustrations of symptoms caused by PPV on indicator plants can be found in Damsteegt et al. (1997, 2007) and Gentit (2006). Symptoms observed may be confused with those of other graft-transmissible agents, or potential physiological disorders.

Performance characteristics

There are no quantitative data published on the analytical specificity and analytical sensitivity of grafting for PPV detection. Bioassays are widely used in certification schemes. However, it should be noted that several PPV isolates are not detected when indexing on *P. persica* cv. GF305, and this cultivar inoculated with PPV-Rec is asymptomatic, or presents very mild symptoms (Glasa et al., 2005).

Limitations of bioassay

Limitations of the bioassay are also the duration of this test (symptom development takes several weeks post-inoculation), that it can only be used to test budwood, and requires dedicated facilities such as temperature-controlled greenhouse space. See also the comment on symptoms.

APPENDIX 12 - CONVENTIONAL RT-PCR USING PRIMERS P3D/P4B AND P3M/P4B (CANDRESSE ET AL., 1998, 2011) FOLLOWED BY SANGER SEQUENCING

The test below differs from the one described in the original publication.

1. General information

1.1. Conventional RT-PCRs followed by Sanger sequencing for the assignment of PPV strains used in ANSES (FR) are performed on nucleic acid extracts that tested positive with a detection test. Two separate amplifications using the first primer pair (P3D/P4b) and the second primer pair (P3M/P4b) should be performed.

1.2. The test can be applied to any kind of plant material.

1.3. Amplicon sequence location: CP gene.

1.4. Oligonucleotides:

Primers	Sequence (5′–3′)	Amplicon size
P3D	ACA TTG CGG AGA CAG CAC TG	467 bp
P3M	ACA TAG CAG AGA CGG CAC TC	
P4b	TGC CTT CAA ACG TGG CAC TG	

2. Methods

2.1. Nucleic acid extraction

Not relevant (same extract as for the previous positive detection tests).

2.2. Reverse transcription polymerase chain reaction—RT-PCR

Reagents	Working concentration	Volume per reaction (μL)	Final concentration
Molecular grade water	–	10.5	–
Reaction Mix (Invitrogen)[a]	2×	12.5	1×
P3D or P3M	20 μM	0.25	0.2 μM
P4b	20 μM	0.25	0.2 μM
SuperScript™ III RT/Platinum™ *Taq* Mix (Invitrogen)[a]	–	0.5	–
Subtotal	–	24.00	–
RNA	–	1.00	–
Total PCR volume	–	25.00	–

[a] From the SuperScript™ III OneStep RT-PCR System with Platinum™ Taq DNA.
Polymerase (Invitrogen).

2.3. RT-PCR cycling conditions: An initial step (42°C for 45 min and 94°C for 5 min) followed by 40 cycles (94°C for 30 s, 62°C for 30 s, and 72°C for 60 s) and one final step at 72°C for 10 min before cooling at 4°C.

2.4. PCR products should be purified, sequenced and the sequence data analysed. Sequence analysis should follow the guidelines described in Appendices 7 and 8 of the EPPO Standard PM 7/129 DNA barcoding as an identification tool for a number of regulated pests (EPPO, 2021). ANSES data was obtained using NCBI (see Section 4).

3. Essential procedural information

3.1. Controls:

For a reliable test result to be obtained, the following (external) controls should be included for each series of amplification of the target organism

- NAC to rule out false positives due to contamination during the preparation of the reaction mix: application of the amplification procedure to molecular grade water that was used to prepare the reaction mix.
- PAC to monitor the efficiency of the amplification: amplification of nucleic acid of the target strains. 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). The PAC should preferably be near to the limit of detection.

3.2. Interpretation of results: the following criteria should be followed:

Verification of the controls:
- NAC no band is visualized.
- PAC a band of 467 bp is visualized.

When these conditions are met:
- A test will be considered positive if a band of 467 bp is visualized.
- 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.

4. Performance characteristics available

The test was not validated but has been used successfully to assign at least one isolate to each of the following strains: D, M, Rec, EA, T, W and An. The sequences analysed were closely related to the following GeneBank accessions (examples given for one isolate per strain): KP198587.1 (PPV-D), KJ994236.1 (PPV-M), JQ794501.1 (PPV-Rec), DQ431465.1 (PPV-EA), EU734794.1 (PPV-T), JN596110.1 (PPV-W), HF674399.1 (PPV-An). For instance, PPV-D isolates were assigned after sequence analysis of amplicons obtained using the first primer pair, whereas PPV-M isolates were assigned using the second one. These primer pairs may also be used to assign isolates to other strains. A weak amplicon was obtained with one isolate of strain C that could not be assigned reliably after sequencing. No amplicon was produced with one isolate of the CR strain. The test was not evaluated with isolates of the CV strain. The test is suitable for single infections and may highlight co-infections.