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EPPO STANDARD ON DIAGNOSTICS

PM 7/146 (2) Tomato brown rugose fruit virus

Specific scope: This Standard describes a diagnostic protocol for detection and identification of tomato brown rugose fruit virus.[1]
This Standard should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.
Specific approval and amendment: Approved in 2020–10. Revised in 2022–07.
Authors and contributors are given in the Acknowledgements section.

1 | INTRODUCTION

Tomato brown rugose fruit virus (ToBRFV; genus *Tobamovirus*) was first observed in 2014 and 2015 on tomatoes in Israel (Luria et al., 2017) and Jordan (Salem et al., 2016) respectively, and has been detected in Palestine (Alkowni et al., 2019), Canada (Sarkes et al., 2020), China, Mexico (Cambrón-Crisantos et al., 2018), the USA (Ling et al., 2019), Egypt (Amer & Mahmoud, 2020) and several EPPO countries (EPPO, 2022). For an up-to-date geographical distribution see EPPO Global Database (EPPO, 2022) The virus is a major concern for growers of tomato and pepper as it reduces the vigour of the plant, causes yield losses, and virus symptoms make the fruits unmarketable. However, the virus may also be present in asymptomatic foliage and fruit.

Tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) are the only confirmed natural cultivated hosts of ToBRFV (Luria et al., 2017; NAPPO, 2018; Panno et al., 2020; Salem et al., 2016, 2019). Tomato hybrids, wild tomato species and other *Capsicum* species (in particular *C. frutescens* and *C. chinense*) are also likely to be hosts; ToBRFV was also detected in weeds (*Chenopodium murale* and *Solanum nigrum*) in Israel (Dombrovsky, pers. comm., 2019).

The host status of *Solanum melongena* (aubergine) is not confirmed.

This Standard includes tests which have been evaluated in laboratories from the EPPO region.

Flow diagrams describing the diagnostic procedure for ToBRFV in plant material and in seeds are presented in Figures 1 and 2.

2 | IDENTITY

Name: Tomato brown rugose fruit virus.
Synonyms: None.
Acronym: ToBRFV.
Taxonomic position: Virus, *Riboviria*, *Virgaviridae*, *Tobamovirus*.
EPPO Code: TOBRFV.
Phytosanitary categorization: EPPO Alert List, EU emergency measures.

Note Virus nomenclature in Diagnostic Protocols is based on the latest release of the official classification by the International Committee on Taxonomy of Viruses (ICTV, Release 2020, https://talk.ictvonline.org/taxonomy/). Accepted species names are italicized when used in their taxonomic context, whereas virus names are not, corresponding to ICTV instructions. The integration of the genus name within the name of the species is currently not consistently adopted by ICTV working groups and therefore species names in diagnostic protocols do not include the genus name. Names of viruses not included in the official ICTV classification are based on first reports.

3 | DETECTION

3.1 | Disease symptoms

ToBRFV causes a wide range of symptoms. Symptoms may range from very severe to mild, or plants can be infected asymptomatically. Leaf symptoms often first appear in the young shoots of the plant.

3.1.1 | Symptoms on tomato

The following virus symptoms may be observed on tomato infected with ToBRFV (Salem et al., 2016; Dombrovsky & Smith, 2017; Cambrón-Crisantos et al., 2018; AHDB Horticulture, 2019):

[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.

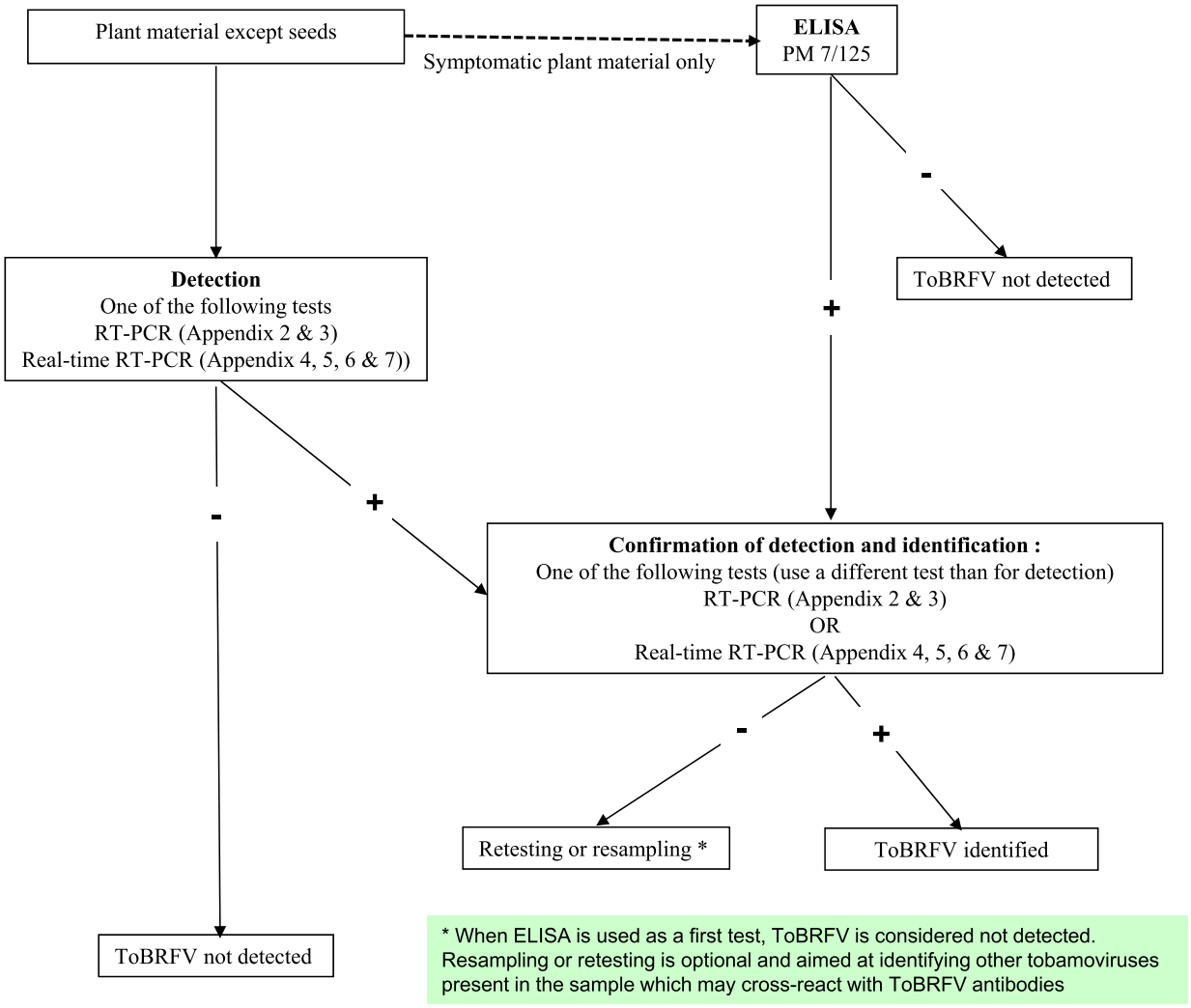

FIGURE 1 Flow diagram describing the diagnostic procedure for tomato brown rugose fruit virus in plant material except seeds.

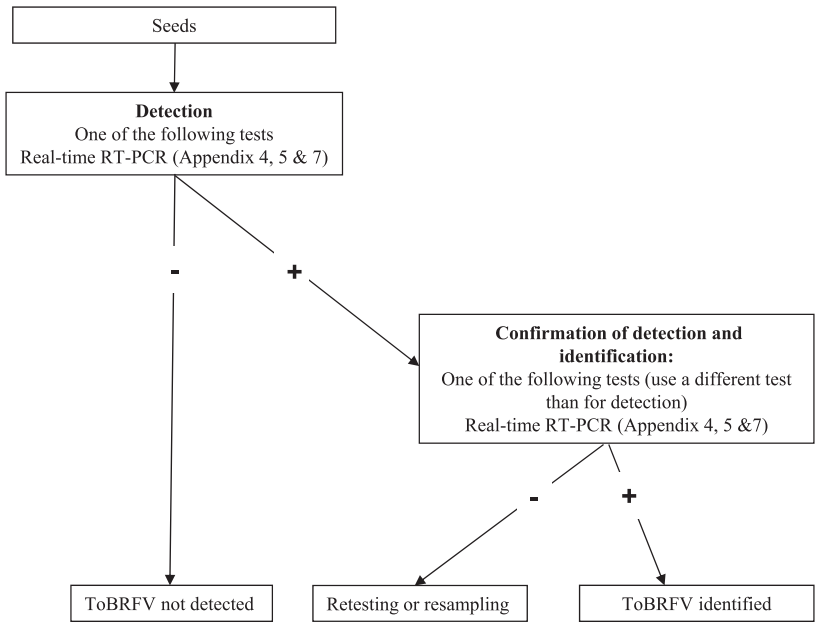

FIGURE 2 Flow diagram describing the diagnostic procedure for tomato brown rugose fruit virus in seeds.

FIGURE 3 Mosaic symptoms caused by ToBRFV on tomato leaves. Courtesy: C. Picard (EPPO).

FIGURE 4 Narrowing (needle-like symptoms) and blistering of the surface of tomato leaf caused by ToBRFV. Courtesy: C. Picard (EPPO).

- *Leaves or plants*
  - Chlorosis, mosaic patterns (chlorotic/pale patches) (Figure 3) and mottling often observed on young leaves at the top of the plant and on side-shoots.
  - Crumpling, puckering or deformation of young leaves.
  - Narrowing of leaves (needle-like symptoms), occasionally observed (Figure 4).
  - Blistering of the leaf surface (Figure 4).
  - Wilting of leaves, followed by yellowing (Figure 5) and plant death.

- *Pedicles (stems), calyx (sepals), and petioles*
  - Brown necrotic lesions (Figures 6 and 7c).

FIGURE 5 Wilting of leaves, followed by yellowing and plant death caused by ToBRFV observed in Germany. Courtesy: H. Scholz-Döbelin (LWK NRW).

FIGURE 6 Necrotic lesions caused by ToBRFV on the sepals of a young tomato fruit. Courtesy: Prof. S. Davino.

- *Fruits*
  - Yellow (chlorotic) spots and marbling of fruits (Figure 7a,b and c).
  - Dark-coloured (necrotic) spots on green fruits (Figure 7b and d).
  - Deformation and uneven ripening of young fruits (e.g. individual fruits can be red in some parts and showing green stripes, blotches or patches in other parts) (Figure 7e).

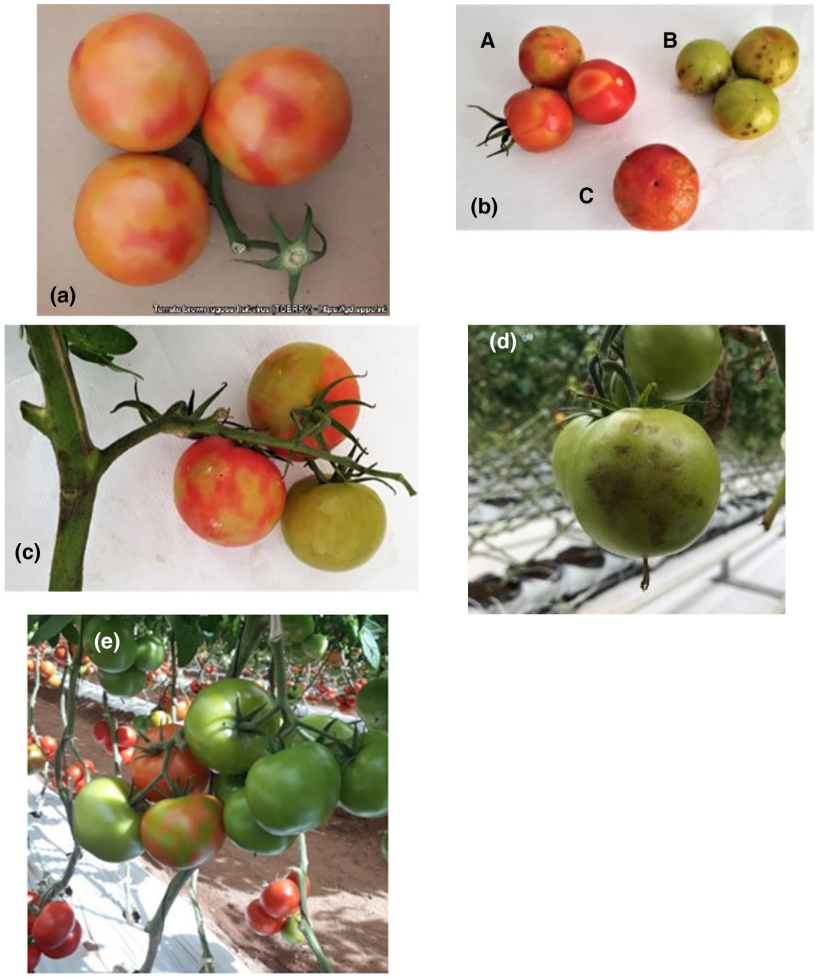

FIGURE 7 Symptoms caused by ToBRFV in tomato fruit. (a) Typical fruit symptoms with yellow (chlorotic) spots. Courtesy: Dr A. Dombrovsky. (b) Yellow and necrotic spots (A, B) and brown rugose (C) on tomato fruits. Courtesy: Dr. P. Keles Ozturk. (c) Yellow spots on tomato fruits and necrotic spots on stems and calyx. Courtesy: Dr. P. Keles Ozturk. (d) Dark-coloured (necrotic) spots on green fruits. Courtesy: D. Godínez. (e) Marbling of fruits and delay in ripening. Courtesy: Dr A. Dombrovsky.

FIGURE 8 Symptoms caused by ToBRFV on tomato fruits. Uneven fruit ripening. Courtesy NVWA.

- Orange fruits not turning red (variety Juanita in Germany; Scholz-Döbelin, pers. comm., 2020).
- Brown rugose (wrinkled) patches (rarely observed).
- Reduced number of fruits per branch.

If any of the above symptoms is observed in a tomato variety harbouring tobamovirus resistance genes (e.g. *Tm-2*^{2}), there is a strong suspicion that ToBRFV is present as these tomato varieties are susceptible to ToBRFV (Luria et al., 2017). However, similar symptoms might be caused by other viruses and symptoms might be different in the case of mixed infections. It should also be noted that physiological stresses may exacerbate symptom expression.

Milder fruit symptoms may also be observed (Figure 8).

Confusion with other diseases of tomato

Tobamoviruses such as tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV) can cause similar (nonspecific) leaf and fruit symptoms that may be confused with ToBRFV symptoms (Alkowni et al., 2019). Furthermore, other viruses such as pepino mosaic virus (PepMV), physostegia chlorotic mottle virus (PhCMoV) and tomato fruit blotch virus (ToFBV) can cause various symptoms on fruits and leaves (Ciuffo et al., 2020; Hanssen & Thomma, 2010; Temple et al., 2022). In some EPPO countries, tomato plants are inoculated with

mild strain(s) of PepMV in an early growth stage at the fruit production site to prevent infection by more severe strains (this technique is called cross-protection) (Agüero et al., 2018; Pechinger et al., 2019). This can lead to mild virus symptoms early in the growing cycle, masking the early ToBRFV symptoms, and therefore delaying the detection of ToBRFV. The uneven ripening seen on fruit infected by ToBRFV is in general more severe than for PepMV (EPPO, 2020). An illustration of mixed infection is provided in Figure 9. Double infection of ToBRFV and another tobamovirus may result in accelerated virus accumulation in the plants. This phenomenon has been observed in susceptible wild-type tomato plants and in tomato plants harbouring the *Tm-2*^{2} resistance gene infected by paprika mild mottle virus (PaMMV) and ToBRFV (Luria et al., 2018).

FIGURE 9 Fruit symptoms due to mixed infection with ToBRFV and PepMV. Courtesy NVWA.

3.1.2 | Symptoms on pepper

Only pepper plants that do not harbour *L* resistance genes/alleles[2] can be infected and can show symptoms (Turina, pers. comm., 2019); plants harbouring those genes/alleles react with a local hypersensitive response and are therefore not systemically infected (Dombrowski, pers. comm., 2019). Symptoms of a hypersensitive response are shown in Figure 10.

Based on pictures of symptoms on pepper from Mexico available from SADER and SENASICA (2019), pepper shows relatively similar symptoms to those described for tomato, but more severe necrosis is seen on fruit. In susceptible pepper varieties (see above), ToBRFV often occurs in mixed infections with other

FIGURE 10 Symptoms of a hypersensitive response (HR) to infection by ToBRFV in pepper plants having *L* resistance genes. (a–c) symptoms developed following mechanical inoculation of leaves: (a) necrotic lesions; (b, c) leaf necrosis. (d–f) HR symptoms developed following mechanical inoculation of roots, showing necrotic spots on stems and growth reduction. Photos from Luria et al., 2017.

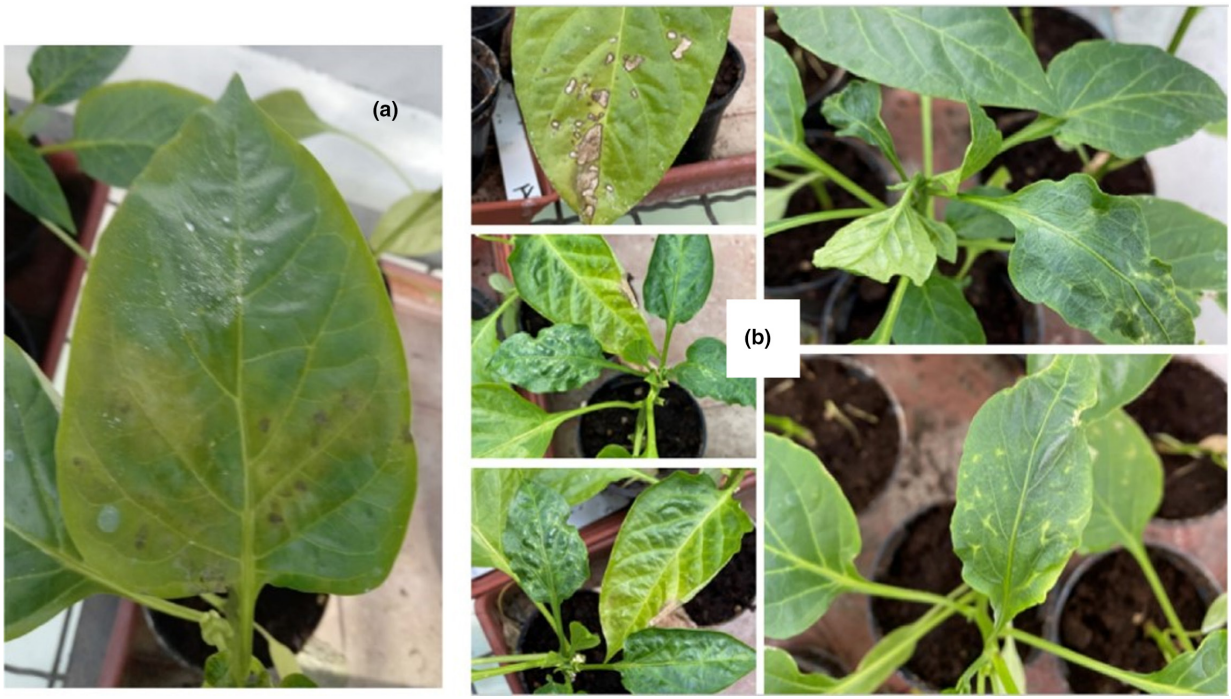

FIGURE 11 Symptoms caused by ToBRFV on pepper not harbouring the L resistance genes/alleles *L3* and *L4* after mechanical inoculation (lesions and necrosis after (a) 26 days or (b) 2 months post inoculation). Courtesy: CREA.

viruses (Sader & Senasica, 2019). Therefore, the observed symptoms are potentially due to a combination of viruses. Symptoms on pepper from an outbreak in Sicily (IT) are described in Panno et al. (2020). The infected variety did not harbour any *L* resistance genes/alleles (Davino, pers. comm., 2020). Symptoms on susceptible pepper plants developing after mechanical inoculation are shown in Figure 11.

Confusion with other diseases of pepper

Other pathogens may cause similar (non-specific) leaf and fruit symptoms that may be confused with ToBRFV symptoms.

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 | Test sample requirements for plant material except fruits and 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. 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. Experience in the EPPO region is currently mainly based on testing of plants for fruit production. In 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 x 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 sub sample 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 to 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).

Sample preparation for molecular tests is described in Appendix 1.

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.

3.2.1.1 | *Tomato crops*

To investigate best practices for sampling tomato crops, studies have been carried out in the Netherlands and the United Kingdom.

The study in the Netherlands compared the detection of ToBRFV between symptomatic and asymptomatic tomato plants grown in commercial greenhouses during an outbreak (NVWA & Naktuinbouw, NL, unpub. data). Samples were taken from leaves from different positions in the plant as well as from sepals and fruit. For symptomatic plants, the highest virus concentrations were found in the young leaves, sepals (and also fruits), whereas detection in older leaves appeared erratic. In symptomless plants grown in the same greenhouses, the detection of ToBRFV in different plant parts varied between plants.

The study in the United Kingdom investigated the reliability of testing different plant parts with respect to timing of infection, comparing detection from plants of commercial varieties inoculated at 8 weeks old and 17 weeks old. Different plant parts (upper/middle/lower leaves, sepals and fruit when ripe) were sampled at regular intervals for up to 20 weeks post inoculation. This study indicated that in plants infected early (inoculated at 8 weeks) the pattern of virus detection was relatively predictable with the virus reliably detected from the upper leaves after approximately 2 weeks. However, in mature plants (inoculated at 17 weeks) virus detection from plant parts is more erratic, but the virus is likely to be detected from fruit and sepals earlier than upper leaves (Skelton et al., 2021).

Based on these data and the fact that virus concentrations are usually the highest in actively growing tissue, it is recommended for both symptomatic and asymptomatic plants to sample young leaflets, sepals and/or fruits.

3.2.1.2 | *Pepper crops*

No data is available for testing of different parts of pepper plants and therefore based on general experience the sampling of young leaves is currently recommended.

3.2.1.3 | *Propagation material*

No data is available on the testing of young plants grown in nurseries. It is not known at which growth stage of the plants the virus can be detected reliably. Seed to seedling transmission in tomato has been found to be low (0.08% transmission rate; Salem et al., 2022). Because there are no data available on the limitations of testing young plants, testing should ideally be carried out as late as practically possible before the plants leave the nursery. It should be noted that the likelihood of spread from an infected plant to other plants is higher for grafted plants because of the handling of the plants during the grafting process.

3.2.2 | Test sample requirements for fruits

ToBRFV has been successfully detected in symptomatic tomato and pepper fruits. Details on sampling strategies are not available. Testing of fruits is mainly performed on imported or marketed symptomatic fruits. Sample preparation is described in Appendix 1.

For fruits with calyces, the sepals should preferably be tested.

3.2.3 | Test sample requirements for seeds

For seed testing it is difficult to recommend sample sizes and bulking rates. For seed lots of tomato and pepper, protocols using weighed samples of approximately 3000 seeds, tested in three subsamples of 1000 seeds, have been validated for real-time RT-PCR (ISF, 2020). In case of 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). Guidance on sampling is provided in 2020/1191 (EU, 2020, last amended by EU Commission Implementing Regulation [EU] 2021/1809). Subsample size may require adaptation if other tests need to be performed with smaller subsample size requirements.

Seed treatments might influence the outcome of a test. 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. Sample preparation is described in Appendix 1.

3.3 | Screening tests

3.3.1 | Molecular methods

Several specific molecular tests have been described for the detection of ToBRFV and those recommended are described in Appendices. For RNA extraction, see Appendix 1.

3.3.1.1 | *Molecular tests for testing plant material (except seeds)*

Most of the molecular tests included in this protocol were evaluated in a Test Performance Study (TPS) organized in the framework of the EU-funded project VALITEST, including one commercial kit (Luigi et al., 2022). Outcomes of the TPS are available through the EPPO database on diagnostic expertise (https://dc.eppo.int/validation_data/validationlist). In this revised version of the protocol, two additional tests have been included: Panno et al. (2019b) which was evaluated in VALITEST, and Bernabé-Orts et al. (2021) which targets a different part of the ToBRFV genome compared to the tests evaluated in the framework of VALITEST.

The recommended tests are:

- Conventional RT-PCR: using the primers from Alkowni et al. (2019) – Appendix 2.
- Conventional RT-PCR: Loewe Biochemica GmbH using the primers from Rodríguez-Mendoza et al. (2019) – Appendix 3.
- Real-time RT-PCR: using the CaTa28 and CSP1325 primers and probes from ISF (2020) – Appendix 4.

- Real-time RT-PCR: using the primers and probe from Menzel and Winter (2021) – Appendix 5.
- Real-time RT-PCR: using primers and probe from Panno et al. (2019b) – Appendix 6.
- Real-time RT-PCR using primers and probe from Bernabé-Orts et al. (2021) – Appendix 7.

The conventional RT-PCR tests described in Ling et al. (2019) and Panno et al. (2019a) are not included in this protocol due to cross-reactions with other tobamoviruses (Anthoine et al., 2020). The test described in Luria et al. (2017) has not been included as other tests have a better analytical sensitivity as well as analytical specificity (F. Constable, pers. comm. 2019).

LAMP tests are being evaluated for plant material and will be considered for a further revision of this protocol.

Generic tobamovirus RT-PCR tests may be used for screening of plant material (except seeds) for tobamoviruses but have not been specifically evaluated for ToBRFV. In any case, because they also detect other species, confirmation of positive tests with sequence analysis or a specific molecular test (see above) is required. No validation data is available for these tests.

3.3.1.2 | *Molecular tests for testing seeds*

Molecular tests included in this protocol were evaluated in a Test Performance Study (TPS) organized in the framework of a Euphresco project (2019-A-327) on tomato and pepper seeds (Giesbers et al., 2021). Outcomes of the TPS are available in the EPPO database on diagnostic expertise. An additional test (Bernabé-Orts et al., 2021) targeting a different part of the ToBRFV genome compared to the other tests evaluated in the framework of the TPS, is included in the protocol. This test has been evaluated by two laboratories.

The recommended tests are:

- Real-time RT-PCR using the CaTa28 and CSP1325 primers and probes from ISF (2020) – Appendix 4.
- Real-time RT-PCR using the primers and probe from Menzel and Winter (2021) - Appendix 5.
- Real-time RT-PCR: using the primers and probe from Bernabé-Orts et al. (2021) – Appendix 7.

The LAMP test from Sarkes et al. (2020) and the isothermal amplification kit (AmplifyRP® XRT for ToBRFV) were evaluated in a Euphresco TPS and are not included in this protocol for the detection of ToBRFV in seeds (Giesbers et al., 2021), because of their lower diagnostic sensitivity compared to the real-time RT-PCR tests recommended above.

The test of Panno et al. (2019b) was not evaluated in the Euphresco TPS. Consequently, although it is recommended for plant material, this test is not recommended for seed testing.

3.3.2 | Other methods

3.3.2.1 | *Serological tests*

ELISA may be used as a screening test on symptomatic plant material. Instructions for performing an ELISA are provided in the EPPO Standard PM 7/125 *ELISA tests for viruses* (EPPO, 2015). However, ToBRFV antisera currently available were either found to cross-react with other tobamoviruses or have limited validation data available. Confirmation of positive samples with a molecular test (see section 3.3.1.1.) is required.

Validation data gathered in the framework of the Euphresco project (Giesbers et al., 2021), showed that serological tests (DAS-ELISA tests from Agdia, DSMZ, Loewe and Prime diagnostics) are not suitable for the detection of ToBRFV in seeds because of their lower diagnostic sensitivity compared to molecular tests. Further validation data is needed before serological tests can be considered for inclusion in this protocol for testing of asymptomatic fruits and leaves.

3.3.2.2 | *Bioassay*

There are no validation data available on the use of bioassay for detection of ToBRFV and for the confirmation of the presence of viable ToBRFV 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 laboratories in the region 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*. Test plants recommended are *Nicotiana glutinosa* or *Nicotiana tabacum* cv. Xanthi NN (ISF, 2020). Plants should be examined for local lesions (positive and negative controls should be included).

4 | IDENTIFICATION

4.1 | Molecular tests

The molecular tests described in section 3.3.1.1 are recommended for identification and confirmation.

The choice of the test will depend on the matrix and the expected virus concentration. For leaf material, both real-time and conventional RT-PCR tests can be used, whereas for seeds real-time RT-PCR tests listed in section 3.3.1.2 are recommended.

4.2 | Other tests

4.2.1 | Sequencing

Sanger sequencing or high-throughput sequencing analysis can be used for further confirmation when the virus concentration allows for its application.

Sequence analysis of amplicons obtained from specific RT-PCR (3.3.1.1), or when relevant generic RT-PCR (3.3.1.1) can be used for identification. Sequence analysis should follow the guidelines described in Appendix 7 and 8 of the EPPO Standard PM 7/129 *DNA barcoding as an identification tool for a number of regulated pests* (EPPO, 2021).

5 | REFERENCE MATERIAL

ToBRFV isolates for reference are available from:

DSMZ Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH Inhoffenstraße 7 B 38124 Braunschweig (DE) (plantvirus@dsmz.de).

Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (CREA), Centro di Ricerca Difesa e Certificazione (CREA-DC), Via C.G. Bertero, 22, 00156 Rome, (IT) (antonio.tiberini@crea.gov.it).

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 is 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: Botermans M (NVWA, NL), Fox A (Fera, GB), Giesbers AKJ (NVWA, NL), Luigi M (CREA, IT), Mehle N (NIB, SI), Oplaat AG (NVWA, NL), Roenhorst JW (EURL for Pests of Plants on Viruses, Viroids and Phytoplasmas), Tomassoli L (CREA, IT) and Ziebell H (JKI, DE).

9 | FEEDBACK ON THIS DIAGNOSTIC PROTOCOL

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

10 | PROTOCOL REVISION

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

ACKNOWLEDGEMENTS

This protocol and its revision were prepared by an Expert Working Group composed of Fox A (Fera, GB lead author), Giesbers AKJ (NVWA, NL), Luigi M (CREA, IT), Mehle N (NIB, SI), Roenhorst JW (EURL for Pests of Plants on Viruses, Viroids and Phytoplasmas), Oplaat AG (NVWA, NL), Tomassoli L (CREA), Ziebell H (JKI, DE). It was reviewed by the Panel on Virology and Phytoplasmology.

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APPENDIX 1 - SAMPLE PREPARATION AND RNA EXTRACTION FOR MOLECULAR METHODS

This appendix describes sample preparation and RNA extraction methods for different hosts and types of plant material. These initial steps are critical for the results of a test and are often more related to the matrix than the specific test. Therefore, they are described in this separate appendix.

RNA extraction using CTAB (e.g. Gambino et al., 2008) or other extraction methods or kits may be used but they 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 −20°C (<1 month) or at −80°C for longer periods.

Care should be taken when handling samples as high concentration of virus may be present.

1. Plant material (leaves or fruits)

1.1. Individual plants and/or small samples

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

1.2. Pooled samples

Pooled samples of tomato leaflets or sepals, pepper leaves, or fruits 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 fruit samples of tomato and pepper, a small piece of the skin and adjacent tissue should be sampled from at least three different locations of each fruit. For pooled samples often larger amounts of plant material are involved. In such cases other buffers can be used for homogenization, such as ELISA buffer, phosphate buffer or GH+ buffer (see Table A1).

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	

Phosphate buffer

Plant tissue is ground in 0.1 M phosphate buffer (Na_2HPO_4/KH_2PO_4, pH 7.2), at a range of 1:10 w/v for leaves and 1:20 w/v for fruits. 100 µL of the obtained sap are added to 380 µL of RLT buffer of the RNeasy Plant Mini kit (Qiagen, Germany) and RNA is then extracted following the manufacturer's instructions.

GH+ buffer

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

For high-throughput RNA extraction, the Sbeadex® maxi plant kit can be used in combination with a Kingfisher KF96 system. In this system 250 µL of the supernatant is transferred to a binding plate containing 450 µL 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 are described. In the Euphresco project (Giesbers et al., 2021), total RNA extract from GH+ buffer resulted in lower average Ct values for the real-time RT-PCR tests, with an average difference of around one cycle compared to phosphate buffer. For the other molecular tests, both extraction buffers gave similar qualitative results 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 to three subsamples of four homogenates.

Briefly, 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 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. These conditions were evaluated during the Euphresco TPS.

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) is added. The seeds are soaked at room temperature for 30–60 min before homogenization (e.g. with an Interscience BagMixer on position 4) for 90 s (tomato) or 4 min (pepper).

Alternatively, dry seeds are ground with a Genogrinder. Three subsamples of 1000 tomato or six subsamples of 500 pepper seeds are transferred to a 50 mL tube and a steel ball (14 mm) is added. Seeds are ground, tubes upside down, at 1700 rpm for 4 min for tomato and 7 min for pepper seeds. After grinding 20 mL GH+ buffer is added, for tomato 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 kit can be used in combination with a Kingfisher KF96 system. In this system 250 µL of the supernatant is transferred to a binding plate containing 600 µL of binding buffer (kit) and 50 µL particle suspension (both included in the kit) and RNA is extracted following the manufacturer's instructions.

Note that when bacopa chlorosis virus (BaCV) is used as internal control, this virus is added to GH+ buffer (BaCV-infected leaf material homogenized in a dilution aiming for a Ct value between 28–32).

APPENDIX 2 - CONVENTIONAL RT-PCR TEST (USING THE PRIMERS FROM ALKOWNI ET AL., 2019)

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

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

1. General information

1.1. This conventional RT-PCR is suitable for the detection and identification of ToBRFV in leaves, sepals and fruit of tomato and pepper.

1.2. The test uses the primers of Alkowni et al., 2019. The original protocol was adapted to a one-step format.

1.3. The ToBRFV-F/ToBRFV-R primers were designed to target the small replicase subunit region of isolate TBRFV-Ps, accession number MK165457 (primer positions: forward 735–755, reverse 1271–1294).

1.4. Oligonucleotides and average amplicon size:

	Primer	Sequence	Amplicon size
Forward primer	ToBRFV-F	5′-AAT GTC CAT GTT TGT TAC GCC-3′	560 bp
Reverse primer	ToBRFV-R	5′-CGA ATG TGA TTT AAA ACT GTG AAT-3′	

1.5. The test has been successfully performed using the OneStep RT-PCR Kit (Qiagen, ref 21 021). Alternative reagents that have been used successfully are Verso 1-Step RT-PCR Kit ReddyMix (Thermo Scientific) (Fera Science Ltd) and One Taq One-Step RT-PCR Kit with Quick-Load buffer (NEB) (JKI).

2. Methods

2.1. Nucleic acid extraction and purification

See Appendix 1, alternative procedures may also be suitable.

2.2. One-step RT-PCR

2.2.1. Master Mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	11.6	
OneStep RT-PCR Buffer (Qiagen)	5×	4.0	1×
dNTPs	10 mM	0.8	0.4 mM
ToBRFV-F	10 µM	0.4	0.2 µM
ToBRFV-R	10 µM	0.4	0.2 µM
OneStep RT-PCR Enzyme mix (Qiagen)	NA	0.8	NA
Subtotal		18.0	
Sample RNA extract	NA	2	NA
Total		20.0	

2.2.2. RT-PCR cycling conditions

Reverse transcription at 50°C for 30 min; denaturation at 95°C for 15 min; 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s; final extension at 72°C for 10 min.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, 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 a known amount of nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue or a synthetic control (e.g. cloned PCR product, artificial RNA or DNA). The PAC should preferably be near to the limit of detection.

As an alternative (or in addition) to the PIC, internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include an endogenous nucleic acid of the matrix using conserved primers, preferably amplifying RNA targets, such as *nad5* (Menzel et al., 2002).

3.2. Interpretation of results

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC and PAC: a band of the expected size (~560 bp) is visualized.
- IPC if used: a band of the expected size (e.g. ~181 bp for *nad5*) is visualized.

When these conditions are met

- A test will be considered positive if a band of the expected size (~560 bp) is visualized.
- 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 general, for viruses and viroids, bands of different sizes may correspond to strains of the target organism and care should be taken when interpreting the results of conventional PCR, in particular the sizes of bands.

4. Performance criteria available

The test was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* within the EU project VALITEST (on leaves) (Luigi et al., 2022).

For VALITEST TPS, the data used for the evaluation of this test were obtained by 22 participants. The panel of samples consisted of 22 blind samples (obtained from freeze-dried sap of tomato and pepper leaves) including:

- 2 non-target samples (tomato and pepper) in duplicate.
- 2 target samples (in tomato) infected at low or medium concentration in duplicate.
- 5 ten-fold dilution points (10^{0}, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) of a target sample (ToBRV infected tomato) in duplicate or triplicate and 3 controls.

The validation data are available at https://dc.eppo.int/validation_data/validationlist.

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

Data from VALITEST preliminary study: the pest was detected in dilutions of 10^{-3} in tomato and 10^{-2} in pepper, based on a dilution series prepared from sap of three infected tomato or pepper plants diluted in sap from healthy plants.

Data from VALITEST TPS in tomato (pepper not evaluated): LOD 95% (i.e. the dilution at which a detection probability of 95% is expected): $10^{-3.4}$.

4.2. Analytical specificity data

- Inclusivity

In the VALITEST preliminary study, evaluated with ToB-SIC21/19; ToB-SIC22/19; ToB-SIC23/19; ToB-SIC24/19; ToB-SIC 25/19 and ToB-PIE105/2019 (originally isolated from *Solanum lycopersicum* and *Capsicum annuum*) belonging to the CREA-DC collection and ToBRFV PV-1236, PV-1241 and PV1244 belonging to the DSMZ collection.

All isolates were detected.

- Exclusivity

No cross-reactions occurred with isolates of any of the viruses or viroids listed below. Please note that data provided below is from different sources as specified in the brackets below.

Tobamoviruses: bell pepper mosaic virus (VALITEST, JKI), cucumber green mottle mosaic virus (JKI), pepper mild mottle virus (VALITEST, Fera and JKI), tobacco mild green mosaic virus (VALITEST), tobacco mosaic virus (VALITEST, Fera and JKI), tomato mosaic virus (VALITEST, Fera and JKI), tomato mottle mosaic virus (JKI), ullucus tobamovirus-1 (Fera).

Common tomato affecting viruses: pepino mosaic virus (all strains) (Fera), southern tomato virus (Fera), tomato spotted wilt virus (Fera and JKI), tomato black ring virus (JKI), tomato bushy stunt virus (JKI), tomato ringspot virus (JKI).

Other viruses: barley stripe mosaic virus (JKI), pea early browning virus (JKI), potato mop-top virus (JKI), soil-borne cereal mosaic virus (JKI).

Pospiviroids: citrus exocortis viroid (Fera), columnea latent viroid (Fera), pepper chat fruit viroid (Fera), potato spindle tuber viroid (Fera), tomato apical stunt viroid (Fera), tomato planta macho viroid (Fera).

Mixed infections: potato virus X with tomato mosaic virus (Fera), potato virus Y with tobacco mosaic virus (Fera).

4.3. Selectivity

No false positive reactions obtained with healthy leaf material of *Capsicum annuum* (pepper) (Fera, JKI, VALITEST), *Chenopodium murale* (JKI), Petunia × hybrida, variety Himmelsröschen (JKI), *Solanum lycopersicum* (tomato) (Fera, VALITEST), *Solanum melongena* (aubergine) (JKI) and *Ullucus tuberosus* (Fera).

4.4. Diagnostic sensitivity and diagnostic specificity

	VALITEST TPS for plant material
	Value (95% confidence interval)
Diagnostic sensitivity	81% (43–100%)
Diagnostic specificity	98% (95–100%)

4.5. Repeatability and reproducibility

	VALITEST TPS for plant material
	Value (95% confidence interval)
Repeatability/accordance	88% (78–98%)
Reproducibility/concordance	87% (85–90%)

APPENDIX 3 - CONVENTIONAL RT-PCR (LOEWE BIOCHEMICA GMBH, USING PRIMERS FROM RODRÍGUEZ-MENDOZA ET AL., 2019)

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

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

1. General information

1.1. This conventional RT-PCR is suitable for the detection and identification of ToBRFV in leaves, sepals and fruit of tomato and pepper.

1.2. This test uses primers designed by Rodríguez-Mendoza et al. (2019). The original protocol was adapted to a one-step format and further optimized for routine use by the Loewe Biochemica GmbH.

1.3. Primers ToBRFV-FMX and ToBRFV-RMX were designed to target part of the putative RNA-dependent RNA polymerase gene of isolate ToBRFV-IL, accession number KX619418 (primer position: forward 2063–2086; reverse 2514–2537).

1.4. Oligonucleotides

	Primer	Sequence	Amplicon size
Forward primer	ToBRFV-FMX	5'-AAC CAG AGT CTT CCT ATA CTC GGA A-3'	~475 bp
Reverse primer	ToBRFV-RMX	5'-CTC WCC ATC TCT TAA TAA TCT CCT-3'	

2. Methods

2.1. Nucleic acid extraction

See Appendix 1. Alternative procedures may also be suitable.

2.2. One-step RT-PCR (Loewe Biochemica GmbH)

2.2.1. Prepare master mix according to manufacturer's instructions.

2.2.2. Reverse transcription PCR cycling parameters according to manufacturer's instructions.

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 an alternative (or in addition) to the PIC, internal positive controls (IPCs) can be used to monitor each individual sample separately. IPC can include an endogenous nucleic acid of the matrix using conserved primers, preferably amplifying RNA targets, such as *nad5* (Menzel et al., 2002).

3.2. Interpretation of results

Verification of the controls

- NIC and NAC: no band is visualized.
- PIC and PAC: a band of the expected size (~475 bp) should be visualized.
- IPC if used: a band of the expected size (e.g. ~181 bp for *nad5*) is visualized.

When these conditions are met

- A test will be considered positive if a band of the expected size (~475 bp) is visualized.
- 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 general, for viruses and viroids bands of different sizes may correspond to strains of the target organism and care should be taken when interpreting the results of conventional PCR, in particular the sizes of bands.

4. Performance criteria available

The test was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* within the EU project VALITEST (on leaves) (Luigi et al., 2022).

For VALITEST TPS, the data used for the evaluation of this test were obtained by 21 participants. The panel of samples consisted of 22 blind samples (obtained from freeze-dried sap of tomato and pepper leaves) including:

- 2 non-target samples (tomato and pepper) in duplicate.
- 2 target samples (tomato) infected at low or medium concentration in duplicate.
- 5 ten-fold dilution points (10^{0}, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) of a target sample (ToBRFV infected tomato) in duplicate or triplicate and 3 controls.

The validation data are available at https://dc.eppo.int/validation_data/validationlist.

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

Data from VALITEST preliminary study: dilutions of 10^{-5} in tomato and 10^{-3} in pepper, based on a dilution series prepared from sap of three infected tomato or pepper plants diluted in sap from the respective healthy plants.

Data from VALITEST TPS in tomato (pepper not evaluated): LOD 95% (i.e. the dilution at which a detection probability of 95% is expected): $10^{-3.3}$.

4.2. Analytical specificity data

4.2.1. Inclusivity

In the VALITEST preliminary study, evaluated with ToB-SIC21/19; ToB-SIC22/19; ToB-SIC23/19; ToB-SIC24/19; ToB-SIC 25/19 and ToB-PIE105/2019 (originally isolated from *Solanum lycopersicum* and *Capsicum annuum*) belonging to the CREA-DC collection and ToBRFV PV-1236, PV-1241 and PV1244 belonging to the DSMZ collection. All isolates were detected.

4.2.2. Exclusivity

In the VALITEST preliminary study, no cross-reactions were observed with isolates of bell pepper mottle virus, pepper mild mottle virus, tobacco mild green mottle virus, tobacco mosaic virus, tomato mosaic virus, obtained from DSMZ (isolates PV-0170, PV-0124, PV-1252, PV-0141 and PV-0165, respectively) and pepper mild mottle virus isolate from Italy (PMMV-218/14 CREA-DC).

4.3. Selectivity data

No false positive reactions obtained with healthy tomato and pepper leaves and tomato fruits.

4.4. Diagnostic sensitivity and diagnostic specificity

	VALITEST TPS for plant material
	Value (95% confidence interval)
Diagnostic sensitivity	85% (57–100%)
Diagnostic specificity	93% (88–99%)

4.5. Repeatability data

	VALITEST TPS for plant material
	Value (95% confidence interval)
Repeatability/ accordance	81% (68–94%)
Reproducibility/ concordance	81% (82–79%)

APPENDIX 4 - REAL-TIME RT-PCR TEST (USING CATA28 AND CSP1325 PRIMERS AND PROBE FROM ISF, 2020)

A. Test as in ISF-ISHI-Veg (2020).

The test below is described as in ISF-ISHI-Veg (2020). Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This one-step duplex (or triplex when BaCV is used) real-time RT-PCR protocol was developed for the detection and identification of ToBRFV in tomato and pepper seeds but can also be used for leaves or sepals.

1.2. The test is based on CaTa28 primers and probe developed by Enza Zaden BV (NL) in combination with the CSP1325 primers and probe developed by CSP Labs (US). This test includes the BaCV primers and probe developed by Naktuinbouw (NL) as internal positive control.

The target sequence of the CaTa28 primers is located within the movement protein gene; using the nucleotide sequence of GenBank accession no NC_028478.1, the forward and reverse primer start at position 5163 and 5283, respectively, and the probe covers positions 5191–5211. The target sequence of the CSP1325 primers is located at the end of the coat protein gene, using the nucleotide sequence of GenBank accession no NC_028478.1, the forward and reverse primers start at positions 6144 and 6223, respectively, and the probe covers positions 6169–6195.

1.3. Oligonucleotides

	Primer/probe	Sequence
Forward primer	CaTa28 Fw	5'-GGT GGT GTC AGT GTC TGT TT-3′
Reverse primer	CaTa28 Pr	5'-GCG TCC TTG GTA GTG ATG TT-3′
Probe		5'-6FAM-AGA GAA TGG AGA GAG CGG ACG AGG-BHQ'1–3'
Forward primer	CSP1325 Fw	5'-CAT TTG AAA GTG CAT CCG GTT T-3′
Reverse primer	CSP1325 Pr	5′-GTA CCA CGT GTG TTT GCA GAC A-3′
Probe		5'-VIC-ATG GTC CTC TGC ACC TGC ATC TTG AGA-BHQ'1–3'
Forward primer*	BaCV-F	5'-CGA TGG GAA TTC ACT TTC GT-3′
Reverse primer*	BaCV-R	5′-AAT CCA CAT CGC ACA CAA GA-3′
Probe*	BaCV-P	5′-TxR - CAA TCC TCA CAT GAT GAG ATG CCG-BHQ'2–3'

*When BaCV is used as IPC.

1.4. The test has been validated using UltraPlex™ 1-Step ToughMix (QuantaBio) using CFX 96 (Bio-Rad).

2. Methods

2.1. Nucleic acid extraction and purification

See Appendix 1. Alternative procedures may also be suitable.

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	NA	Up to 20	
UltraPlex 1-Step ToughMix (QuantaBio)	4×	6.25	1×
CaTa28 Fw	10 µM	0.75	0.3 µM
CaTa28 Rv	10 µM	0.75	0.3 µM
CaTa28 Pr	10 µM	0.50	0.2 µM
CSP1325 Fw	10 µM	0.75	0.3 µM
CSP1325 Rv	10 µM	0.75	0.3 µM
CSP1325 Pr	10 µM	0.50	0.2 µM
BaCV-F*	10 µM	0.75	0.3 µM
BaCV-R*	10 µM	0.75	0.3 µM
BaCV-P*	10 µM	0.50	0.2 µM
Subtotal		20.00	
RNA		5.00	
Total		25.00	

*When BaCV is used as IPC.

2.2.2. Real-time RT-PCR cycling conditions

reverse transcription at 50°C for 10 min; denaturation at 95°C for 3 min; 40 cycles of denaturation at 95°C for 10 s 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 (IPC) can be used to monitor each individual sample separately. In ISF-ISHI-Veg (2020), bacopa chlorosis virus (BaCV) is used as an IPC. IPC can also 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. Alternatively, COX (e.g. Weller et al., 2000 or Papayiannis et al., 2011) can also be used as IPC.

Comment: A study was conducted by the EURL (NVWA) to investigate the impact of different IPC (BaCV, *nad5*, COX) on the performance of the ISF test. The comparison of the results at three different virus concentrations (10^{-6}, 10^{-7}, 10^{-8}) show that using different internal controls is unlikely to have an effect on the (qualitative) outcome of the test

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should be negative.
- The PIC and PAC (as well as IPC, if applicable) should be positive.

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.

It should be noted that a preliminary cut-off value at 32 has been indicated in ISF-ISHI-Veg (2020). As a Ct cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

4. Performance characteristics available

A validation report has been prepared by ISF in March 2020 and is available on ISF website (https://worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-validation-reports/).

A slightly modified test was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* in a TPS organized in the framework of the Euphresco project 2019-A-327 (see Appendix 3 of the technical project report for the details on the test):

In this TPS, the data used for the evaluation of this test were obtained by 16 participants. The panel of samples consisted of 37 blind samples and 3 controls (1000 seeds per sample) including:

- 6 and 5 non-target samples (tomato and pepper respectively).
- 20 and 4 target samples (tomato) infected at medium or high concentration respectively.
- 5 target samples (pepper) infected at medium concentration.

The validation data are available at https://dc.eppo.int/validation_data/validationlist.

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).

4.1. Analytical sensitivity (relative)

- Data from ISF

Two seed samples from *Solanum lycopersicum* and *Capsicum annuum* were used to prepare healthy seed extracts. The seed extracts were spiked with ToBRFV-infected leaf material (5 mg of dried leaves) and used to prepare three individual 10-fold dilution series up to six dilutions per seed sample. A pre-test was performed to determine the starting concentration of the ToBRFV-infected leaf material used for spiking with the aim to reach the analytical sensitivity (relative) of the ELISA at the second or third dilution step (details available in the report). A total of 42 spiked samples (2 seed samples × 3 dilution series × 7 concentrations) were tested in triplicate.

Samples at a dilution of 10^{-8} were consistently detected when using RNeasy Plant Mini Kit (Qiagen) for RNA extraction. Samples at a dilution of 10^{-7} were consistently detected when using Sbeadex Plant Maxi kit (LGC Genomics) for RNA extraction.

- Data from Euphresco project preliminary studies

Using a serial dilution of infected seed extract into healthy seed extract, dilutions of 10^{-5} and 10^{-4} were detected using GH+ or phosphate buffers (respectively) for RNA extraction (tomato seeds).

4.2. Analytical specificity

- Data from ISF

Multiple company data from leaf and/or seed samples from various origins infected with the target virus ToBRFV or nontarget virus/viroid was compiled.

- Inclusivity: All 17 available ToBRFV isolates, originating from eight different countries (Israel, Saudi Arabia, Mexico, Germany, Egypt, United Kingdom, Jordan, USA), were detected with both primer/probe sets.
- Exclusivity: Exclusivity was evaluated on bell pepper mottle virus, columnea latent viroid, cucumber green mottle mosaic virus, cucumber mosaic virus, dahlia latent viroid, kyuri green mottle mosaic virus, paprika mild mottle virus (2 strains), pepino mosaic virus (4 strains), pepper chat fruit viroid, pepper mild mottle virus (4 strains), tobacco mild green mosaic virus, tobacco mosaic virus (3 strains), tomato apical stunt viroid, tomato chlorotic dwarf viroid, tomato mosaic virus (7 strains), tomato mottle mosaic virus (2 strains), tomato planta macho viroid, tropical soda apple mosaic virus.

None of the non-ToBRFV isolates reacted with either of the two sets of primers (CaTa28 and CSP1325).

- Data from Euphresco project preliminary studies

No cross-reactions were observed with isolates of bell pepper mottle virus (BPeMV), pepper mild mottle virus (PMMV), tobacco mild green mottle virus (TMGMV), tobacco mosaic virus (TMV), tomato mosaic virus (ToMV).

- Data from EURL for Pests of Plants on Viruses, Viroids and Phytoplasmas

It should be noted that based on the result of the Proficiency Test on leaf material, organized by the EURL (NVWA), it cannot be excluded that cross reactions may occur with other tobamoviruses when present at high concentrations as was the case with non-diluted samples prepared from indicator plants inoculated with TMV.

4.3. Diagnostic sensitivity and specificity

	Euphresco TPS for seed material	
	Tomato	**Pepper**
Diagnostic sensitivity	98%	98%
Diagnostic specificity	98%	97%

4.4. Repeatability

- Data from ISF: 100%.

For both *S. lycopersicum* and *C. annuum* ToBRFV could be detected in all three replicates up to a dilution of 10^{-8} when using the RNeasy Plant Mini Kit (Qiagen).

4.5. Reproducibility

- Data from ISF

100% at 10^{-7} with Sbeadex Plant Maxi kit (LGC Genomics).
100% at 10^{-8} with RNeasy Plant Mini Kit (Qiagen).
Evaluated in two laboratories.

4.6. Other information
When compared with ELISA this Duplex real-time RT-PCR test was 1000× more sensitive using the RNeasy Plant Mini Kit (Qiagen) and 100× more sensitive with Sbeadex Plant Maxi kit (LGC Genomics).

B. Adapted ISF-ISHI-Veg test for plant material (except seeds) testing (Master Mix and PCR conditions).

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

1. General information

1.1. This one-step duplex real-time RT-PCR protocol based on ISF-ISHI-Veg (2020) was developed for the detection and identification of ToBRFV in leaf, sepal and fruit material. Primers and probes concentrations are changed in comparison to the original protocol.
1.2. The test is based on CaTa28 primers and probe developed by Enza Zaden BV (NL) in combination with the CSP1325 primers and probe developed by CSP Labs (US).

The target sequence of the CaTa28 primers is located within the movement protein gene; using the nucleotide sequence of GenBank accession no NC_028478.1, the forward and reverse primers start at positions 5163 and 5283, respectively, and the probe covers positions 5191–5211. The target sequence of the CSP1325 primers is located at the end of the coat protein gene; using the nucleotide sequence of GenBank accession no NC_028478.1, the forward and reverse primers start at positions 6144 and 6223, respectively, and the probe covers positions 6169–6195.

1.3. Oligonucleotides

	Primer/probe	Sequence
Forward primer	CaTa28 Fw CaTa28 Rv	5'-GGT GGT GTC AGT GTC TGT TT-3′
Reverse primer	CaTa28 Pr	5'-GCG TCC TTG GTA GTG ATG TT-3′
Probe		5'-6FAM- AGA GAA TGG AGA GAG CGG ACG AGG-BHQ'1–3'
Forward primer	CSP1325 Fw	5'-CAT TTG AAA GTG CAT CCG GTT T-3′
Reverse primer	CSP1325 Rv CSP1325 Pr	5′-GTA CCA CGT GTG TTT GCA GAC A-3′
Probe		5'-VIC-ATG GTC CTC TGC ACC TGC ATC TTG AGA-BHQ'1–3'

1.4. The test has been validated using a TaqMan® RNA-to-Ct™ 1-Step Kit (Thermo Fisher Scientific) and an iTaq™ Universal Probes One-Step kit (Bio-Rad) using CFX96 optical reaction module with C1000 Touch thermal cycler (Bio-Rad).

2. Methods

2.1. Nucleic acid extraction and purification
See Appendix 1. Alternative procedures may also be suitable.

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	NA	5.90	
TaqMan® RT-PCR mix (RNA-to-Ct™ 1-Step master, Thermo Fisher Scientific)	2×	10.00	1×
CaTa28 Fw	10 µM	0.30	0.15 µM
CaTa28 Rv	10 µM	0.30	0.15 µM
CaTa28 Pr	10 µM	0.20	0.10 µM
CSP1325 Fw	10 µM	0.30	0.15 µM
CSP1325 Rv	10 µM	0.30	0.15 µM
CSP1325 Pr	10 µM	0.20	0.10 µM
TaqMan® RT enzyme mix (RNA-to-Ct™ 1-Step enzyme mix, Thermo Fisher Scientific)	40×	0.50	1×
Subtotal		18.00	
RNA		2.00	
Total		20.00	

2.2.2. Real-time RT-PCR cycling conditions: reverse transcription at 48°C for 15 min; denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s 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 (IPC) can be used to monitor each individual sample separately. IPC can include endogenous nucleic acid of the matrix using conserved primers preferably amplifying RNA targets such as *nad5* (Botermans et al., 2013). Alternatively, a BaCV spike prepared from a plant infected with bacopa chlorosis virus (BaCV) can be added during the RNA extraction (Naktuinbouw, unpubl. data), see Appendix 1. BaCV-specific primers and probes should be used (Naktuinbouw, the Netherlands; ISF, 2020) see Part A.

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should be negative.
- The PIC and PAC (as well as IPC, if applicable) should be positive.

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.

It should be noted that weak cross reactions can appear with e.g. some non-target tobamoviruses and therefore a cut-off value is required (N. Mehle, unpubl.). A preliminary cut-off value of 35 has been indicated in the test description established for the VALITEST test performance study. As a Ct cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

4. Performance characteristics available

The test was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* within the EU project VALITEST on leaves (Luigi et al., 2022).

For VALITEST TPS, the data used for the evaluation of this test were obtained by 24 participants. The panel of samples consisted of 22 blind samples (obtained from freeze-dried sap of tomato and pepper leaves) including:

- 2 non-target samples (tomato and pepper) in duplicate.
- 2 target samples (tomato) infected at low or medium concentration in duplicate.
- 5 ten-fold dilution points (10^{0}, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) of a target sample (ToBRFV infected tomato) in duplicate or triplicate and 3 controls.

The validation data are available at https://dc.eppo.int/validation_data/validationlist.

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).

4.1. Analytical sensitivity data

Data from VALITEST preliminary study: 10^{-8} in tomato and 10^{-5} in pepper based on a dilution series prepared from sap of three infected tomato or pepper plants diluted in sap from the respective healthy plants.

Data from VALITEST TPS in tomato (pepper not evaluated): LOD 95% (i.e. the dilution at which a detection probability of 95% is expected): $10^{-4.6}$.

4.2. Analytical specificity data

4.2.1. Inclusivity

In the VALITEST preliminary study, evaluated with ToB-SIC21/19; ToB-SIC22/19; ToB-SIC23/19; ToB-SIC24/19; ToB-SIC 25/19 and ToB-PIE105/2019 (originally isolated from *Solanum lycopersicum* and *Capsicum annuum*) belonging to the CREA-DC collection and ToBRFV PV-1236, PV-1241 and PV1244 belonging to the DSMZ collection. All isolates were detected.

4.2.2. Exclusivity

In the VALITEST preliminary study, no cross-reactions were observed with isolates of bell pepper mottle virus, tobacco mild green mottle virus, tobacco mosaic virus, tomato mosaic virus, pepper mild mottle virus, obtained from DSMZ (isolates PV-0170, PV-0124, PV-1252, PV-0141 and PV-0165, respectively).

- Data from EURL for Pests of Plants on Viruses, Viroids and Phytoplasmas

It should be noted that based on the result of the Proficiency Test on leaf material, organized by the EURL (NVWA), it cannot be excluded that cross reactions may occur with other tobamoviruses when present at high concentrations as was the case with non-diluted samples prepared from indicator plants inoculated with TMV.

4.3. Selectivity data

No false positive reactions obtained with healthy tomato and pepper leaves and tomato fruits.

4.4. Diagnostic sensitivity and diagnostic specificity

	VALITEST TPS for plant material
	Value (95% confidence interval)
Diagnostic sensitivity	88% (59–100%)
Diagnostic specificity	86% (80–92%)

4.5. Repeatability data

	VALITEST TPS for plant material
	Value (95% confidence interval)
Repeatability/accordance	78% (65–90%)
Reproducibility/ concordance	73% (69–74%)

APPENDIX 5 - REAL-TIME RT-PCR TEST (MENZEL & WINTER, 2021)

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

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

1. General information

1.1. This one-step real-time RT-PCR protocol is suitable for the detection and identification of ToBRFV in leaves, sepals, fruit and seed of tomato and pepper.

1.2. The test is based on primers and probe published by Menzel and Winter (2021).

1.3. Primers and probe target a fragment from the end of the coat protein gene to the middle of 3-NTR (position 6133–6228 for Genbank accession no. NC_028478).

1.4. Oligonucleotides

	Primer/probe	**Sequence**
Forward primer	ToBRFV qs1 ToBRFV qas2	5'-CAA TCA GAG CAC ATT TGA AAG TGC A-3′
Reverse primer	ToBRFV pl	5'-CAG ACA CAA TCT GTT ATT TAA GCA TC-3′
Probe		5'-6FAM- ACA ATG GTC CTC TGC ACC TG-BHQ1-3'

1.5. The test has been successfully performed using the TaqMan® RNA-to-Ct™ 1-Step Kit (Thermo Fisher Scientific), iTaq™ Universal Probes One-Step Kit (Bio-Rad), AgPath-ID One-Step RT-qPCR mix (Applied Biosystems) and Superscript IV (Invitrogen) in a one-tube assay with Fermentas Maxima Probe qPCR Mix (Thermo Fisher Scientific) and a range of different real-time PCR systems including Bio-Rad (CFX96 optical reaction module with C1000 Touch thermal cycler) and Eppendorf realplex 4.

2. Methods

2.1. Nucleic acid extraction and purification

See Appendix 1. Alternative procedures may also be suitable.

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	NA	5.8	NA
TaqMan® RT-PCR mix (RNA-to-Ct™ 1-Step Kit, Thermo Fisher Scientific)	2×	10.0	1×
Forward Primer ToBRFV qs1	10 µM	0.6	0.3 µM
Reverse Primer ToBRFV qas2	10 µM	0.6	0.3 µM
Probe ToBRFV p1	10 µM	0.5	0.25 µM
TaqMan® RT Enzyme Mix (RNA-to-Ct™ 1-Step enzyme mix, Thermo Fisher Scientific)	40×	0.5	1×
Subtotal		18.0	
RNA		2.0	
Total		20.0	

2.2.2. Real-time RT-PCR cycling conditions

reverse transcription at 48°C for 15 min, denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of 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 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. Alternatively, a BaCV spike prepared from a plant infected with bacopa chlorosis virus (BaCV) can be added during the RNA extraction, see Appendix 1 (Naktuinbouw, unpubl data). BaCV-specific primers and probes should be used (Naktuinbouw, the Netherlands; ISF, 2020) see Appendix 4 Part A.

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should be negative.
- The PIC and PAC (as well as IPC, if applicable) should be positive.

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.

It should be noted that weak cross reactions can appear with e.g. some non-target tobamoviruses and therefore a cut-off value is required (N. Mehle, unpubl.). A preliminary cut-off value of 35 has been indicated in the test description established for the VALITEST test performance study. As a Ct cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

4. Performance characteristics available

The test was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* within EU project VALITEST (on leaves) (Luigi et al., 2022) and within the Euphresco project 2019-A-327 (on seeds). The validation data are available at https://dc.eppo.int/validation_data/validationlist.

For VALITEST TPS, the data used for the evaluation of this test were obtained by 25 participants. The panel of samples consisted of 22 blind samples (obtained from freeze-dried sap of tomato and pepper leaves) including:

- 2 non-target samples (tomato and pepper) in duplicate.
- 2 target samples (tomato) infected at low or medium concentration in duplicate.
- 5 ten-fold dilution points (10^0, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) of a target sample (ToBRFV infected tomato) in duplicate or triplicate and 3 controls.

For Euphresco TPS, the data used for the evaluation of this test were obtained by 14 participants. The panel of samples consisted of 37 blind samples and 3 controls (1000 seeds per sample) including:

- 6 and 5 non-target samples (tomato and pepper respectively).
- 20 and 4 target samples (tomato) infected at medium or high concentration respectively.
- 5 target samples (pepper) infected at medium concentration.

Additional data on analytical specificity were obtained from Menzel & Winter (Menzel & Winter, 2021), where the test has been carried out by using Superscript IV (Invitrogen) in a one-tube assay with Fermentas Maxima Probe qPCR Mix (ThermoFisher Scientific).

4.1. Analytical sensitivity data

4.1.1. Data on plant material

Data from VALITEST preliminary study: 10^{-8} in tomato and 10^{-5} in pepper based on a dilution series prepared from sap of three infected tomato or pepper plants diluted in sap from the respective healthy plants.

Data from VALITEST TPS in tomato (pepper not evaluated): LOD 95% (i.e. the dilution at which a detection probability of 95% is expected): 10^{-5}.

4.1.2. Data on seeds

Data from Euphresco preliminary studies: using a serial dilution of infected seed extract into healthy seed extract, dilutions of 10^{-5} and 10^{-4} were detected using GH+ or phosphate buffers, respectively, for RNA extraction (tomato seeds).

4.2. Analytical specificity data

4.2.1. Inclusivity

In the VALITEST preliminary study, eight out of eight ToBRFV isolates gave positive results; i.e. the isolates ToB-SIC21/19; ToB-SIC22/19; ToB-SIC23/19; ToB-SIC24/19; ToB-SIC 25/19 and ToB-PIE105/2019 (originally isolated from *Solanum lycopersicum* and *Capsicum annuum*) belonging to the CREA-DC collection and ToBRFV PV-1236, PV-1241 and PV1244 belonging to the DSMZ collection.

4.2.2. Exclusivity

According to Menzel and Winter (2021), no cross-reactions occurred with the following non-target viruses (accession number of tested isolates from DSMZ collection is given in parentheses): barley stripe mosaic virus (PV-0330), beet soil-borne virus (PV-0576), beet virus Q (PV-0961), bell pepper mottle virus (PV-0170), cucumber green mottle mosaic virus (PV-0375), obuda pepper virus (PV-1176), odontoglossum ringspot virus (PV-1048, PV-0625), paprika mild mottle virus (PV-0606), pea early browning virus (PV-0298), peanut clump virus (PV-0291), pepino mosaic virus (PV-1022), pepper mild mottle virus (PV-0166), piper chlorosis virus (PV-1126), ribgrass mosaic virus (PV-0436), soil-borne cereal mosaic virus (PV-0552), soil-borne wheat mosaic virus (PV-0748), streptocarpus flower break virus (PV-1058), sunn-hemp mosaic virus (PV-0156), tobacco mild green mosaic virus (PV-0887), tobacco mosaic virus (PV-0107), tobacco rattle virus (PV-0354), tomato aspermy virus (PV-0220), tomato black ring virus (PV-0191), tomato bushy stunt virus (PV-0269), tomato chlorosis virus (PV-1023), tomato leaf curl New Delhi virus (PV-1109), tomato mosaic virus (PV-0135), tomato ringspot virus (PV-0380), tomato spotted wilt virus (PV-0182), tomato yellow leaf curl virus (PV-0588), tomato yellow ring virus (PV-0526), tropical soda apple mosaic virus (PV-1223), turnip vein clearing virus (PV-0148), youcai mosaic virus (PV-0527).

In addition, in VALITEST and Euphresco preliminary studies, no cross-reactions were observed with isolates of bell pepper mottle virus, pepper mild mottle virus, tobacco mild green mottle virus, tobacco mosaic virus, tomato mosaic virus.

4.3. Diagnostic sensitivity and diagnostic specificity

	VALITEST TPS for plant material Value (95% confidence interval)	Euphresco TPS for seed material	
		Tomato	Pepper
Diagnostic sensitivity	88% (56–100%)	99%	97%
Diagnostic specificity	89% (79–99%)	99%	100%

4.4. Repeatability and reproducibility data

	VALITEST TPS for plant material	Euphresco TPS
	Value (95% confidence interval)	For seed material
Repeatability/ accordance	79% (66–92%)	Not evaluated
Reproducibility/ concordance	76% (72–78%)	Not evaluated

APPENDIX 6 - REAL-TIME RT-PCR TEST (PANNO ET AL., 2019B)

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

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

1. General information

1.1. This one-step real-time RT-PCR protocol is suitable for the detection and identification of ToBRFV in leaves, sepals and fruit of tomato and pepper.

1.2. The test is based on primers and probe published by Panno et al., 2019b.

1.3. Primers and probe target a fragment from the middle of the movement protein gene to the middle of the coat protein gene (CP) (position 5520–5620 for Genbank accession no. NC_028478).

1.4. Oligonucleotides

	Primer/probe	Sequence
Forward primer	ToB5520F	5'-GTA AGG CTT GCA AAA TTT CGT TCG-3′
Reverse primer	ToB5598R	5′- CTT TGG TTT TTG TCT GGT TTC GG-3′
Probe	ToB-probe	5′- FAM-GTT TAG TAG TAA AAG TGA GAA T-MGB-3'

1.5. The test has been successfully performed using the TaqMan® RNA-to-Ct™ 1-Step Kit (Thermo Fisher Scientific) (VALITEST preliminary studies), iTaq™ Universal Probes One-Step Kit (Bio-Rad) (VALITEST preliminary studies), QuantiNova Probe RT-PCR kit (Qiagen) (Panno et al., 2019b) and a range of different real-time PCR systems including Bio-Rad (CFX96 optical reaction module with C1000 Touch thermal cycler), and Rotor-Gene Q2plex HRM Platform Thermal Cycler (Qiagen) (Panno et al., 2019b).

2. Methods

2.1. Nucleic acid extraction and purification

See Appendix 1. Alternative procedures may also be suitable.

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	NA	5	NA
TaqMan® RT enzyme mix (RNA-to-Ct™ 1-Step enzyme mix, Thermo Fisher Scientific),	2×	10.0	1×
Forward Primer ToB5520F	10 µM	1	0.5 µM
Reverse Primer ToB5598R	10 µM	1	0.5 µM
Probe ToB-probe	10 µM	0.5	0.25 µM
TaqMan® RT enzyme mix (RNA-to-Ct™ 1-Step enzyme mix, Thermo Fisher Scientific)	40×	0.5	1×
Subtotal		18.0	
RNA		2.0	
Total		20.0	

2.2.2. Real-time RT-PCR cycling conditions: reverse transcription at 48°C for 15 min, denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min.

3. Essential procedural information

3.1. Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of 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 conserved primers preferably amplifying RNA targets such as *nad5* (Botermans et al., 2013).

3.2. Interpretation of results

Verification of the controls
- NIC and NAC should be negative.
- The PIC and PAC (as well as IPC, if applicable) should be positive.

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.

It should be noted that weak cross-reactions can appear with e.g. some non-target tobamoviruses and therefore a cut-off value is required (N. Mehle, unpubl.). A preliminary cut-off value of 35 has been indicated in the test description established for the VALITEST test performance study. As a Ct cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

4. Performance characteristics available

The test was validated according to PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* within the EU project VALITEST (on leaves) (Luigi et al., 2022). The validation data are available at https://dc.eppo.int/validation_data/validationlist.

For VALITEST TPS, the data used for the evaluation of this test were obtained by 22 participants. The panel of samples consisted of 22 blind samples (obtained from freeze-dried sap of tomato and pepper leaves) including:

- 2 non-target samples (tomato and pepper) in duplicate.
- 2 target samples (tomato) infected at low or medium concentration in duplicate.
- 5 ten-fold dilution points (10^{0}, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) of a target sample (ToBRFV infected tomato) in duplicate or triplicate and 3 controls.

Additional data on exclusivity were obtained from Panno et al. (2019) on a slightly modified test and is reported below.

4.1. Analytical sensitivity data

Data from VALITEST preliminary study: 10^{-8} in tomato and 10^{-5} in pepper based on a dilution series prepared from sap of three infected tomato or pepper plants diluted in sap from the respective healthy plants.

Data from VALITEST TPS in tomato (pepper not evaluated): LOD 95% (i.e. the dilution at which a detection probability of 95% is expected): $10^{-4.2}$.

4.2. Analytical specificity data

4.2.1. Inclusivity

In the VALITEST preliminary study, eight out of eight ToBRFV isolates gave positive results; i.e. the isolates ToB-SIC21/19; ToB-SIC22/19; ToB-SIC23/19; ToB-SIC24/19; ToB-SIC 25/19 and ToB-PIE105/2019 (originally isolated from *Solanum lycopersicum* and *Capsicum annuum*) belonging to the CREA-DC collection and ToBRFV PV-1236, PV-1241 and PV1244 belonging to the DSMZ collection.

4.2.2. Exclusivity

No cross-reactions were observed with isolates of bell pepper mottle virus (VALITEST), cassava green mottle virus (Panno et al. (2019b)), paprika mild mottle virus (Panno et al. (2019b)), pepper mild mottle virus (Panno et al. (2019b), VALITEST), tobacco mild green mottle virus (Panno et al. (2019b), VALITEST), tobacco mosaic virus (Panno et al. (2019b), VALITEST), tomato mosaic virus (Panno et al. (2019b), VALITEST), tomato mottle mosaic virus (Panno et al. (2019b)), zucchini green mottle mosaic virus (Panno et al. (2019b)).

4.3. Selectivity

No false positive reactions obtained with healthy tomato and pepper leaves and with tomato fruits (VALITEST preliminary studies).

4.4. Diagnostic sensitivity and diagnostic specificity

	VALITEST TPS for plant material
	Value (95% confidence interval)
Diagnostic sensitivity	84% (48–100%)
Diagnostic specificity	90% (74–100%)

4.5. Repeatability & reproducibility data

	VALITEST TPS for plant material
	Value (95% confidence interval)
Repeatability/accordance	82% (72–91%)
Reproducibility/concordance	81% (78–83%)

APPENDIX 7 - REAL-TIME RT-PCR TEST (BERNABÉ-ORTS ET AL., 2021, ABIOPEP[3] S.L.)

The test below is described as it was carried out to generate the validation data provided in section 4 and published in Bernabé-Orts et al. (2021). Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General information

1.1. This one-step real-time PCR (RT-PCR) protocol was developed for the detection and identification of ToBRFV in leaves and seed of tomato and pepper.

1.2. The test is based on primers and probe developed by the company Abiopep[3] S.L. (ES) and published by Bernabé-Orts et al. (2021).

1.3. Primers and probe target a fragment from the replicase gene (position 3213–3335 for Genbank accession MK648157).

1.4. Oligonucleotides:

	Primer/probe	Sequence (5′-3′)
Forward primer	AB-620 Fw	CAGATGTGTCGTTGGTCA-GAT
Reverse primer	AB-621 Rev	CATCACTACGGTGTAATA-CTTC
Probe	AB-622 Pr	FAM-CGTAGCTTTGTCAA-GGCATACCCAAA-BHQ1

1.5. The test has been successfully used using KAPA PROBE FAST Universal One-Step qRT-PCR Master Mix (2X) kit (KAPA BIOSYSTEMS) and a StepOnePlus Real-Time PCR System (Applied Biosystems) thermal cycler (Bernabé-Orts et al., 2021). CREA used the test with the APA PROBE FAST Universal One-Step qRT-PCR Master Mix (2×) kit and TaqMan® RNA-to-Ct™ 1-Step Kit (Thermo Fisher Scientific. NIB used the test with TaqMan® RNA-to-Ct™ 1-Step Kit (Thermo Fisher Scientific) and the AgPath-ID One-Step RT-PCR (Thermo Fisher) (NIB)).

2. Methods

2.1. Nucleic Acid Extraction and Purification

Nucleic acid extraction from individual samples (tomato leaves) was performed using the NucleoSpin RNA plant kit (MACHEREY-NAGEL) following the manufacturer's instructions (Bernabé-Orts et al., 2021). In the framework of the Euphresco project RNA extraction was performed as in Appendix 1.

[3]Abiopep S.L. Parque Científico de Murcia. Ctra. de Madrid Km 388, Complejo Espinardo. Edificio R 2ª Planta, 30100. Espinardo Murcia. Spain.

2.2. Real-time quantitative PCR

2.2.1. Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	6.0	N.A.
KAPA PROBE FAST qPCR Master mix (KAPABIOSYSTEMS)	2×	10.0	1×
AB-620 Fw	10 µM	0.4	200 nM
AB-621 Rev	10 µM	0.4	200 nM
AB-622 Pr	10 µM	0.4	200 nM
ROX HIGH reference dye (KAPABIOSYSTEMS)	50×	0.4	1×
KAPA RT MIX (KAPABIOSYSTEMS)	50×	0.4	1×
RNA		2	
Total		20	

2.2.2. Real-time RT-PCR cycling conditions reverse transcription at 42°C for 5 min; denaturation at 95°C for 3 min; 40 cycles of denaturation at 95°C for 3 s and annealing and elongation at 60°C for 30 seconds

Note: If other enzymes are used reaction conditions may need to be changed.

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 conserved primers preferably amplifying RNA targets such as *nad5* (Botermans et al., 2013).

3.2. Interpretation of results

Verification of the controls:
- NIC and NAC should be negative.
- The PIC and PAC (as well as IPC, if applicable) should be positive.

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 that is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

It should be noted that weak cross reactions can appear with e.g. some non-target tobamoviruses and therefore a cut-off value is required (N. Mehle, unpubl.). A preliminary cut-off value of 35 has been indicated in the test description established by Abiopep (ES). As a Ct cut-off value is equipment, material and chemistry dependent it needs to be verified in each laboratory when implementing the test.

4. Performance criteria available

The validation data generated by Abiopep (ES) and presented below have been published in Bernabé-Orts et al. (2021). In addition, a slightly modified test (different reagents) was evaluated by two laboratories (CREA & NIB).

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

4.1. Analytical sensitivity:

- Validation data according to Bernabé-Orts et al. (2021):

Using a serial dilution of RNA extracts obtained from several infected tomato plants diluted in healthy plant RNA extract, the analytical sensitivity was 10^{-4}.

- Validation data according CREA (IT)

Based on a dilution series prepared from sap of infected tomato leaves diluted in sap from healthy leaves, the analytical sensitivity was 10^{-6}.

4.2. Analytical specificity

- Validation data according to Bernabé-Orts et al. (2021):

Inclusivity: An in silico alignment of the target region from 10 ToBRFV isolates showed sequence conservation among these isolates.

Exclusivity: No cross-reactions occurred experimentally with the following non-target viruses: pepper mild mottle virus (PV-0093), tobacco mild green mosaic virus (PV-0124), tobacco mosaic virus (PV-1252), and tomato mosaic virus (PV-0141) from DSMZ collection. An in silico alignment of the target region from non-target viruses showed sequence divergence with tobacco mild green mosaic virus, tobacco mosaic virus, tomato mosaic virus and pepper mild mottle virus isolates.

- Validation data according CREA (IT)

Inclusivity evaluated with 7 ToBRFV isolates: 100%.

- Validation data according NIB (SI)

Exclusivity: 100%. No cross-reactions occurred with the following non-target viruses: cucumber green mottle mosaic virus (NIB V 271, NIB V 320), tobacco mosaic virus (NIB V 037), tomato mosaic virus (NIB V 036, NIB V 049, NIB V 072, NIB V 104) from NIB collection and bell pepper mottle virus (PV-0170), obuda pepper virus (PV-1176), odontoglossum ringspot virus (PV-1048), paprika mild mottle virus (PV-0606), ribgrass mosaic virus (PV-0145), streptocarpus flower break virus (PV-1058), sunn-hemp mosaic virus (PV-0156), tomato mottle mosaic virus (PV-1267), tobacco mild green mosaic virus (PV-0124), tobacco mosaic virus (PV-0137, PV-0943), youcai mosaic virus (PV-0527) from DSMZ collection.

4.3. Selectivity according to Bernabé-Orts et al. (2021)

Selectivity was evaluated using *Solanum lycopersicum*, *Capsicum annuum* and *Nicotiana benthamiana* leaves and *Solanum lycopersicum* and *Capsicum annuum* seeds with no noticeable impact on the performance of the test.

4.4. Diagnostic sensitivity & diagnostic specificity

Calculated based on the results of 2 laboratories (CREA & NIB) using the samples provided in the Euphresco TPS.

	On seed material	
	Tomato	**Pepper**
Diagnostic sensitivity	96%	89%
Diagnostic specificity	100%	100%

4.5. Repeatability according to Bernabé-Orts et al. (2021)

In each experiment, all the samples included were tested at least in triplicate. In all the cases the test performed similarly with no remarkable oscillations in the Ct values between the different experiments.

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CORRIGENDUM

Corrigendum for PM 7/146 (2) Tomato brown rugose fruit virus

In Appendix 1, Section 2.2 Homogenization using GH+ buffer, paragraph 2 states the following:

'*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) is added.*

However, it has been pointed out to us that this is only correct for tomato. For pepper it should be 40 mL of GH+ buffer. The above line should therefore be replaced by (new text in bold).

'*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* ***for tomato or 40 mL for pepper*** *(Table A1) is added.*

REFERENCE

EPPO (2022) PM 7/146 (2) Tomato brown rugose fruit virus. *EPPO Bulletin*, 52, 665–692.