DOI: 10.1111/epp.70086

EPPO STANDARD ON DIAGNOSTICS

PM 7/113 (2) *Potexvirus pepini* (pepino mosaic virus)

Specific scope: This Standard describes a Diagnostic Protocol for the detection and identification of pepino mosaic virus in all plant parts including seeds.[1]
This Standard should be used in conjunction with PM 7/76 Use of EPPO Diagnostic Standards.
Specific approval and amendment: First approved in 2012-09. Revision approved in 2026-06. Main revisions include updates of the available tests and their validation data, as well as addition of two new tests: a conventional RT-PCR (Mumford & Metcalfe, 2001) and a real-time RT-PCR tests for strain identification of Pepino mosaic virus (Gutiérrez-Aguirre et al., 2009). The revision also includes updates of the flow diagram and of the Sections on samples preparation for serological and molecular tests, and RNA extraction.

Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Pepino mosaic virus (PepMV) was originally described from pepino (*Solanum muricatum*) in Peru in 1974. From 1999 on, the United Kingdom, Spain and the Netherlands reported outbreaks of PepMV in tomato crops. For an updated geographical distribution consult EPPO Global Database (EPPO, 2026a). In 2004, the EU implemented Decision 2004/200/EC, to prevent the introduction and spread of PepMV within the EU. This decision was repealed in 2020 and PepMV was added to Part F of Annex IV to Commission Implementing Regulation (EU) 2019/2072 listing PepMV as regulated non-quarantine pest (Commission Implementing Regulation (EU) 2020/1549). Symptoms of PepMV may be variable, ranging from latent infections (no visible symptoms) to severe leaf and fruit symptoms. The type and severity of symptoms on tomato depend not only on the genetic characteristics of the virus strain, but also on the environmental conditions prevailing during the growing season, as well as the virus concentration and physiological condition of the plants. In general, PepMV symptoms depend on the specified virus isolate in combination with the tomato cultivar. Particular point mutations in the genomes of PepMV isolates have been identified as being involved in the severity of symptoms (Hasiow-Jaroszewska & Borodynko, 2012; Hasiow-Jaroszewska, Borodynko, & Pospieszny, 2009a). Mixed infections of different genotypes may occur (Pagán et al., 2006). It has been suggested that coinfections by two genotypes (EU and CH2) or infection with a recombinant PepMV isolate can result in more severe PepMV symptoms (Hanssen et al., 2008).

Currently clusters of PepMV isolates are distinguished and considered as separate genotypes or strains sharing complete nucleotide sequence identities ranging from 78% to 95%: the Peruvian pepino strain (LP), the European tomato strain (EU), the Chile-2 strain (CH2), the American strains (US1, US2 (a recombinant between US1 and CH1)), and the Peruvian strains (PES), identified from wild *Solanum* species in Peru (Hanssen et al., 2008; Hasiow-Jaroszewska, Pospieszny, & Borodynko, 2009b; Ling et al., 2007; Maroon-Lango et al., 2005; Moreno-Pérez et al., 2014). Complete nucleotide sequence comparisons between multiple isolates of the different strains show that the LP and EU strains are the most closely related with around 95% nucleotide sequence identity.

Sequence analyses of PepMV isolates over time have shown that in Europe and in North America, the EU isolates have been replaced by CH2 isolates (Moreno-Pérez et al., 2014).

PepMV is efficiently transmitted by mechanical means, i.e. fruit harvesting, pruning, and other cultural practices lead to rapid spread in tomato crops. In addition, bumblebees have been associated with PepMV transmission in glasshouses (Shipp et al., 2008). The transmission rate of PepMV with seeds has been reported as approximately 0.005–0.057% (Hanssen & Thomma, 2010). Available evidence suggests that PepMV does not infect the embryo or endosperm but contaminates the seed coat (Hasiow-Jaroszewska, Borodynko, & Pospieszny, 2009a). Long distance spread of PepMV is thought to be through contaminated seeds or infected transplants (Hanssen & Thomma, 2010). Infected fruits are also likely to contribute to long distance virus transmission (EPPO, 2026b).

The reported host range of PepMV appears to be relatively broad with plants from different families

[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.*

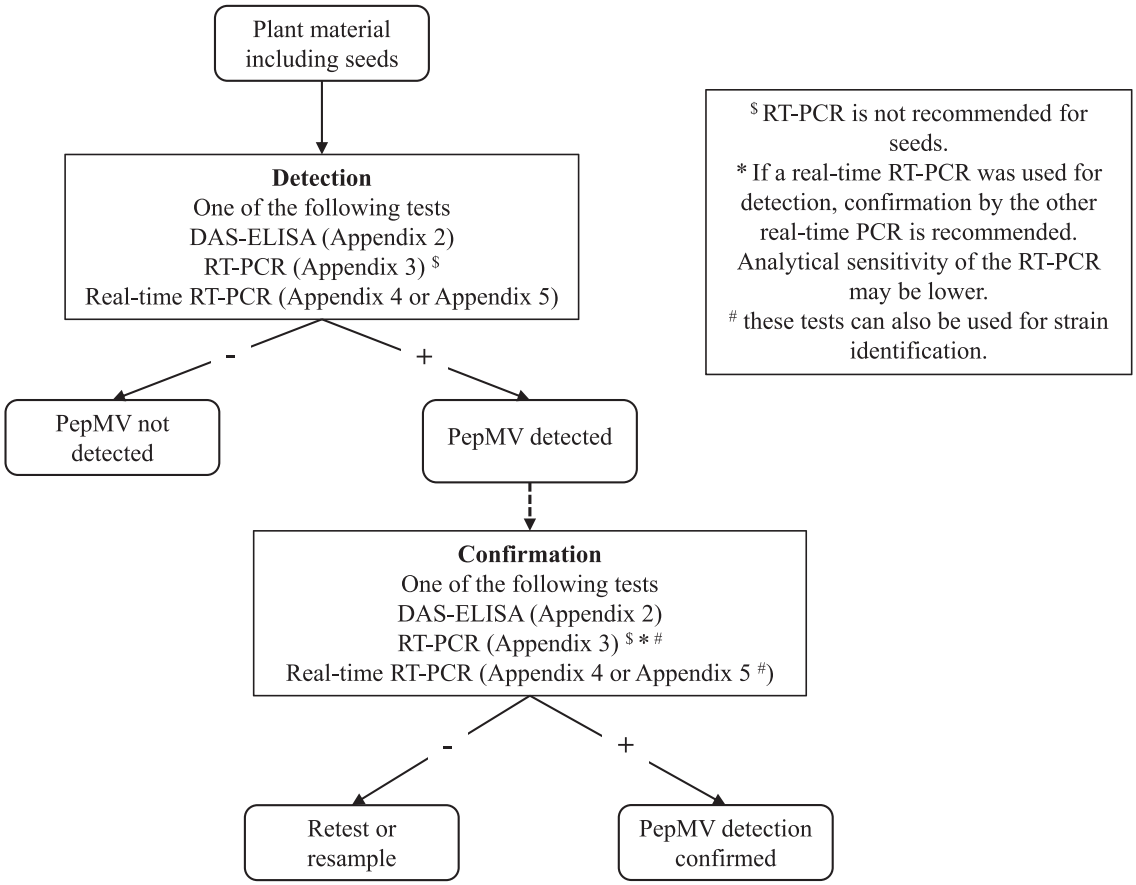

FIGURE 1 Flow diagram describing the diagnostic procedure for pepino mosaic virus in plant material including seeds. This flow diagram is intended to provide an overview of the diagnostic process and may not cover all possible scenarios.

including both cultivated and wild plant species. PepMV infects many members of the Solanaceae family, and *Solanum* spp. appear to include its main hosts in terms of economic damage. However, a number of vegetable, ornamental and wild plant species can be infected with PepMV at least experimentally. Therefore, PepMV may be a risk for other crops. Consult the EPPO Global Database (EPPO, 2026a) for the list of currently known hosts.

Licenced PepMV vaccines are currently commercially available. The effect of the vaccine is based on the phenomenon of cross-protection. Tomato plants inoculated with a mild isolate are cross-protected from the damage induced by a more severe PepMV strain (Agüero et al., 2018). It should be noted that the vaccinating isolates cannot be distinguished from the other strains using the tests described in this Standard.

A datasheet providing more information on the biology is also available in EPPO Global Database (EPPO, 2026b).

A flow diagram describing the diagnostic procedure for PepMV in plant material and in seeds is presented in Figure 1.

2 | IDENTITY

Name: Pepino mosaic virus
Acronym: PepMV
Taxonomic position: Viruses and viroids: Riboviria: Orthornavirae: Kitrinoviricota: Alsuviricetes: Tymovirales: Alphaflexiviridae: Potexvirus: *Potexvirus pepini*
EPPO Code: PEPMV0
Phytosanitary categorization: EPPO A2 no. 369; EU RNQP (Annex IV[2]).

Note Virus nomenclature in Diagnostic Standards is based on the latest release of the official classification by the International Committee on Taxonomy of Viruses (ICTV, Release 2024, https://talk.ictvonline.org/taxonomy/). All accepted viral species have a binomial species name which is used in a taxonomic context, whereas their common English virus names and acronyms are used throughout the EPPO

[2]Commission Implementing Regulation (EU) 2019/2072.

Diagnostic Standards. Names of viruses not included in the official ICTV classification are based on first reports.

3 | DETECTION

3.1 | Symptoms

Symptoms induced by PepMV depend on the strain of PepMV, the host plant, host cultivar as well as environmental conditions. Symptoms may be noticeable as yellow mosaic in young leaves of pepino plants or as yellow/pale-yellow/whitish spots, mild interveinal chlorosis, nettle heads or minor leaf deformation on infected tomato plants (Figures 2 and 3). Leaves may also become chlorotic or display blistering (Figure 4). Fruit from infected tomato plants may have yellow discoloration or marbling (Figures 5 and 6), sometimes showing fruit cracking or malformation. Sepal necrosis can be observed (Hanssen et al., 2009). Unripe fruit may turn dark brown with cracking. Leaf and stem necrosis have also been observed occasionally.

3.2 | Test sample requirements

The virus concentration in different plant parts can vary significantly. Based on available data the following recommendations are made regarding test sample requirements.

3.2.1 | Plant material including fruits

Tables 1, 3 and 4 of ISPM 31 *Methodologies for sampling of consignments* (IPPC, 2008) provide information on the number of units to be sampled to detect varying levels of infection depending on the size of a lot and for different confidence levels. These tables are considered useful to determine sample sizes for both consignments and places of production. Experimental data to recommend a specific sample size is lacking. The following recommendations prepared for detection of tomato brown rugose fruit virus in tomatoes and peppers (EPPO Diagnostic Standard PM 7/146 (2) *Tomato brown rugose fruit virus* (EPPO, 2022a)) are also recommended for pepino mosaic virus: In

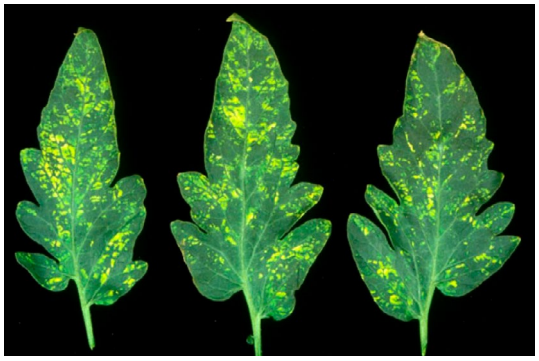

FIGURE 2 Bright yellow spots on tomato leaves. Courtesy: R. van der Vlugt, WUR (NL).

FIGURE 4 Leaf blistering symptoms on tomato plants. Courtesy: R. van der Vlugt, WUR (NL).

FIGURE 3 Pale yellow to whitish areas on tomato leaves with deformation. Courtesy: NVWA-NIVIP (NL).

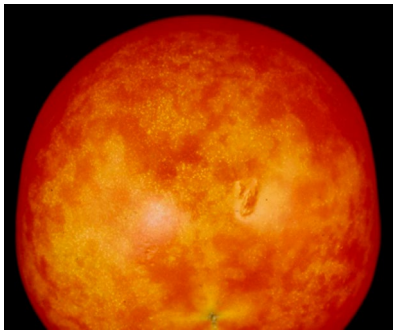

FIGURE 5 Yellow discoloration of tomato fruit. Courtesy: R. van der Vlugt, WUR (NL).

FIGURE 6 Discoloured patches on tomato fruits. Courtesy: A. Minoto, Centro di Saggio, CERSAA, Albenga (IT).

the Netherlands and the United Kingdom, for asymptomatic plants, sampling is based on the collection of 200 leaflets/sepals (from fruits) per site of production and cultivar. This corresponds to the number of units that should be sampled to achieve a 99% confidence level to detect an infection level × efficacy of detection of approximately 2%. Young leaflets should be collected from the top or side shoots of different plants. For molecular testing leaflets/sepals can be pooled, however the number of leaflets /sepals that can be tested in a subsample should be validated. For example, in the United Kingdom a maximum of 10 leaflets are pooled for one test corresponding to 20 subsamples to be tested for one sample of 200 leaflets (A. Fox pers. comm.). In France 20–40 leaflets are pooled for one test (P. Gentit, pers. comm.). In Italy and the Netherlands, pooling up to 50 and 60 leaflets or sepals, respectively, has been shown to be effective (L. Tomassoli, pers. comm.; Naktuinbouw, unpub. data).

For symptomatic plants, sampling for laboratory testing is based on at least three symptomatic young leaflets collected from the top of the plants or shoots. These recommendations are also applicable to the detection of PepMV.

Details on sample preparation can be found in Appendix 1.

3.2.2 | Seeds

Tables 1, 3 and 4 of ISPM 31 *Methodologies for sampling of consignments* (IPPC, 2008) provide information on the number of units to be sampled to detect varying levels of infection depending on the size of a lot and for different confidence levels

The recommended minimum sample size for ELISA tests (Appendix 2) is 3000 seeds with a maximum subsample size of 250 seeds (ISF, 2023). For a test with an efficacy of detection of 100%, a sample of 3000 seeds allows an infection level of 0.1% to be detected with a confidence level of 95% in lots of more than 200 000 seeds (IPPC, 2008). For molecular tests, subsamples sizes of 1000 seeds can be used (ISF, 2023).

Depending on the size of the batch being tested and the expected lowest level of seed infection that can be detected with a predetermined level of confidence smaller samples (e.g. <3000 seeds) may be used.

Seed treatments might influence the outcome of a test. Therefore, tests should be performed on seeds that are not primed, pelleted and/or coated unless it has been verified that these treatments have no significant effect on the performance of the test. Internal controls can be used to monitor possible inhibition

Details on sample preparation can be found in Appendix 1.

3.3 | Detection tests

3.3.1 | Recommended tests

The following tests are recommended for the detection of PepMV in plant material including seeds except the conventional RT-PCR which is not recommended in seeds.

- DAS-ELISA (PM 7/125; Appendix 2)
- Conventional RT-PCR (Mumford & Metcalfe, 2001; Appendix 3)
- Real-time RT-PCR (Ling et al., 2007; Appendix 4)
- Real-time RT-PCR (Gutiérrez-Aguirre et al., 2009; Appendix 5)

In critical cases, confirmation is performed. If a molecular test was used for detection, confirmation by DAS-ELISA is not recommended as the analytical sensitivity of DAS-ELISA may be lower. Therefore, a different molecular test should be used. If real-time RT-PCR was used for detection, confirmation by conventional RT-PCR is not recommended. The other real-time RT-PCR test should be used in this instance.

3.3.1.1 | *Serological test: DAS-ELISA*

DAS-ELISA is a recommended test for the detection of PepMV and can readily be used to detect the virus in plant and fruit tissue as well as seeds. Commercial kits for the

serological detection of PepMV are available. Further information on DAS-ELISA can be found in Appendix 2 and in PM 7/125 (2) *ELISA tests for viruses* (EPPO, 2025).

3.3.1.2 | *Molecular tests*

Several molecular tests have been described for the detection of PepMV. The recommended tests (see above) were selected based on the availability of reagents and validation data, and the performance of the tests, in particular regarding the analytical sensitivity and analytical specificity.

3.3.2 | Other detection tests

3.3.2.1 | *Bioassay*

PepMV can easily be transferred mechanically from infected leaf or fruit material to test plants. Detailed guidance on mechanical inoculation of test plants can be found in PM 7/153 *Mechanical inoculation of test plants* (EPPO, 2022b). Bioassays can only be used for virus detection and propagation. A bioassay may be used to confirm infectivity of PepMV derived from seed extracts (ISF, 2023). As the analytical sensitivity is low and can be very variable, bioassay is not recommended to detect PepMV in seeds. This test is therefore not described in this Diagnostic Standard.

3.3.2.2 | *High-throughput sequencing analysis*

High-Throughput Sequencing (HTS) can be used for the detection of PepMV in symptomatic plant material (see the test described in the annex 1 of the supporting information S2 of PM 7/151 *Considerations for the use of high throughput sequencing in plant health diagnostics* (EPPO, 2022c), and the associated validation data available in the EPPO Database on Diagnostic Expertise (https://dc.eppo.int/validation_data/dwvalidation?id=512)). The use of an HTS test should follow the guidelines provided in the EPPO Standard PM 7/151 (EPPO, 2022c).

4 | IDENTIFICATION

4.1 | Identification of PepMV

The tests recommended in section 3 (except bioassays) for detection also enable identification of PepMV at the species level.

4.2 | Identification of pepino mosaic virus strains

Many molecular tests have been developed for the identification of PepMV strains. Most tests are based on the amplification of viral nucleic acids, either using specific or generic primers to different target regions (Gutiérrez-Aguirre et al., 2009; Mumford & Metcalfe, 2001). Different tests are able to distinguish between different strains in mixed infections (multiplex RT-PCR; real-time RT-PCR) (Alfaro-Fernandez et al., 2009).

The following tests are recommended for the strain identification of pepino mosaic virus:

- In plant material excluding seeds, RT-PCR followed by amplicon sequencing (Mumford & Metcalfe, 2001; Appendix 3) (when the virus concentration allows for its application). Sequence analysis of amplicons obtained from the RT-PCR test 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 under revision).
- In plant material including seeds, real-time RT-PCR (Gutiérrez-Aguirre et al., 2009; Appendix 5)

High-throughput sequencing may be used to obtain complete or near complete genome sequences when the virus concentration allows for its application, and analysis of the obtained sequences can be used for identification of the strain(s). The application of HTS test should follow the guidelines in the EPPO Standard PM 7/151 *Considerations for the use of high throughput sequencing in plant health diagnostics* (EPPO, 2022c).

5 | REFERENCE MATERIAL

Reference material infected with each of the major strains of PepMV can be obtained from

- DSMZ-German collection of micro-organisms and cell cultures GmbH, Leibniz, D, www.webshop.dsmz.de. E-mail: plantvirus@dsmz.de.
- Prime Diagnostics, Wageningen Plant Research, Wageningen, NL, www.primediagnostics.com. E-mail: prime.diagnostics@wur.nl.
- Scientia Terrae VZW, Fortsesteenweg 30A, B-2860, St-Katelijne-Waver, Belgium, https://scientia.be/en. E-mail: contact@scientia.be.

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 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 (https://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

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9 | FEEDBACK ON THIS DIAGNOSTIC STANDARD

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

10 | STANDARD REVISION

A regular review process is in place to identify the need for revision of Diagnostic Standards. Standards 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 Diagnostic Standard was originally drafted by: H.J. Vetten (DE), R.A. Mumford (GB) and R. van der Vlugt (NL). The revision was prepared by: H. Ziebell, Julius Kühn-Institut, Institut für Epidemiologie und Pathogendiagnostik (DE), N. Mehle, National Institute of Biology (SI), A. Tiberini, CREA-DC Council for Agricultural Research and Economics, Research Centre for Plant Protection and Certification (IT), M. Hejlova and H. Orsagova, Central Institute for Supervising and Testing in Agriculture, Division of Plant Pest Diagnostics (CZ), J. Piecinska and A. Rosinska, Main Inspectorate of Plant Health and Seed Inspection Service, Central Laboratory (PL), J. Mihailova, State Plant Protection Service (LV). The Standard was reviewed by the Panel on Diagnostics in Virology and Phytoplasmology.

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APPENDIX 1 - SAMPLE PREPARATION FOR SEROLOGICAL AND MOLECULAR TESTS, AND RNA EXTRACTION

This appendix describes sample preparation for serological and molecular tests, and RNA extraction methods for molecular tests, for different types of plant material.

1. Sample preparation for ELISA

Sample preparation of plant material is described in PM 7/125 (2) *ELISA tests for viruses* (EPPO, 2025).

When testing seed samples, care should be taken to avoid cross contamination e.g. place a paper or plastic sheet on the bench when grinding and handling the samples. It is recommended that healthy controls are processed at the end to evaluate potential cross-contamination.

Preparing subsamples can be performed on the basis of the mean weight of 5–10 subsamples of 250 tomato seeds. Each subsample is placed in a special extraction bag with a filter (e.g., Bioreba) to which approximately 10 mL 0.1 M phosphate buffer (Table A1) is added. Bags can be closed by heat-sealing. Make sure all seeds are immersed in buffer and refrigerate bags (4 ±2°C) overnight. Place the seed- and buffer-containing extraction bag on a flat, hard surface and grind the seeds in the bag using a hand or use a semi-automated homogenizer (e.g., Bioreba). After grinding for 1 min, the liquid in the bag should turn milky. Continue with grinding for another minute until the milky discoloration of the buffer becomes more intense [Note that complete grinding of seeds is not accomplished with this method - it is not required]. Transfer the milky liquid (approximately 7 mL) in a 10-mL vial or tube. The extracts should either be kept on ice and used the same day or stored at approximately −20°C until use.

TABLE A1 Extraction buffer (0.1 M phosphate buffer, pH 7.2).

		Final concentration
Solution A	Na_2HPO_4	0.1 M
Solution B	KH_2PO_4	0.1 M

Note: Prepare 0.1 M phosphate buffer (pH 7.2) by mixing approximately 500 mL of 0.1 M Na_2HPO_4 with approximately 250 mL 0.1 M KH_2PO_4 solution until the pH is 7.2.

2. Sample preparation for molecular tests

Sample preparation and RNA extraction procedures for different hosts and types of plant material are critical

for the results of a test and the selection of a given procedure is generally more dependent on the matrix than on a specific test. RNA extraction using CTAB (e.g. Gambino et al., 2008) or other extraction methods or kits used, should be validated in combination with the molecular test to be used. Extracted RNA should be stored refrigerated for short-term storage (<8 h), at approximately −20°C (<1 month) or at approximately −80°C for long term storage. Care should be taken to avoid cross-contaminations when handling samples especially when high virus concentrations can be expected.

2.1 Plant material (leaves or fruits)

2.1.1 Individual plants

For testing small numbers of individual plants, the RNeasy Plant Mini kit (Qiagen) or other RNA extraction kits can be used according to the manufacturer's instructions.

2.1.2 Pooled samples

Pooled samples of tomato leaflets, sepals or fruits should consist of equal amounts of each plant. For leaf material, this can be achieved e.g. by stacking leaves and preparing leaf discs using a disposable 4 mm leaf punch, cutting or tearing the top parts.

For fruit samples, a piece of the skin and adjacent tissue should be sampled from at least three different locations of each fruit.

For pooled samples involving large amounts of plant material, ELISA buffer, phosphate buffer or GH+ buffer as specified in Table A2 can be used for homogenization. It is recommended that the molecular test is validated/verified using the selected buffer.

2.1.2.1 Procedure using phosphate buffer or ELISA buffer

Plant tissue is ground in 0.1 M phosphate buffer (Na_2HPO_4 /KH_2PO_4, pH 7.2) or ELISA buffer, at a range of 1:10 [w/v]. 100 µL of the obtained homogenate are added to 380 µL of the RLT buffer of the RNeasy Plant Mini kit (Qiagen) and RNA is then extracted following the manufacturer's instructions.

2.1.2.2 Procedure using GH+ buffer

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 (see Table A2). Samples are incubated for 10 min at 65°C. After centrifugation extract at 12 000 *g* for 2 min, 500 µL of supernatant is loaded on the QIAshredder spin column (Qiagen) and centrifuged. Thereafter, manufacturer's instructions in the RNeasy Plant Mini kit (Qiagen) should be followed.

TABLE A2 GH+ buffer.

	Amount	Final concentration
Guanidine hydrochloride	573.18 g	6 M
Sodium acetate (4M, 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	1L	

2.1.2.3 Procedure for high-throughput RNA extraction

For high throughput RNA extraction, the Sbeadex Maxi Plant DNA Purification Kit (LGC Biosearch Technologies) can be used in combination with a Kingfisher KF96 system. The samples are processed as described above (see 2.1.2.2) and 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.

2.2 Seeds

Sample preparation, including grinding, and RNA extraction from seeds can be challenging. Two procedures using different homogenization buffers are described that have been used for detection of ToBRFV in seeds of tomato and pepper and allow a similar sample preparation for detection of PepMV and other RNA viruses In the Euphresco project 2019-A-327 (Giesbers et al., 2021), using GH+ buffer homogenates resulted in lower average C_t values for the real-time RT-PCR tests when testing for ToBRFV, with an average difference of around one cycle compared to phosphate buffer. For the other molecular tests, both extraction buffers gave similar C_t values regarding the lowest dilution to be detected.

2.2.1 Homogenization in phosphate buffer

Twelve subsamples of 250 seeds are prepared. The subsamples are immersed in 10 mL of 0.1 M phosphate buffer (Table A1), incubated at approximately 4°C overnight, and then ground with, for example, a FastPrep homogenizer at 5 m s^{-1} 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 as explained below. The homogenates can be processed separately or combined to three subsamples each of four homogenates.

600 µL of the supernatant is added to 600 µL of RLT buffer (without 2-mercapto-ethanol). Two aliquots of 600 µL of this mix are loaded one after the other on the same QIAshredder spin column and centrifuged. Subsequently, the manufacturer's protocol is followed until the elution step. RNA is eluted from the RNeasy

Mini Spin columns by applying 50 µL of RNase-free hot water (65°C) followed by centrifugation. To maximize RNA recovery, an additional elution step is performed using the same procedure.

2.2.2 Homogenization using GH+ buffer

The RNeasy Plant Mini Kit (Qiagen) is used with the following modifications: the RLT buffer is replaced by GH+ buffer and the centrifugation temperature is decreased to 4°C at all steps to optimize RNA extraction from seeds.

- The three subsamples of 1000 seeds are transferred to a grinding bag (e.g. Interscience BagPage 100 mL) and 20 mL of GH+ buffer (Table A2) 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.

- Alternatively, dry seeds are ground (e.g. with a Genogrinder). Three subsamples of 1000 seeds are transferred to a 50 mL tube and a steel ball (14 mm) is added. When using a Genogrinder, seeds are ground, tubes upside down, at 1700 rpm for 4 min. After grinding, 20 mL GH+ buffer is added. Tubes are shaken by hand to obtain homogenous solutions.

For each subsample, 1.0 mL 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 of the RNeasy Plant Mini Kit (Qiagen) are followed (with centrifugation at 4°C as stated above).

For high-throughput RNA extractions, the Sbeadex Maxi Plant DNA Purification Kit (LGC Biosearch Technologies) can be used in combination with a Kingfisher KF96 system. The samples are processed as described above, following centrifugation, 250 µL of the supernatant is transferred to a binding plate containing 600 µL of binding buffer (kit) and 50 µL of particle suspension (both included in the kit), and subsequently RNA is extracted following the manufacturer's instructions.

APPENDIX 2 - DAS-ELISA

DAS-ELISA can be used for detection and identification (to species level) of PepMV on tomato leaf material, fruit and seeds. Details on sample preparation and execution of DAS-ELISA tests can be found in Appendix 1 and PM 7/125 *ELISA tests for viruses*. Several PepMV antibodies are available from various suppliers which may differ in the reactivity against individual virus strains.

Performance characteristics available

1. Prime Diagnostic antibodies

- **Prime Diagnostics validation sheet (Prime Diagnostics, 2023)**

Analytical sensitivity: Detection limit below 24 pg purified PepMV per well.
Analytical specificity: 100%.
Inclusivity: detection of PepMV (10 isolates of PepMV-tom (PepMV-EU), 4 different isolates of PepMV-CH2, PepMV-US1, isolate PepMV-Peru1).
Exclusivity: no cross-reaction with 20 isolates of viruses infecting tomato: cucumber mosaic virus (CMV), hosta virus X, potato aucuba mosaic virus (PAMV), potato virus X (PVX), tomato black ring virus (TBRV), potato virus X (PVX), tomato aspermy virus, tomato brown rugose fruit virus (ToBRFV), tobacco mosaic virus (TMV), tomato mosaic virus (ToMV), tomato spotted wilt virus (TSWV).
Repeatability: 100%.
Reproducibility: no data.

- **NVWA-NIVIP (NL)**:

Validation of this antiserum was carried out according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity*:
Analytical sensitivity: 10^{-4} for infected tomato leaf material diluted in non-infected leaf material for 4 isolates of different strains (see inclusivity).
Analytical specificity:
Inclusivity: 100%, isolates of the following strains detected: PepMV-tom (PepMV-EU), PepMV-CH2, PepMV-US1, and a Peruvian isolate (BB1137).
Exclusivity: 100%, no cross-reactions observed for CMV, PAMV, PVX, PVY and TSWV.
Selectivity: Dilution in plant sap of leaves from tomato and *Nicotiana occidentalis* P1 gave slightly lower OD values in comparison to dilution in buffer.

- **ANSES (FR) (Saison & Tassus, 2012)**

Analytical sensitivity: 1000× diluted extract of infected tomato seeds in extract of healthy tomato seeds.
Diagnostic sensitivity (DSE) was evaluated on 14 samples (3 repetitions each) including naturally infected seeds and tomato leaves and healthy seed extract spiked with infected lyophilized tomato leaves (4 different strains of PepMV-CH2, PepMV-US1, PepMV-EU, PepMV-LP). DSE = 95% (false negative results for 2 repetitions of 1 sample).
Diagnostic specificity (DSP) was evaluated on 19 samples (3 repetitions each) including 8 non-infected tomato seed samples, and samples infected by citrus yellow

mosaic virus (CiYMV), papaya mosaic virus (PapMV), white clover mosaic virus (WClMV), cymbidium mosaic virus (CymMV), PVX, tomato torrado virus (ToTV), tomato chlorosis virus (ToCV), tomato infectious chlorosis virus (TICV) and TMV. DSP=95% (false positive results for 1 out of the 3 repetitions of 2 healthy tomato seeds samples and on sample infected with CymMV (a potexvirus specific to vanilla)).

Repeatability: 94%.

Reproducibility: No data.

2. Bioreba antibodies

- **Bioreba validation report** (Bioreba, 2022).

Analytical sensitivity: no data.

Diagnostic sensitivity: 100%.

Analytical specificity: 100%.

Inclusivity: detection of: PepMV 7061 AZ-2 (US, Tobacco), PepMV 1297 AZ-2 (US, Tomato), PepMV 1338 TX-1 (US, Tomato), PepMV TX-1 (US, Tobacco), PepMV CH1/CH2 (US, Tomato), PepMV CH1/CH2 (US, Tobacco) PepMV 1279 (Switzerland, Tomato), PepMV various seed lots mildly or heavily infected (Europe, Tomato).

Exclusivity: no cross-reaction known, tested with CMV (Cucumber mosaic virus), GLRaV-7 (Grapevine leafroll-associated virus 7), LMV (Lettuce mosaic virus), PVX (Potato virus X), TMV (Tobacco mosaic virus), ToMV (Tomato mosaic virus).

Diagnostic specificity: 100%.

Selectivity: No matrix effect observed with *Nicotiana occidentalis* (Tobacco), *Solanum lycopersicum* (Tomato, leaf & seed), *Solanum muricatum* (Pepino).

- **ANSES (FR) (Saison & Tassus, 2012)**

Analytical sensitivity: 1000× diluted extract of infected tomato seeds in extract of healthy tomato seeds.

Diagnostic sensitivity (DSE) was evaluated on 14 samples (3 repetitions each) (see details above for PRI). DSE=95% (false negative results for 2 repetitions of 1 sample).

Diagnostic specificity (DSP) was evaluated on 19 samples (3 repetitions each) (see details above for PRI). DSP=98% (false positive results for 1 out of the 3 repetitions of 1 healthy tomato seeds sample).

Repeatability: 94%.

Reproducibility: No data.

3. Loewe antibodies

- **NRL (CZ):** Loewe antibodies

Analytical sensitivity: 1000 × diluted infected tobacco leaves in extraction buffer.

Analytical specificity:

Inclusivity: 100%: three different isolates of PepMV detected.

Exclusivity: 100%: no detection of CMV, kyuri green mottle mosaic virus (KGMMV), tomato bushy stunt virus (TBSV), tomato black ring virus (TBRV), TMV, ToMV.

Repeatability: 100%.

Reproducibility: 100%.

APPENDIX 3 - Conventional RT-PCR (Mumford & Metcalfe, 2001)

The test below differs from the one described in the original publication (see 1.2).

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 is carried out (see PM 7/98).

1. General Information

1.1 This test can be used for the detection of PepMV in tomato leaf material and fruits. For identification of PepMV strains, amplicons should be sequenced.

1.2 This conventional RT-PCR test was initially described by Mumford and Metcalfe (2001). It was adapted and validated by the NRL of the Czech Republic.

1.3 The target sequence of the primers is located between the triple gene block (TGB) and the 3′UTR amplifying the complete ORF for the CP resulting in an amplicon of 844 bp.

1.4 Oligonucleotides:

	Name	Sequence	Amplicon size*
Forward primer	TGBf	5′- CAC ACC AGA AGT GCT TAA AGC A -3′	844 bp
Reverse primer	UTRr	5′- CTC TGA TTA AAG TTT CGA GTG -3′	

* Including primer sequences.

1.5 The test has been validated using QIAGEN OneStep RT-PCR Kit and performed on different PCR cyclers including TProfessional Trio (Biometra) and XP Cycler (Bioer).

2. Methods

2.1 Nucleic Acid Extraction

See Appendix 1.

RNA should preferably be stored at approximately −20°C for short term (<1 month) or at approximately −80°C for long term storage.

2.2 Conventional OneStep RT- PCR

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	13.0	N.A.
OneStep RT-PCR Buffer (*Qiagen*)	5×	5.0	1×
QIAGEN dNTP mix	10 mM	1.0	0.4 mM
Forward primer (TGBf)	10 µM	1.5	0.6 µM
Reverse primer (UTRr)	10 µM	1.5	0.6 µM
QIAGEN OneStep RT-PCR Enzyme Mix	25×	1.0	1×
Subtotal		23.0	
RNA extract		2.0	
Total		25.0	

2.2.2 PCR conditions: Reverse transcription at 50°C for 30 min, initial denaturation at 95° for 15 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min, store 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 nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- 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: 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).
- 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). The PAC should preferably be near to the limit of detection.

As alternative or in addition to the PIC, internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include endogenous nucleic acid of the matrix using primers preferably amplifying RNA targets such as *nad5* (Menzel et al., 2002). However, for seed samples, *nad5* might not perform consistently. In this case, COX or ribosomal 5S (Kolchinsky et al., 1991; Weller et al., 2000) can be used as IPC.

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:** In order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC, PAC a band of the expected size [844 bp] is visualized. 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 the expected size [844 bp] is visualized. If relevant, a band of the expected size is visualized for the IPC.
- A test will be considered negative, if no band or a band of a different size than expected is visualized.
- Tests should be repeated if any contradictory or unclear results are obtained.

It should be noted that in virology bands of different sizes may correspond to strains of the target organism and care should be taken when interpreting conventional PCR products.

For strain identification, amplicons should be sequenced.

Guidance for sequence analysis is given in Appendices 7 and 8 of EPPO Standard PM 7/129 *DNA Barcoding as*

an identification tool for a number of regulated plant pests (EPPO, 2021; under revision).

4. Performance characteristics available

The test was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* by the NRL of the Czech Republic.

4.1 Analytical sensitivity

100 000 × diluted (in water) RNA extract from tomato fruits.

4.2 Analytical specificity

Inclusivity: 100%, four different isolates were detected (two originated from Spain, one from Morocco, one from the Netherlands).

Exclusivity: 100%, four isolates of TSWV, and one isolate of the following viruses: pepper mild mottle virus (PMMoV), tomato apical stunt viroid (TASVd), TBRV, tomato chlorotic dwarf viroid (TCDVd), ToMV, tomato ringspot virus (ToRSV), and tomato yellow leaf curl Sardinia virus (TYLCSV). No cross-reactions observed.

4.3 Repeatability

100%

4.4 Reproducibility

100%

APPENDIX 4 - Real-time RT-PCR test adapted from Ling et al. (2007)

The test below is described in Ling et al. *(2007). It was adapted 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 test can be used for the detection of PepMV in tomato leaf material, fruit and seeds. The test was designed for the 5 clusters of PepMV strains: EU, CH1, CH2, LP, and US2.

1.2 This method is based on the test published by Ling et al. (2007), with modifications (Van der Vlugt et al., 2007). A commercial kit based on this test is also available from www.qualiplante.eu.

1.3 The target sequence of the PepMV primers is located within the triple gene block 2 (TGB 2) protein gene; using the nucleotide sequence of GenBank accession no DQ_000984 (positions 5126-5232, isolate CH1).

1.4 Primers amplify a fragment of 107 bp.

1.5 Oligonucleotides:

	Name	Sequence	Strain specificity
Forward primer 1	KL05-48	5′-ACT CCT AGA GCT GAC CTC AC -3′	CH1, US1, EU, LP, PES
Forward primer 2	KL05-49	5′-ACT CCT AGA GCT GAT CTT AC -3′	CH2, US2
Probe	KL05-50	5′-FAM-TGT CAG CTT GCA TTT ACT TCC AAA A-BHQ-1 -3′	All PepMV isolates
Reverse primer 1	KL05-51	5′-TCT CCA GCA ACA GGT TGG TA -3′	CH1, US1, EU, LP, PES
Reverse primer 2	KL05-52	5′-TCA CCT GCA ACT GGT TGA TA -3′	CH2, US2

1.6 The test has been validated using several chemicals as: TaqMan™ RNA-to-Ct™ *1-Step* Kit (ThermoFisher), AgPath-ID™ One-Step RT-qPCR mix (Thermo Fisher), and using a range of different real-time systems including CFX Maestro v2.2 (Bio-Rad), QuantStudio™, 7 Pro (Applied Biosystems) and ViiA7™ (Applied Biosystems).

2. Methods

2.1 Nucleic acid extraction

See Appendix 1.

RNA should preferably be stored at approximately −20°C for short term (<1 month) or at approximately −80°C for long term storage.

2.2 One-step real-time RT-PCR

2.2.1 Master Mix (with TaqMan RNA-to-Ct™ *1-Step* kit, ThermoFisher):

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	5.6	N.A.
Mastermix (from the kit)	2×	10.0	1×
KL05-48	10 µM	0.6	0.3 µM
KL05-49	10 µM	0.6	0.3 µM
KL05-51	10 µM	0.6	0.3 µM
KL05-52	10 µM	0.6	0.3 µM
KL05-50	10 µM	0.5	0.25 µM
RT-enzyme (from the kit)	40×	0.5	1×
Subtotal		19.0	
RNA extract		1.0	
Total		20.0	

2.2.2 Real-time RT-PCR cycling conditions: Reverse transcription at 48°C for 30 min; denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s and 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 (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- 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: 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).
- 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). The PAC should preferably be near to the limit of detection.

As alternative or in addition to the PIC, internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include endogenous nucleic acid of the matrix using primers preferably amplifying RNA targets such as *nad5* (Botermans et al., 2013). However, for seed samples, *nad5* might not perform consistently. In this case, COX (e.g. Weller et al., 2000 or Papayiannis et al., 2011) can be used as IPC.

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:** In order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC should give no amplification.
- The PIC and PAC (and if relevant IPC) amplification curves should be exponential.

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

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

4.1 Validation data from Ling et al. (2007)

Validation data were obtained using the FullVelocity™ QRT-PCR Master Mix (Stratagene, La Jolla, CA) and a Mx3000P real-time PCR system (Stratagen, La Jolla, CA).

Analytical sensitivity
20 pg of infected total RNA and 1 artificially contaminated seed in 1000 healthy seeds (no lower concentrations were tested).

Analytical specificity
Isolates of 4 strains (US1, US2, CH1, CH2) and 25 field isolates from USA and Canada detected.

4.2 Validation data available from Gutiérrez-Aguirre et al. (2009)

Validation data were obtained using the high capacity cDNA archive kit (Applied Biosystems, Foster City, USA) combined with the TaqMan™ universal PCR master Mix (Applied Biosystems) for a 2-step test real-time RT-PCR and the Brilliant QPCR Core Reagent kit (Stratagene, La Jolla, CA, USA) for a one-step real-time RT-PCR, on an ABI PRISM 7900HT sequence detection system (Applied Biosystems).

Analytical sensitivity
It was demonstrated that the LOD was down to a theoretical limit of detection equivalent to 10–100 genome copies. The test could detect one naturally infested seed in 5000.

Analytical specificity
The test is able to detect 15 isolates covering LP, EU, US1, US2 and CH2.

4.3 Validation data available from CREA-DC (IT)

The test was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* by the CREA-DC:

Validation data were originally obtained using the TaqMan™ One step RT-PCR Master Mix (Thermo Fisher) but compared and confirmed using the TaqMan™ RNA-to-C_t™ *1-Step* kit (Thermo Fisher); thermocyclers used, include Applied Biosystem ABIPRISM 7500 Fast, Applied Biosystem Step One Plus, BioRad CFX96.

Analytical sensitivity
The LOD was determined using the 10-fold dilution series (leaves, fruits and seeds) obtained from PepMV infected samples, and diluted in RNA from healthy material of corresponding matrix The LODs were 10^{-10}, 10^{-10}, 10^{-7}, in leaves fruits and seeds, respectively.

Analytical specificity
Inclusivity: 100%, the inclusivity of the test was assessed using 16 different PepMV isolates: 6 leaf samples collected from different Italian regions (CREA collection: CREA-DC 322, CREA-DC 439, CREA-DC 441, CREA-DC 448, CREA-DC 463, CREA-DC 464); 3 fruit samples collected from different Italian regions (CREA collection: CREA-DC 252, CREA-DC 442, CREA-DC 448); 4 seed samples collected from different Italian regions (CREA collection: CREA-DC 461, CREA-DC 488, CREA-DC 534, CREA-DC 612); 3 PEPEIRA reference leaf samples (PEPEIRA-CH2; PEPEIRA-EU; PEPEIRA-US1). In all cases, the detection of PepMV was successful.
Exclusivity: 100%, the exclusivity was assessed by testing the following species: PVX (DSMZ isolate PV-0014; CREA-DC 572); PVY (CREA isolate CREA-DC 278); TMV (CREA isolate CREA-DC 575); ToCV (CREA isolate CREA-DC 607), ToBRFV (CREA isolate CREA-DC 587); TSWV (CREA isolate CREA-Dc 453). No cross reactions were observed.

Selectivity
No relevant differences in C_t values were found when six tomato varieties were tested (in four varieties it was possible to test leaves, fruit and seed).

Repeatability
The repeatability was evaluated by analysing three replicates of RNA samples (three samples of each matrix: leaves, seeds) of one variety, containing medium and low PepMV concentrations in the same run. The repeatability was 100% (standard deviation (SD) of the mean C_t values obtained was always <1.5 C_t).

Reproducibility
The reproducibility was evaluated in the framework of a national test performance study involving CREA and two other laboratories (regional phytosanitary service). The reproducibility was 87% (SD below 1.5 C_t).

APPENDIX 5 - REAL-TIME RT-PCR TESTS FOR STRAIN IDENTIFICATION OF PepMV (Gutiérrez-Aguirre et al., 2009)

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 These one-step real-time RT-PCR protocols can be used for the detection and strain identification of PepMV in leaves, fruit and seeds of tomato.
1.2 The tests were developed, published and validated by Gutiérrez-Aguirre et al. (2009).
1.3 Primers and probes target a fragment of the coat protein (CP) and the replicase gene (Rep).
1.4 Oligonucleotides that target Rep gene:

Eur-rep	Sequence	Position in genome	Strain specificity
Forward	5′-TGG GTC AAG AAA GTG GAA AAA TTA G -3′	3516-3586 to AF484251	EU, LP
Reverse	5′-TGC ATG AAA GCT GCA ATG GT -3′		
Probe	5′-FAM-TGC TGT CAA GTC AAA GCC TGG CCA-TAMRA -3′		

Ch2-rep	Sequence	Position in genome	Strain specificity
Forward	5′-TGA CTC CAA TAA GAT CTC ATT ATT CCT AAA -3′	3475-3621 to DQ000985	CH2
Reverse	5′-AAG TAC CTG GCC ATA GTA CCA TAC AAC -3′		
Probe	5′-FAM-TGG AGC TAT CAA ATC AAA ACC TGG TCA GAC C-TAMRA -3′		

1.5 Oligonucleotides that target CP gene:

Eur-cp	Sequence	Position in genome	Strain specificity
Forward	5′-TGG AAC ATA CTT CTC GAC AGC AA -3′	6035-6133 to AF484251	EU, LP
Reverse	5′-TCC ATC GAA GAA GTC AAA TGC A -3′		
Probe	5′-FAM-ATT CCA CCA GCA AAT TGG GCC AAA CTT-TAMRA -3′		

Ch2&US2-cp	Sequence	Position in genome	Strain specificity
Forward	5′-TGG GTT TAG CAG CCA ATG AGA -3′	5832-5903 to DQ000985	CH2, US2
Reverse	5′-AAC TTT GCA CAT CAG CAT AAG CA -3′		
Probe	5′-FAM-CGG ACC TGC CAT GTG GGA CCT C-TAMRA -3′		

US1-cp	Sequence	Position in genome	Strain specificity
Forward	5′-AGC GCG TGC TTA TGC TGA T -3′	5878-5958 to AY509926	US1
Reverse	5′-CGT GAG AGT GCT GGA TTT GAA G -3′		
Probe	5′-FAM-CCG CAC AAC TCA TAG GTG CCA CCC-TAMRA -3′		

1.6 Tests have been successfully performed using the Brilliant QPCR Core Reagent kit (Stratagene, La Jolla, CA, USA) and AgPath-ID One-Step RT-qPCR mix (Thermo Fisher).

2. Methods

2.1 Nucleic acid extraction

See Appendix 1.

RNA should preferably be stored at approximately −20°C for short term (<1 month) or at approximately −80°C for long term storage.

2.2 One-step real-time RT-PCR

2.2.1 Master Mix with AgPath-ID One-Step RT-qPCR mix (note that a separate master mix should be prepared for each strain).

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	1.0	N.A.
RT-PCR buffer (from the kit)	2×	5.0	1×
Forward primer	10 µM	0.9	0.9 µM
Reverse primer	10 µM	0.9	0.9 µM
Probe	2.5 µM	0.8	0.2 µM
RT-PCR enzyme (from the kit)	25×	0.4	1×
Subtotal		9.0	
RNA extract		1.0	
Total		10.0	

2.2.2 Real-time RT-PCR cycling conditions with AgPath-ID One-Step RT-qPCR mix: reverse

transcription at 48°C for 10 min; denaturation at 95°C for 10 min; 45 cycles of denaturation at 95°C for 15 s and 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 (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- 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: 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).
- 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). The PAC should preferably be near to the limit of detection.

As alternative or in addition to the PIC, internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include endogenous nucleic acid of the matrix using primers preferably amplifying RNA targets such as *nad5* (Botermans et al., 2013). However, for seed samples, *nad5* might not perform consistently. In this case, COX (e.g. Weller et al., 2000 or Papayiannis et al., 2011) can be used as IPC.

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:** In order to assign results from PCR-based test the following criteria should be followed:

Verification of the controls

- NIC and NAC should give no amplification.
- The PIC and PAC (and if relevant IPC) amplification curves should be exponential.

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

Validation data for all tests from Gutiérrez-Aguirre et al. (2009).

4.1 Analytical sensitivity

A theoretical limit of detection for all five tests is 10 genome copies.

For the Eur-cp, CH2-rep and CH2&US2-cp primers and probes, it has been shown that one naturally infected seed can be detected in 5000 seeds.

4.2 Analytical specificity

All 5 tests were evaluated using 15 PepMV samples of the following strains: 3× LP, 3× EU, 2× US1, 2× CH2, 3× CH2 or US2, 1× mixed infection of EU and CH2, 1× mixed infections of US1 and CH2.

Inclusivity (PepMV samples of the respective strains detected in tests are listed): 100%.

- Eur-rep: 3× LP, 3× EU, 1× mix of EU and Ch2.
- CH2-rep: 2× CH2, 3× CH2 or US2, 1× mix of EU and CH2, 1× mix of US1 and CH2.
- Eur-cp: 3× LP, 3× EU, 1× mix of EU and CH2.
- CH2&US2-cp: 2× CH2, 3× CH2 or US2, 1× mix of EU and CH2, 1× mix of US1 and CH2.
- US1-cp: 2× US1, 1× mix of US1 and CH2.

Exclusivity (PepMV strains): Observed cross-reactions (listed are PepMV samples of the respective strain in which cross-reactions were detected in tests):

- Eur-rep: none.
- CH2-rep: none.
- Eur-cp: cross reaction with: 2× US1, 1× mix of US1 and CH2.
- CH2&US2-cp: none.
- US1-cp: cross reaction with 2× CH2, 3× CH2 or US2, 1× mix of EU and CH2 (in all cases $C_t \geq 35$).

Exclusivity: Concerning potential cross reactions with closely related viruses, none of the tests detected the PVX RNA isolated from a PVX-infected *Nicotiana tabacum*. In addition, in silico alignments of the 5 tests indicate no significant sequence identity to any other virus in the NCBI database at that time.

Selectivity
No background reactions were observed with healthy tomato leaves, fruit, and seed.

Repeatability
No data.

Reproducibility
No data.