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EPPO STANDARD ON DIAGNOSTICS

PM 7/163 (1) *Tobamovirus maculatessellati* (tomato mottle mosaic virus)

Specific scope: This Standard describes a Diagnostic Protocol for **tomato mottle mosaic virus**.[1]
This Standard should be used in conjunction with PM 7/76 Use of EPPO Diagnostic Standards.
Specific approval and amendment: Approved in 2026–06.
Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Tomato mottle mosaic virus (ToMMV, genus *Tobamovirus*) was first reported in 2013 from infected tomato crops (*Solanum lycopersicum*) in Mexico (Li et al., 2013). It was subsequently found in other countries in North and South America, in several countries in Asia, and also in Europe and Mauritius. The virus can cause severe infections in tomatoes and peppers (*Capsicum* spp.). For an updated geographical distribution consult EPPO Global Database (EPPO, 2025a). Some other natural hosts in the Solanaceae, Fabaceae and Chenopodiaceae families have also been identified (EPPO, 2022a).

Tobamoviruses are easily transmitted mechanically through cultural practices which cause wounds or microlesions. ToMMV has been detected in many tomato and pepper seed lots and is thought to be seed-borne, as is the case for other tobamoviruses such as tobacco mosaic virus and tomato mosaic virus (EPPO, 2022a). In addition, it has been shown that water and soil contaminated with ToMMV could also be the source of tomato infection (Mehle et al., 2024b).

Tomato and pepper are the primary cultivated hosts of tobamoviruses. Resistance in tomato, mediated by the *Tm-2*^{2} gene, confers partial protection, while similar resistance is presumed to be associated with *L* genes in pepper. However, under specific environmental conditions, such as high temperatures, the resistance may be less effective (EPPO, 2022a).

Flow diagrams describing the diagnostic procedure for ToMMV are presented in Figures 1 and 2.

[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.*

2 | IDENTITY

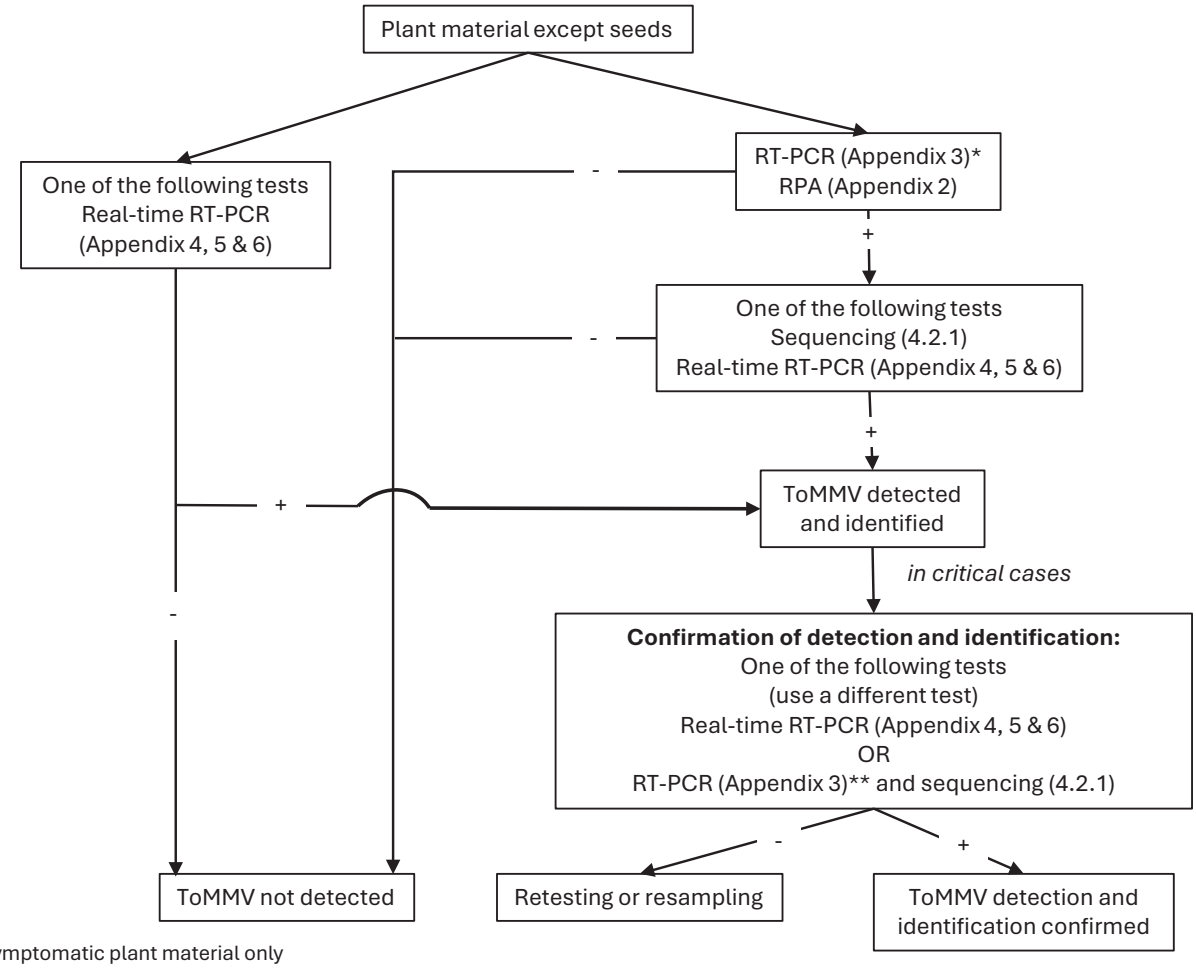
Name: Tomato mottle mosaic virus.
Acronym: ToMMV.
Taxonomic position: Viruses and viroids: Riboviria: Orthornavirae: Kitrinoviricota: Alsuviricetes: Martellivirales: Virgaviridae: Tobamovirus; *Tobamovirus maculatessellati*.
EPPO Code: TOMMV0.
Phytosanitary categorization: EPPO Alert list from 2020 to 2024.
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 Diagnostic Standards. Names of viruses not included in the official ICTV classification are based on first reports.
Note on phytosanitary categorization: ToMMV was added to the EPPO Alert List in 2020. Based on a PRA and more information collected on the current situation in the EPPO region, the Working Party on Phytosanitary Regulations decided to not recommend the addition of ToMMV to the List of pests recommended for regulation and, in June 2024, to delete this pest from the EPPO Alert list, considering that sufficient alert had been given.

3 | DETECTION

3.1 | Symptoms

As is the case for other tobamoviruses, symptoms can vary depending on factors such as virus isolate, plant species, cultivar, environmental conditions, physiological conditions, co-infection with other viruses or pathogens.

The following symptoms of ToMMV are reported in the literature:



* symptomatic plant material only
** RT-PCR is less sensitive than real-time RT-PCR and RPA. Therefore, when a low virus titre is observed, RT-PCR should not be used for confirmation.

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

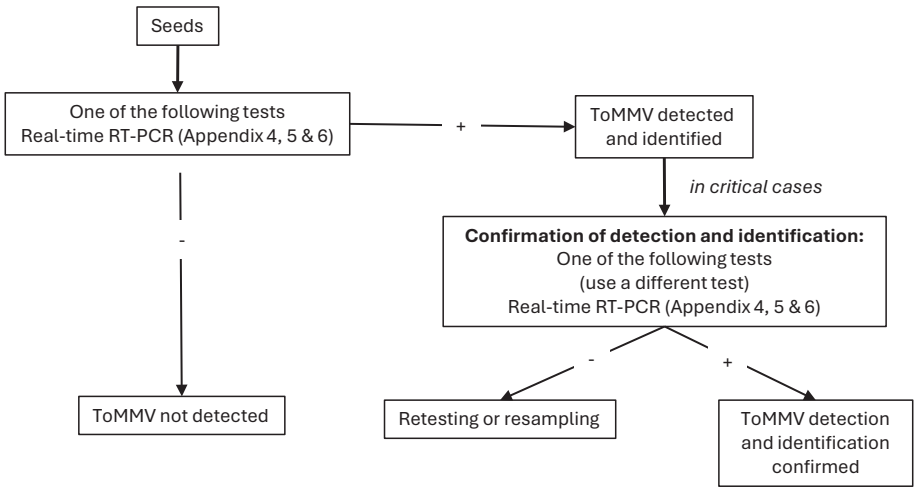


FIGURE 2 Flow diagram describing the diagnostic procedure for ToMMV in seeds. This flow diagram is intended to provide an overview of the diagnostic process and may not cover all possible scenarios.

FIGURE 3 Tomato 'Moneymaker' mechanically inoculated with ToMMV showing leaf narrowing and blistering. Copyright: Padmanabhan et al. (2025).

- **Symptoms on tomato:**
  - Plants: stunting (Ambrós et al., 2017, Sui et al., 2018 in experiments); complete loss of flowers due to flower abortion is observed when young plants were infected (and therefore no fruit production) (Sui et al., 2017).
  - Shoots: chlorosis (Maudarbaccus et al., 2021).
  - Leaves: mosaic, mottling, narrowing, blistering (Figure 3), necrosis/necrotic spots (Figure 4), chlorosis, crinkling, epinasty, deformation and leaf curl (Ambrós et al., 2017; ICAR-IIHR, 2017; Li et al., 2013, 2017; Maudarbaccus et al., 2021; Sui et al., 2017; Turina et al., 2016; Webster et al., 2014; Zhan et al., 2018);
  - Fruit: necrotic lesions and fruit necrosis (Sui et al., 2017; Figure 4); uneven ripening and necrotic spots on fruits (Maudarbaccus et al., 2021).
- **Symptoms on pepper:**
  - Plants: fast apical yellowing, necrosis (observed on *C. annuum*; Ambrós et al., 2017) and stunting (observed on *C. annuum* var. *annuum* (*Grossum* group) (Li et al., 2017)).
  - Leaves: mottle, shrinking, necrosis (Li et al., 2014); crinkling, mosaic and necrosis (Zhan et al., 2018); chlorosis, mosaic, necrosis (observed on *C. frutescens*, Yunnan, isolate YYMLJ) and mottle, necrosis (observed on *C. annuum* var. *annuum* (*Grossum* group) in Lhasa, TiLhaLJ) (Li et al., 2017).
  - Fruit: no symptoms reported.
- **Symptoms on other hosts**
  - For symptoms reported in other hosts, see EPPO (2022a).

Confusion with other diseases of tomato/pepper

Other tobamoviruses may cause similar (non-specific) symptoms that may be confused with ToMMV symptoms.

3.2 | Detection in symptomatic/asymptomatic plant material except seeds

3.2.1 | 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.

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.

For asymptomatic plants, experimental data to recommend a specific sample size is lacking. Therefore, the following recommendation is based on the one prepared for the detection of tomato brown rugose fruit virus (TOBRFV) (EPPO, 2022b).

Sampling can be based on the collection of one leaf/sepal from each plant from a total of 200 plants 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 leaves should be collected from the top or side shoots of plants. It is likely that distribution of ToMMV will follow a similar pattern to tobacco mosaic virus and tomato brown rugose fruit virus. Research work carried out on these viruses suggests the highest likelihood of detection can be obtained from sampling young leaflets or sepals (calyxes) when available (Samuel, 1934; Skelton et al., 2023).

For molecular testing of asymptomatic plants, leaves/sepals (calyxes) can be pooled, however, the number of leaves/sepals (calyxes) that can be tested in a sub sample should be validated. For example, in Slovenia 30 leaves are pooled for one test (N. Mehle, pers. comm.), in Italy 20 leaves are pooled for one test (A. Tiberini, pers. comm.), and in the United Kingdom 10 leaves or calyxes are pooled for one test (A. Fowkes, pers. comm.).

For symptomatic plants, for laboratory testing it is recommended to collect at least three symptomatic young leaves/sepals from the top of the plant or from side shoots.

FIGURE 4 Brown necrotic lesions on leaves and fruits of a tomato plant with Tm-2^2 gene infected by ToMMV in an experiment. Copyright: Sui et al., 2017.

3.2.2 | Molecular tests

Several specific molecular tests have been described for the detection of ToMMV and those recommended are described in Appendices 2–6. For sample preparation and RNA extraction, see Appendix 1.

Molecular tests included in this Standard were evaluated in a Test Performance Study (TPS) organized in the framework of the Euphresco project 2022-A-394 (Validation of molecular diagnostic methods for the detection and identification of tomato mottle mosaic virus (ToMMV-detect) (hereafter referred to as Euphresco TPS)). Outcomes of the Euphresco TPS are available through the EPPO database on Diagnostic Expertise (https://dc.eppo.int/validation_data/validationlist) and in Mehle et al., (2024a).

The following tests are recommended for the detection of ToMMV in tomato leaves/sepals or pepper leaves:

- Recombinase-Polymerase Amplification (RPA) for ToMMV from Agdia (2022) described in Appendix 2.
- Conventional RT-PCR for tobamoviruses from Levitzky et al. (2019) described in Appendix 3.
- Real-time RT-PCR for ToMMV from Tiberini et al. (2022) described in Appendix 4.
- Real-time RT-PCR for ToMMV from Fowkes et al. (2022) described in Appendix 5.
- Real-time RT-PCR for ToMMV from DAFF & DEECA (unpublished) described in Appendix 6.

Multiplex real-time PCR tests were developed but are not included in this Standard as the singleplex real-time RT-PCR tests have slightly better diagnostic sensitivity and specificity (see Mehle et al., 2024a for the description of the tests and validation data).

The diagnostic sensitivity of the conventional RT-PCR for tobamoviruses by Levitzky et al. (2019) is lower compared to the real-time RT-PCR tests and RPA test (Mehle et al., 2024a), which is why the use of this conventional RT-PCR is only recommended for symptomatic plant material.

The LAMP test with primers by Kimura et al. (2023) and the conventional RT-PCR tests by Loewe (Cat. No. 09181) and by Sui et al. (2017) are not included in this Standard due to their lower diagnostic sensitivity compared to the recommended tests (Mehle et al., 2024a).

3.2.3 | Other tests

3.2.3.1 | *Serological tests*

The currently available ToMMV antibodies have not been included in the Euphresco TPS and have limited validation data. In general, the analytical sensitivity of ELISA is known to be lower than that of molecular tests, so they can probably be used for the detection of ToMMV in symptomatic material, but further validation data is required. Instructions for performing an ELISA are included in PM 7/125 *ELISA tests for viruses* (EPPO, 2025b).

3.2.3.2 | *Bioassay*

There is no validation data available on the use of bioassay for detection of ToMMV and for the confirmation of the presence of viable ToMMV on plant material and seeds. In general, analytical sensitivity of bioassays is known to be lower than for ELISA and molecular tests.

However, experience in some laboratories indicates that it may be used for detection from symptomatic material. Buffers that can be used for mechanical inoculation are described in PM 7/153 *Mechanical inoculation of test plants* (EPPO, 2022d). Test plants that can be used and symptoms to be expected on mechanically inoculated test plants are listed in EPPO (2022d).

3.2.3.3 | *High-throughput sequencing*

High-Throughput Sequencing (HTS) can be used for the detection of ToMMV 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).

3.3 | Detection in seeds

3.3.1 | Test sample requirements

For seed testing, there is limited information on which to base recommended sample sizes and bulking rates. For seed lots of tomato and pepper, protocols using weighed samples of approximately 3000 seeds, tested in three sub-samples of 1000 seeds, have been validated for the real-time RT-PCR in Appendix 4 (Tiberini et al., 2022). 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 with an efficacy of detection of 100% (IPPC, 2008). The real-time RT-PCR from Appendix 6 (DAFF & DEECA, unpublished) has been validated for samples of 20 000 seeds and sub-samples of 400 seeds.

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.

3.3.2 | Molecular tests

The following tests are recommended for the detection of ToMMV in tomato/pepper seeds:

- Real-time RT-PCR for ToMMV from Tiberini et al. (2022) described in Appendix 4.
- Real-time RT-PCR for ToMMV from Fowkes et al. (2022) described in Appendix 5.
- Real-time RT-PCR for ToMMV from DAFF & DEECA (unpublished) described in Appendix 6.

4 | IDENTIFICATION

4.1 | Molecular tests

4.1.1 | Real-time PCR tests

The real-time RT-PCR tests described in Sections 3.2.2 and 3.3.2 (Appendices 4–6) allow both detection and identification of ToMMV. They are recommended for identification and confirmation.

4.1.2 | Sanger Sequencing

Sanger sequencing can be used for identification (when the RT-PCR from Appendix 3 is used) and/or confirmation when the virus concentration allows for its application. 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).

4.2 | Other tests

High-throughput sequencing (HTS) tests can also be used to identify ToMMV if the virus concentration permits. The application of a 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 can be obtained from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Inhoffenstrasse 7B, 38124, Braunschweig, Germany (https://www.dsmz.de/catalogues/catalogue-plant-viruses-and-antisera.html).

Sequences are available in EPPO-Q-bank (https://qbank.eppo.int/).

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 (http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

8 | FURTHER INFORMATION

Further information on this organism can be obtained from:

Heiko Ziebell, Julius Kühn-Institut, Messeweg 11-12, 38104 Braunschweig (DE); heiko.ziebell@julius-kuehn.de.

Kai-Shu Ling, Vegetable Research, USDA, 2700 Savannah highway Charleston, SC 29414 (US); kai.ling@usda.gov.

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 | STANDARDS 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.

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This Standard was originally drafted by: N. Mehle, National Institute of Biology (NIB) (SI), A. Tiberini, Council for Agricultural Research and Economics (CREA) (IT), A. Fowkes & A. Fox, Fera Science Ltd (FERA) (GB).

The Standard was reviewed by the Panel on Diagnostics in Virology and Phytoplasmology.

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APPENDIX 1 - SAMPLE PREPARATION AND RNA EXTRACTION FOR MOLECULAR TESTS

This Appendix describes sample preparation and RNA extraction procedures for different hosts and types of plant material. These initial steps 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), other extraction methods or kits may be used. The RNA-extraction procedure should be validated in combination with the molecular test to be used.

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

Care should be taken to avoid cross-contamination when handling samples especially when high virus concentrations can be expected.

1. Plant material

1.1 Individual plants and/or small samples

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

1.2 Pooled samples

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

For pooled samples involving large amounts of plant material, ELISA buffer, phosphate buffer or GH+ buffer (see Table A1) can be used for homogenization. It is recommended that the molecular test is validated/verified using the selected buffer.

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.

1.2.2 Procedure using GH+ buffer

Plant tissue is ground in an extraction bag in GH+ buffer (range 1:2–1:5 [w/v]) (see Table A1). Samples are

TABLE A1 GH+ buffer.

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

incubated for 10 min at 65°C. After centrifugation at 12000 ***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.

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 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. Seeds

Sample preparation, including grinding and RNA extraction, from seeds can be challenging. Two procedures using different homogenization buffers, that have been used for detection of ToBRFV in seeds of tomato and pepper, are described and allow a similar sample preparation for detection of ToMMV 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.

2.1 Homogenization in phosphate buffer

For tomato and pepper, 12 subsamples of 250 seeds are prepared. The subsamples are immersed in 10 mL (for tomato) and 20 mL (for pepper) of 0.1 M phosphate buffer (Na_2HPO_4/KH_2PO_4, pH 7.2), 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 10000 *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 into three subsamples each of four homogenates.

600 µL of the supernatant is added to 600 µL of RLT buffer (without 2-mercapto-ethanol). This mixture is loaded in two aliquots of 600 µL 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 warm water (65°C) followed by centrifugation. To maximize RNA recovery, an additional elution step is performed using the same procedure.

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.

Two homogenization procedures can be used:

- For both tomato and pepper three subsamples of 1000 seeds are transferred to a grinding bag (e.g. Interscience BagPage 100 mL) and 20 mL of GH+ buffer (Table A1) for tomato or 40 mL for pepper is added. The seeds are soaked at room temperature for 30–60 min before homogenization, (e.g. with an Interscience BagMixer on position 4) for 90 s (tomato) or 4 min (pepper).
- Alternatively, dry seeds are ground (e.g. with a Genogrinder). Three subsamples of 1000 tomato or six subsamples of 500 pepper seeds are transferred to a 50 mL tube and a steel ball (14 mm) is added. Seeds are ground, tubes up-side down, at 1700 rpm for 4 min for tomato and 7 min for pepper seeds. After grinding 20 mL GH+ buffer is added for tomato and for pepper samples. Tubes are shaken by hand to obtain homogenous solutions. For pepper, three times two homogenates of the same sample are combined and mixed before further processing to make three subsamples.

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 16000 *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 RNA is extracted following the manufacturer's instructions.

APPENDIX 2 - RECOMBINASE-POLYMERASE AMPLIFICATION – RPA (AGDIA, 2022)

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

1. General Information

1.1 This test can be used for the detection of ToMMV in tomato and pepper plant material (excluding seeds).
1.2 The test was developed by Agdia in 2022, and it is appropriate for on-site detection as well as for use in laboratories.
1.3 Oligonucleotides: Primers and probe (probe label: FAM) are available in a kit from Agdia (AmplifyRP XRT for ToMMV, rapid RNA amplification test Kit; Product No. XCS 22800).
1.4 AmpliFire Isothermal Fluorometer AFR 60400 (Agdia) or real-time PCR thermocycler QuantStudio3 (Applied Biosystems) or another thermocycler (more information regarding applicable thermocyclers available on request from Agdia).

2. Methods

2.1 Nucleic Acid Extraction

The test can be applied to plant material (excluding seeds) after RNA extraction (see point 2.1.1), or to crude homogenates (see point 2.1.2), without RNA extraction. In the latter case the test can be completed in less than 30 min.

2.1.1 Nucleic Acid Extraction

See Appendix 1, alternative procedures may also be suitable.

The RNA samples included in the Euphresco-TPS were extracted using the RNeasy Plant Mini Kit (Qiagen) (for details see Mehle et al., 2024a).

2.1.2 Crude homogenates, without RNA extraction

0.15 g of leaf or petiole tissue are placed in the extraction bag with GEB2 extraction buffer (AmplifyRP XRT for ToMMV, Agdia) and thoroughly macerated.

2.2 RPA

2.2.1 Master Mix

5 µL RNA or crude homogenate is added to the tube containing the PD1 diluent (AmplifyRP XRT for ToMMV, Agdia) and mixed well. From this tube, 25 µL of the mix (sample and PD1 diluent) is transferred to the reaction pellet (clear tube; AmplifyRP XRT for ToMMV, Agdia).

2.2.2 RPA reaction conditions

Amplification: 42°C; 20 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 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 or host tissue extract spiked with the target organism).
- Negative amplification control (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.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue. The PAC should preferably be near to the limit of detection.

Other possible controls
Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 **Interpretation of results**: in order to assign results from this test the following criteria should be followed

Verification of the controls

- NIC and NAC should produce no fluorescence.
- PIC, PAC (and if relevant IC) should produce fluorescence.

When these conditions are met:

- A test will be considered positive if it produces fluorescence.
- A test will be considered negative, if it produces no fluorescence.
- Tests should be repeated if any contradictory or unclear results are obtained

4. Performance characteristics available

Validation on crude homogenates of leaves was carried out by Agdia (FR) Agdia (2022), and validation on RNA extracts was carried out by 6 laboratories in the framework of the Euphresco TPS in accordance with PM 7/98 (Mehle et al., 2024a). 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 Analytical sensitivity

- Data from Agdia:

Limit of detection: 1:62 500 dilution of infected tissue (pathogen titre unknown).

- Data from Euphresco TPS:

Analytical sensitivity evaluated using dilutions of RNA from ToMMV-infected leaf material in RNA from tobamovirus-free tomato leaf material was at least 2.5 $\times 10^5$ dilutions (level of agreement between 6 laboratories: 100%).

3.4 Analytical specificity

- Data from Agdia:

Inclusivity: 100% evaluated on two isolates of ToMMV (ToMMV PV-1267 and ToMMV-MX5). Additionally, in-silico analysis confirmed the detection of 26 ToMMV isolates of different origins.

Exclusivity: no cross-reaction observed.

- Data from Euphresco TPS:

Inclusivity: 100% evaluated on two isolates of ToMMV (one from seed and one from leaf material).

Exclusivity: from 93% (1 laboratory false positive result for one tobacco mosaic virus (TMV) isolate, 1 laboratory false positive result for paprika mild mottle virus (PaMMV), 2 laboratories inconclusive result for PaMMV) to 100% (2 laboratories). Exclusivity was evaluated on 3 healthy tomato samples (two of seeds, one of leaves) and 11 isolates of 9 other tobamovirus species (1 isolate of cucumber green mottle mosaic virus (CGMMV), obuda pepper virus (ObPV), odontoglossum ringspot virus (ORSV), paprika mild mottle virus (PaMMV), pepper mild mottle virus (PMMoV), tobacco mild green mosaic virus (TMGMV) and tomato brown rugose fruit virus (ToBRFV), and 2 isolates of tomato mosaic virus (ToMV) and tobacco mosaic virus (TMV)).

4.3 Repeatability

- Data from Agdia:

97% (evaluated on 3 replicates of 22 samples).

4.4 Reproducibility

- Data from Agdia:

97% (evaluated with 3 replicates of 22 samples by 3 operators).

- Data from Euphresco TPS:

92% (evaluated with 13 non-target RNA samples and 9 RNA samples with different dilutions of ToMMV RNA in 6 laboratories).

4.5 Other data

- Data from Agdia:

Diagnostic sensitivity: 100%.
Diagnostic specificity: 100%.
Selectivity: no matrix effect observed with pea leaves, pea petioles, pea seeds, pea stems, pepper fruit, pepper leaves, pepper petioles, pepper seeds, pepper stems, petunia leaves, petunia petioles, petunia stems, tomato fruit, tomato leaves, tomato petioles, tomato seeds, tomato stems.

- Data from Euphresco TPS:

Diagnostic sensitivity: 80.0%.
Diagnostic specificity: 95.2%.
The test was evaluated by 6 laboratories using a panel of samples composed of 9 target samples (dilutions of two ToMMV isolates including dilutions below the LOD) and 13 non-targets (including 3 healthy tomato samples and 11 isolates of 9 other tobamovirus species).

APPENDIX 3 - CONVENTIONAL RT-PCR (LEVITZKY ET AL., 2019)

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 (see PM 7/98) is carried out.

1. General Information

1.1 This test can be used for the detection of ToMMV and some other tobamoviruses in symptomatic tomato and pepper plant material (excluding seeds). In combination with amplicon sequencing, it can be used also for identification of ToMMV.

1.2 The test was developed by Levitzky et al. (2019) and adapted by Fera science Ltd (FERA, GB) in 2023.

1.3 The target sequence is located on the movement protein gene, coat protein gene and the 3′UTR of the ToMMV genome.

1.4 Amplicon location: based on the nucleotide sequence of GenBank accession no NC_022230, the forward and reverse primer start at position 5480 and 6264, respectively.

1.5 Oligonucleotides:

	Name	Sequence	Amplicon size*
Forward primer	F-5476	5′-GAA GAA GTT GTT GAT GAG TTC AT-3′	807 bp
Reverse primer	R-6287	5′-GAT TTA AGT GGA GGG AAA AAC AC-3′	

* including primer sequences.

1.6 Cycler: GeneAmp PCR System 9700, 2720 Thermal Cycler (Applied Biosystems).

2. Methods

2.1 Nucleic Acid Extraction

See Appendix 1, alternative procedures may also be suitable.

The RNA samples included in the Euphresco TPS were extracted using the RNeasy Plant Mini Kit (Qiagen) with some modifications.

2.2 One step Reverse Transcription PCR

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	16	N.A.
RT-PCR buffer (*OneStep RT-PCR kit, Qiagen*)	5×	5	1×
dNTPs (*OneStep RT-PCR kit, Qiagen*)	10 mM each	1	0.4 mM each
PCR Forward primer (*F-5476*)	10 µM	0.5	0.2 µM
PCR Reverse primer (*R-6287*)	10 µM	0.5	0.2 µM
RT-PCR Enzyme mix (*OneStep RT-PCR kit, Qiagen*)		1	
Subtotal		24	
RNA extract		1	
Total		25	

2.2.2 RT-PCR conditions: Reverse transcription at 50°C for 30 min; denaturation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min and elongation at 72°C for 30 s. Followed by a final elongation of 72°C for 10 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 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 or host tissue extract spiked with the target organism).
- Negative amplification control (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.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As an 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 of endogenous nucleic acid, using primers that amplify conserved non-pest target nucleic acid that is also present in the sample. IPC can include endogenous nucleic acid of the matrix using conserved primers preferably amplifying RNA targets such as nad5 (Menzel et al., 2002).
- 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.

Laboratories should take additional care to prevent risks of cross contamination when using high concentration positive controls (e.g. cloned products, gBlocks, and whole genome amplicons) directly or when preparing dilutions of them.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 **Interpretation of results**: in order to assign results from this PCR-based test the following criteria should be followed

Conventional PCR tests

Verification of the controls

- NIC and NAC: no band is visualized
- PIC, PAC a band of the expected size [807 bp] is visualized. If relevant, a band of the expected size is visualized for the IC and IPC.

When these conditions are met:

- A test will be considered positive for tobamovirus if a band of the expected size [807 bp] is visualized. For identification of ToMMV, Sanger sequencing of PCR product is needed.
- 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.

4. Performance characteristics available

Validation was carried out by 8 laboratories in the framework of the Euphresco TPS in accordance with PM 7/98 (Mehle et al., 2024a). Within this TPS, the performance characteristics of the test (including the sequencing of the RT-PCR products) was evaluated for the detection and identification of ToMMV only (and not for other viruses). 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 Analytical sensitivity

Analytical sensitivity evaluated using dilutions of RNA from ToMMV-infected leaf material in RNA from tobamovirus-free tomato leaf material was at least 2.5 $\times 10^5$ dilutions (level of agreement between 8 laboratories: 100%).

4.2 Analytical specificity

Inclusivity: from 50% (5 laboratories false negative result for ToMMV from seeds) to 100% (3 laboratories), evaluated on two isolates of ToMMV (one from seeds and one from leaf material; the estimated concentration of ToMMV in seeds was lower than the concentration of ToMMV in leaf material).

Exclusivity: 100%, evaluated on 3 healthy tomato samples (two of seeds, one of leaves) and 11 isolates of 9 other tobamovirus species (1 isolate of CGMMV, ObPV, ORSV, PaMMV, PMMoV, TMGMV and ToBRFV, and 2 isolates of ToMV and TMV).

4.3 Repeatability

Data not available.

4.4 Reproducibility

94% (evaluated with 13 non-target RNA samples and 9 RNA samples with different dilutions of RNA from ToMMV-infected leaf material in 8 laboratories).

4.5 Other data

Diagnostic sensitivity: 62.5%.
Diagnostic specificity: 100%.
The test was evaluated by 8 laboratories using a panel of samples composed of 9 target samples (dilutions of two ToMMV isolates including dilutions below the LOD) and 13 non-target (including 3 healthy tomato samples and 11 isolates of 9 other tobamovirus species; see data on exclusivity).

APPENDIX 4 - REAL-TIME RT-PCR (TIBERINI ET AL., 2022)

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

1. General Information

1.1 This test can be used for the detection and identification of ToMMV in tomato and pepper plant material (including seeds).
1.2 The test was developed by Tiberini et al. (2022).
1.3 The target sequence is located on the gene coding for the movement protein.
1.4 Amplicon location: based on the nucleotide sequence of GenBank accession no NC_022230, the forward and reverse primer start at position 5173 and 5255, respectively, and the probe covers positions 5197–5220.
1.5 Oligonucleotides:

	Name	Sequence	Amplicon size*
Forward primer	ToMMV CataAT Fw	5′-CAG CAT CTG CTT GGT CGA TAA-3′	105 bp
Reverse primer	ToMMV CataAT Rv	5′-GGA ACG ATC TTA AAC TGG AAC CT-3′	
Probe	ToMMV CataAT Pr**	5′-TexasRed-AAT GCA AAG AGC GGA TGA AGC GAC-BHQ2-3′	

* Including primer sequences.

** It has been validated that the probe labelled with HEX (instead of TexasRed) can also be used (Mehle, unpublished).

1.6 Real-time PCR system: CFX Maestro v2.2 (Bio-Rad), QuantStudio™ 7 Pro and ViiA7™ (both Applied Biosystems).
1.7 The Design & Analysis Software v2.5.1 (Applied Biosystems Foster city, CA, USA) was used for fluorescence acquisition and determination of C_t values. The baseline and fluorescence threshold are set manually.

2. Methods

2.1 Nucleic Acid Extraction

See Appendix 1, alternative procedures may also be suitable.

The RNA samples included in the Euphresco-TPS were extracted using the RNeasy Plant Mini Kit (Qiagen) with some modifications, that is, for the seeds the RLT buffer was replaced by the GH+ buffer (for other minor modifications see Mehle et al., 2024a).

The same RNA extraction procedure was also used for the validation data obtained at CREA-DC (IT) and NIB (SI).

2.2 One step real-time Reverse Transcription Polymerase Chain Reaction – real-time RT-PCR

2.2.1 Master Mix

Option 1:

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	2.95	N.A.
Mastermix from kit*	2×	5	1×
Forward primer (*ToMMV CataAT Fw*)	10 µM	0.3	0.3 µM
Reverse primer (*ToMMV CataAT Rv*)	10 µM	0.3	0.3 µM
Probe (*ToMMV CataAT Pr*)	10 µM	0.2	0.2 µM
RT enzyme*	40×	0.25	1×
Subtotal		9	
RNA extract		1	
Total		10	

* The test was validated using two different kits by Tiberini et al. (2022) and Mehle et al. (2024a): TaqMan™ RNA-to-Ct™ 1-Step Kit, ThermoFisher Scientific and AgPath-ID One-Step RT-qPCR, Applied Biosystems.

2.2.2 PCR conditions

With TaqMan™ RNA-to-Ct™ 1-Step Kit (ThermoFisher Scientific): 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.
With AgPath-ID One-Step RT-qPCR (Applied biosystems): 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.
Note: Both TaqMan™ RNA-to-Ct™ 1-Step and AgPath-ID One-Step RT-qPCR master mix contain

ROX. Therefore, if the probe is labelled with TexasRed, the ROX™ reference dye should be excluded prior to the analysis to avoid interference with the TexasRed dye.

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 or host tissue extract spiked with the target organism).
- Negative amplification control (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.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As an 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 of endogenous nucleic acid, using primers that amplify conserved non-pest target nucleic acid that is also present in the sample. IPC can include endogenous nucleic acid of the matrix using conserved 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.
- 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.

Laboratories should take additional care to prevent risks of cross contamination when using high concentration positive controls (e.g. cloned products, gBlocks, and whole genome amplicons) directly or when preparing dilutions of them.

Other possible controls

Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results

: in order to assign results from this real-time 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 IC and 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.

Note: Samples showing an exponential amplification curve with a relatively high C_t value can be considered inconclusive as this could be due to contamination with a low titre of ToMMV; however, a cross-reaction with a non-target tobamovirus could not be completely excluded. In the framework of the Euphresco TPS, the range of C_t cut-off values above which a result was considered inconclusive was 33 to 37 (depending on the laboratories). 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.

4. Performance characteristics available

Validation was carried out in accordance with PM7/98 by the Council for Agricultural Research and Economics - Research Centre for Plant Protection and Certification (CREA-DC, IT), and the National Institute of Biology (NIB, SI) (Tiberini et al., 2022) and by 10 laboratories in the framework of the Euphresco TPS (Mehle et al., 2024a).

The ToMMV primers were used in a duplex reaction with primers targeting ToBRFV. The duplex PCR was validated in Tiberini et al. (2022) and in Mehle et al. (2024a) and some validation data that are also relevant for the singleplex PCR are reported below. 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 Analytical sensitivity

- Data from Tiberini et al. (2022):

For both TaqMan™ RNA-to-Ct™ 1-Step and AgPath-ID One-Step RT-qPCR master mix, analytical sensitivity evaluated using 10-fold dilution series (leaves and seeds) from samples infected with ToMMV and ToBRFV was 10^{-6} for ToMMV in leaves and seeds.

- Data from Euphresco TPS:

Analytical sensitivity evaluated using dilutions of RNA from ToMMV-infected leaf material in RNA from tobamovirus-free tomato leaf material was at least 2.5 $\times 10^6$ dilutions (level of agreement between 10 laboratories: 100%).

4.2 Analytical specificity

- Data from Tiberini et al. (2022):

Inclusivity: 100% evaluated on three isolates of ToMMV in a duplex test (mastermix with ToMMV and ToBRFV primers and probes).

The in-silico analysis targeting all the full genome sequences of ToMMV available in GenBank indicated only up to two nucleotide polymorphisms in forward and/or reverse primers. However, these single point mutations were observed in a group of isolates including the DSMZ isolate PV-1267 that was successfully detected experimentally.

Exclusivity was assessed in a duplex test (mastermix with ToMMV and ToBRFV primers and probes) with testing the following tobamovirus species: bell pepper mottle virus - BPMV (DSMZ isolate BN-4708), CGMMV (DSMZ isolate PV-0375, and NIB isolates NIB V 271 and NIB V 320), ObPV (DSMZ isolate PV-1176), ORSV (DSMZ isolate PV-1048), PaMMV (DSMZ isolate PV-0606), PMMoV (DSMZ isolate PV-0165, CREA isolate CREA-552), ribgrass mosaic virus - RMV (DSMZ isolate PV-0145), streptocarpus flower break virus - SFBV (DSMZ isolate PV-1058), sunn-hemp mosaic virus - SHMV (DSMZ isolate PV-0156), ToMV (DSMZ isolate PV-0141, and NIB isolates NIB V 036, NIB V 049, NIB V 072, NIB V 104), TMGMV (DSMZ isolate PV-0124), TMV (DSMZ isolates PV-1252, PV-0137 and PV-0943, and NIB isolate NIB V 037), youcai mosaic virus - YMoV (DSMZ isolate PV-0527). No cross-reactions were observed for the ToMMV test, except for PaMMV which gave C_t values ≥29.3.

- Data from Euphresco TPS:

Inclusivity: 100% evaluated on two isolates of ToMMV (one from seed and one from leaf material).

Exclusivity: from 93% (7 laboratories inconclusive result for PaMMV) to 100% (3 laboratories) evaluated on 3 healthy tomato samples (two of seeds, one of leaves) and 11 isolates of 9 other tobamovirus species (1 isolate of CGMMV, ObPV, ORSV, PaMMV, PMMoV, TMGMV and ToBRFV, and 2 isolates of ToMV and TMV).

4.3 Repeatability

- Data from Tiberini et al. (2022):

100% (evaluated in a duplex test (mastermix with ToMMV and ToBRFV primers and probes)) on 3 replicates of 2 RNA samples containing various concentrations of ToMMV.

4.4 Reproducibility

- Data from Tiberini et al. (2022):

100% (evaluated in a duplex test (mastermix with ToMMV and ToBRFV primers and probes) for two dilutions of a ToMMV-positive RNA samples, with medium and low target concentrations. It was assessed at both CREA and NIB, analysing different target RNA samples and using different reagents. In each laboratory, different real-time RT-PCR runs, four (CREA) and six (NIB), were performed on different days. In addition, at NIB two different instruments were used.)

- Data from Euphresco TPS:

96% (evaluated with 13 non-target RNA samples and 9 RNA samples with different dilutions of ToMMV RNA in 10 laboratories).

4.5 Other data

- Data from Euphresco TPS:

Diagnostic sensitivity: 80.0%.
Diagnostic specificity: 95.2%.
The test was evaluated by 10 laboratories using a panel of samples composed of 9 target samples (dilutions

of two ToMMV isolates including dilutions below the LOD) and 13 non-target (including 3 healthy tomato samples and 11 isolates of 9 other tobamovirus species; see data on exclusivity).

APPENDIX 5 - REAL-TIME RT-PCR (FOWKES ET AL., 2022)

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

1. General Information

1.1 This test can be used for the detection and identification of ToMMV in tomato and pepper plant material (including seeds).
1.2 The test was developed by Fowkes et al. (2022).
1.3 The target sequence is located on the gene coding for the movement protein.
1.4 Amplicon location: based on the nucleotide sequence of GenBank accession no NC_022230, the forward and reverse primer start at position 5162 and 5212, respectively, and the probe covers positions 5183-5210.
1.5 Oligonucleotides:

	Name	Sequence	Amplicon size*
Forward primer	ToMMV-F	5′-CGT GGT GGT GTC AGC ATC TG-3′	69
Reverse primer	ToMMV-R	5′-CGA TCC GAG TGT CGC TTC A-3′	
Probe	ToMMV-Pe	5′-FAM- TTG GTC GAT AAA AGA ATG CAA AGA GCG G -TAMRA-3′	

* Including primer sequences.

1.6 Real-time PCR system: QuantStudio™ 6, QuantStudio™ 12K Flex and ViiA7™ (Applied Biosystems).
1.7 Automatic baseline determined by machine.

2. Methods

2.1 Nucleic Acid Extraction

See Appendix 1, alternative procedures may also be suitable.

The RNA samples included in the Euphresco-TPS were extracted using the RNeasy Plant Mini Kit (Qiagen) with some modifications, that is, for the seeds the RLT buffer was replaced by the GH+ buffer (for other minor modifications see Mehle et al., 2024a).

For the validation data obtained at FERA (GB), the RNA was extracted using a Kingfisher method based on magnetic bead extraction (Invimag Virus DNA/RNA mini-kit (Invitek GmbH)).

2.2 One step real-time RT-PCR

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	6.45	N.A.
Master mix (iTaq Universal Probes One-Step Kit, *Bio-Rad*)	2×	10	1×
Forward primer (*ToMMV-F*)	7.5 µM	1	0.375 µM
Reverse primer (*ToMMV-R*)	7.5 µM	1	0.375 µM
Probe (*ToMMV-Pe*)	5 µM	0.5	0.25 µM
RT enzyme (iTaq Universal Probes One-Step Kit (*Bio-Rad*))		0.05	1×
Subtotal		19	
RNA extract		1	
Total		20	

2.2.2 PCR conditions

Reverse transcription at 50°C for 10 min; denaturation at 95°C for 2 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 or host tissue extract spiked with the target organism).
- Negative amplification control (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.

- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

  As an 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 of endogenous nucleic acid, using primers that amplify conserved non-pest target nucleic acid that is also present in the sample. IPC can include endogenous nucleic acid of the matrix using conserved 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.
- 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.

Laboratories should take additional care to prevent risks of cross contamination when using high concentration positive controls (e.g. cloned products, gBlocks, and whole genome amplicons) directly or when preparing dilutions of them.

Other possible controls.
Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 **Interpretation of results**: in order to assign results from this real-time 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 IC and 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.

Note: Samples showing an exponential amplification curve with a relatively high C_t value can be considered inconclusive as they could be due contamination with a low titre of ToMMV; however, a cross-reaction with a non-target tobamovirus could not be completely excluded. In the framework of the Euphresco TPS, the range of C_t cut-off values above which a result was considered inconclusive was 33–37 (depending on the laboratories). 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.

4. Performance characteristics available

Validation was carried out in accordance with PM7/98 by Fera science Ltd (FERA, GB) and by 13 laboratories in the framework of the Euphresco TPS (Mehle et al., 2024a). 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 Analytical sensitivity

- Data from FERA (GB):

The LOD was determined using the 10-fold dilution series of ToMMV-infected leaf material in water and healthy seed extract, respectively. For leaf material diluted in water the LOD was 10^{-5} and for leaf material diluted in seed extract the LOD was 10^{-4}.

- Data from Euphresco TPS:

Analytical sensitivity evaluated using dilutions of RNA from ToMMV-infected leaf material in RNA from tobamovirus-free tomato leaf material was at least 2.5×10^6 dilutions (level of agreement between 13 laboratories: 100%).

4.2 Analytical specificity

- Data from FERA (GB):

Inclusivity: 100% evaluated on 3 isolates of ToMMV (Fera diagnostic samples 2020023426, 2021005757 and DSMZ PV-1267).

In addition, the in-silico analysis targeting all the full genome sequences of ToMMV available in GenBank indicated:

- for KU594507: one difference in forward primer.
- for MW582804, KX898034: one difference in probe.
- A common polymorphism is seen in both the probe and reverse binding regions: T instead of C. This is seen in e.g. OK334224 which is from Fowkes et al. (2022).

Exclusivity: 100% evaluated on other tobamoviruses: ToMV (Fera diagnostic sample 20220027690 B, C D, E, F., 2020029550, 2020027562, 2020027564, 2021024410, 2022000021), PMMoV (Fera diagnostic sample 2022027690G, 2020027561, 2021024450, Fera positive control 04/09/2013), potato spindle tuber viroid (PSTVd) (Fera diagnostic sample 2020027563, Fera positive control 19-1 A), TMV (Fera positive control 19-1), ToBRFV (Fera diagnostic sample 2020013163, 2020015326, 2020015325, 2020013592, 2020013589, Fera positive control 19-1 A, 19-1 H) and ulluco tobamovirus (Fera positive control 2). Other viruses and viroids included in the evaluation of the exclusivity: citrus exocortis viroid (CEVd) (Fera positive control 16-1 D), Columnea latent viroid (CLVd) (Fera positive control 16-1 F), pepper chat fruit viroid (PCFVd) (Fera positive control 17-1 B), Pepino mosaic virus (PepMV) EU (Fera positive control 25.4.2016), PepMV Ch1 (Fera positive control 5), PepMV Ch2 (Fera positive control 16-2), potato virus X (PVX) (Fera diagnostic sample 21816077), potato virus Y (PVY) (Fera positive control 19-8), southern tomato virus (STV) (Fera positive control 19-2), tomato apical stunt viroid (TASVd) (Fera positive control 19-1 M), tomato planta macho viroid (TPMVd syn MPVd) (DSMZ PV-1230), tomato spotted wilt virus (TSWV) (Fera positive control 2), tomato yellow leaf curl virus (TYLCV) (Fera positive control 17-6).

- Data from Euphresco TPS:

Inclusivity: 100% evaluated on two isolates of ToMMV (one from seed and one from leaf material).

Exclusivity: from 86% (1 laboratory inconclusive result for both healthy seed samples) to 93% (12 laboratories inconclusive result for PaMMV), evaluated on 3 healthy tomato samples (two of seeds, one of leaves) and 11 isolates of 9 other tobamovirus species (1 isolate of CGMMV, ObPV, ORSV, PaMMV, PMMoV, TMGMV and ToBRFV, and 2 isolates of ToMV and TMV).

4.3 Repeatability

- Data from FERA (GB):

100% (evaluated on 8 replicates of a ToMMV positive seed sample diluted 10^{-4}, on 8 replicates of a ToMMV positive water sample diluted 10^{-5}, and on 2 replicates of a ToMMV positive undiluted sample).

4.4 Reproducibility

- Data from FERA (GB):

100% (evaluated on 8 replicates of a ToMMV positive seed sample diluted 10^{-4}, on 8 replicates of a ToMMV positive water sample diluted 10^{-5}. and on 2 replicates of a ToMMV positive undiluted sample, by 2 operators and with 3 different PCR cyclers).

- Data from Euphresco TPS:

97% (evaluated with 13 non-target RNA samples and 9 RNA samples with different dilutions of ToMMV RNA in 13 laboratories).

4.5 Other data

- Data from Euphresco TPS: The test was evaluated by 13 laboratories using a panel of samples composed of 9 target samples (dilutions of two ToMMV isolates including dilutions below the LOD) and 13 non-targets (including 3 healthy tomato samples and 11 isolates of 9 other tobamovirus species; see data on exclusivity).

Diagnostic sensitivity: 80.0%.
Diagnostic specificity: 92.3%.

APPENDIX 6 - REAL-TIME RT-PCR (DAFF & DEECA, AUSTRALIA UNPUBLISHED)

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

1. General Information

1.1 This test can be used for the detection and identification of ToMMV in tomato and pepper plant material (including seeds).

1.2 The primer and probe set was developed by California Seed & Plant Labs (CSP Labs). The test was adapted and validated by the Department of Agriculture, Fisheries and Forestry (DAFF) and the Department of Energy, Environment and Climate Action (DEECA), Australia, in 2022.

1.3 The target sequence is located on the coat protein gene of the ToMMV genome.

1.4 Amplicon location: based on the nucleotide sequence of GenBank accession no KU594507, the forward and reverse primer start at position 6037 and 6124, respectively, and the probe covers positions 6076-6089.

1.5 Oligonucleotides:

	Name	Sequence	Amplicon size*
Forward primer	CSP 1572-F	5′-CCC GAC TAC AGC CGA AAC AT-3′	109 bp
Reverse primer	CSP 1572-R	5′-TTA ACA GCG GAC CTG ATC GC-3′	
Probe	CSP 1572-P	5′-6FAM- TGC CAC TCG CAG AGT GGA CGA TGC TAC G -BHQ1-3′	

* Including primer sequences.

1.6 Real-time PCR system: Biorad CFX and BMS mic-qPCR.

1.7 Software and settings for data analyses: CFX manager v3.1 or micPCR v2.8.12 software; in both cases an automatic threshold was used.

2. Methods

2.1 Nucleic Acid Extraction

See Appendix 1, alternative procedures may also be suitable.

The RNA samples included in the Euphresco-TPS were extracted using the RNeasy Plant Mini Kit (Qiagen) with some modifications, that is, for the seeds the RLT buffer was replaced by the GH+ buffer (for other minor modifications see Mehle et al., 2024a).

For the validation data obtained at DAFF & DEECA laboratories, the RNA was extracted with the protocol modified from Hoshino et al. (2006).

2.2 One step real-time Reverse Transcription Polymerase Chain Reaction – real-time RT-PCR

2.2.1 Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	7	N.A.
RT-PCR buffer (AgPath-ID One-Step RT-qPCR, *Applied biosystems*)	2×	12.5	1×
Forward primer (*CSP 1572-F*)	10 µM	1	0.4 µM
Reverse primer (*CSP 1572-R*)	10 µM	1	0.4 µM
Probe (*CSP 1572-P*)	10 µM	0.5	0.2 µM
RT-PCR enzyme (AgPath-ID One-Step RT-qPCR, *Applied biosystems*)	25×	1	1×
Subtotal		23	
RNA extract		2	
Total		25	

2.2.2 PCR conditions

Reverse transcription at 48°C for 30 min; denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 10 s, and annealing and elongation at 60°C for 30 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 or host tissue extract spiked with the target organism).
- Negative amplification control (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.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product). The PAC should preferably be near to the limit of detection.

As an 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 of endogenous nucleic acid, using primers that amplify conserved non-pest target nucleic acid that is also present in the sample. IPC can include endogenous nucleic acid of the matrix using conserved 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.
- 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.

Laboratories should take additional care to prevent risks of cross contamination when using high concentration positive controls (e.g. cloned products, gBlocks, and whole genome amplicons) directly or when preparing dilutions of them.

Other possible controls.
Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 **Interpretation of results**: in order to assign results from this real-time PCR-based test the following criteria should be followed

The C_t cut-off value given below is as established in Department of Agriculture, Fisheries and Forestry (DAFF) & Department of Energy, Environment and Climate Action (DEECA) (AU). 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

- NIC and NAC should give no amplification.
- The PIC and PAC (and if relevant IC and IPC) amplification curves should be exponential with a C_t value below 37.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve with a C_t value below 37*.
- 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.

*Note: Samples showing an exponential amplification curve with a relatively high C_t value can be considered inconclusive as they could be due contamination with a low titre of ToMMV; however, a cross-reaction with a non-target tobamovirus could not be completely excluded. In the framework of the Euphresco TPS, the range of C_t cut-off values above which a result was considered inconclusive was 33 to 37 (depending on the laboratories). 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.

4. Performance characteristics available

Validation was carried out in accordance with PM7/98 by Department of Agriculture, Fisheries and Forestry (DAFF) & Department of Energy, Environment and Climate Action (DEECA) (AU) and by 13 laboratories in the framework of the Euphresco TPS. 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 Analytical sensitivity

- Data from Euphresco TPS:

Analytical sensitivity evaluated using dilutions of RNA from ToMMV infected plant material in RNA from tobamovirus-free tomato leaves was at least 2.5×10^5 dilutions (level of agreement between 13 laboratories: 100%).

4.2 Analytical specificity

- Data from DAFF & DEECA:

Exclusivity: 100% evaluated on ToBRFV (DSMZ Cat. No. PV-1236), TMV (DSMZ Cat. No. PV-0107), ToMV (DSMZ Cat. No. PV-0135), PaMMV (DSMZ Cat. No. PV-0606), PMMoV (DSMZ Cat. No. PV-0165), BPMV (DSMZ Cat. No. PV-0170), RMV (DSMZ Cat. No. PV-0145).

- Data from Euphresco TPS:

Inclusivity: 100% evaluated on two isolates of ToMMV (one from seed and one from leaf material).

Exclusivity: from 86% (12 laboratories with an inconclusive result for PaMMV, one laboratory with an inconclusive result also for PMMoV; one laboratory with an inconclusive result for PaMMV also had one for a ToMV isolate) to 100% (1 laboratory) evaluated on 3 healthy tomato samples (two of seeds, one of leaves) and 11 isolates of 9 other tobamovirus species (1 isolate of CGMMV, ObPV, ORSV, PaMMV, PMMoV, TMGMV and ToBRFV, and 2 isolates of ToMV and TMV).

4.3 Repeatability

- Data from DAFF & DEECA:

100% (evaluated on several replicates of a dilution of ToMMV at LOD in seed homogenates)

4.4 Reproducibility

- Data from DAFF & DEECA:

100% (evaluated on a dilution of ToMMV at LOD by 3 laboratories)

- Data from Euphresco TPS:

97% (evaluated with 13 non-target RNA samples and 9 RNA samples with different dilutions of ToMMV RNA in 13 laboratories)

4.5 Other data

Data from Euphresco TPS: Evaluated by 13 laboratories using a panel of samples composed of 9 target samples (dilutions of two ToMMV isolates including dilutions below the LOD) and 13 non-target (including 3 healthy tomato samples and 11 isolates of 9 other tobamovirus species; see data on exclusivity).
Diagnostic sensitivity: 79.2%.
Diagnostic specificity: 92.3%.