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Test Performance Study (TPS) - Report

Report on the results of the test performance study on detection and identification of tomato mottle mosaic virus (ToMMV) using molecular tests

TPS code	**Euphresco-ToMMV_TPS2024**

Version of report	V2.0

	Name	Function	Organization	Date	Signature
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Contents

1 Content of application

In recent decades, diseases caused by emerging viruses have become increasingly important in terms of their economic impact. Currently, the most economically important emerging virus is the tobamovirus tomato brown rugose fruit virus (ToBRFV), which poses a threat to tomato production and, to a lesser extent, pepper production. These crops can also be infected by another tobamovirus, tomato mottle mosaic virus (ToMMV), which was first described in Mexico in 2013 (Li et al., 2013) and is since then reported in several other countries (EPPO, 2024). The symptoms caused by ToMMV can be severe, but vary depending on factors such as host cultivar and environmental conditions. The biological characteristics of tobamoviruses allow them to spread rapidly and give them a high epidemic potential, which can consequently have a significant impact on agriculture.

A crucial step in successfully controlling tobamoviruses and preventing further spread is the accurate and timely detection of the virus. Molecular tests for the detection and identification of ToBRFV have been validated as part of the EU VALITEST project and the Euphresco 2019-A-327 project. There is also a great need for harmonized and validated protocols for ToMMV. There are several published tests for the detection and identification of ToMMV. The aim of this Euphresco project was to conduct a test performance study (TPS) to compare the performance of different molecular tests that can be used for the detection and identification of ToMMV. The results of the TPS provide insight into how these tests perform in different laboratories, i.e. on different equipment, with different reagents and with different operators. They also allow a better evaluation of the accuracy and reproducibility of the tests.

This document represents report of the test performance study on detection and identification of tomato mottle mosaic virus (ToMMV) using molecular tests which was organized by the National Institute of Biology, Department of Biotechnology and Systems Biology in the framework of the Euphresco project 2022-A-394 (Validation of molecular diagnostic methods for the detection and identification of tomato mottle mosaic virus (ToMMV-detect)).

2 Disease and pathogen

Tomato mottle mosaic virus (ToMMV, Tobamovirus), currently referred to as *Tobamovirus maculatessellati*, was first described in 2013 when it infected tomato crops (*Solanum lycopersicum*) in Mexico (Li et al., 2013). It was subsequently found in America, Asia and Europe, causing infections in tomatoes and peppers (*Capsicum* spp.) (EPPO, 2024). Several other natural hosts in the Solanaceae, Leguminosae and Chenopodiaceae families have also been identified. ToMMV is a tobamovirus that shares similarities with another tobamovirus, tomato brown rugose fruit virus, which causes high yield losses in tomato crops. ToMMV was added to the EPPO Alert List in 2020 and removed from this list in 2024.

The symptoms caused by ToMMV can vary significantly depending on factors such as co-infection with other viruses, the specific plant species, cultivars and environmental conditions. In tomatoes, ToMMV can lead to stunted growth, with a complete loss of flowers, which in turn results in the absence of fruit production when young plants are infected. Shoots may exhibit chlorosis, while leaves can display a range of symptoms, including mosaic and mottling patterns, necrosis or necrotic spots, chlorosis, crinkling, blistering, epinasty, leaf deformation and curling. The fruits of infected tomato plants may develop necrotic lesions and fruit necrosis, and they may also ripen unevenly or show necrotic spots.

In *Capsicum annuum*, ToMMV can cause rapid apical yellowing and necrosis, along with stunted growth in certain varieties. The leaves of infected Capsicum plants may become mottled, shrinking, and exhibit chlorosis, necrosis, crinkling, and mosaic patterns. (EPPO, 2022)

Tobamoviruses are easily transmitted mechanically through common cultural practices causing wounds or microlesions. ToMMV was detected in many seed lots of tomato and Capsicum, and is thought to be seed-borne, similar to other tobamoviruses such as tobacco mosaic virus and tomato mosaic virus. ToMMV may also be transmitted to new host plants via soil contaminated with infested plant debris, and water-mediated transmission of ToMMV is also likely, especially in systems with circulating water. (EPPO, 2022)

3 Selection of tests for TPS

3.1 Definition of the scope of testing

The scope of this test performance study:

- **Detection and identification of tomato mottle mosaic virus (ToMMV) using molecular tests**.

For practical reasons, mainly related to the limited budget for the organisation of the TPS, it was decided that the TPS starting material will be extracted RNA (and not plant material).

3.2 Collection of available tests

In 2023, the collection of available tests that can be used for the defined scope (detection and identification of ToMMV) and for the selected type of material for the TPS (RNA extract) was carried out in the following steps:

- Survey among the partners of the Euphresco project ToMMV-detect
- Literature search
- Search on the websites of various companies that could commercially produce tests of our interest

3.3 Selection of tests

As it was not possible to include all available tests in the TPS, it was decided to include only those tests for which a clear diagnostic protocol and at least some validation data were available.

There were some cases where different tests contained the same primers and probes but slightly different protocols. In such cases, the version of the test with the most validation data was selected for the TPS.

3.4 Pre-testing of selected tests

All selected protocols/ tests (Table 1; Appendix 1) have been pre-tested in our laboratory on a limited number of samples (two ToMMV-positive and two ToBRFV-positive samples and two non-template controls), and where ambiguities or errors were found, these have been discussed with the protocol provider (Table 1) and corrected.

3.5 Selected tests for TPS

Selected tests for TPS are listed in Table 1. Appendix 1 contains all protocols for the selected tests in EPPO PM7 format and includes validation data that was available prior to this TPS. These protocols and

validation data were produced by various partners of the Euphresco project ToMMV-detect and by some other TPS participants (Table 1). In addition, we have prepared an executive summary containing all the key data required to conduct these tests (Appendix 2)

Table 1: Selected tests for TPS

Method	Test - reference	Protocol prepared by
RT-PCR	Levitzky et al. (2019) + sequencing of the PCR product	Fera science Ltd (FERA, UK)
	Loewe (Cat. No. 09181)	Loewe® Biochemica GmbH (DE)
	Sui et al. (2017)	USDA APHIS PPQ, Plant Pathogen Confirmatory Laboratory (USA)
Real time RT-PCR (RT-qPCR)	DAFF DEECA	Department of Agriculture, Forestry and Fisheries (DAFF) & Department of Energy, Environment and Climate Action (DEECA) (AU)
	Fowkes et al. (2022)	Fera science Ltd (FERA, UK)
	ISF	Naktuinbouw (NL)
	Tiberini et al. (2022) singleplex or duplex	National Institute of Biology (NIB, SI) & Council for Agricultural Research and Economics - Research Centre for Plant Protection and Certification (CREA-DC, IT)
Recombinase-Polymerase Amplification (RPA)	Agdia RPA (XCS 22800)	Agdia (FR)
Loop-mediated amplification (LAMP)	Kimura et al. (2023)	National Institute of Biology (NIB, SI)

4 Preliminary study

4.1 Materials and Methods

4.1.1 Samples

Virus isolates of ToMMV and other tobamoviruses used in this study were obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ) or were kindly provided by colleagues from Julius Kühn-Institut - JKI (Germany; Heiko Ziebell), GEVES (France; Sophie Perrot) or Bejo Zaden BV (Netherlands; Wilfried Jonkers) (Table 2). A total of 15 tobamovirus species were included in this study. ToMMV was represented by two isolates. Other tobamoviruses were represented with one isolate, except ToMV with four and PMMoV and TMV with two isolates. Tobamovirus-free tomato leaf and seed material (hereinafter: healthy samples) were also included in a study (Table 2).

Table 2. Isolates used for preliminary study. Targeted ToMMV isolates are in red shaded rows.

Virus	Virus – name	ID	Collection	Origin	Plant species	Matrix/type of sample received at NIB	NIB ID
Genus *Tobamovirus*							
BPeMV	bell pepper mottle virus	PV-0170	DSMZ (Germany)	Netherlands	*Solanum melongena*; Lab host: *Nicotiana benthamiana*	dry-lyophilized leaf material	NIB V 363
CGMMV	cucumber green mottle mosaic virus	JKI32360	JKI (Germany)	Germany	Cucumber; Lab host: *N. benthamiana*	$CaCl_2$ dried leaf material	NIB V 403
ObPV	obuda pepper virus	PV-1176	DSMZ (Germany)	Hungary	*Capsicum annuum*; Lab host: *N. tabacum* cv. Xanthi nc	dry-lyophilized leaf material	NIB V 364
ORSV	odontoglossum ringspot virus	PV-1048	DSMZ (Germany)	Germany	Orchis sp.; Lab host: *N. benthamiana*	dry-lyophilized leaf material	NIB V 365
PaMMV	paprika mild mottle virus	PV-0606	DSMZ (Germany)	Greece	Capsicum sp.; Lab host: *N. benthamiana*	dry-lyophilized leaf material	NIB V 366
PMMoV	pepper mild mottle virus	PAS 487	GEVES (France)	France	*C. annuum*	dry-lyophilized leaf material	NIB V 408
PMMoV	pepper mild mottle virus	PAS 486	GEVES (France)	France	*C. annuum*	dry-lyophilized leaf material	NIB V 409
RMV	ribgrass mosaic virus	PV-0145	DSMZ (Germany)	unknown	*Plantago media*; Lab host: *N. tabacum* cv. Samsun eN	dry-lyophilized leaf material	NIB V 367
SFBV	streptocarpus flower break virus	PV-1058	DSMZ (Germany)	unknown	*Streptocarpus* sp.; Lab host: *N. benthamiana*	dry-lyophilized leaf material	NIB V 368
SHMV	sunn-hemp mosaic virus	PV-0156	DSMZ (Germany)	unknown	*Phaseolus vulgaris.*; Lab	dry-lyophilized leaf material	NIB V 369

Virus	Virus – name	ID	Collection	Origin	Plant species	Matrix/type of sample received at NIB	NIB ID
					host: *P. vulgaris cv. Black Turtle* l		
TMGMV	tobacco mild green mosaic virus	JKI32436	JKI (Germany)	Madagascar	*C. annuum*; Lab host: *N. benthamiana*	$CaCl_2$ dried leaf material	NIB V 404
TMV	tobacco mosaic virus	JKI32434	JKI (Germany)	Ohio, USA	Lab host: *Chenopodium quinoa*	$CaCl_2$ dried leaf material	NIB V 405
TMV	tobacco mosaic virus	PAS 601	GEVES (France)	France	*S. lycopersicum*	dry-lyophilized leaf material	NIB V 413
ToBRFV	tomato brown rugose fruit virus	PV-1236	DSMZ (Germany)	Germany	*S. lycopersicum*	dry-lyophilized leaf material	NIB V 331
ToMMV	tomato mottle mosaic virus	PV-1267	DSMZ (Germany)	California (USA)	*S. lycopersicum*	dry-lyophilized leaf material	NIB V 373
ToMMV	tomato mottle mosaic virus	/	Bejo Zaden BV (Netherlands)	China	*S. lycopersicum*	seeds	NIB V 414
ToMV	tomato mosaic virus	JKI23645	JKI (Germany)	Madagascar	*Brassica rapa*; lab host: *C. quinoa*	$CaCl_2$ dried leaf material	NIB V 406
ToMV	tomato mosaic virus	PAS 603	GEVES (France)	France	*S. lycopersicum*	dry-lyophilized leaf material	NIB V 410
ToMV	tomato mosaic virus	PAS 598	GEVES (France)	France	*S. lycopersicum*	dry-lyophilized leaf material	NIB V 411
ToMV	tomato mosaic virus	PAS 599	GEVES (France)	France	*S. lycopersicum*	dry-lyophilized leaf material	NIB V 412
YMoV	youcai mosaic virus	PV-0527	DSMZ (Germany)	Germany	Impatiens spp.	dry-lyophilized leaf material	NIB V 374
Healthy samples							
/	/	/	NIB (Slovenia)	Slovenia	*S. lycopersicum*	seeds	D1977/23 + D93/23

Virus	Virus – name	ID	Collection	Origin	Plant species	Matrix/type of sample received at NIB	NIB ID
/	/	/	NIB (Slovenia)	Slovenia	*S. lycopersicum*	seeds	D1977/23
/	/	/	NIB (Slovenia)	Slovenia	*S. lycopersicum*	leaf material	/

4.1.2 RNA extraction

Total RNA was extracted from leaf material (approximately 200 mg of fresh leaf material or dry-lyophilized leaf material representing 200 mg fresh leaf weight) using RNeasy Plant mini kit (Qiagen) with following modifications: extraction was performed without using 2-mercaptoethanol and the final RNA elution was performed with two consecutive additions of 50 µL of RNase-free water pre-warmed to 65°C (total elution volume 100 µL).

The RNeasy Plant Mini Kit (Qiagen) was also used for total RNA extraction from seeds, but with the following modifications: the RLT buffer was replaced with GH+ buffer (EPPO PM7/146(2) Appendix 1), and the centrifugation temperature was lowered to 4°C for all steps. Detailed procedure: Samples of 1000 seeds were placed in a grinding bag (Bioreba extraction bag with synthetic interlayer (universal long)) and ground with a hand Homex homogeniser or Homex 6 (Bioreba): firstly, seeds were crushed without buffer, then 5mL GH+ buffer was added and seeds further crushed, and finally 15 mL GH+ buffer was added and the homogenate mixed thoroughly. For each sample, 1 mL of the seed homogenate was transferred into a 1.5 mL tube and 30 µL of dithiothreitol (DTT, 5 M) was added, followed by incubation in a thermoshaker at 850 rpm and 65°C for 15 min. After centrifugation at 16,000 g for 10 min, 750 µL of supernatant was loaded on the QIA shredder spin column and centrifuged. Thereafter the manufacturer's instructions of the RNeasy Plant Mini Kit (Qiagen) were followed (with centrifugation at 4 °C as stated above). RNA was eluted from the RNeasy Mini Spin columns by two consecutive additions of 50 µL of RNase-free water pre-warmed to 65°C (total elution volume 100 µL).

Extracted RNA was stored at -20°C until its use in the molecular tests.

To monitor the RNA extraction procedure, a real-time RT-PCR (RT-qPCR) amplifying plant nad5 transcript (nad5; Botermans et al., 2013) was included in the analysis.

4.1.3 Confirmation of the virus identity

The presence or absence of tobamoviruses in the extracted RNA was confirmed by generic tobamovirus nested PCR (Dovas et al., 2004), and the identity of the tobamovirus isolates was determined by Sanger sequencing of the nested PCR products.

4.1.4 Analytical specificity

Inclusivity was determined by analysing two ToMMV isolates, one from tomato leaf material and one from tomato seed material (Table 2 and 4).

Exclusivity was determined by analysing 19 other tobamovirus isolates (14 different virus species) and 3 tobamovirus-free samples (Table 2 and 4).

4.1.5 Analytical sensitivity

To determine the analytical sensitivity of all tests examined, analyses of serial dilutions of ToMMV NIB V 373 RNA in RNA from tobamovirus-free tomato leaves were performed. In addition, two RNA dilutions of ToMMV NIB V 414 in water were analysed with all tests.

4.1.6 Specification of the molecular tests used in the preliminary study

All tests selected for the TPS were performed according to the procedures described in Appendix 1. The equipment and chemicals used for the preliminary study of these tests are listed in Table 3.

Table 3: Equipment and reagents used for preliminary study

Method	Test - reference	Equipment	Chemicals	Comments
RT-PCR	Levitzky et al. (2019) + sequencing of the PCR product	Deep well PCR Biorad C1000	OneStep RT-PCR kit (Qiagen)	Sequencing performed at Macrogen
	Loewe (Cat. No. 09181)	Deep well PCR Biorad C1000	RT-PCR Loewe (Cat. No. 09181/100)	/
	Sui et al. (2017)	ProFlex PCR system (ABI)	OneStep RT-PCR kit (Qiagen)	/
Real time RT-PCR (RT-qPCR)	DAFF DEECA	QuantStudio 7 PRO (ABI)	AgPath-ID One-Step RT-qPCR (ThermoFisher Scientific)	/
	Fowkes et al. (2022)	QuantStudio 7 PRO (ABI)	iTaq Universal Probes One-Step Kit (BioRad)	/
	ISF	QuantStudio 7 PRO (ABI)	Ultraplex 1-Step ToughMix (QuantaBio)	Version of the ToughMix: Low ROX (4x)
	Tiberini et al. (2022) - singleplex	QuantStudio 7 PRO (ABI)	AgPath-ID One-Step RT-qPCR (ThermoFisher Scientific)	Probe labelled with Hex
	Tiberini et al. (2022) - duplex	QuantStudio 7 PRO (ABI)	AgPath-ID One-Step RT-qPCR (ThermoFisher Scientific)	Probe labelled with Hex
Recombinase-Polymerase Amplification (RPA)	Agdia RPA (XCS 22800)	AmpliFire® fluorometer	AmplifyRP® XRT for ToMMV	/
Loop-mediated amplification (LAMP)	Kimura et al. (2023)	Genie II (Optigen)	Isothermal Master Mix (Optigene Ltd., Horsham, UK)	/

4.2 Results

4.2.1 Evaluation of RNA extraction and virus identity

As the amount of RNA extracts of some samples was limited, dilutions in RNAse-free water were prepared (see Table 4) and used for further testing. The performance of the RNA extraction was checked by RT-qPCR using a set of oligonucleotide primers and a probe for nad5. In all cases, nad5 gave a Cq value between 22 and 32 (Table 4), so RNA extraction could be considered successful for all samples. Generic nested PCR for tobamovirus and Sanger sequencing of its product confirmed the presence of assign viruses in all RNA sample dilutions tested (Table 4).

4.2.2 Analytical specificity

Inclusivity:

The ToMMV isolate from leaf material was detected with all tests examined, while the ToMMV isolate from seeds were detected with all RT-qPCRs examined, RPA, LAMP and with RT-PCR by Sui et al. (2017). The ToMMV isolate from seeds did not give a positive signal in the RT-PCR of Lewitzky et al. (2019) and Loewe (cat. no. 09181), which is most likely due to a relatively low ToMMV titer (RT-qPCRs gave Cq values between 28 and 30; Table 4).

Exclusivity:

The analysis of 19 tobamovirus isolates (14 different virus species) and 3 tobamovirus-free (healthy) samples showed no cross-reactions with non-targets in the LAMP (Kimura et al., 2023) and RT-PCRs by Loewe (cat. no. 09181) and Sui et al. (2017) (Table 4).

As expected, the RT-PCR by Lewitzky et al. (2019) showed a positive signal with some other tobamoviruses, but not with all tobamoviruses. However, Sanger sequencing of the RT-PCR product by Lewitzky et al. (2019) proved useful for distinguishing ToMMV from other tobamoviruses, but it should be noted that in a few cases we were unable to obtain the sequence required for virus identification (Table 4).

RPA (Agdia) gave a false positive signal for the PaMMV isolate, but the same isolate also gave a weak signal (Cq > 33) in all RT-qPCRs. In addition, the RT-qPCR DAFF-DEECA also gave a high Cq value (39) for one tobamovirus-free tomato seed sample. Therefore, with respect to RT-qPCRs, it was decided that any signals above the Cq value we obtained for ToMMV-free samples should be considered inconclusive (Table 4).

PaMMV is reported to have sequence homology of about 64 % with different ToMMV isolates (Li et al., 2017); in view of this, cross-reaction between the two targets does not seem likely to occur. Since a cross-reaction was only observed for the most sensitive tests (see next section) and these tests target different regions of the virus genome, we cannot even exclude a contamination of this PaMMV isolate with a low titer of ToMMV. In addition, the samples of non-target tobamoviruses included in this study are from artificially inoculated test plants. A high virus concentration is expected in these samples; such a virus titer is hardly found in naturally infected leaf samples and not at all in seed samples, but further analysis and investigation are required.

4.2.3 Analytical sensitivity

Based on the dilution of the isolate ToMMV NIB V 373 in RNA from healthy tomato leaves, RPA (Agdia) and RT-qPCR Tiberini et al. (2022) were found to be the most sensitive, other RT-qPCRs and LAMP ten times less sensitive, RT-PCR Sui et al. (2017) 100 times less sensitive and the least sensitive were RT-PCR Levitzky et al. (2019) and RT-PCR Loewe (1,000 times less sensitive compared to RPA (Agdia) and RT-qPCR Tiberini et al. (2022)) (Table 5). For the evaluation of these results, in the case of RT-qPCRs, the Cq values considered positive were as described in the section above. In the case of RT-PCR Levitzky et al. (2019), the success in obtaining the Sanger sequence were taken into account.

Both tested dilutions of ToMMV NIB V 414 (ToMMV isolate from seeds) were detected with all RT-qPCRs, RPA and LAMP. The RT-PCR by Sui et al. (2017) only detected the dilution with the higher titer of ToMMV, while the RT-PCR by Levitzky et al. (2019) and the RT-PCR by Loewe did not detect ToMMV in either dilution (Table 5).

Table 4. Results of internal evaluation of the analytical specificity. Legend: pos=positive; neg=negative; inc = inconclusive

					RT-PCR			RT-qPCR[b]					RPA	LAMP
Virus	**NIB ID**	**Dilution factor**	**Nad5 (Cq)**	**Tobamovirus confirmed[a]**	**Levitzky et al. (2019)[a]**	**Loewe (Cat. No. 09181)**	**Sui et al. (2017)**	**DAFF DEECA**	**Fowkes et al. (2022)**	**ISF[c]**	**Tiberini et al. (2022) singleplex**	**Tiberini et al. (2022) duplex[c]**	**Agdia RPA (XCS 22800)**	**Kimura et al. (2023)[d]**
BPeMV	NIB V 363	1x	24	pos (BPeMV)	pos (BPeMV)	neg	neg	neg	neg	neg	neg	neg	neg	neg
CGMMV	NIB V 403	25x	31	pos (CGMMV)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
ObPV	NIB V 364	25x	23	pos (ObPV)	pos (ObPV)	neg	neg	neg	neg	neg	neg	neg	neg	neg
ORSV	NIB V 365	25x	28	pos (ORSV)	inc (ORSV)	neg	neg	neg	neg	neg	neg	neg	neg	neg
PaMMV	NIB V 366	25x	26	pos (PaMMV)	pos (PaMMV)	neg	neg	inc (34)	inc (36)	inc (33)	inc (35)	inc (37)	pos	neg
PMMoV	NIB V 408	25x	26	pos (PMMoV)	pos (PMMoV)	neg	neg	neg	neg	neg	neg	neg	neg	neg
PMMoV	NIB V 409	1x	25	pos (PMMoV)	pos (PMMoV)	neg	neg	neg	neg	neg	neg	neg	neg	neg
RMV	NIB V 367	1x	22	pos (RMV)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
SFBV	NIB V 368	1x	23	pos (SFBV)	pos (SFBV)	neg	neg	neg	neg	neg	neg	neg	neg	neg
SHMV	NIB V 369	1x	25	pos (SHMV)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
TMGMV	NIB V 404	25x	24	pos (TMGMV)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
TMV	NIB V 405	25x	32	pos (TMV)	pos (TMV)	neg	neg	neg	neg	neg	neg	neg	neg	neg
TMV	NIB V 413	25x	26	pos (TMV)	pos (TMV)	neg	neg	neg	neg	neg	neg	neg	neg	neg

					RT-PCR			RT-qPCR[b]					RPA	LAMP
Virus	**NIB ID**	**Dilution factor**	**Nad5 (Cq)**	**Tobamovirus confirmed[a]**	**Levitzky et al. (2019)[a]**	**Loewe (Cat. No. 09181)**	**Sui et al. (2017)**	**DAFF DEECA**	**Fowkes et al. (2022)**	**ISF[c]**	**Tiberini et al. (2022) singleplex**	**Tiberini et al. (2022) duplex[c]**	**Agdia RPA (XCS 22800)**	**Kimura et al. (2023)[d]**
ToBRFV	NIB V 331	25x	24	pos (ToBRFV)	pos (ToBRFV)	neg	neg	neg	neg	neg	neg	neg	neg	neg
ToMMV	NIB V 373	25x	26	pos (ToMMV)	pos (ToMMV)	pos	pos	pos (14)	pos (17)	pos (12)	pos (15)	pos (15)	pos	pos (21)
ToMMV	NIB V 414	2x	22	pos (ToMMV)	neg/ inc (nd)[e]	neg	pos	pos (28)	pos (30)	pos (28)	pos (29)	pos (29)	pos	pos (41)
ToMV	NIB V 410	25x	24	pos (ToMV)	pos (ToMV)	neg	neg	neg	neg	neg	neg	neg	neg	neg
ToMV	NIB V 406	25x	23	pos (ToMV)	pos (nd)	neg	neg	neg	neg	neg	neg	neg	neg	neg
ToMV	NIB V 411	1x	25	pos (ToMV)	pos (ToMV)	neg	neg	neg	neg	neg	neg	neg	neg	neg
ToMV	NIB V 412	1x	23	pos (ToMV)	pos (ToMV)	neg	neg	neg	neg	neg	neg	neg	neg	neg
YMoV	NIB V 374	1x	24	pos (YMoV)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
healthy tomato seed	D1977/23 + D93/23	25x	30	neg	neg	neg	neg	inc (39)	neg	neg	neg	neg	neg	neg
healthy tomato seed	D1977/23	25x	29	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
healthy tomato leaves	/	1x	30	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
Cq values considered positive based on the analysis of these samples					/	/	/	< 34	< 36	< 33	< 35	< 37	/	/

[a]In bracket, result of Sanger sequencing is given (nd – not determined due low sequence quality); [b]In bracket, Cq value is given; [c]Results for ToMMV only is given ; [d]In bracket, Tp in minutes is given; [e]different results for the same sample tested in two parallels (note that each sample was tested in two parallels and only in case of different results the results of both parallels are given)

Table 5. Results of internal evaluation of the analytical sensitivity. Legend: pos=positive; neg=negative; inc = inconclusive

			RT-PCR			RT-qPCR[b]					RPA	LAMP
Virus	**NIB ID**	**Dilution factor**	**Levitzky et al. (2019)[a]**	**Loewe (Cat. No. 09181)**	**Sui et al. (2017)**	**DAFF DEECA**	**Fowkes et al. (2022)**	**ISF[c]**	**Tiberini et al. (2022) singleplex**	**Tiberini et al. (2022) duplex[c]**	**Agdia RPA (XCS 22800)**	**Kimura et al. (2023)[d]**
ToMMV NIB V 373 RNA diluted in RNA from leaves of healthy *Solanum lycopersicum*												
ToMMV	NIB V 373	2.5 x 10^1	pos (ToMMV)	pos	pos	pos (14)	pos (17)	pos (12)	pos (15)	pos (15)	pos	pos (21)
		2.5 x 10^2	pos (ToMMV)	pos	pos	pos (17)	pos (20)	pos (16)	pos (18)	pos (18)	pos	pos (24)
		2.5 x 10^3	pos (ToMMV)	pos	pos	pos (20)	pos (24)	pos (19)	pos (21)	pos (21)	pos	pos (27)
		2.5 x 10^4	pos (ToMMV)	pos	pos	pos (24)	pos (27)	pos (22)	pos (24)	pos (24)	pos	pos (31)
		2.5 x 10^5	pos (nd)	neg	pos	pos (27)	pos (30)	pos (26)	pos (28)	pos (27)	pos	pos (41)
		2.5 x 10^6	inc (nd)	neg	neg	pos (30)	pos (33)	pos (30)	pos (31)	pos (31)	pos	pos (59)
		2.5 x 10^7	neg	neg	neg	inc (34)	inc (36)	inc (33)	pos (34)	pos (34)	pos	neg
		2.5 x 10^8	neg	neg	neg	inc (37)	neg	inc (38)	neg/ inc (39)[e]	inc (38)	neg	neg
RNA from healthy tomato leaves used as a diluent			neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
ToMMV NIB V 414 RNA diluted in water												
ToMMV	NIB V 414	2x 10^0	neg/ inc (nd)[e]	neg	pos	pos (28)	pos (30)	pos (28)	pos (29)	pos (29)	pos	pos (41)
		2 x 10^1	neg	neg	neg	pos (31)	pos (32)	pos (32)	pos (32)	pos (32)	pos	pos (49)
Water used as a diluent			neg	neg	neg	neg	neg	neg	neg	neg	neg	neg

[a]In bracket, result of Sanger sequencing is given (nd – not determined due low sequence quality); [b]In bracket, Cq value is given.; [c]Results for ToMMV only is given ; [d]In bracket, Tp in minutes is given; [e]different results for the same sample tested in two parallels (note that each sample was tested in two parallels and only in case of different results the results of both parallels are given)

5 Test performance study

5.1 Evaluated parameters

The test performance study was conducted according to the EPPO guidelines (PM7/122) (EPPO, 2022), also taking into account the guidelines described in Vučurović et al. (2022). The key performance characteristics of the tests evaluated in the TPS were analytical and diagnostic sensitivity and specificity, and reproducibility (PM7/98(5); EPPO, 2021).

5.2 Test panel composition and preparation

The sample and control aliquots were prepared from homogeneous RNA extracts pretested in the preliminary study (see section 4). For each sample type and for each control, 40 aliquots were prepared. Subsequently, 40 sample panels labeled L1-L40 were prepared. Each sample panel contained 22 RNA samples (S-1 to S-22), one positive control (PC - pos ToMMV) and one negative control (NC - neg tomato) (Table 6). The sample panels were immediately stored at -20°C until shipment to the participants.

Table 6. Test panel provided to the participants of TPS (for details about samples see Tables 2, 4 and 5)

Virus	NIB ID	Dilution factor	Sample ID	Health status of ToMMV
healthy tomato seed	D1977/23 + D93/23	25x	S-12	neg
healthy tomato seed	D1977/23	25x	S-15	neg
CGMMV	NIB V 403	25x	S-3	neg
ObPV	NIB V 364	25x	S-19	neg
ORSV	NIB V 365	25x	S-17	neg
PaMMV	NIB V 366	25x	S-13	neg
PMMoV	NIB V 408	25x	S-11	neg
TMGMV	NIB V 404	25x	S-5	neg
TMV	NIB V 405	25x	S-8	neg
TMV	NIB V 413	25x	S-22	neg
ToBRFV	NIB V 331	25x	S-9	neg
ToMV	NIB V 410	25x	S-16	neg
ToMV	NIB V 406	25x	S-18	neg
ToMMV	NIB V 373	2.5×10^8	S-21	pos
ToMMV	NIB V 373	2.5×10^7	S-6	pos
ToMMV	NIB V 373	2.5×10^6	S-4	pos
ToMMV	NIB V 373	2.5×10^5	S-10	pos
ToMMV	NIB V 373	2.5×10^4	S-20	pos
ToMMV	NIB V 373	2.5×10^3	S-2	pos
ToMMV	NIB V 373	2.5×10^2	S-14	pos
ToMMV	NIB V 414	$2x\ 10^1$	S-7	pos
ToMMV	NIB V 414	2x	S-1	pos
healthy tomato leaves	/	1x	NC	neg
ToMMV	NIB V 373	25x	PC	pos

5.3 Assigned reference values

Reference values can be assigned to the test items in various ways with the two most commonly employed: (i) assigning reference values on the true health status of the test items and (ii) assigning reference values based on the results of tests expected to be used by the participants.

For this TPS, assigned reference values were based on health status of the test items and controls: samples were defined as positive when they contained ToMMV, and negative if they contained other tobamoviruses or if they were without any tobamovirus (Table 6).

5.4 Homogeneity and stability testing

For the homogeneity and stability test, four randomly selected batches of aliquots of all samples prepared for the TPS (sample panels) were selected and tested with all or some of the tests. Stability testing was performed under conditions mimicking transportation and storage conditions by incubating three randomly selected sample panels (L17, L6 and L9) at room temperature for up to one week and then at -20 °C. One of these sample sets was analysed during the time period available for the participants to perform the analyses. These tests were performed to verify that the stability of the samples was maintained throughout the TPS. The samples were stable in all cases (Appendix 3). Some results for samples with low ToMMV titers and for non-targets differ between batches. These variations are not due to an impairment of the stability or homogeneity of the samples, but reflect a low titer of the target or weak cross-reactions, which sometimes turn out to be positive and sometimes negative.

5.5 Randomization

The samples were coded in order to ensure a full blind testing of samples. Coding of samples was randomly. Sample de-coder is given in Table 6. For all sample panels, the same sample codes have been used.

5.6 Distribution of the samples

A total of 33 sample panels were distributed to 15 TPS participants (Table 7). All 33 sample panels were sent in May 2024. Thirteen participants received the samples within one to seven days. Two participants took longer to receive the samples. Both participants who received samples after 7 days provide datasets with expected results, thus eventhough we tested stability only for 7 days shipping, this deviation did not have effect on the stability of the samples. All participants confirmed that they had stored the samples at -20 °C upon receipt. All participants also confirmed that the condition of the samples on receipt was considered satisfactory and that they had also received the TPS protocols and the TPS important instructions.

Table 7: Summary of panels of samples provided to TPS participants (In the table, sample panel name is indicated). Note: for each method one panel of samples was provided to participant (one panel of samples for all RT-qPCRs, one for all RT-PCRs, one for RPA and one for LAMP)

		Method			
		RT-PCRs	RT-qPCRs	RPA	LAMP
Lab ID	A	L19	L12		L8
	B	L10	L25	L35	L24
	C				L15
	D	L18	L28		L3
	E		L29		
	F		L38		
	G	L33	L2		
	H	L34		L22	
	I		L20		
	J	L16	L23	L5	
	K		L36		
	L	L4	L37	L31	L13
	M		L1	L11	L26
	N		L30		
	O	L14	L27	L7	

5.7 Consumables

Participants were asked to use/purchase their own consumables, chemicals and reagents. Details of the chemicals and reagents required are included in Appendix 1 for each test. However, participants were allowed to make some changes if they could demonstrate that these changes would not affect the results. In this regard, they were asked to test at least three dilutions of ToMMV and at least three non-targets in advance and obtain the expected results. And only in such cases were they allowed to use a modified protocol, but were asked to submit the list of all modifications together with the results of the TPS.

5.8 Equipment and materials

Standard laboratory equipment (including those required for PCR, real time PCR, and LAMP) and standard laboratory material was needed. Agdia's RPA test can be performed using the AmpliFire® fluorometer (or equivalent) and three demonstration units were available from Agdia Inc. and were shipped to/between different participants.

5.9 Methods

The technical instructions provided to the participants are contained in Appendix 1 and a summary of all the key data required to carry out these tests is contained in Appendix 2. The technical instructions

contain detailed protocols for each test, including the proposed analysis settings. Participants were asked to record and report any deviations.

Note that all participants who were registered to perform RT-PCRs and/or RT-qPCRs were required to perform all RT-PCRs and/or all RT-qPCRs. Accepted exceptions were only laboratories from countries where it is not possible to order certain kits or where it may take too long for the order to be processed or the kits/chemicals to be delivered.

5.10 Participants

Number of registered participants: 15. Participating laboratories were from 13 countries (Table 8, Figure 1).

Table 8. List of TPS participants (in alphabetical order) (note that there are no links between Lab ID and this list of TPS participants)

Name of the participating laboratory	**Country**
Agdia, Inc. Technical Support	USA
AGES - Austrian Agency for Health and Food Safety	Austria
Agriculture Victoria Research - Crop Health Services	Australia
All-Russian Plant Quarantine Center	Russia
BIOREBA AG	Switzerland
CREA-DC Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria – Centro di Ricerca Difesa e Certificazione	Italy
Groupe d\'Etude et de contrôle des Variétés Et des Semences (GEVES) - BioGEVES	France
JKI/EPV	Germany
LOEWE Biochemica GmbH	Germany
Ministry for Primary Industries/Plant Health and Environment Laboratory	New Zealand
Naktuinbouw	The Netherlands
National Institute of Biology	Slovenia
Plant Clinic, Fera Science Ltd	United Kingdom
Plant Protection and Inspection Services-Ministry of Agriculture and Rural Development/Seeds Lab	Israel
United States Department of Agriculture, Animal Plant Health Inspection Service, Plant Protection and Quarantine, Science and Technology – Plant Pathogen Confirmatory Diagnostics Laboratory	USA

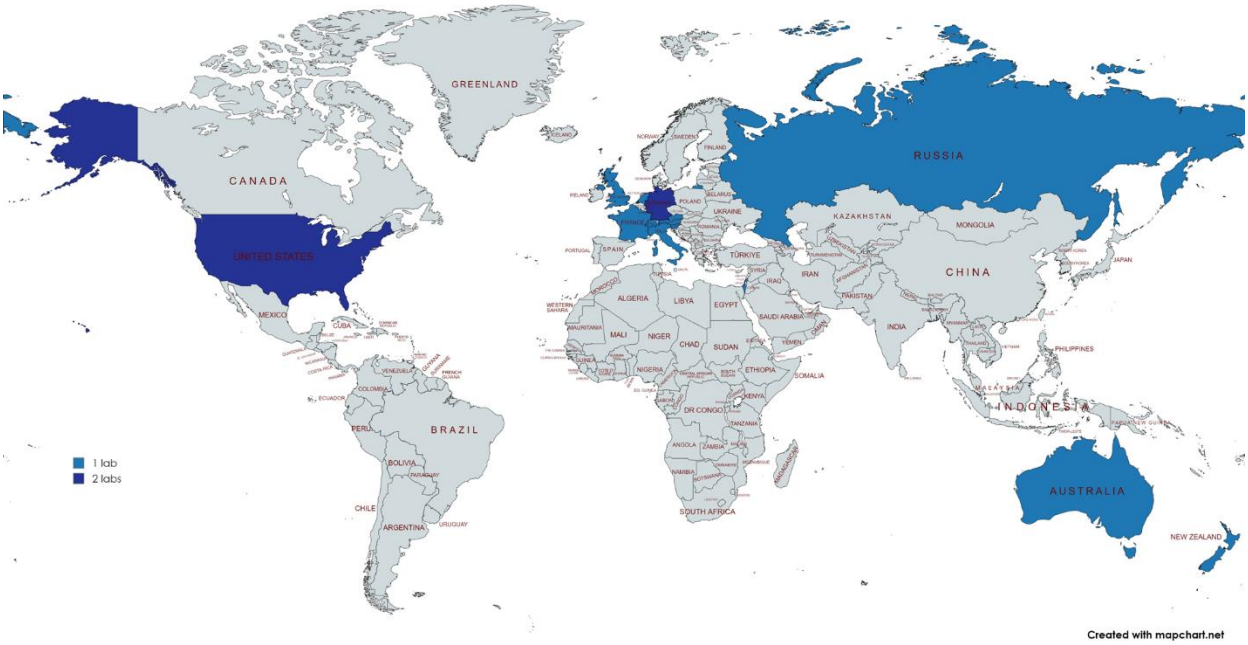

Figure 1: Countries of participants of the TPS are represented in blue colour.

5.11 Results

5.11.1 Collected results

Results were collected via an excel file in which the participants reported the results and if any deviations was done from the recommended protocols. Raw data for all methods submitted by participants are collected in Appendix 4.

The number of data sets submitted for each test is given in Table 9. Some laboratories did not submit results for all requested data sets: three laboratories did not submit results for RT-PCR Loewe and one laboratory did not submit results for RT-qPCR ISF because it either did not receive the chemicals/reagents in time or has no possibility to order the chemicals/reagents in the country where the laboratory is located (see Appendix 4).

5.11.2 Outlier results

Some laboratories reported that they performed some tests with a deviation from the prescribed protocol (Appendix 4). All such data sets were compared with data sets obtained with unmodified protocols, and whenever the deviations from the protocol appeared to have an impact on the results, the data sets were excluded from further analysis (Appendix 4). Based on this, 3 data sets were excluded (Table 9).

Table 9: Number of submitted data sets and the valid data sets per individual test and method.

Method and test	Number of submitted data sets	Number of valid data sets	
		(N°)	Percentage (%)
RT-PCR			
Levitzky et al. (2019)	8	8	100
Loewe (Cat no. 09181)	5	4	80
Sui et al. (2017)	8	7	88
RT-qPCR			
DAFF DEECA	13	13	100
Fowkes et al. (2022)	13	13	100
ISF	12	12	100
Tiberini et al. (2022): - Singleplex - Duplex	 10 9	 10 9	 100 100
RPA			
Agdia (XCS 22800)	6	6	100
LAMP			
Kimura et al. (2023)	6	5	83
Total	***90***	***87***	***97***

5.11.3 Performance of individual tests

Performances of individual tests over all valid data sets are shown from Table 10 to Table 19 for each individual tests as well as across all test items. Performance is described in terms of the number and percentage of results which were inconclusive, true negative (TN), false positive (FP), false negative (FN), true positive (TP) and the number and percentage of concordant and non-concordant results. Inconclusive results were treated as non-concordant.

Colour coding is consistent among tables with concordant results coloured in green, inconclusive results coloured in yellow and non-concordant results coloured in red.

Before analysing the RT-qPCR results, we modified some of the results submitted by the participants (the analysis based on the unmodified results can be found in Appendix 5). This was necessary because participants used very different approaches to sample determination and were unable to perform the necessary analyses to determine the Cq cut-off value for each test. The Cq cut-off value is required for all RT-qPCRs tested as cross-reactions with some other tobamoviruses are known to result in high Cq values (Appendix 1). The Cq cut-off value depends on the equipment, material and chemistry and can therefore vary from laboratory to laboratory. However, as this could not be verified separately for all TPS participants, the following decision was made: Any Cq values that were equal to or higher than the Cq values obtained by the participant for PaMMV or healthy samples were considered inconclusive, i.e. they could be due to either cross-reaction with a non-target virus, environmental contamination or a low titer of ToMMV. In the absence of signal for PaMMV and healthy samples, all Cq values of 37 or higher were considered inconclusive results. Below is a range of the lowest Cq values above which the

results of various laboratories are considered inconclusive (detailed data on modification of the results from participants is provided in Appendix 4):

- RT-qPCR DAFF DEECA: ≥31-37
- RT-qPCR Fowkes et al. (2022): ≥33-37
- RT-qPCR ISF: ≥28-35
- RT-qPCR Tiberini et al. (2022) – singleplex: ≥33-37
- RT-qPCR Tiberini et al. (2022) – duplex: ≥32-37

Table 10: Performance of RT-PCR Levitzky et al. (2019).
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

Sample description	Sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
healthy tomato seed	S-15	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
CGMMV (NIB V 403) 25x	S-3	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
ObPV (NIB V 364) 25x	S-19	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
ORSV (NIB V 365) 25x	S-17	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
PMMoV (NIB V 408) 25x	S-11	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
ToBRFV (NIB V 331) 25x	S-9	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
ToMV (NIB V 406) 25x	S-18	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	0			8	0	0,0	0,0	0,0	100,0	0,0	0	8	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	0			6	2	0,0	0,0	0,0	75,0	25,0	2	6	25,0	75,0
ToMMV (NIB V 373) 2.5 x 10^6	S-4	0			3	5	0,0	0,0	0,0	37,5	62,5	5	3	62,5	37,5
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			0	8	0,0	0,0	0,0	0,0	100,0	8	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	8	0,0	0,0	0,0	0,0	100,0	8	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			0	8	0,0	0,0	0,0	0,0	100,0	8	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			1	7	0,0	0,0	0,0	12,5	87,5	7	1	87,5	12,5
ToMMV (NIB V 414) 2 x 10^1	S-7	0			7	1	0,0	0,0	0,0	87,5	12,5	1	7	12,5	87,5
ToMMV (NIB V 414) 2x	S-1	0			5	3	0,0	0,0	0,0	62,5	37,5	3	5	37,5	62,5
healthy tomato leaves	NC	0	8	0			0,0	100,0	0,0	0,0	0,0	8	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	8	0,0	0,0	0,0	0,0	100,0	8	0	100,0	0,0
	Total	**0**	**112**	**0**	**30**	**50**	**0,0**	**58,3**	**0,0**	**15,6**	**26,0**	**162**	**30**	**84,4**	**15,6**

Table 11: Performance of RT-PCR Loewe (Cat no. 09181).
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

Sample description	Sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
healthy tomato seed	S-15	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
CGMMV (NIB V 403) 25x	S-3	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
ObPV (NIB V 364) 25x	S-19	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
ORSV (NIB V 365) 25x	S-17	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
PMMoV (NIB V 408) 25x	S-11	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
ToBRFV (NIB V 331) 25x	S-9	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
ToMV (NIB V 406) 25x	S-18	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	0			4	0	0,0	0,0	0,0	100,0	0,0	0	4	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	0			4	0	0,0	0,0	0,0	100,0	0,0	0	4	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^6	S-4	0			4	0	0,0	0,0	0,0	100,0	0,0	0	4	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1			2	1	25,0	0,0	0,0	50,0	25,0	1	3	25,0	75,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	4	0,0	0,0	0,0	0,0	100,0	4	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			0	4	0,0	0,0	0,0	0,0	100,0	4	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			0	4	0,0	0,0	0,0	0,0	100,0	4	0	100,0	0,0
ToMMV (NIB V 414) 2 x 10^1	S-7	0			4	0	0,0	0,0	0,0	100,0	0,0	0	4	0,0	100,0
ToMMV (NIB V 414) 2x	S-1	0			3	1	0,0	0,0	0,0	75,0	25,0	1	3	25,0	75,0
healthy tomato leaves	NC	0	4	0			0,0	100,0	0,0	0,0	0,0	4	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	4	0,0	0,0	0,0	0,0	100,0	4	0	100,0	0,0
	Total	**1**	**56**	**0**	**21**	**18**	**1,0**	**58,3**	**0,0**	**21,9**	**18,8**	**74**	**22**	**77,1**	**22,9**

Table 12: Performance of RT-PCR Sui et al. (2017).
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

sample description	sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
healthy tomato seed	S-15	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
CGMMV (NIB V 403) 25x	S-3	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
ObPV (NIB V 364) 25x	S-19	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
ORSV (NIB V 365) 25x	S-17	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
PMMoV (NIB V 408) 25x	S-11	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
ToBRFV (NIB V 331) 25x	S-9	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
ToMV (NIB V 406) 25x	S-18	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	0			7	0	0,0	0,0	0,0	100,0	0,0	0	7	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	0			7	0	0,0	0,0	0,0	100,0	0,0	0	7	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1			5	1	14,3	0,0	0,0	71,4	14,3	1	6	14,3	85,7
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			2	5	0,0	0,0	0,0	28,6	71,4	5	2	71,4	28,6
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	7	0,0	0,0	0,0	0,0	100,0	7	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			0	7	0,0	0,0	0,0	0,0	100,0	7	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			0	7	0,0	0,0	0,0	0,0	100,0	7	0	100,0	0,0
ToMMV (NIB V 414) 2 x 10^1	S-7	1			5	1	14,3	0,0	0,0	71,4	14,3	1	6	14,3	85,7
ToMMV (NIB V 414) 2x	S-1	0			4	3	0,0	0,0	0,0	57,1	42,9	3	4	42,9	57,1
healthy tomato leaves	NC	0	7	0			0,0	100,0	0,0	0,0	0,0	7	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	7	0,0	0,0	0,0	0,0	100,0	7	0	100,0	0,0
	Total	**2**	**98**	**0**	**30**	**38**	**1,2**	**58,3**	**0,0**	**17,9**	**22,6**	**136**	**32**	**81,0**	**19,0**

Table 13: Performance of RT-qPCR DAFF DEECA.
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

sample description	sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
healthy tomato seed	S-15	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
CGMMV (NIB V 403) 25x	S-3	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ObPV (NIB V 364) 25x	S-19	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ORSV (NIB V 365) 25x	S-17	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	12	1	0			92,3	7,7	0,0	0,0	0,0	1	12	7,7	92,3
PMMoV (NIB V 408) 25x	S-11	1	12	0			7,7	92,3	0,0	0,0	0,0	12	1	92,3	7,7
TMGMV (NIB V 404) 25x	S-5	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToBRFV (NIB V 331) 25x	S-9	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMV (NIB V 406) 25x	S-18	1	12	0			7,7	92,3	0,0	0,0	0,0	12	1	92,3	7,7
ToMMV (NIB V 373) 2.5 x 10^8	S-21	12			1	0	92,3	0,0	0,0	7,7	0,0	0	13	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	11			1	1	84,6	0,0	0,0	7,7	7,7	1	12	7,7	92,3
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1			0	12	7,7	0,0	0,0	0,0	92,3	12	1	92,3	7,7
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 414) 2 x 10^1	S-7	1			0	12	7,7	0,0	0,0	0,0	92,3	12	1	92,3	7,7
ToMMV (NIB V 414) 2x	S-1	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
healthy tomato leaves	NC	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100	0
	Total	**39**	**168**	**0**	**2**	**103**	**12,5**	**53,8**	**0,0**	**0,6**	**33,0**	**271**	**41**	**86,9**	**13,1**

Table 14: Performance of RT-qPCR Fowkes et al. (2022).
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

sample description	sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	1	12	0			7,7	92,3	0,0	0,0	0,0	12	1	92,3	7,7
healthy tomato seed	S-15	1	12	0			7,7	92,3	0,0	0,0	0,0	12	1	92,3	7,7
CGMMV (NIB V 403) 25x	S-3	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ObPV (NIB V 364) 25x	S-19	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ORSV (NIB V 365) 25x	S-17	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	12	1	0			92,3	7,7	0,0	0,0	0,0	1	12	7,7	92,3
PMMoV (NIB V 408) 25x	S-11	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToBRFV (NIB V 331) 25x	S-9	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMV (NIB V 406) 25x	S-18	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	4			9	0	30,8	0,0	0,0	69,2	0,0	0	13	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	11			1	1	84,6	0,0	0,0	7,7	7,7	1	12	7,7	92,3
ToMMV (NIB V 373) 2.5 x 10^6	S-4	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			1	12	0,0	0,0	0,0	7,7	92,3	12	1	92,3	7,7
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 414) 2 x 10^1	S-7	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 414) 2x	S-1	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
healthy tomato leaves	NC	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
	Total	**29**	**168**	**0**	**11**	**104**	**9,3**	**53,8**	**0,0**	**3,5**	**33,3**	**272**	**40**	**87,2**	**12,8**

Table 15: Performance of RT-qPCR ISF.
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

sample description	sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	1	11	0			8,3	91,7	0,0	0,0	0,0	11	1	91,7	8,3
healthy tomato seed	S-15	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
CGMMV (NIB V 403) 25x	S-3	1	11	0			8,3	91,7	0,0	0,0	0,0	11	1	91,7	8,3
ObPV (NIB V 364) 25x	S-19	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
ORSV (NIB V 365) 25x	S-17	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	12	0	0			100,0	0,0	0,0	0,0	0,0	0	12	0,0	100,0
PMMoV (NIB V 408) 25x	S-11	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	1	11	0			8,3	91,7	0,0	0,0	0,0	11	1	91,7	8,3
ToBRFV (NIB V 331) 25x	S-9	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
ToMV (NIB V 406) 25x	S-18	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	12			0	0	100,0	0,0	0,0	0,0	0,0	0	12	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	11			0	1	91,7	0,0	0,0	0,0	8,3	1	11	8,3	91,7
ToMMV (NIB V 373) 2.5 x 10^6	S-4	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
ToMMV (NIB V 414) 2 x 10^1	S-7	3			0	9	25,0	0,0	0,0	0,0	75,0	9	3	75,0	25,0
ToMMV (NIB V 414) 2x	S-1	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
healthy tomato leaves	NC	1	11	0			8,3	91,7	0,0	0,0	0,0	11	1	91,7	8,3
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
	Total	**42**	**152**	**0**	**0**	**94**	**14,6**	**52,8**	**0,0**	**0,0**	**32,6**	**246**	**42**	**85,4**	**14,6**

Table 16: Performance of RT-qPCR Tiberini et al. (2022) singleplex.
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

sample description	sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
healthy tomato seed	S-15	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
CGMMV (NIB V 403) 25x	S-3	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ObPV (NIB V 364) 25x	S-19	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ORSV (NIB V 365) 25x	S-17	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	7	3	0			70,0	30,0	0,0	0,0	0,0	3	7	30,0	70,0
PMMoV (NIB V 408) 25x	S-11	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ToBRFV (NIB V 331) 25x	S-9	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ToMV (NIB V 406) 25x	S-18	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	3			7	0	30,0	0,0	0,0	70,0	0,0	0	10	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	8			2	0	80,0	0,0	0,0	20,0	0,0	0	10	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^6	S-4	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
ToMMV (NIB V 414) 2 x 10^1	S-7	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
ToMMV (NIB V 414) 2x	S-1	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
healthy tomato leaves	NC	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
	Total	**18**	**133**	**0**	**9**	**80**	**7,5**	**55,4**	**0,0**	**3,8**	**33,3**	**213**	**27**	**88,8**	**11,3**

Table 17: Performance of RT-qPCR Tiberini et al. (2022) duplex.
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

sample description	sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
healthy tomato seed	S-15	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
CGMMV (NIB V 403) 25x	S-3	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
ObPV (NIB V 364) 25x	S-19	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
ORSV (NIB V 365) 25x	S-17	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	6	3	0			31,6	33,3	0,0	0,0	0,0	3	6	33,3	66,7
PMMoV (NIB V 408) 25x	S-11	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
ToBRFV (NIB V 331) 25x	S-9	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	0	8	1			0,0	88,9	11,1	0,0	0,0	8	1	88,9	11,1
ToMV (NIB V 406) 25x	S-18	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	5			4	0	26,3	0,0	0,0	44,4	0,0	0	9	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	6			2	1	31,6	0,0	0,0	22,2	11,1	1	8	11,1	88,9
ToMMV (NIB V 373) 2.5 x 10^6	S-4	0			0	9	0,0	0,0	0,0	0,0	100,0	9	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			0	9	0,0	0,0	0,0	0,0	100,0	9	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	9	0,0	0,0	0,0	0,0	100,0	9	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			0	9	0,0	0,0	0,0	0,0	100,0	9	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			1	8	0,0	0,0	0,0	11,1	88,9	8	1	88,9	11,1
ToMMV (NIB V 414) 2 x 10^1	S-7	1			0	8	5,3	0,0	0,0	0,0	88,9	8	1	88,9	11,1
ToMMV (NIB V 414) 2x	S-1	0			0	9	0,0	0,0	0,0	0,0	100,0	9	0	100,0	0,0
healthy tomato leaves	NC	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	9	0,0	0,0	0,0	0,0	100,0	9	0	100,0	0,0
	Total	**18**	**119**	**1**	**7**	**71**	**8,3**	**55,1**	**0,5**	**0,0**	**32,9**	**190**	**26**	**88,0**	**12,0**

Table 18: Performance of RPA Agdia (XCS 22800).
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

sample description	sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	0	6	0			0,0	100,0	0,0	0,0	0,0	6	0	100,0	0,0
healthy tomato seed	S-15	0	6	0			0,0	100,0	0,0	0,0	0,0	6	0	100,0	0,0
CGMMV (NIB V 403) 25x	S-3	0	6	0			0,0	100,0	0,0	0,0	0,0	6	0	100,0	0,0
ObPV (NIB V 364) 25x	S-19	0	6	0			0,0	100,0	0,0	0,0	0,0	6	0	100,0	0,0
ORSV (NIB V 365) 25x	S-17	0	6	0			0,0	100,0	0,0	0,0	0,0	6	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	2	3	1			33,3	50,0	16,7	0,0	0,0	3	3	50,0	50,0
PMMoV (NIB V 408) 25x	S-11	0	6	0			0,0	100,0	0,0	0,0	0,0	6	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	6	0			0,0	100,0	0,0	0,0	0,0	6	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	6	0			0,0	100,0	0,0	0,0	0,0	6	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	5	1			0,0	83,3	16,7	0,0	0,0	5	1	83,3	16,7
ToBRFV (NIB V 331) 25x	S-9	0	6	0			0,0	100,0	0,0	0,0	0,0	6	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	0	6	0			0,0	100,0	0,0	0,0	0,0	6	0	100,0	0,0
ToMV (NIB V 406) 25x	S-18	0	6	0			0,0	100,0	0,0	0,0	0,0	6	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	2			4	0	33,3	0,0	0,0	66,7	0,0	0	6	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1			3	2	16,7	0,0	0,0	50,0	33,3	2	4	33,3	66,7
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1			0	5	16,7	0,0	0,0	0,0	83,3	5	1	83,3	16,7
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			0	6	0,0	0,0	0,0	0,0	100,0	6	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	6	0,0	0,0	0,0	0,0	100,0	6	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			0	6	0,0	0,0	0,0	0,0	100,0	6	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			0	6	0,0	0,0	0,0	0,0	100,0	6	0	100,0	0,0
ToMMV (NIB V 414) 2 x 10^1	S-7	0			1	5	0,0	0,0	0,0	16,7	83,3	5	1	83,3	16,7
ToMMV (NIB V 414) 2x	S-1	0			0	6	0,0	0,0	0,0	0,0	100,0	6	0	100,0	0,0
healthy tomato leaves	NC	0	6	0			0,0	100,0	0,0	0,0	0,0	6	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	6	0,0	0,0	0,0	0,0	100,0	6	0	100,0	0,0
	Total	**6**	**80**	**2**	**8**	**48**	**4,2**	**55,6**	**1,4**	**5,6**	**33,3**	**128**	**16**	**88,9**	**11,1**

Table 19: Performance of LAMP Kimura et al. (2023).
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

sample description	sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	0	5	0			0,0	100,0	0,0	0,0	0,0	5	0	100,0	0,0
healthy tomato seed	S-15	0	5	0			0,0	100,0	0,0	0,0	0,0	5	0	100,0	0,0
CGMMV (NIB V 403) 25x	S-3	0	5	0			0,0	100,0	0,0	0,0	0,0	5	0	100,0	0,0
ObPV (NIB V 364) 25x	S-19	1	4	0			20,0	80,0	0,0	0,0	0,0	4	1	80,0	20,0
ORSV (NIB V 365) 25x	S-17	1	4	0			20,0	80,0	0,0	0,0	0,0	4	1	80,0	20,0
PaMMV (NIB V 366) 25x	S-13	1	4	0			20,0	80,0	0,0	0,0	0,0	4	1	80,0	20,0
PMMoV (NIB V 408) 25x	S-11	0	5	0			0,0	100,0	0,0	0,0	0,0	5	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	5	0			0,0	100,0	0,0	0,0	0,0	5	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	5	0			0,0	100,0	0,0	0,0	0,0	5	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	5	0			0,0	100,0	0,0	0,0	0,0	5	0	100,0	0,0
ToBRFV (NIB V 331) 25x	S-9	0	5	0			0,0	100,0	0,0	0,0	0,0	5	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	1	4	0			20,0	80,0	0,0	0,0	0,0	4	1	80,0	20,0
ToMV (NIB V 406) 25x	S-18	1	4	0			20,0	80,0	0,0	0,0	0,0	4	1	80,0	20,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1			4	0	20,0	0,0	0,0	80,0	0,0	0	5	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1			4	0	20,0	0,0	0,0	80,0	0,0	0	5	0,0	100,0
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1			2	2	20,0	0,0	0,0	40,0	40,0	2	3	40,0	60,0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			1	4	0,0	0,0	0,0	20,0	80,0	4	1	80,0	20,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	5	0,0	0,0	0,0	0,0	100,0	5	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			0	5	0,0	0,0	0,0	0,0	100,0	5	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			0	5	0,0	0,0	0,0	0,0	100,0	5	0	100,0	0,0
ToMMV (NIB V 414) 2 x 10^1	S-7	1			4	0	20,0	0,0	0,0	80,0	0,0	0	5	0,0	100,0
ToMMV (NIB V 414) 2x	S-1	1			1	3	20,0	0,0	0,0	20,0	60,0	3	2	60,0	40,0
healthy tomato leaves	NC	0	5	0			0,0	100,0	0,0	0,0	0,0	5	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	5	0,0	0,0	0,0	0,0	100,0	5	0	100,0	0,0
	Total	**10**	**65**	**0**	**16**	**29**	**8,3**	**54,2**	**0,0**	**13,3**	**24,2**	**94**	**26**	**78,3**	**21,7**

5.11.4 Comparison of the tests and other diagnostic parameters

A summary of performance of the tests included in the TPS are presented in Table 20 and 21. Graphical summaries of diagnostic parameters are given in Figures 2 to 6.

All parameters were calculated considering all samples included in the sample panel.

Please note that this performance is directly related to the test panel and that the calculation of all parameters was based on health status and that for the RT-qPCRs some qualitative results for samples from participants were modified as described above (the comparison based on participants' unmodified qualitative results for the RT-qPCRs can be found in Appendix 5).

In addition to the performance parameters reported in previous chapters, the following performance parameters were calculated as follows:

- diagnostic sensitivity, an estimation of the ability of the method to detect the target = 100 x true positive / expected positives*
- diagnostic specificity, an estimation of the ability of the method not to detect the non-target = 100 x true negative / expected negatives*
- false positive rate = 1 – diagnostic specificity
- false negative rate = 1- diagnostic sensitivity
- relative accuracy = (true positive + true negative) / total data points
- positive predictive value = probability that subjects with a positive screening test truly have the disease = true positive / (true positive + false positive)**
- negative predictive value = probability that subjects with a negative screening test truly don't have the disease = true negative / (false negative + true negative)**

*Diagnostic sensitivity and diagnostic specificity could be calculated differently than it was used in this study. Those two parameters are heavily dependent on how expected value is assigned to the samples and how inconclusive results are treated (for other options see Vučurović et al., 2022).

**As it was impossible to make justified interpretation of the results reported as inconclusive, these were excluded from analysis of positive/ negative predicted value.

Reproducibility was calculated using the following formula: number of recurring results per sample/total number of results per sample.

Table 20: Comparison of performance parameters determined for individual tests included in TPS over all submitted data sets. Legend: TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

	RT-PCR			RT-qPCR					RPA	LAMP
Diagnostic parameter	Levitzky et al. (2019)	Loewe (Cat. No. 09181)	Sui et al. (2017)	DAFF DEECA	Fowkes et al. (2022)	ISF	Tiberini et al. (2022) singleplex	Tiberini et al. (2022) duplex	Agdia RPA (XCS 22800)	Kimura et al. (2023)
Total data sets	8	4	7	13	13	12	10	9	6	5
Expected positives	80	40	70	130	130	120	100	90	60	50
Expected negatives	112	56	98	182	182	168	140	126	84	70
Total data points	192	96	168	312	312	288	240	216	144	120
INC	0	1	2	39	29	42	18	18	6	10
TN	112	56	98	168	168	152	133	119	80	65
FP	0	0	0	0	0	0	0	1	2	0
FN	30	21	30	2	11	0	9	7	8	16
TP	50	18	38	103	104	94	80	71	48	29
INC %	0,0	1,0	1,2	12,5	9,3	14,6	7,5	8,3	4,2	8,3
TN %	58,3	58,3	58,3	53,8	53,8	52,8	55,4	55,1	55,6	54,2
FP %	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,5	1,4	0,0
FN %	15,6	21,9	17,9	0,6	3,5	0,0	3,8	3,2	5,6	13,3
TP %	26,0	18,8	22,6	33,0	33,3	32,6	33,3	32,9	33,3	24,2
Concordant	162	74	136	271	272	246	213	190	128	94
Non-concordant	30	22	32	41	40	42	27	26	16	26
Concordant %	84,4	77,1	81,0	86,9	87,2	85,4	88,8	88,0	88,9	78,3
Non-concordant %	15,6	22,9	19,0	13,1	12,8	14,6	11,3	12,0	11,1	21,7
diagnostic sensitivity %	62,5	45,0	54,3	79,2	80,0	78,3	80,0	78,9	80,0	58,0
diagnostic specificity %	100,0	100,0	100,0	92,3	92,3	90,5	95,0	94,4	95,2	92,9
false positive rate %	0,0	0,0	0,0	7,7	7,7	9,5	5,0	5,6	4,8	7,1
false negative rate %	37,5	55,0	45,7	20,8	20,0	21,7	20,0	21,1	20,0	42,0
relative accuracy %	84,4	77,1	81,0	86,9	87,2	85,4	88,8	88,0	88,9	78,3
positive predictive value %	100,0	100,0	100,0	100,0	100,0	100,0	100,0	98,6	96,0	100,0
negative predictive value %	78,9	72,7	76,6	98,8	93,9	100,0	93,7	94,4	90,9	80,2

Figure 2 shows the concordance rates of all the tests examined. The highest concordance rates, almost 89 %, are observed for the RPA-Agdia test and the RT-qPCR by Tiberini et al. (2022) - Singleplex. The concordance rates for all other RT-qPCRs were between 85 and 88 %. The concordance rate for the RT-PCR test by Levitzky et al. (2019) is 84 %, for the RT-PCR by Sui et al. (2017) 81 %, while the concordance rates for the LAMP test by Kimura et al. (2023) and the RT-PCR by Loewe are only 78 % and 77 % respectively.

Figure 3 highlights the distribution of the true negatives (TN%), false positives (FP%), false negatives (FN%), true positives (TP%), and inconclusive results (INC%). The largest proportion of true negatives (TN%) is observed in the RT-PCR tests. In contrast, the highest percentages of true positives (TP%) are seen in the RT-qPCR tests and the RPA Agdia test. Regarding inconclusive results (INC%), the RT-PCR tests show the lowest percentages, indicating fewer cases where the test results were indeterminate.

Figure 4 shows the comparison between the diagnostic specificity and sensitivity of the individual tests. The highest diagnostic specificity is observed for the RT-PCR tests, all of which achieve 100 %. The RPA Agdia and the RT-qPCR by Tiberini et al. (2022; singleplex and duplex) follow closely behind with a specificity between 94 and 95 %. The LAMP test by Kimura et al. (2023) has a specificity of 93 %, while the other RT-qPCR tests have a slightly lower specificity, with the RT-qPCR test by ISF being the lowest at 90.5 %. In terms of diagnostic sensitivity, the RT-qPCR tests and the RPA Agdia have the highest values with a diagnostic sensitivity between 78 and 80 %. The RT-PCR tests and the LAMP test by Kimura et al. (2023) have a much lower sensitivity; of these tests, the RT-PCR by Levitzky et al. (2019) is the most sensitive at 62.5 % and the RT-PCR by Loewe is the least sensitive at 45 %.

Figure 5 shows the probability of detection for tests across a range of dilutions of the isolate ToMMV NIB V 373 in RNA extracted from healthy tomato leaves. As the dilution factor increases, the probability of detection generally decreases for all tests. The RT-qPCR tests, together with the RPA-Agdia test, consistently show the highest probability of detection, even at higher dilution factors. The RT-PCR tests, in particular Loewe and Sui et al. (2017), as well as the LAMP test by Kimura et al. (2023) show a more significant decrease in the probability of detection with increasing dilution, indicating that they are less effective at detecting lower concentrations of the target RNA.

Figure 6 shows the probability of detection for tests at two RNA dilution levels (2x and 20x) of the ToMMV isolate from seeds (NIB V 414). The most reliable tests are the RT-qPCRs of Fowkes et al. (2022) and Tiberini et al. (2022; Singleplex), which consistently achieved a detection rate of 100 at both dilutions, demonstrating their great ability to detect the target RNA even at higher dilution levels. This is followed by the RT-qPCR tests from DAFF DEECA, Tiberini et al. (2022; duplex) and the RPA-Agdia test, which all showed a similarly high probability of detection (100 % for dilution level 2x and between 83 and 92 % for dilution level 20x). The ISF RT-qPCR test was still effective, but had a slightly lower probability of detection compared to the other RT-qPCR tests and the RPA-Agdia test (100 % for dilution level 2x and 75 % for dilution level 20x). The RT-PCR tests and the LAMP test showed a significant decrease in the probability of detection. The most unreliable test among these tests was the RT-PCR Loewe, which had a detection probability of only 25 % at the 2x dilution and could no longer detect the target RNA at the 20x dilution.

Reproducibility, i.e. the ability of a test to give consistent results when applied to aliquots of the same sample tested under different conditions, is shown in Table 21. Overall, the reproducibility values for the tests included in the TPS were high, ranging from 87 % to 97 %. The highest reproducibility is observed for the RT-PCR Loewe and the RT-qPCRs DAFF DEECA, Fowkes et al. (2022) and ISF, where it

reaches 97 %. The lowest reproducibility of 87 % was observed for the LAMP test by Kimura et al. (2023). At the sample level, the lowest reproducibility was observed for highly diluted ToMMV samples and for samples of the non-target tobamovirus PaMMV.

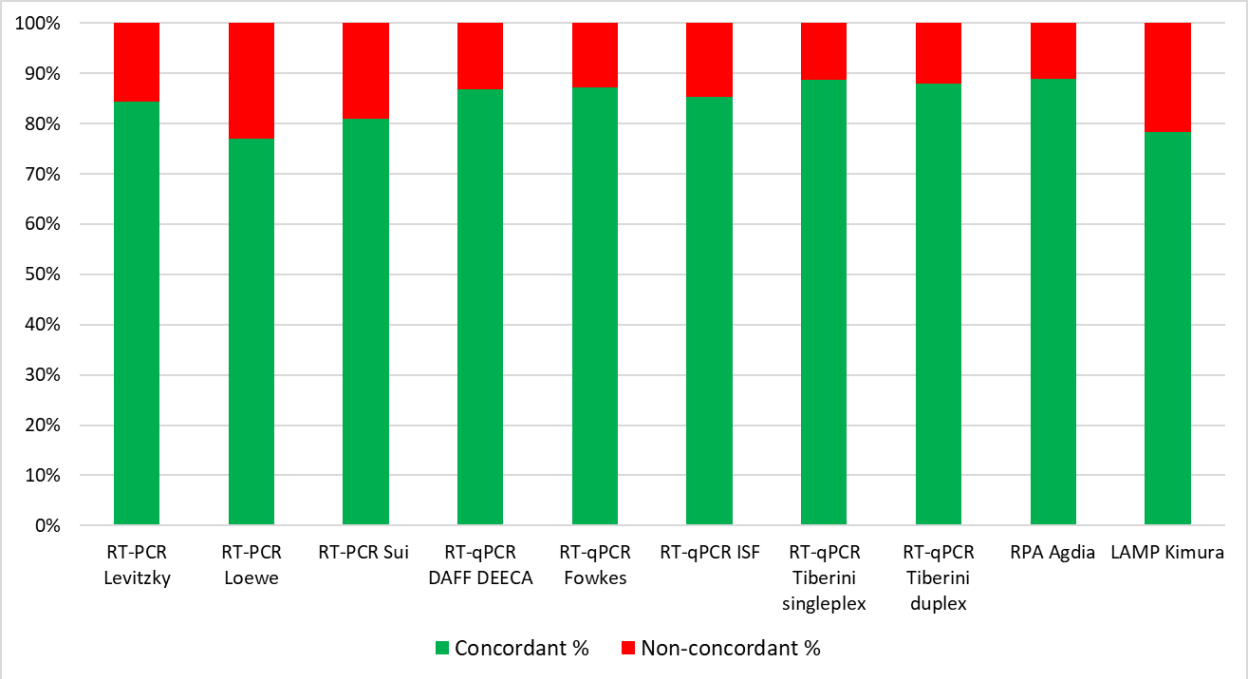

Figure 2: Graphical representation of concordance rates for studied tests.

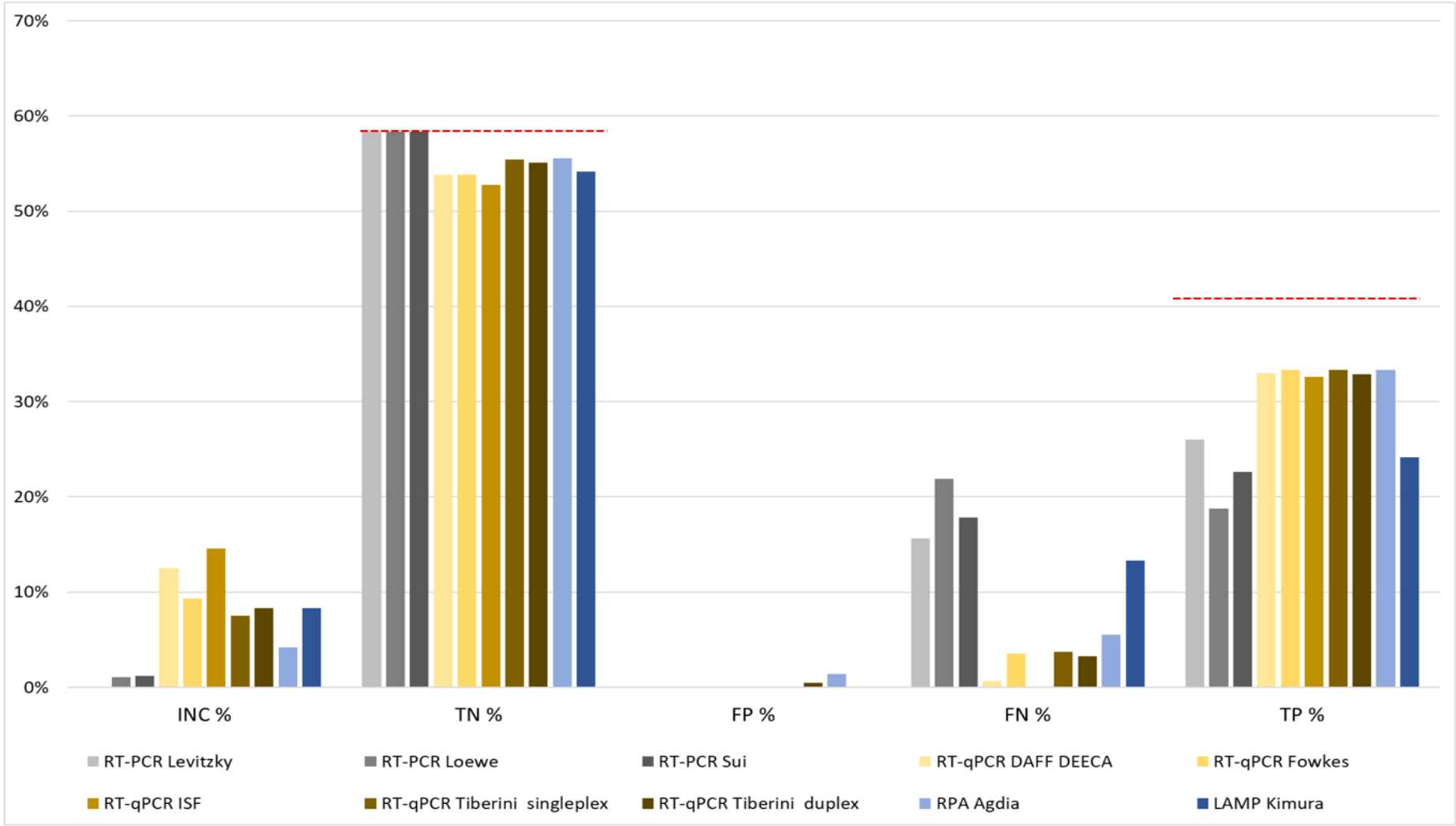

Figure 3. Graphical representation of the true-negative (TN), false-positive (FP), false-negative (FN), true-positive (TP) and inconclusive (INV) results of the tests examined. The red dotted line represents the expected percentage of TN and TP.

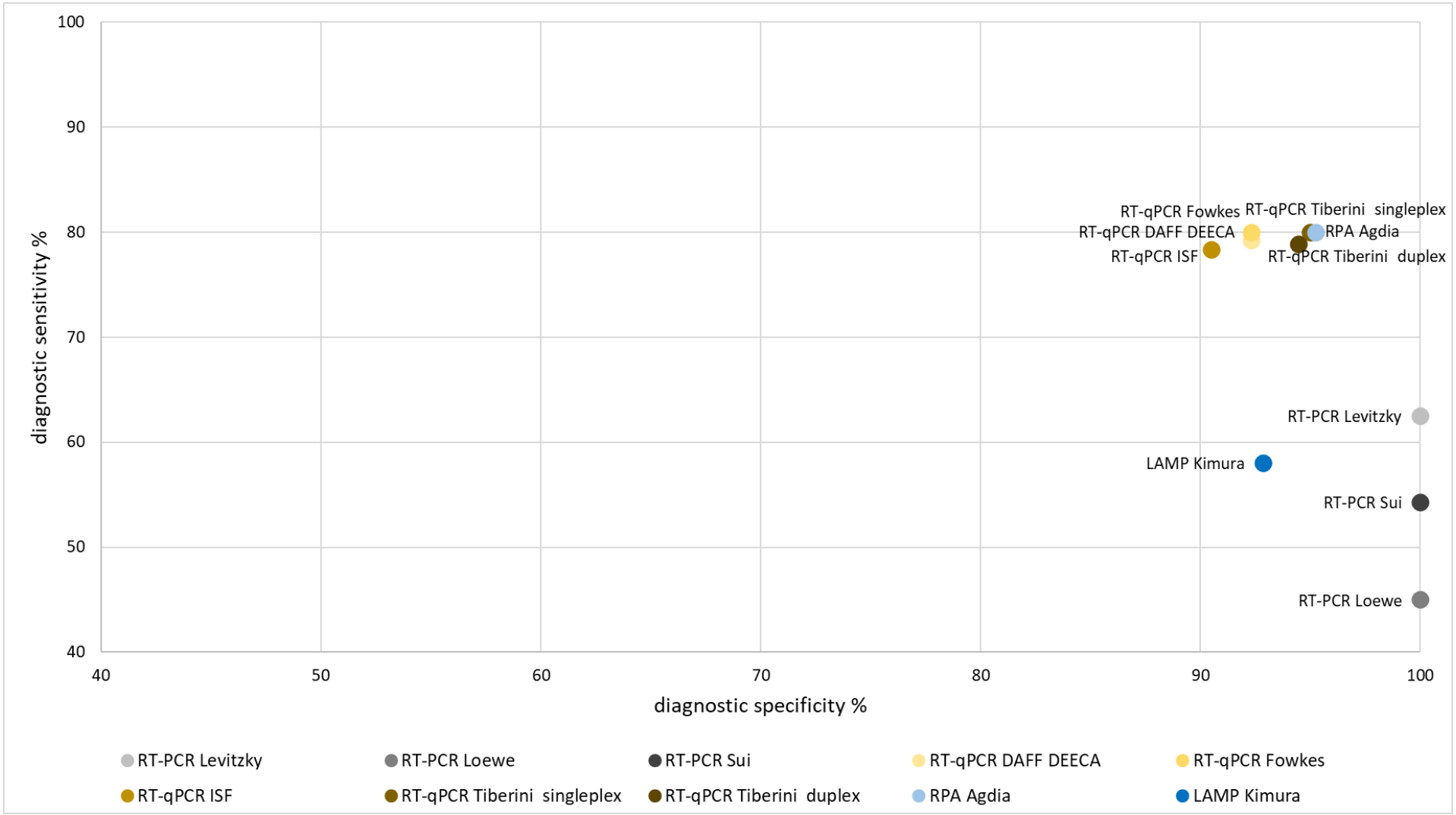

Figure 4. Graphical representation of the value of diagnostic specificity and diagnostic sensitivity for tests examined.

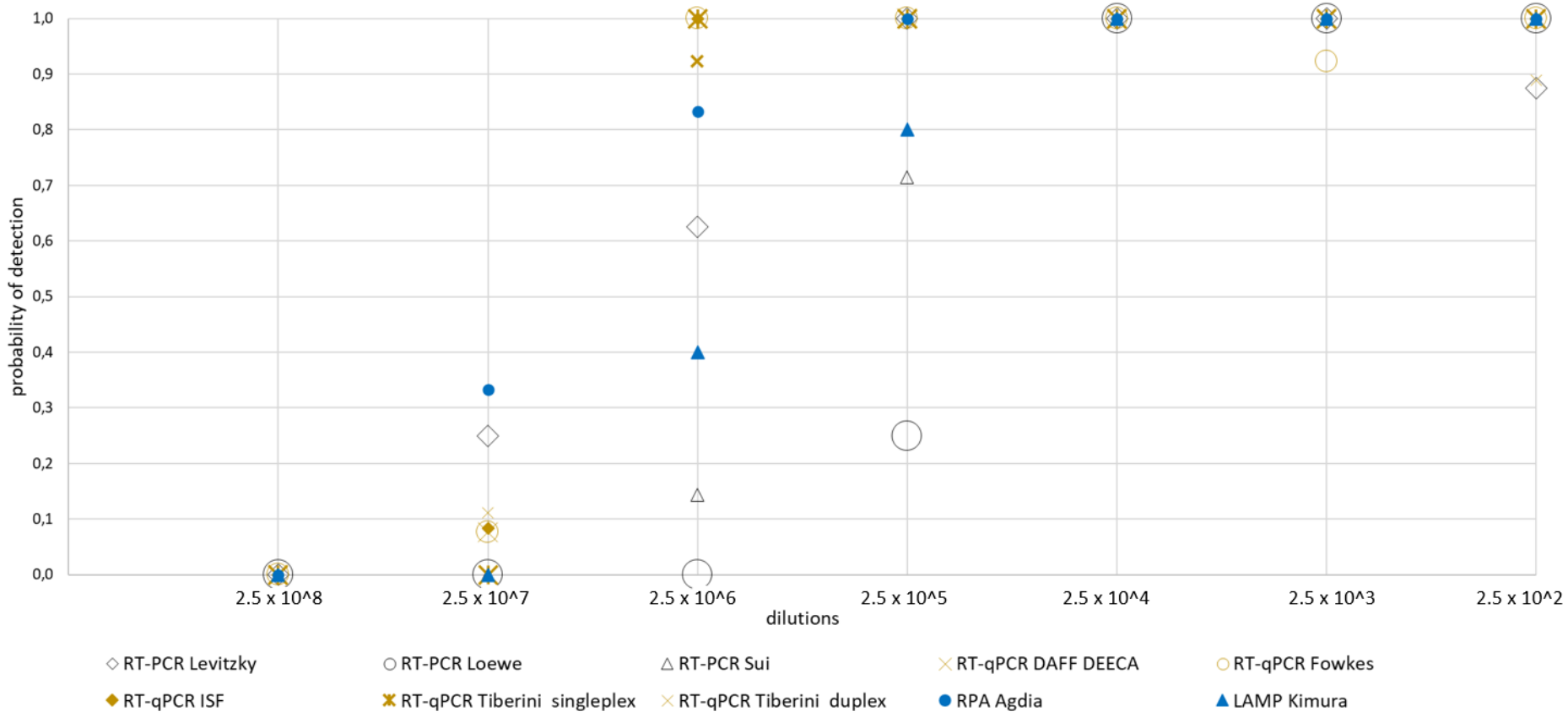

Figure 5. Graphical representation of the probability of detection for all tests examined based on the results of the dilution of the isolate ToMMV NIB V 373 in RNA from healthy tomato leaves (dilution factors are indicated on the x-axes).

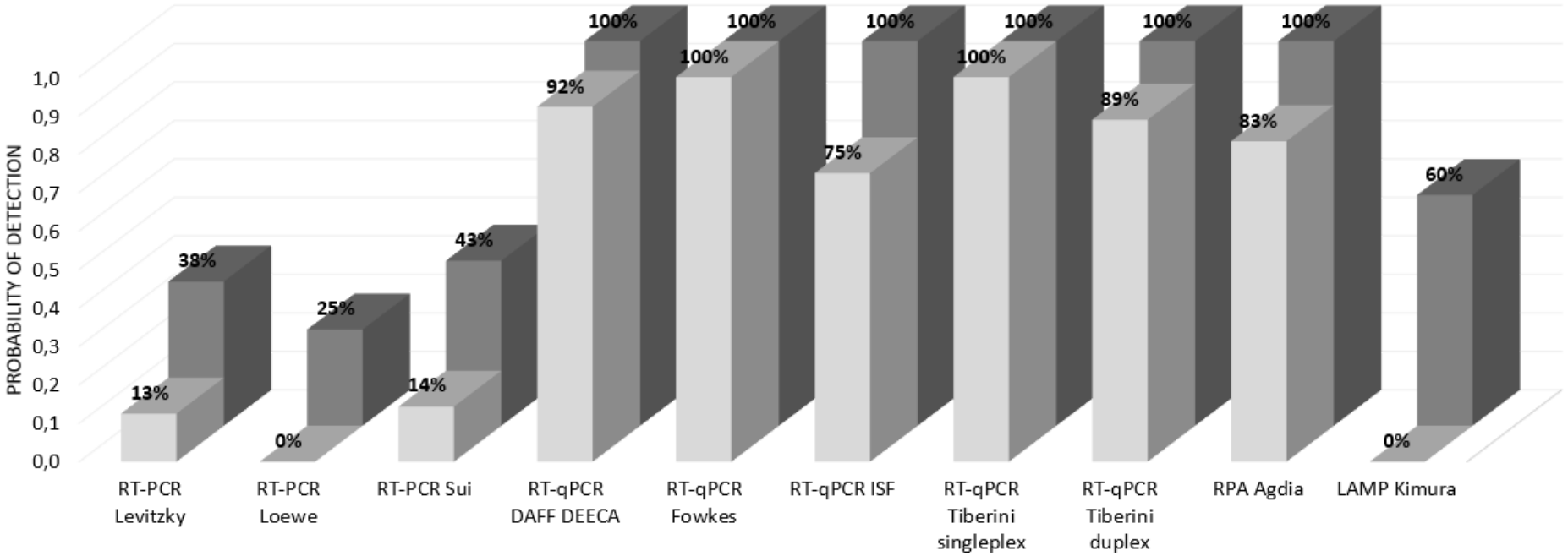

Figure 6. Graphical representation of the probability of detection for all tests examined based on the results of two RNA dilutions of the ToMMV isolate from seed (NIB V 414) (the results for the dilution factor 2x are shown in dark grey and the results for the dilution factor 20x in light grey).

Table 21: Summary of the reproducibility (%) of tests.

	RT-PCR			RT-qPCR					RPA	LAMP
sample description	Levitzky et al. (2019)	Loewe (Cat. No. 09181)	Sui et al. (2017)	DAFF DEECA	Fowkes et al. (2022)	ISF	Tiberini et al. (2022) singleplex	Tiberini et al. (2022) duplex	Agdia RPA (XCS 22800)	Kimura et al. (2023)
healthy tomato seed	100	100	100	100	92	92	100	100	100	100
healthy tomato seed	100	100	100	100	92	100	100	100	100	100
CGMMV (NIB V 403) 25x	100	100	100	100	100	92	100	100	100	100
ObPV (NIB V 364) 25x	100	100	100	100	100	100	100	100	100	80
ORSV (NIB V 365) 25x	100	100	100	100	100	100	100	100	100	80
PaMMV (NIB V 366) 25x	100	100	100	92	92	100	70	67	50	80
PMMoV (NIB V 408) 25x	100	100	100	92	100	100	100	100	100	100
TMGMV (NIB V 404) 25x	100	100	100	100	100	100	100	100	100	100
TMV (NIB V 405) 25x	100	100	100	100	100	100	100	100	100	100
TMV (NIB V 413) 25x	100	100	100	100	100	92	100	100	83	100
ToBRFV (NIB V 331) 25x	100	100	100	100	100	100	100	100	100	100
ToMV (NIB V 410) 25x	100	100	100	100	100	100	100	89	100	80
ToMV (NIB V 406) 25x	100	100	100	92	100	100	100	100	100	80
ToMMV (NIB V 373) 2.5 x 10^8	100	100	100	92	69	100	70	56	67	80
ToMMV (NIB V 373) 2.5 x 10^7	75	100	100	85	85	92	80	67	50	80
ToMMV (NIB V 373) 2.5 x 10^6	63	100	71	92	100	100	100	100	83	40
ToMMV (NIB V 373) 2.5 x 10^5	100	50	71	100	100	100	100	100	100	80
ToMMV (NIB V 373) 2.5 x 10^4	100	100	100	100	100	100	100	100	100	100
ToMMV (NIB V 373) 2.5 x 10^3	100	100	100	100	92	100	100	100	100	100
ToMMV (NIB V 373) 2.5 x 10^2	88	100	100	100	100	100	100	89	100	100
ToMMV (NIB V 414) 2 x 10^1	88	100	71	92	100	75	100	89	83	80
ToMMV (NIB V 414) 2x	63	75	57	100	100	100	100	100	100	60
Average:	**94**	**97**	**94**	**97**	**97**	**97**	**96**	**93**	**92**	**87**

6 Conclusions

RT-PCR tests showed the highest diagnostic specificity, while RT-qPCR tests and RPA Agdia showed the highest diagnostic sensitivity. Reproducibility is high for all tests, with some showing slightly better consistency, particularly the RT-PCRs and RT-qPCRs.

The highest concordance rates (relative accuracy) were obtained with the RPA-Agdia and the RT-qPCR singleplex Tiberini et al. (2022). Closely followed by other RT-qPCR tests, then the RT-PCR tests by Levitzky et al. (2019) and Sui et al. (2017), the LAMP test by Kimura et al. (2023) and finally the RT-PCR test by Loewe.

Disclaimers:

- The results presented in this report only reflect the specific case study and the associated performance results of the commercial reagents at the time when they were included in the study.
- For the RT-qPCRs, it should be noted that prior to the TPS it was known that a Cq cut-off value was required for all RT-qPCRs tested, as cross-reactions with some other tobamoviruses are known to result in high Cq values. The Cq cut-off value depends on the equipment, material and chemistry and can therefore vary from laboratory to laboratory. However, in most participating laboratories these Cq cut-off values were not determined, so we decided to consider the detected Cq values which could be due to a cross-reaction with a non-target virus (and not just a low target concentration) as inconclusive results in this study.

References

Dovas CI, Efthimiou K, Katis NI (2004) Generic detection and differentiation of tobamoviruses by a spot nested RT-PCR-RFLP using DI-containing primers along with homologous DG-containing primers. J. Virol. Methods 117, 137–144. https://doi.org/10.1016/j.jviromet.2004.01.004.

EPPO (2022) EPPO Technical Document No. 1088. Pest risk analysis for Tomato mottle mosaic virus. EPPO, Paris. Available at https://gd.eppo.int/taxon/TOMMV0/documents.

EPPO (2024) EPPO Global Database. https://gd.eppo.int (accessed on 13.8.2024)

EPPO (2022) PM 7/122 (2) Guidelines for the organization of interlaboratory comparisons by plant pest diagnostic laboratories. EPPO Bulletin;52:604–618

EPPO (2021) PM7/98(5) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. EPPO Bulletin;51:468–498.

Fowkes AR, Botermans M, Frew L, de Koning PPM, Buxton-Kirk A, Westenberg M, et al. (2022) First report of Tomato mottle mosaic virus in *Solanum lycopersicum* seeds in The Netherlands and intercepted in seed imported from Asia. New Disease Reports, 45, e12067. https://doi.org/10.1002/ndr2.12067

Kimura K, Miyazaki A, Suzuki T, Yamamoto T, Kitazawa Y, Maejima K, Namba S, Yamaji Y (2023) A Reverse-Transcription Loop-Mediated Isothermal Amplification Technique to Detect Tomato Mottle Mosaic Virus, an Emerging Tobamovirus. Viruses 15(8):1688. https://doi.org/10.3390/v15081688

Levitzky N, Smith E, Lachman O, Luria N, Mizrahi Y, et al. (2019) The bumblebee Bombus terrestris carries a primary inoculum of Tomato brown rugose fruit virus contributing to disease spread in tomatoes. PLOS ONE 14(1): e0210871. https://doi.org/10.1371/journal.pone.0210871

Li RG, Gao S, Fei ZJ, Ling KS (2013) Complete genome sequence of a new tobamovirus naturally infecting tomatoes in Mexico. Genome Announcements 1(5), e00794-13. http://genomea.asm.org/content/1/5/e00794-13.full

Li Y, Wang Y, Hu J, Xiao L, Tan G, Lan P, Liu Y, Li F (2017) The complete genome sequence, occurrence and host range of tomato mottle mosaic virus Chinese isolate. Virol. J. 14, 15. https://doi.org/10.1186/s12985-016-0676-2.

Sui X, Zheng Y, Li R, Padmanabhan C, Tian T, Groth-Helms D, Keinath AP, Fei Z, Wu Z, Ling K-S (2017). Molecular and Biological Characterization of Tomato mottle mosaic virus and Development of RT-PCR Detection. Plant Disease 101:5, 704-711.

Tiberini A, Manglli A, Taglienti A, Vučurović A, Brodarič J, Ferretti L, Luigi M, Gentili A, Mehle N (2022) Development and Validation of a One-Step Reverse Transcription Real-Time PCR Assay for Simultaneous Detection and Identification of Tomato Mottle Mosaic Virus and Tomato Brown Rugose Fruit Virus. Plants 11(4):489. https://doi.org/10.3390/plants11040489

Vučurović A, Mehle N, Anthoine G, Dreo T, Ravnikar M (2022) Critical Points for the Organisation of Test Performance Studies in Microbiology; Plant Pathogens as a Case Study. In: Plant Pathology in the 21st Century, Springer: 97 p. https://doi.org/10.1007/978-3-030-99811-0

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Appendix 1: Protocols of the tests included in the TPS

Appendix 2: Summary with all important data required for carrying out tests within the framework of TPS

Appendix 3: Results of homogeneity and stability testing

Appendix 4: Rawdata submitted by participants

Appendix 5: Comparison of the tests based on participants' unmodified qualitative results for the RT-qPCRs

Appendix 1: Protocols of the tests included in the TPS

RT-PCR_Levitzky_tobamo

RT-PCR test Levitzky et al. (2019)

The test below is described as in Levitzky et al. (PLOS ones 2019, 14, e021081). 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 RT-PCR protocol was developed for detection of tomato brown rugose fruit virus in plant tissue or bumblebee-hive components.

1.2. Additional validation work showed that this virus is able to detect other tobamoviruses.

1.3. The target sequence of the ToMMV primers covers the movement protein gene, coat protein gene and the 3'UTR; using the nucleotide sequence of GenBank accession no NC_022230, the forward and reverse primer start at position 5480 and 6264, respectively.

1.4. Oligonucleotides

	Primer/probe	Sequence
Forward primer	F-5476	5'- GAA GAA GTT GTT GAT GAG TTC AT-3'
Reverse primer	R-6287	5'- GAT TTA AGT GGA GGG AAA AAC AC-3'

1.5. The test was originally validated on Verso One-Step Reddymix (Thermo), after this product was discontinued Qiagen OneStep RT-PCR (Qiagen) was used instead.

1.6. The test has been validated using GeneAmp PCR System 9700, 2720 Thermal Cycler (Applied Biosystems).

2. Methods

2.1. Nucleic acid extraction and purification

See Appendix 1 of EPPO PM7/146.

2.2. One-step RT-PCR

2.2.1.Master Mix:

- with Qiagen OneStep RT-PCR

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water		16	
Mastermix (from the kit)	5×	5	1×
ToMMV F	10 µM	0.5	0.4 µM
ToMMV R	10 µM	0.5	0.4 µM
dNTP (from the kit)		1	400 µM each dNTP
RT-enzyme (from the kit)		1	
Subtotal		24.00	
RNA		1.00	
Total		25.00	

2.2.2.RT-PCR cycling conditions:

- with Qiagen OneStep RT-PCR conditions are:
  Reverse transcription at 50 °C for 30 min; denaturation at 95 °C for 15 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min and elongation at 72 °C for 30 s. Followed by a final elongation of 72 °C for 10 min.

3. Essential procedural information

3.1. **Controls**

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (*e.g.,* naturally infected host tissue).
- 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.

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 a band.
- A test will be considered negative if it does not produce a band.
- Tests should be repeated if any contradictory or unclear results are obtained.
- Sequencing is required to identify species.

4. Performance characteristics available

The validation data is not publicly available at present.

4.1. Analytical sensitivity data

The LOD was determined using the 10-fold dilution series (leaf material in water) obtained from tomato brown rugose fruit virus. A weak band was identified at 10^{-6}, a stronger band was seen at 10^{-5}.

4.2 Analytical specificity data

4.2.2 Inclusivity

Full range of detection across all tobamoviruses tested. Additionally, the following accessions were detected and reported in Fowkes et al., (2022) OK334226, OK3344230, OK3344231, OK3344232, OK334229, OK334228, OK334227, OK334226.

ToMMV Fera diagnostic samples include 2021029729 and 2021008940 and DSMZ PV-1267, pepper mild mottle virus (Fera positive control 04/09/2013, 17-2), tomato mosaic virus (Fera diagnostic sample 21816077), tobacco mosaic virus (Fera positive control 19-1).

4.2.3 Exclusivity

Specificity work was done using Reddymix mastermix. No specificity difference expected in change of mastermix.

Virus and viroids tested were CEVd (Fera positive control 16-1 D), CLVd (Fera positive control 16-1 D), PCFVd (Fera positive control 17-1 B), PepMV EU (Fera positive control 3, 25.4.2016), PepMV Ch1 (Fera positive control 5, 6), PepMV Ch2 (Fera positive control 16-2, 17-6), PSTVd (Fera positive control 16-1 D, 19-1 A), PVX (Fera diagnostic sample 15-1), PVY (Fera positive control 11/09/2015), STV (Fera positive control 19-2), TASVd (Fera positive control 16-1 H), TPMVd (DSMZ PV-1230), TSWV (Fera positive control 2), TYLCV (Fera positive control 17-6).

4.3 Selectivity

No selectivity data is available.

4.4 Repeatability

Not available.

4.5 Reproducibility

Not available.

RT-PCR_Loewe_ToMMV

RT-PCR test Loewe

The test below is reverse transcription-polymerase chain reaction kit for ToMMV detection developed by Loewe® Biochemica GmbH.

1. General information

1.1. This test can be used for detection and identification of Tomato mottle mosaic virus (ToMMV) using the reverse primer designed by Sui et. al, (Sui, Xuelian et al. "Molecular and Biological Characterization of Tomato mottle mosaic virus and Development of RT-PCR Detection." Plant disease vol. 101,5 (2017): 704-711. doi:10.1094/PDIS-10-16-1504-RE). The forward primer has been designed by LOEWE® and the original protocol was adapted to a one-step format and further optimized for routine use by Loewe® Biochemica GmbH.

- 1.2. This test has been successfully used for testing fresh, frozen or dried leaf and material from tomato.
- 1.3. Primers ToMMV-F5600 and ToMMV-R were designed to target the movement protein sequence and the coat protein sequence, respectively.
- 1.4. Oligonucleotides

	Primer	Sequence	Amplicon size
Forward primer	ToMMV-F5600	confidential	~460 bp
Reverse primer	ToMMV-R	5'-CAC TCT GCG AGT GGC ATC CAA T-3'	

2. Methods

- 2.1. Nucleic acid extraction

We recommend using commercial kits for plant RNA extraction. Alternative procedures may also be suitable.

- 2.2. One-step RT-PCR (Loewe Biochemica GmbH)
  - 2.3.1. Prepare master mix according to manufacturer's instructions.
  - 2.3.2. 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. The LOEWE® complete kit contains a DNA-based positive amplification control.

    - 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 (~460 bp) should be visualized.

When these conditions are met

- A test will be considered positive if a band of the expected size (~460 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

Validation data was generated by in-house validation procedures.

4.1. Analytical sensitivity data
The virus can be detected in as less as 0.01 ng of total RNA isolated from infected plants.

4.2. Analytical specificity data

    - 4.2.1. Inclusivity

Evaluated with isolate of ToMMV PV-1267 (DSMZ; Solanum lycopersicum; California, US).

- 4.2.2. Exclusivity

No cross-reactions were observed with the following viruses (belonging to the tobamovirus family or occurring on same host plants):

BPeMV (Bell pepper mottle virus)
CeMV (Celery mosaic virus)
CGMMV (Cucumber green mottle mosaic virus)
CMV (Cucumber mosaic virus)
CYSDV (Cucurbit yellow stunting disorder virus)
KGMMV (Kyuri green mottle mosaic virus)
ORSV (Odontoglossum ringspot virus)
PaMMV (Paprika mild mottle virus)
PepMV (Pepino Mosaic Virus)
PMMoV (Pepper mild mottle virus)
PVX (Potato virus X)
PVY (Potato virus Y)
RMV (Ribgrass mosaic virus)
SFBV (Streptocarpus flower break virus)
TAV (Tomato aspermy virus)
TBRV (Tomato black ring virus)
TBSV (Tomato bushy stunt virus)
TCSV (Tomato chlorotic spot orthotospovirus)
TMGMV (Tobacco mild green mosaic virus)
TMV (Tobacco mosaic virus)
ToBRFV (Tomato brown rugose fruit virus)
ToLCNDV (Tomato Leafcurl New Delhi Virus (DNA))
ToMV (Tomato mosaic virus)
TRV (Tobacco rattle virus)
TSWV (Tomato spotted wilt virus)
TVCV (Turnip vein clearing virus)
TYLCV (Tomato Yellow Leafcurl Virus (DNA))
YMoV (Youcai mosaic virus)
ZGMMV (Zucchini green mottle mosaic virus)

4.3. Selectivity data
No cross-reactions were observed with the following plant species

Capsicum annuum, Chenopodium quinoa, Nicotiana benthamiana, Petunia hybrid, Physalis spp., Solanum lycopersicum, Solanum melongena, Solanum tuberosum

- 4.4. Repeatability data

100% of repeatability was obtained testing isolated RNA from infected samples (0.01ng) on different occasions.

RT-PCR_Sui_ToMMV

APHIS-PPQ-S&T Plant Pathogen Confirmatory Diagnostics Laboratory

Detection of Tomato mottle mosaic virus (ToMMV) using conventional RT-PCR assays

The purpose of this work instruction is to describe a

1. Primer Sequences

Table 1. Sequences of primers (Sui et al., 2017) to amplify a region located within the coat protein coding sequences of the Tomato mottle mosaic virus.

Target	Primer Name	Sequence and Location	Amplicon size
ToMMV (coat protein sequence)	ToMMV-F	5'- CGACCCTGTAGAATTAATAAATATT-3' (5775-5799)	289bp
	ToMMV-R	5'- CACTCTGCGAGTGGCATCCAAT-3' (6063-6042)	

Note: Primer stock and working solutions should be prepared after receipt of new reagents.Reagent solutions are stored in small aliquots in the freezer until needed. New reagents need to be tested using approved and validated positive and negative controls prior to testing samples.

2. Preparation on Master Mix

Table 3. Preparation of PCR Master Mix

Primer	Volume (µL)	Final Concentration
5 × Qiagen OneStep RT-PCR Buffer	5.0	1x
Qiagen dNTPs (10 mM)	1.0	0.4 mM
ToMMV-F (10 µM)	1.0	0.4 µM
ToMMV-R (10 µM)	1.0	0.4 µM
Qiagen OneStep RT-PCR Enzyme Mix	1.0	---
RNaseOUT	1.0	1.6 U/µL
Water	13.0	---
Template	2.0	---
Total Reaction	**25.0**	N/A

*Master Mix preparation must be done in a decontaminated PCR workstation/enclosure.

3. Controls

a. Buffer Extraction Control
b. Non-Template Water Control (NTC)
c. Healthy Control (if available – the buffer extraction control can be used in place of a healthy control)

d. Positive Control

Positive and Healthy controls must be validated before use.

4. RT-PCR Reaction

1. Turn on the thermocycler and allow the machine to run through its self-testing procedures. Place the PCR tubes into the thermocycler.
2. Program the following settings for PCR conditions into the machine or select the correct saved program.

- 50°C for 30 minutes
- 95°C for 15 minutes
- 35 cycles of the following:
    - 94°C for 1 min
    - 55°C for 45 seconds
    - 72°C for 1 min
- 72°C for 10 minutes
- Hold at 4°C

Note: Use maximum temperature ramping rate between steps

5. Assessment of Results

1. **Assess the Controls**
    a. **Ladders** must be distinct and well resolved to be valid. If the ladders are not distinct and/or well resolved, determine the cause and correct, then rerun the gel with the remainder of the PCR reactions for the samples and controls.

    b. **Non-template control** (NTC), **Buffer Extraction Control and Healthy Control** should not contain any band (other than primer dimers). If a band is present, then the entire run is invalid. This indicates contamination of the PCR run. All samples must be retested using this WI.

    c. **Positive control** – The positive control should produce a band of ~289 bp. If such a band is not present, the run is invalid, and all samples and controls must be retested using this WI. This indicates that the PCR reaction failed, typically a reagent was not added into the master mix or the control was not added to the reaction tube.

2. **Assess the Sample Results -** ToMMV sample reactions can only be evaluated after all controls are determined to be valid.
    a. **NEGATIVE:** If a sample does not produce a ~289 bp amplicon, then it is determined as NEGATIVE for ToMMV.

    b. **POSITIVE:** If a sample produces a ~289 bp amplicon, it is determined positive for ToMMV, which can be confirmed by subsequent sequencing.

    c. **INCONCLUSIVE:** If a sample produces non-specific amplicons or amplicons at sizes other than the expected ~289bp, the RT-PCR must be repeated paying close attention to the conditions specified in this WI. If once repeated, unspecific bands are seeing, the sample is INCONCLUSIVE.

RT-qPCR_DAFF DEECA_ToMMV

This protocol is submitted on behalf of the project team from the Department of Agriculture, Forestry and Fisheries (DAFF), Department of Energy, Environment and Climate Action (DEECA) Australia, for inclusion to the EUPHRESCO project 'Validation of molecular diagnostic methods for the detection and identification of tomato mottle mosaic virus (ToMMV) (2022-A-394)'

1. General Information

- This RT-qPCR protocol was developed for detection of ToMMV in *Solanum lycopersicum* (tomato) and *Capsicum sp.* (pepper, chili) seeds in samples of 20,000 seeds sub-divided into sub-samples of 400 seeds.
- ToMMV testing requires the use an RT-qPCR assay:
  - RT-qPCR CSP1572 (CSP Labs) (Table 1)
- Validation work showed that the test is suitable for seed subsamples of 400 seeds and is specific and selective for ToMMV.
- The real-time RT-PCR (RT-qPCR) assay were validated using the testing conditions presented in the Methods (Section 2) and set up as per Table 2.
- The RT-qPCR assay (CSP Labs, Primers CSP1572-F/R) targets ORF3 of the ToMMV genome.

2. Methods

- Nucleic acid extraction and purification.

Nucleic acid extraction from tomato and capsicum seeds for RT-(q)PCR: (modified from Hoshino et al. (2006).

Grinding:

- Weigh out approximately 400 seeds and place in homex grind bag/extraction vessel. If fewer seeds are used, adjust volumes proportionally.
- Crush seeds and place on ice.
- Add 5ml of grinding buffer 1 and 10µL of 2-mercaptoethanol to grind bag. Homogenize using the Homex.
- Add 5ml of grinding buffer 2 to grind bag and mix. Place bag containing sample and 10 ml total buffer on ice.
- Add 50ml of extraction buffer to grind bag and mix. Pour into 50ml centrifuge tube ready for extraction.

Extraction:

- Take 1.5ml of sample from 50ml centrifuge tube and place in 2ml microcentrifuge tube. Incubate at 65°C for 10 minutes in water bath or heating block.
- Add 500µl of 5M potassium acetate and vortex thoroughly. Place on ice for 30 minutes.
- Centrifuge at 10,000 rpm for 10 minutes at 10°C.
- Take 900µl of supernatant and place in a clean 1.5 ml microcentrifuge tube. Add 540µl of isopropyl alcohol and mix upside-down. Place on ice for 30 minutes.

- Centrifuge at 10,000 rpm for 10 minutes at 10°C.
- Remove supernatant and rinse pellet in 70% ethanol.
- Pour off ethanol and dry pellet on heat block at 70°C or in biological safety cabinet.
- Resuspend pellet in 50 µl of RNase-free water and vortex.

Grinding Buffer 1 (for nucleic acid extraction from tomato and capsicum seed).

Reagent	Final Concentration	Amount required per litre
1.0M Tris-HCl pH 8.0	0.2M	200ml
NaCl	1.0M	58.44g

Grinding Buffer 2 (for nucleic acid extraction from tomato and capsicum seed).

Reagent	Final Concentration	Amount required per litre
0.5M EDTA pH 8.0	0.1M	200ml
Sodium lauryl sulphate	2.5%	25g
PVP-40	6.60%	66g

For buffer preparation, mix all the above listed chemicals thoroughly in distilled water using stirring bead and magnetic stirrer. Adjust final volume to 1000 ml.

2.2. Test components, conditions, and set up.

Table 1: RT-qPCR CSP1572 (sequences supplied by, CSP Labs).

Primers	Sequence (5'-3') / Tm = 60 ˚C	Amplicon (bp)	LOD	Assay
CSP 1572-F	CCCGACTACAGCCGAAACAT	~74	≤37 Ct	48 ˚C (for 30 min) 94 ˚C (for 5 min) 40 cycles (94˚C for 10 sec) 60 ˚C (for 30 sec)
CSP 1572-R	TTAACAGCGGACCTGATCGC			
CSP 1572-P	(6FAM)-TGCCACTCGCAGAGTGGACGATGCTACG-(BHQ10)			

Table 2: RT-qPCR reaction set up. (Follow manufacturer's instructions if changing enzyme)

RT-qPCR Reagents (AgPath-ID™ One-Step RT-PCR Reagents – Thermo-Fisher) – 25 µL reaction

- 2.0 µl RNA template
- 12.5 µl 2x RT-PCR Buffer
- 1 ul 25X RT-PCR Enzyme Mix
- 7.0 µl nuclease-free water
- 1.0 µl forward primer (10 µM)
- 1.0 µl reverse primer (10 µM)
- 0.5 µl probe (10 µM)

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 extractions and amplifications of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC): perform a nucleic acid extraction and amplification using a sample of uninfected matrix or, if matrix is not available, then a sample of clean extraction buffer.
- Positive isolation control (PIC): perform a nucleic acid extraction and amplification using a known sample of the target organism or a matrix sample that contains the target organism (*e.g.,* naturally infected host tissue).
- Negative amplification control (NAC): perform an amplification using the molecular grade water that was used to prepare the reaction mix in place of a sample.

- Positive amplification control (PAC): perform an amplification using a known sample of nucleic acids 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 concentration should be near to the limit of detection (LOD).

As alternative or in addition to the PIC, internal positive controls (IPCs) can be used to monitor each individual sample separately. A sequence in the nucleic acids of the matrix can be used as the IPC using conserved primers that amplify mRNA targets such as *nad5* (Botermans *et al.*, 2013). For seed samples, *nad5* might not perform consistently, and a leaf tissue sample spike can be used as an IPC or alternatively COX can be used(e.g. Weller *et al.*, 2000 or Papayiannis *et al.*, 2011).

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 the test assay produces a Ct value <37.

4. Performance characteristics available

The validation data is not publicly available at present.

4.1. Analytical sensitivity data

The LOD was determined by using several 10-fold dilution series of RNAs extracted from ToMMV infected seed (Lovelock et al. 2020). ToMMV had been identified and confirmed in this seed previously.

The LOD was determined as the Ct value that showed 100% replicate consensus at the lowest dilution. i.e. all replicates produced a Ct value (variance under ±1 Ct) at the dilution tested. The LOD was confirmed by replication (independent sample creation and extraction), reproduction (independent operators), and ring testing (independent laboratories).

4.2 Analytical specificity data

4.2.2 Inclusivity

Previously identified ToMMV infected seed samples were used as positive controls (Lovelock et al., 2020).

4.2.3 Exclusivity

Seven Solanaceae infecting *Tobamovirus* isolates were obtained from DMSZ and included for specificity and selectivity assessment. No non-target viruses were detected, including:

- Tomato brown rugose fruit virus (ToBRFV) RNA (German Isolate, DZMZ Cat. No. PV-1236, Menzel et al., 2019)
- Tobacco mosaic virus (TMV) (German Isolate, DZMZ Cat. No. PV-0107, Zaitlin et al., 1975)
- Tomato mosaic virus (ToMV) (German Isolate, DZMZ Cat. No. PV-0135, Hollings et al., 1976)
- Paprika mild mottle virus (PaMMV) (Greece Isolate, DZMZ Cat. No. PV-0606, Hamada et al., 2003)
- Pepper mild mottle virus (PMMoV) (German Isolate, DZMZ Cat. No. PV-0165, Wetter et al., 1984)
- Bell pepper mottle virus (BPeMV) (Netherlands Isolate, DZMZ Cat. No. PV-0170, Wetter et al., 1988)
- Ribgrass mosaic virus (RMV) (German Isolate, DZMZ Cat. No. PV-0145, Oshima et al., 1975).

4.3 Selectivity

No non-target amplification was recorded when a Ct<37 cut off was applied.

4.4 Repeatability

The test LOD was shown to be repeatable (variance under ±1 Ct) using several independently created, extracted, and tested 10-fold dilution series of ToMMV infected material in seed homogenate.

4.5 Reproducibility

When performed by a second operator the test was shown to be reproducible (variance under ± 3 Ct) using several independently created, extracted, and tested 10-fold dilution series.

The test was performed on 20 de-identified real-life diagnostic samples, with test outcomes matching previously reported data for these samples.

4.6 Ring testing

Samples of diluted nucleic acid extracts at concentrations near the LOD were shared among three independent labs. All teams extracted RNA from the samples and performed the RT-qPCRs test. All teams confirmed the Ct <37 cut off was suitable using the recommended assay and sampling/extraction process.

References:

CSP Labs: the primer/probe sets in Table 1 were designed by CSP Labs.

Hoshino S et al. (2006) Detection of Potato spindle tuber viroid (PSTVd) in Tomato and Potato Seeds. Res. Bull. Pl/ Prot/ Japan 42: 75–79.

Levitzky, N, Smith, E, Lachman, O, Luria, N, Mizrahi, Y, Bakelman, H, Sela, N, Laskar, O, Milrot, E & Dombrovsky, A, 2019, 'The bumblebee *Bombus terrestris* carries a primary

inoculum of Tomato brown rugose fruit virus contributing to disease spread in tomatoes' PloS one, volume14, issue 1, p.e0210871.

Lovelock, D.A., Kinoti, W. M., Bottcher, C., Wildman, O., Dall., D., Rodoni, B. C. & Constable, F. E. (2020) Tomato mottle mosaic virus intercepted by Australian biosecurity in Capsicum annum seed. Australasian Plant Disease Notes 15. DOI: 10.1007/s13314-020-0378-x.

RT-qPCR_Fowkes_ToMMV

Real-time RT-PCR test Fowkes et al. (2022)

The test below is described as in Fowkes et al. (New Disease Report 2022, 45, e12067). 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 was developed for detection ToMMV in tomato and pepper leaves and seeds.

1.2. The target sequence of the ToMMV primers is located within the movement protein gene; using the nucleotide sequence of GenBank accession no NC_022230, the forward and reverse primer start at position 5162 and 5212, respectively, and the probe covers positions 5183-5210.

1.3. Oligonucleotides

	Primer/probe	Sequence
Forward primer	ToMMV-F	5'-CGT GGT GGT GTC AGC ATC TG-3'
Reverse primer	ToMMV-R	3'-CGA TCC GAG TGT CGC TTC A-5'
Probe	ToMMV-Pe	5'-FAM- TTG GTC GAT AAA AGA ATG CAA AGA GCG G - TAMRA-3'

1.4. The test has been validated using i) iTaq Universal Probes One-Step Kit (Bio-Rad) and real-time PCR systems QuantStudio™ 6, QuantStudio™ 12K Flex and ViiA7™ (Applied Biosystems).

2. Methods

2.1. Nucleic acid extraction and purification

See Appendix 1 of EPPO PM7/146.

2.2. One-step real-time RT-PCR

2.2.1. Master Mix:

- with iTaq Universal Probes One-Step Kit

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water		6.45	
Mastermix (from the kit)	2×	10.00	1×
ToMMV F	7.5 µM	1.00	0.375 µM
ToMMV R	7.5 µM	1.00	0.375 µM
ToMMV Pe	5 µM	0.50	0.125 µM
RT-enzyme (from the kit)		0.05	1x
Subtotal		19.00	
RNA		1.00	
Total		20.00	

2.2.2. Real-time RT-PCR cycling conditions:

- with iTaq Universal Probes One-Step Kit:
  Reverse transcription at 50 °C for 10 min; denaturation at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 15 s and annealing and elongation at 60 °C for 60 s.

3. Essential procedural information

3.1. **Controls**

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (*e.g.,* naturally infected host tissue).
- 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.

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.

4. Performance characteristics available

The validation data is not publicly available at present.

4.1. Analytical sensitivity data

The LOD was determined using the 10-fold dilution series (leaf material in water and leaf material diluted in healthy seed extract) obtained from ToMMV. In leaf material diluted in water the LOD was 10^{-5} and for leaf material diluted in seed extract was 10^{-4}.

4.2. Analytical specificity data

4.2.2 Inclusivity

The assay has been tested against isolates of ToMMV initially identified by RT-PCR (Levitzky *et al*., 2019), (Fera diagnostic samples 2020023426,2021005757, DSMZ PV-1267).

In addition, the *in-silico* analysis versus all the full genome sequences of ToMMV available in GenBank highlighted:

KU594507- one difference in forward.
MW582804, KX898034- one difference in probe
Common polymorphism in probe T instead of C, as in reverse. This is seen in OK334224 which is from Fowkes et al. (2022). The virus was initially identified by RT-PCR (Levitzky *et al* 2019), OK334226, OK344230-32).

4.2.3 Exclusivity

The exclusivity was assessed by testing the assay against other tobamoviruses and common viruses and viroids in tomato.

Including ToMV (Fera diagnostic sample 20220027690 B, C D, E, F., 2020029550, 2020027562, 2020027564, 2021024410, 2022000021), PMMoV (Fera diagnostic sample 2022027690 G, 2020027561, 2021024450, Fera positive control 04/09/2013), PSTVd (Fera diagnostic sample 2020027563, Fera positive control 19-1 A), TMV (Fera positive control 19-1), ToBRFV (Fera diagnostic sample 2020013163, 2020015326, 2020015325, 2020013592, 2020013589, Fera positive control 19-1 A, 19-1 H) and ulluco tobamovirus (Fera positive control 2).

Common viruses and viroids includes CEVd (Fera positive control 16-1 D), CLVd (Fera positive control 16-1 F), PCFVd (Fera positive control 17-1 B), PepMV EU (Fera positive control 25.4.2016), PepMV Ch1 (Fera positive control 5), PepMV Ch2 (Fera positive control 16-2), PVX (Fera diagnostic sample 21816077), PVY (Fera positive control 19-8), STV (Fera positive control 19-2), TASVd (Fera positive control 19-1 M), TPMVd (DSMZ PV-1230), TSWV (Fera positive control 2), TYLCV (Fera positive control 17-6).

4.1. **Selectivity**

The samples used in specification testing were obtained from virus and viroid positive aubergine seed, capsicum seed, tomato seed, leaf, and fruit. No cross reactions were identified.

4.3. **Repeatability**

The repeatability of the assay was evaluated at Fera by analysing eight wells of RNA at the limit of detection.

Dilution	Mean Ct ± SD	Positive replicates
Seed, 10-4	35.25 ± 0.89	8/8
Water, 10-5	33 ± 0	8/8
Neat	14 ± 0	2/2

4.4. **Reproducibility**

The repeatability of the assay was evaluated at Fera by analysing eight wells of RNA at the limit of detection across two different users on different days and machines.

Instrument and User	Dilution	Mean Ct ± SD	Positive replicates
User 1			
QuantStudio 12	Seed 10-4	35.25 ± 0.89	8/8
	Water 10-5	33 ± 0	8/8
	Neat	14 ± 0	2/2
ViiA7	Seed 10-4	35.25 ± 0.71	8/8
	Water 10-5	33.50 ± 0.93	8/8
	Neat	15 ± 0	2/2
User 2			
Quantstudio 6	Seed 10-4	35 ± 0.76	8/8
	Water 10-5	32.25 ± 0.46	8/8
	Neat	14 ± 0	2/2

RT-qPCR_ISF_ToMMV

Real-time RT-PCR tests ISF

The test below is described as in ISF protocol (publication planned for 2023). Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

1. General Information

1.1. Taqman RT-PCR is an extremely sensitive method in comparison with ELISA. Cross contamination between contaminated and ToMMV-free seed lots during seed processing and/or in the laboratory is a considerable risk.

1.2. This one-step real-time RT-PCR protocol was developed for detection of ToMMV in tomato and pepper seeds. The target sequence of the CaTa9 test is located within the 126 kDa replicase gene; the target sequences of the ToMMV2 and CSP1572 tests are located within the coat protein gene. The CaTa9 test should be used in combination with one of the tests targeting the coat protein. In addition, an internal positive control (IPC) should be used, a Taqman test targeting the Nad5 gene was validated. Alternative IPCs may be used provided that a verification is carried out (PM7/98).

1.3. Oligonucleotides are indicated in Table 1.

Table 1 Primers and probes

	Primer/probe	Sequence
Forward primer	CaTa9 Fw	5'-ATG TGG AGG AAC CCT CTA TGA-3'
Reverse primer	CaTa9 Rw	5'-AAT CTC CTC GCT CCT TGT AAA C-3'
Probe	CaTa9 Pr	5'-6FAM-TCA ATG GCC CGT GGT GAG TTA CAA-BHQ1-3'
Forward primer	ToMMV2 Fw	5'-GAA ACA TTG GAT GCC ACT CG-3'
Reverse primer	ToMMV2 Rv	5'-CTC TGG TTG TAG AAA CCT GTT CC-3'
Probe	ToMMV2 Pr	5'-6FAM-CGA TGC TAC GGT TGC GAT CAG GTC-BHQ1-3'
Forward primer	CSP1572 Fw	5'-CCC GAC TAC AGC CGA AAC AT-3'
Reverse primer	CSP1572 Rv	5'-TTA ACA GCG GAC CTG ATC GC-3'
Probe	CSP1572 Pr	5'-6FAM-TGC CAC TCG CAG AGT GGA CGA TGC TAC G-BHQ1-3'
Forward primer	Nad 5-F	5'-GAT GCT TCT TGG GGC TTC TTG TT-3'
Reverse primer	Nad 5-R	5'-CTC CAG TCA CCA ACA TTG GCA TAA-3'
Probe	Nad 5-Pr	5'-VIC-AGG ATC CGC ATA GCC CTC GAT TTA TGT G-NFQ-MGB-3'

1.4. The test has been validated using Ultraplex 1-Step ToughMix (QuantaBio).

1.5. Controls to be used are described in Table 2.

Table 2 Controls

Control type	Description
Negative Process Control (NPC)	Tomato seed-free of ToMMV
Positive Process Control (PPC)	Tomato seed with ToMMV
Positive Amplification control (PAC)	ToMMV RNA aiming for a Cq (Cycle quantification) value between 28 and 32
Internal Process Control (IPC)	Nad5 plant RNA.
Negative Template Control (NTC)	Contains all PCR reagents but no target or spike DNA, RNA or PEC nucleic acids

2. **Methods**

2.1. Seed extraction

Alternative procedures may also be suitable provided that a verification is carried out (PM7/98). Analysis is done on 3000 seeds for both tomato and pepper, results are given on 3 x 1000 subsamples. For the pepper seed grinding due to seed size the 3000 seeds are divided into 6 subsamples of 500 pepper seeds which are pooled 2:1 after the homogenisation with seed extraction buffer.

2.1.1. Add the positive extraction control (PEC) to the seed extraction buffer (Table A.1).
2.1.2. Weigh per sample 3 subsamples of 1000 tomato seeds or 6 subsamples of 500 pepper seeds.
2.1.3. Add 14 mm stainless steel bead to 50 ml Greiner tube. Add 1000 tomato or 500 pepper seeds to tube.
2.1.4. Position tubes upside down in Geno Grinder. Grind seeds with Geno Grinder (7 min. at 1,500 rpm).
2.1.5. Spin 50 ml tubes for 5 min. at minimum of 5,000 g.
2.1.6. Open 50 ml tubes carefully and add 20 ml GH+ buffer (Table 3). If an alternative IPC spike is used, this should be spiked into the GH+ buffer at this point.

Table 3 GH+ seed extraction buffer

GH+ extraction buffer (6M)	Adjust to 1 liter with demineralized water	
guanidine-hydrochloride	573	g
NaAC-buffer pH5.2 (4M)	50	ml
EDTA (di-natrium)	9.3	g
PVP-10	25	g

2.1.7. Shake closed tubes with force (vortexing is not sufficient) during 10 seconds.
2.1.8. Spin tubes with seed extract for 5 min. at 5,000 g.
2.1.9. Tomato: Transfer for subsamples of 1000 seeds 1000 µl extract (supernatant) carefully to 1,5 ml safe lock tube. Pepper: Pool two subsamples of 500 µl extract in one 1,5 ml safe lock tube.
2.1.10. Preheat thermoshaker (setting: 65°C, 850 rpm).
2.1.11. Add 30 µl DTT (5 M stock) to 1 ml of seed extract per subsample and vortex.
2.1.12. Incubate the subsamples in thermoshaker for 15 minutes at 65 °C and 850 rpm.
2.1.13. Centrifuge the tubes for 10 minutes at 16,000 g at 4 ºC.
2.1.14. Continue with the RNA isolation.

2.2. RNA extraction

2.2.1. Use 100 µL from each subsample for further analysis and start RNA isolation within 1 hour after grinding.
2.2.2. Use the commercial RNA isolation kit. RNeasy plant mini kit (Qiagen) and Powerplant kits (Qiagen) have been validated for this test. Process the subsamples according to the supplier's instructions.
2.2.3. Eluate the RNA in 100 µL elution buffer.

2.3. Preparation of the RT-qPCR

2.3.1. Prepare the RT-qPCR mixture as indicated in Table 4. For each run, include a negative template control (NTC) and at least one positive amplification control (PAC) that give a Cq value between 28 and 32.
2.3.2.

Table 4 Reaction composition

Reagent	**Working concentration**	**Volume per reaction (µL)**	**Final concentration**
Molecular grade water		6.8	
Ultraplex 1-Step ToughMix	4×	5.00	1×
CaTa9 Fw	10µM	0.6	300 nM
CaTa9 Rw	10µM	0.6	300 nM
CaTa9 Pr	10µM	0.4	200 nM
ToMMV Fw or CSP1572 Fw	10µM	0.6	300 nM
ToMMV Rw or CSP1572 Rw	10µM	0.6	300 nM
ToMMV Pr or CSP1572 Pr	10µM	0.4	200 nM
Nad 5-F	5µM	0.4	100 nM
Nad 5-R	5µM	0.4	100 nM
Nad 5-Pr	5µM	0.2	50 nM
Subtotal		16.00	
RNA		4.00	
Total		20.00	

2.3.3. Perform the PCR reaction in a real-time PCR instrument: 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.

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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.
- Internal positive controls (IPCs) used to monitor each individual sample separately, the Nad 5 test was validated in a triplex real-time PCR with two tests targeting the ToMMV RNA. Alternative IPCs, such as DLVd or BaCV, may be used provided that a verification is carried out (PM7/98).

3.2. Interpretation of results

Verification of the controls

- NIC and NAC should be negative.
- The PAC and the IPC 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.
- A Ct cut-off value should be applied. As a Ct cut-off value is equipment, material, and chemistry dependent it needs to be verified in each laboratory when implementing the test.

RT-qPCR_Tiberini_singleplex_duplex_ToMMV

Real-time RT-PCR test Tiberini et al. (2022)

The test below is described as in Tiberini et al. (Plants 2022, 11, 489. https://doi.org/10.3390/plants11040489). 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 was developed for simultaneous detection and identification of ToMMV and ToBRFV in tomato and pepper leaves and seeds. It can also be used as a single test for the detection and identification of ToMMV. A single test for the detection and identification of ToBRFV is already included in Appendix 5 of EPPO PM 7/146.

1.2. The test is based on ToMMV primers and probe developed by Tiberini et al. (2022) in combination with the ToBRFV primers and probe developed by Menzel and Winter (2021).

1.3. The target sequence of the ToMMV primers is located within the movement protein gene; using the nucleotide sequence of GenBank accession no NC_022230, the forward and reverse primer start at position 5173 and 5255, respectively, and the probe covers positions 5197–5220. ToBRFV 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	ToMMV CataAT Fw	5'-CAG CAT CTG CTT GGT CGA TAA-3'
Reverse primer	ToMMV CataAT Rv	5'-GGA ACG ATC TTA AAC TGG AAC CT-3'
Probe	ToMMV CataAT Pr*	5'-TexasRed-AAT GCA AAG AGC GGA TGA AGC GAC-BHQ2-3'
Forward primer	ToBRFV qs1	5'- CAA TCA GAG CAC ATT TGA AAG TGC A -3'
Reverse primer	ToBRFV qas2	5'- CAG ACA CAA TCT GTT ATT TAA GCA TC -3'
Probe	ToBRFV p1	5'-6FAM- ACA ATG GTC CTC TGC ACC TG-BHQ1-3'

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

1.5. The test has been validated using i) TaqMan® RNA-to-Ct™ 1-Step Kit (Thermo Fisher) and real-time PCR system CFX Maestro v2.2 (Bio-Rad) and ii) AgPath-ID One-Step RT-qPCR mix (Thermo Fisher) and real-time PCR systems QuantStudio™ 7 Pro and ViiA7™ (both Apploed Biosystems)

2. Methods

2.1. Nucleic acid extraction and purification

See Appendix 1 of EPPO PM7/146.

2.2. One-step real-time RT-PCR

2.2.1.Master Mix:

- with TaqMan® RNA-to-Ct™ 1-Step Kit:

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water		2.15	
Mastermix (from the kit)	2×	5.00	1×
ToMMV CataAT Fw	10 µM	0.30	0.3 µM
ToMMV CataAT Rv	10 µM	0.30	0.3 µM
ToMMV CataAT Pr	10 µM	0.20	0.2 µM
ToBRFV qs1	10 µM	0.30	0.3 µM
ToBRFV qas2	10 µM	0.30	0.3 µM
ToBRFV p1	10 µM	0.20	0.2 µM
RT-enzyme (from the kit)	40x	0.25	1x
Subtotal		9.00	
RNA		1.00	
Total		10.00	

- with AgPath-ID One-Step RT-qPCR mix:

Reagent	Working	Volume per	Final

	concentration	reaction (µL)	concentration
Molecular grade water		1.00	
RT-PCR buffer (from the kit)	2×	5.00	1×
ToMMV CataAT Fw	10 µM	0.30	0.3 µM
ToMMV CataAT Rv	10 µM	0.30	0.3 µM
ToMMV CataAT Pr	10 µM	0.20	0.2 µM
ToBRFV qs1	10 µM	0.30	0.3 µM
ToBRFV qas2	10 µM	0.30	0.3 µM
ToBRFV p1	10 µM	0.20	0.2 µM
RT-PCR enzyme (from the kit)	25x	0.40	1x
Subtotal		8.00	
RNA		2.00	
Total		10.00	

2.2.2. Real-time RT-PCR cycling conditions:

- with TaqMan® RNA-to-Ct™ 1-Step Kit:
  Reverse transcription at 48°C for 30 min; denaturation at 95°C for 10 min; 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 60 s.
- with AgPath-ID One-Step RT-qPCR mix:
  Reverse transcription at 48°C for 10 min; denaturation at 95°C for 10 min; 45 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 60 s.
  Note: if the probe is labelled with TexasRed, the ROX™ reference dye should be excluded prior to analysis to avoid interference with the TexasRed dye.

3. Essential procedural information

3.1. Controls

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue).
- 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.

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. As an example, high Ct values for both ToMMV and ToBRFV were obtained for PaMMV artificially inoculated test plant (Ct ≥ 29.3 for ToMMV, Ct ≥ 33.5 for ToBRFV) (Tiberini et al., 2022). 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 validation data for one-step duplex real-time RT-PCR are extracted from Tiberini et al. (2022).

4.1. Analytical sensitivity data

The LOD was determined using the 10-fold dilution series (leaves and seeds) obtained from ToMMV and ToBRFV mixed infected samples. In leaves, the LODs were 10^{-5} for ToMMV and 10^{-6} for ToBRFV, whereas in seeds both viruses could be detected up to the 10^{-5} dilution.

Note: in both ToBRFV and ToMMV single assays, the LOD was 10-fold lower than in the duplex assay (except for ToBRFV in leaves confirming a LOD of 10^{-6}).

4.2. Analytical specificity data

4.2.2 Inclusivity

The inclusivity of the duplex assay was assessed using three different ToBRFV (CREA isolate MR50, and Volcani center isolates S21 and S22) and three different ToMMV (DSMZ isolate PV-1267, and IBMCP isolates S1 and S2) isolates. In all cases, the identification of ToBRFV and ToMMV was successful.

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

4.2.3 Exclusivity

The exclusivity was assessed by testing the following tobamovirus species: BPeMV (DSMZ isolate BN-4708), CGMMV (DSMZ isolate PV-0375, and NIB isolates NIB V 271 and NIB V 320), ObPV (DSMZ isolate PV-1176), ORSV (DSMZ isolate PV-1048), PaMMV (DSMZ isolate PV-0606), PMMoV (DSMZ isolate PV-0165, CREA isolate CREA-552), RMV (DSMZ isolate PV-0145), SFBV (DSMZ isolate PV-1058), SHMV (DSMZ isolate PV-0156), ToMV (DSMZ isolate PV-0141, and NIB isolates NIB V 036, NIB V 049, NIB V 072, NIB V 104), TMGMV (DSMZ isolate PV-0124), TMV (DSMZ isolates PV-1252, PV-0137 and PV-0943, and NIB isolate NIB V 037), YMoV (DSMZ isolate PV-0527).

No cross-reaction was observed, except for PaMMV and RMV. PaMMV resulted with high Ct values for both ToMMV and ToBRFV (Ct ≥ 29.3 for ToMMV, Ct ≥ 33.5 for ToBRFV). RMV cross-reaction occur only with ToBFRV (Ct ≥ 35.2).

4.3. Selectivity

No relevant differences in Ct values were found when five tomato and six pepper cultivars spiked with ToMMV and ToBRFV were tested; in one tomato variety (pom-241 ‘sv5197’) both leaf and fruit matrices were tested.

4.4. Repeatability

The repeatability of the duplex assay was evaluated at CREA and NIB by analyzing three replicates of RNA samples containing various concentrations of ToBRFV or ToMMV in the same run.
Repeatability in both labs and within each sample was 100% (standard deviation (SD) of the mean Ct values obtained was always less than 1.5 Ct).

4.5. Reproducibility

Reproducibility was analyzed for two dilutions of a ToBRFV- and ToMMV-positive RNA samples, with medium and low target concentrations. It was assessed at both CREA and NIB, analyzing different target RNA sample and using different reagents. In each laboratory, different real-time RT-PCR runs, four (CREA) and six (NIB), were performed on different days. In addition, at NIB two different instruments were used.
Reproducibility was 100% (SD below 1.5 Ct).

RPA_Agdia_ToMMV

Recombinase-Polymerase Amplification (RPA) by Agdia (2022)

The test below is a rapid and portable isothermal amplification kit for ToMMV detection developed by Agdia Inc. To date, it is the only commercial method for ToMMV available in the market..

1. **General information**
   - AmplifyRP® XRT for ToMMV is a rapid RNA amplification and detection platform designed for testing peppers and tomatoes for Tomato mottle mosaic virus. This kit includes lyophilized reaction pellets containing the necessary reagents to amplify ToMMV RNA at a single operating temperature (42 °C).
   - AmplifyRP® XRT is a real-time isothermal nucleic acid amplification and detection system that rapidly amplifies small portions of DNA or RNA and offers unrivaled detection capabilities in an easy-to-use testing format. It offers comparable sensitivity and specificity to published PCR methods while eliminating laborious and costly nucleic acid extractions.
   - The test can be performed virtually anywhere using the battery operated AmpliFire® fluorometer. Assay parameters are loaded via barcode and results are automatically displayed as (+) or (-). Real Time PCR thermocyclers or other fluorometers can also be suitable to perform the test.
   - Prior molecular diagnostic experience is not required to perform AmplifyRP® XRT tests. Total assay time is less than 30 minutes when used with the AmpliFire® as a real-time assay.

2. **Methods (for full instructions, please refer to user guide)**

   Sample Preparation - Plant Tissue
   - Symptomatic or asymptomatic tissue may be tested. Agdia recommends sampling leaves or petioles. Collect 0.15 g of leaf or petiole tissue from the suspect area.
   - Place the tissue inside the provided mesh extraction bag containing GEB2 extraction buffer. Extract the tissue by thoroughly macerating it with a blunt object such as a pen.
   - Remove one colored PD1 filled tube for each sample being tested.. Inspect the tube to ensure all liquid is at the bottom before use. Transfer 5 µL of sample extract into the tube containing PD1 diluent and mix well.
   - The test can be also performed using purified ARN. In this case transfer 5 µL of the solution containing extracted ARN into into the tube containing PD1 diluent and mix well.

   Test Protocol for Real-Time Detection In AmpliFire®

   - Transfer 25 µL from the colored tube containing your sample extract into the reaction pellet (clear tube).
   - Press “Start” on the AmpliFire. Immediately follow the prompts to add your reactions, press “OK”, and put the lid down.
   - After 20 minutes of total run time the instrument will beep, indicating the test is complete. The test results will be visible next to the well designation on the screen, and should be interpreted as follows: (+) = Positive for ToMMV (-) = ToMMV not detected (!) = Invalid

3. **Analytical specificity data**

   Diagnostic Sensitivity
   True Positives 46
   Correct Diagnoses 46
   Percent 100%
   Analytical Sensitivity
   Limit of Detection: Approximately 400 ag/µL of RNA transcripts
   Limit of Detection: 1:62,500 dilution of infected tissue (pathogen titer unknown)

   Analytical Specificity
   Diagnostic Specificity
   True Negatives 79
   Correct Diagnoses 79
   Percent 100%
   Selectivity:
   No Matrix Effect Observed With:
   Pea leaves, Pea petioles, Pea seeds, Pea stems, Pepper fruit, Pepper leaves, Pepper petioles, Pepper seeds, Pepper

stems, Petunia leaves, Petunia petioles, Petunia stems, Tomato fruit, Tomato leaves, Tomato petioles, Tomato seeds, Tomato stems.

Inclusivity:
Isolates and Geographic Regions Detected:

ToMMV PV-1267 (CA, USA)	ToMMV PV-1342 (Mauritius)1
ToMMV-10-100 (FL, USA)1	ToMMV-19-02305 (Netherlands)1
ToMMV-CA16-01 (USA)1	ToMMV-CpB1 (Brazil)1
ToMMV-Hainan (China)1	ToMMV-HN (China)1
ToMMV-Hn18 (China)1	ToMMV-Hn19 (China)1
ToMMV-Hn23 (China)1	ToMMV-LN (China)1
ToMMV-MX5 (Mexico)	ToMMV-NVWA 36783676 (Netherlands)1
ToMMV-NVWA36783860 (China)1	ToMMV-NVWA411068131
ToMMV-NVWA57856601	ToMMV-NY-13 (USA)1
ToMMV-SC13-05 (USA)1	ToMMV-SY11026 (China)1
ToMMV-TiLhaLJ (China)1	ToMMV-ToMMV_83 (Viet Nam)1
ToMMV-VLC-1 (Spain)1	ToMMV-WD-YMZ1 (China)1
ToMMV-YYMFQ12 (China)1	ToMMV-YYMLJ (China)1

1Predicted detection by in silico analysis only

Exclusivity:
Cross-reacts With: None Known

Repeatability
Number of Samples 22
Replicates per Sample 3
Average percent agreement between replicates 97.0%

Reproducibility
Number of Samples 22
Replicates per Sample 3
Number of Operators 3
Average percent agreement between replicates between operators: 97.0%

LAMP_Kumura_ToMMV

Reverse-Transcription Loop-Mediated Isothermal Amplification (LAMP) by Kimura et al. (2023)

The test below is described as in Kimura et al. (Viruses 2023, 15, 1688, https://doi.org/10.3390/v15081688).

The test was modified from the chemistry that was used in the Kimura et al. paper (RNA amplification reagent kit cat no. NE6051 Nippon Gene, Japan) to the chemistry Isothermal Master Mix (Optigene, UK) because the original chemistry was not available for purchase in Slovenia.

1. General Information

1.1 This protocol was developed for detection of ToMMV based on reverse-transcription loop-mediated isothermal amplification (RT-LAMP)

1.2 The test is based on LAMP primer set developed by Kimura et al. (2023)

1.3 The target sequences of the ToMMV LAMP primers are located within the end of movement and start of coat protein genes. Primers were designed based on the sequence of the ToMMV isolate YYMLJ (GenBank accession no. KR824950). Using the nucleotide sequence of GenBank accession no KF477193, the forward outer primer and backward outer primer start at position 5585 and 5798, respectively. The forward inner primer covers positions 5689–5665 (F1c) and 5606–5627 (F2). The backward inner primer covers positions 5691–5710 (B1c) and 5770–5751 (B2). The forward loop primer and backward loop primer start at position 5652 and 5719, respectively.

1.4 Oligonucleotides

	Primer Name	Sequence (5' → 3')
Forward outer primer	ToMMV_2-3_FOP	ACTAATGAAAGAAAAGGGCGG
Backward outer primer	ToMMV_2-3_BOP	ATATTTATTAATTCTACAGGGTCG
Forward inner primer	ToMMV_2-3_FIP (F1c * + F2)	CGTCTCGGTATCATCTTCAATCAA ATCTAATTTCCGTAAGAAACAAG
Backward inner primer	ToMMV_2-3_BIP (B1c * + B2)	CAGTCGCGGGATCTGATTCGATGC TGATGATAAAAACACG
Forward loop primer	ToMMV_2_LF	TCACTAACTCCATAACTCTCCTGGT
Backward loop primer	ToMMV_2_LB	ATGTCTTACGCTATTACTTCTCCGT

* F1c and B1c indicate the complementary sequences of F1 and B1.

2. Methods

2.1. Nucleic acid extraction and purification

See Appendix 1 of EPPO PM7/146.

2.2. LAMP

2.2.1.Master Mix (Optigene Ltd., Horsham, UK):

Reagent		Working concentration	Volume per reaction (µL)	Final concentration
Isothermal Master Mix (Optigene Ltd., Horsham, UK)		2x	12.5	1x
FOP	10x primer mix	See 2.2.2	2.5	See 2.2.2

BOP		See 2.2.2		See 2.2.2
FIP		See 2.2.2		See 2.2.2
BIP		See 2.2.2		See 2.2.2
LF		See 2.2.2		See 2.2.2
LB		See 2.2.2		See 2.2.2
Transcriptor RT reaction buffer (Roche, Switzerland)		5x	1	1x
Transcriptor RT (Roche, Switzerland)		20 U/µL	0.25	5 U
Water			3.75	
Subtotal			20	
RNA			5	
Total			25	

2.2.2. Preparation of 10x primer mix:

Primer Mix (10x)	**Working concentration**	**Final concentration**	**Volume (µL)**
FOP	100 µL	2 µL	1
BOP	100 µL	2 µL	1
FIP	100 µL	16 µL	8
BIP	100 µL	16 µL	8
LF	100 µL	8 µL	4
LB	100 µL	8 µL	4
Water			24
Total			50

2.3. LAMP amplification

LAMP amplification conditions: 65°C for 60 min; melting curve analysis: 98–80°C, 0.05°C per second.

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.

3.2. **Interpretation of results**

Verification of the controls

- NIC and NAC should be negative.
- The PIC and PAC should be positive. Amplification curve should be exponential. Using GenieII, we determined the Tm (melting temperature) between 83.5 and 84.5°C using a limited number of samples. A similar Tm range can be expected when analysed with a different device, but still needs to be verified.

When these conditions are met

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

4. **Performance characteristics available**

Some validation data is available in Kimura et al. (2023).

Appendix 2: Summary with all important data required for carrying out tests within the framework of TPS

General Instruction for the TPS

Number of samples that each TPS participant will receive is 24 (22 test items + 2 controls).

Each sample should be tested in two technical replicates, therefore the calculations which are provided in this document are for a total of 48 samples.

If participants decide to include additional samples (e.g. additional controls) the number of samples in the calculation tables (in orange highlighted cells) should be changed accordingly, then needed amounts will be recalculated authomatically.

RT-PCR test Levitzky et al. (2019)

	Primer	Sequence (5' → 3')	Region/ gene	Size
Forward primer	F-5476	GAA GAA GTT GTT GAT GAG TTC AT		
Reverse primer	R-6287	GAT TTA AGT GGA GGG AAA AAC AC	MP	~800bp

Master Mix: OneStep RT-PCR kit (Qiagen)

Reagent	Working concentration	Volume per reaction (µL)	Volume per reaction (µL)+10%	For 48 reactions
Molecular grade water	/	16	17.6	844.8
Qiagen OneStep RT-PCR Buffer (from the kit)	5×	5	5.5	264
F-5476	10 µM	0.5	0.55	26.4
R-6287	10 µM	0.5	0.55	26.4
Qiagen dNTPs (10 mM) (from the kit)	/	1	1.1	52.8
Qiagen OneStep RT-PCR Enzyme Mix (from the kit)	/	1	1.1	52.8
Subtotal	/	24	26.4	1267.2
RNA	/	1	/	
Total	/	25	/	

5 ×

Cycling conditions

Reverse transcription	50°C for 30 min	
denaturation	95°C for 15 min	
denaturation	94°C for 60 s	35 cycles
annealing	53°C for 60 s	
elongation	72°C for 30 s	
final elongation	72°C for 10 min	
Hold at 4°C		

Interpretation of the results:
A test will be considered positive for tobamoviruses if it produces a band (~800bp).
A test will be considered negative if it does not produce a band.
Tests should be repeated if any contradictory or unclear results are obtained.
Sequencing of the PCR product is required to identify species.

RT-PCR Loewe (Cat. No. 09181/100)

	Primer	Sequence (5' → 3')	Amplicon size
Forward primer	ToMMV-F5600	confidential	
Reverse primer	ToMMV-R	CAC TCT GCG AGT GGC ATC CAA T	~460 bp

Reagent	Volume per reaction (µL)	Volume per reaction (µL)+10%	For	48	reactions
PCR-grade water	9.2	10.12	485.76		
RNA-PCR Reaction Buffer	7.5	8.25	396		
DTT 100mM	1	1.1	52.8		
Premix	5	5.5	264		
Reverse-Transcriptase	0.1	0.11	5.28		
DNA-Polymerase	0.2	0.22	10.56		
Subtotal	23	25.3	1214.4		
RNA	2				
Total	25				

Cycling conditions

Reverse transcription	42°C for 45 min	
Initial denaturation	95°C for 12 min	
denaturation	95°C for 15 s	
annealing	58°C for 20 s	
elongation	72°C for 30 s	40 cycles
final elongation	72°C for 1 min	
Cool down	4 -8°C	

Interpretation of the results:

For a positive sample the 460 bt fragment (*Tomato mottle mosaic virus*) must be visible.

For negative samples no 460 bp fragment must be visible.

When no 460 bp fragment is visible, the specific RNA of Tomato mottle mosaic virus is not detected in the analysed sample.

RT-PCR Sui et al., 2017

	Primer	Sequence (5' → 3')	Location	Amplicon size (nt)	Region
Forward primer	ToMMV-F	CGACCCTGTAGAATTAATAAATATT	5775-5799	289	CP
Reverse primer	ToMMV-R	CACTCTGCGAGTGGCATCCAAT	6063-6042		

Master Mix: OneStep RT-PCR Kit (Qiagen)

Primer	Volume (µL)	Working Concentration	Volume per reaction (µL)+10%	For	48	reactions
Water	13	/	14.3	686.4		
Qiagen OneStep RT-PCR Buffer (from the kit)	5	5x	5.5	264		
Qiagen dNTPs (10 mM) (from the kit)	1	/	1.1	52.8		
ToMMV-F	1	10 µM	1.1	52.8		
ToMMV-R	1	10 µM	1.1	52.8		
Qiagen OneStep RT-PCR Enzyme Mix (from the kit)	1	/	1.1	52.8		
RNaseOUT*	1	/	1.1	52.8		
Subtotal	23	/	25.3	1214.4		
Template	2	/	/			
Total Reaction	25	N/A	/			

*not provided in the kit RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, Catalog number: 10777019)

Cycling conditions

Reverse transcription	50°C for 30 min	
denaturation	95°C for 15 min	
denaturation	95°C for 60 s	35 cycles
annealing	55°C for 45 s	
elongation	72°C for 60 s	
final elongation	72°C for 10 min	
Hold at 4°C		

Note: Use maximum temperature ramping rate between steps

Interpretation of the results:
If a sample produces a ~289 bp amplicon, it is determined positive for ToMMV, which can be confirmed by subsequent sequencing.
If a sample does not produce a ~289 bp amplicon, then it is determined as NEGATIVE for ToMMV.
If a sample produces non-specific amplicons or amplicons at sizes other than the expected ~289bp, the RT-PCR must be repeated. If once repeated, unspecific bands are seeing, the sample is INCONCLUSIVE.

RT-qPCR DAFF DEECA

	Primers	Sequence (5'-3')	Region/ gene
Forward primer	CSP 1572-F	CCCGACTACAGCCGAAACAT	
Reverse primer	CSP 1572-R	TTAACAGCGGACCTGATCGC	
Probe	CSP 1572-P	(6FAM)-TGCCACTCGCAGAGTGGACGATGCTACG- (BHQ1)	CP

Master Mix: One-step real-time RT-PCR AgPath-ID One-Step RT-qPCR (Applied Biosystems™/ThermoFisher Scientific)

Reagent	**Working concentration**	**Volume per reaction (µL)**	**Volume per reaction (µL)+10%**	**For 48 reactions**
Molecular grade water	/	7	7.7	369.6
RT-PCR buffer (from the kit)	2×	12.5	13.75	660
ToMMV CSP 1572-F	10 µM	1	1.1	52.8
ToMMV CSP 1572-R	10 µM	1	1.1	52.8
ToMMV CSP 1572-P	10 µM	0.5	0.55	26.4
RT-PCR enzyme (from the kit)	25x	1	1.1	52.8
Subtotal	/	23	25.3	1214.4
RNA	/	2	/	
Total	/	25	/	

Cycling conditions

Reverse transcription	48°C for 30 min	
denaturation	94°C for 5 min	
denaturation	94°C for 10 s	40 cycles
annealing and elongation	60°C for 30 s	

Evaluation of the results:

A test is considered positive if it produces an exponential amplification curve. If the exponential amplification curve is obtaine provide Cq value.

A test is considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential. If no exponential curve is obtained Cq values should not be provided.

RT-qPCR Fowkes et al. (2022)

	Primer/probe	Sequence (5' → 3')	Region/ gene
Forward primer	ToMMV-F	CGT GGT GGT GTC AGC ATC TG	MP
Reverse primer	ToMMV-R	CGA TCC GAG TGT CGC TTC A	
Probe	ToMMV-Pe	FAM- TTG GTC GAT AAA AGA ATG CAA AGA GCG G -TAMRA	

Master Mix: One-step real-time RT-PCR iTaq Universal Probes One-Step Kit (BioRad)

Reagent	Working concentration	Volume per reaction (µL)	Volume per reaction (µL)+10%	For	48	reactions
Molecular grade water	/	7.2	7.92		380.16	
Mastermix (from the kit)	2×	10	11		528	
ToMMV F	10 µM	0.75	0.825		39.6	
ToMMV R	10 µM	0.75	0.825		39.6	
ToMMV Pe	10 µM	0.25	0.275		13.2	
RT-enzyme (from the kit)	/	0.05	0.055		2.64	
Subtotal	/	19	20.9		1003.2	
RNA	/	1	/			
Total	/	20	/			

*the amounts and probes to be added in the mix are adpted to working concentration 10 µM

Cycling conditions

Reverse transcription	50°C for 10 min	
denaturation	95°C for 2 min	
denaturation	95°C for 15 s	40 cycles
annealing and elongation	60°C for 60 s	

Evaluation of the results:

A test is considered positive if it produces an exponential amplification curve. If the exponential amplification curve is obtaine provide Cq value.

A test is considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential. If no exponential curve is obtained Cq values should not be provided.

RT-qPCR ISF

	Primer/probe	Sequence (5' → 3')	Region/ gene
Forward primer	CaTa9 Fw	ATG TGG AGG AAC CCT CTA TGA	126 kDa replicase
Reverse primer	CaTa9 Rw	AAT CTC CTC GCT CCT TGT AAA C	
Probe	CaTa9 Pr	6FAM-TCA ATG GCC CGT GGT GAG TTA CAA-BHQ1	
Forward primer	ToMMV2 Fw	GAA ACA TTG GAT GCC ACT CG	CP
Reverse primer	ToMMV2 Rv	CTC TGG TTG TAG AAA CCT GTT CC	
Probe	ToMMV2 Pr	6FAM-CGA TGC TAC GGT TGC GAT CAG GTC-BHQ1	
Forward primer	CSP1572 Fw	CCC GAC TAC AGC CGA AAC AT	CP
Reverse primer	CSP1572 Rv	TTA ACA GCG GAC CTG ATC GC	
Probe	CSP1572 Pr	6FAM-TGC CAC TCG CAG AGT GGA CGA TGC TAC G-BHQ1	
Forward primer	Nad 5-F	GAT GCT TCT TGG GGC TTC TTG TT	/
Reverse primer	Nad 5-R	CTC CAG TCA CCA ACA TTG GCA TAA	
Probe	Nad 5-Pr	VIC-AGG ATC CGC ATA GCC CTC GAT TTA TGT G-NFQ-MGB	

Master Mix: One-step real-time RT-PCR Ultraplex 1-Step ToughMix (QuantaBio **or** VWR, three different versions of the kit are available, please check what is requred for your instrument-options: without ROX, ROX, low Rox)

Reagent	Working concentration	Volume per reaction (µL)	Volume per reaction (µL)+10%	For 48 reactions
Molecular grade water	/	6.8	7.48	359.04
Ultraplex 1-Step ToughMix (from the kit)	4×	5	5.5	264
CaTa9 Fw	10µM	0.6	0.66	31.68
CaTa9 Rw	10µM	0.6	0.66	31.68
CaTa9 Pr	10µM	0.4	0.44	21.12
ToMMV Fw or CSP1572 Fw	10µM	0.6	0.66	31.68
ToMMV Rw or CSP1572 Rw	10µM	0.6	0.66	31.68
ToMMV Pr or CSP1572 Pr	10µM	0.4	0.44	21.12
Nad 5-F	5µM	0.4	0.44	21.12
Nad 5-R	5µM	0.4	0.44	21.12
Nad 5-Pr	5µM	0.2	0.22	10.56
Subtotal	/	16	17.6	844.8
RNA	/	4	/	
Total	/	20	/	

Cycling conditions

Reverse transcription	50°C for 10 min	
denaturation	95°C for 3 min	40 cycles
denaturation	95°C for 10 s	
annealing and elongation	60°C for 60 s	

Evaluation of the results:

A test is considered positive if it produces an exponential amplification curve. If the exponential amplification curve is obtaine provide Cq value.

A test is considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential. If no exponential curve is obtained Cq values should not be provided.

RT-qPCR Tiberini et al. (2022) Singleplex

	Primer/probe	Sequence (5' → 3')	Region/ gene
Forward primer	ToMMV CataAT Fw	CAG CAT CTG CTT GGT CGA TAA	
Reverse primer	ToMMV CataAT Rv	GGA ACG ATC TTA AAC TGG AAC CT	
Probe	ToMMV CataAT Pr*	TexasRed-AAT GCA AAG AGC GGA TGA AGC GAC-BHQ2	MP

*probe labelled with HEX (instead of TexasRed) can also be used

Master Mix: One-step real-time RT-PCR TaqMan® RNA-to-Ct™ 1-Step Kit (ThermoFisher Scientific)

Reagent	**Working concentration**	**Volume per reaction (µL)**	**Volume per reaction (µL)+10%**	For 48 reactions
Molecular grade water		2.95	3.245	155.76
Mastermix (from the kit)	2×	5	5.5	264
ToMMV CataAT Fw	10 µM	0.3	0.33	15.84
ToMMV CataAT Rv	10 µM	0.3	0.33	15.84
ToMMV CataAT Pr	10 µM	0.2	0.22	10.56
RT-enzyme (from the kit)	40x	0.25	0.275	13.2
Subtotal	/	9	9.9	475.2
RNA	/	1	/	
Total	/	10	/	

Cycling conditions

Reverse transcription	48°C for 30 min	
denaturation	95°C for 10 min	
denaturation	95°C for 15 s	40 cycles
annealing and elongation	60°C for 60 s	

Master Mix: One-step real-time RT-PCR AgPath-ID One-Step RT-qPCR (Applied Biosystems™/ThermoFisher Scientific)

Reagent	**Working concentration**	**Volume per reaction (µL)**	per reaction	For 48 reactions
Molecular grade water		1.8	1.98	95.04
RT-PCR buffer (from the kit)	2×	5	5.5	264
ToMMV CataAT Fw	10 µM	0.3	0.33	15.84
ToMMV CataAT Rv	10 µM	0.3	0.33	15.84
ToMMV CataAT Pr	10 µM	0.2	0.22	10.56
RT-PCR enzyme (from the kit)	25x	0.4	0.44	21.12
Subtotal	/	8	8.8	422.4
RNA	/	2	/	
Total	/	10	/	

Cycling conditions

Reverse transcription	48°C for 10 min	
denaturation	95°C for 10 min	
denaturation	95°C for 15 s	45 cycles
annealing and elongation	60°C for 60 s	

Evaluation of the results:

A test is considered positive if it produces an exponential amplification curve. If the exponential amplification curve is obtaine provide Cq value.

A test is considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential. If no exponential curve is obtained Cq values should not be provided.

RT-qPCR Tiberini et al. (2022) Duplex

Primers

	Primer/probe	Sequence (5' → 3')	Region/ gene
Forward primer	ToMMV CataAT Fw	CAG CAT CTG CTT GGT CGA TAA	
Reverse primer	ToMMV CataAT Rv	GGA ACG ATC TTA AAC TGG AAC CT	
Probe	ToMMV CataAT Pr*	TexasRed-AAT GCA AAG AGC GGA TGA AGC GAC-BHQ2	MP
Forward primer	ToBRFV qs1	CAA TCA GAG CAC ATT TGA AAG TGC A	
Reverse primer	ToBRFV qas2	CAG ACA CAA TCT GTT ATT TAA GCA TC	
Probe	ToBRFV p1	6FAM- ACA ATG GTC CTC TGC ACC TG-BHQ1	CP, 3-NTR

Master Mix: One-step real-time RT-PCR TaqMan® RNA-to-Ct™ 1-Step Kit (ThermoFisher Scientific)

Reagent	**Working concentration**	**Volume per reaction (µL)**	**Volume per reaction (µL)+10%**	**For 48 reactions**
Molecular grade water		2.15	2.365	114
Mastermix (from the kit)	2×	5	5.5	264
ToMMV CataAT Fw	10 µM	0.3	0.33	15.8
ToMMV CataAT Rv	10 µM	0.3	0.33	15.8
ToMMV CataAT Pr	10 µM	0.2	0.22	10.6
ToBRFV qs1	10 µM	0.3	0.33	15.8
ToBRFV qas2	10 µM	0.3	0.33	15.8
ToBRFV p1	10 µM	0.2	0.22	10.6
RT-enzyme (from the kit)	40x	0.25	0.275	13.2
Subtotal	/	9	9.9	475
RNA	/	1	/	
Total	/	10	/	

Cycling conditions

Reverse transcription	48°C for 30 min	
denaturation	95°C for 10 min	
denaturation	95°C for 15 s	40 cycles
annealing and elongation	60°C for 60 s	

Master Mix: One-step real-time RT-PCR AgPath-ID One-Step RT-qPCR (Applied Biosystems™/ThermoFisher Scientific)

Reagent	**Working concentration**	**Volume per reaction (µL)**	**Volume per reaction (µL)+10%**	**For 48 reactions**
Molecular grade water		1	1.1	52.8
RT-PCR buffer (from the kit)	2×	5	5.5	264
ToMMV CataAT Fw	10 µM	0.3	0.33	15.84
ToMMV CataAT Rv	10 µM	0.3	0.33	15.84
ToMMV CataAT Pr	10 µM	0.2	0.22	10.56
ToBRFV qs1	10 µM	0.3	0.33	15.84
ToBRFV qas2	10 µM	0.3	0.33	15.84
ToBRFV p1	10 µM	0.2	0.22	10.56
RT-PCR enzyme (from the kit)	25x	0.4	0.44	21.12
Subtotal	/	8	8.8	422.4
RNA	/	2	/	
Total	/	10	/	

Cycling conditions

Reverse transcription	48°C for 10 min	
denaturation	95°C for 10 min	
denaturation	95°C for 15 s	45 cycles
annealing and elongation	60°C for 60 s	

Evaluation of the results:

A test is considered positive if it produces an exponential amplification curve. If the exponential amplification curve is obtaine provide Cq value.

A test is considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential. If no exponential curve is obtained Cq values should not be provided.

AmplifyRP® XRT for ToMMV (Agdia)

Recombinase-Polymerase Amplification (RPA) by Agdia (2022)The test below is a rapid and portable isothermal amplification kit for ToMMV detection developed by Agdia Inc

User manual (follow the link below)

user guide

1. General information

- AmplifyRP® XRT for ToMMV is a rapid RNA amplification and detection platform designed for testing peppers and tomatoes for Tomato mottle mosaic virus. This kit includes lyophilized reaction pellets containing the necessary reagents to amplify ToMMV RNA at a single operating temperature (42 °C).
- AmplifyRP® XRT is a real-time isothermal nucleic acid amplification and detection system that rapidly amplifies small portions of DNA or RNA and offers unrivaled detection capabilities in an easy-to-use testing format. It offers comparable sensitivity and specificity to published PCR methods while eliminating laborious and costly nucleic acid extractions.
- The test can be performed virtually anywhere using the battery operated AmpliFire® fluorometer. Assay parameters are loaded via barcode and results are automatically displayed as (+) or (-). Real Time PCR thermocyclers or other fluorometers can also be suitable to perform the test.
- Prior molecular diagnostic experience is not required to perform AmplifyRP® XRT tests. Total assay time is less than 30 minutes when used with the AmpliFire® as a real-time assay.

2. Methods (for full instructions, please refer to user guide) Sample Preparation - Plant Tissue

- Symptomatic or asymptomatic tissue may be tested. Agdia recommends sampling leaves or petioles. Collect 0.15 g of leaf or petiole tissue from the suspect area.
  Place the tissue inside the provided mesh extraction bag containing GEB2 extraction buffer. Extract the tissue by thoroughly macerating it with a blunt object such as a pen.
- Remove one colored PD1 filled tube for each sample being tested.. Inspect the tube to ensure all liquid is at the bottom before use. Transfer 5 µL of sample extract into the tube containing PD1 diluent and mix well.
- The test can be also performed using purified ARN. In this case transfer 5 µL of the solution containing extracted ARN into into the tube containing PD1 diluent and mix well.

Test Protocol for Real-Time Detection In AmpliFire®

- Transfer 25 µL from the colored tube containing your sample extract into the reaction pellet (clear tube).
- Press “Start” on the AmpliFire. Immediately follow the prompts to add your reactions, press “OK”, and put the lid down.
- After 20 minutes of total run time the instrument will beep, indicating the test is complete. The test results will be visible next to the well designation on the screen, and should be interpreted as follows: (+) = Positive for ToMMV (-) = ToMMV not detected (!) = Invalid

LAMP Kimura et al. (2023)

Primer Name	Sequence (5' → 3')	Length (nt)	Position*
ToMMV_2-3_FOP	ACTAATGAAAGAAAAGGGCGG	21	5585–5605 (F3)
ToMMV_2-3_BOP	ATATTTATTAATTCTACAGGGTCG	24	5798–5775 (B3)
ToMMV_2-3_FIP (F1c ** + F2)	CGTCTCGGTATCATCTTCAATCAAA TCTAATTTCCGTAAGAAACAAG	47	5689–5665 (F1c) 5606–5627 (F2)
ToMMV_2-3_BIP (B1c ** + B2)	CAGTCGCGGGATCTGATTCG ATGCTGATGATAAAAACACG	40	5691–5710 (B1c) 5770–5751 (B2)
ToMMV_2_LF	TCACTAACTCCATAACTCTCCTGGT	25	5652–5628 (LF)
ToMMV_2_LB	ATGTCTTACGCTATTACTTCTCCGT	25	5719–5743 (LB)

* Genome position refers to the nucleotide sequence of Tomato mottle mosaic virus (ToMMV) isolate MX5(GenBank accession no. KF477193). ** F1c and B1c indicate the complementary sequences of F1 and B1.

Primer mix

Primer Mix (10x) *	µL	Final concentration	Working concentration
FOP	1	2 µM	100 µM
BOP	1	2 µM	100 µM
FIP	8	16 µM	100 µM
BIP	8	16 µM	100 µM
LF	4	8 µM	100 µM
LB	4	8 µM	100 µM
H_2O	24	/	/
total	50	/	/

* prepare 10x Primer Mix before the analyses as in the table above and use it to prepare reaction mixture

Master Mix: Optigen

Reaction mixture		Working concentration	Volume per reaction (µL)	Volume per reaction (µL)+10%	For 48 reactions
Water		/	3.75	4.125	198
Isothermal Master Mix (Optigene Ltd., Horsham, UK)		2x	12.5	13.75	660
FOP	10x primer mix	/	2.5	2.75	132
BOP					
FIP					
BIP					
LF					
LB					
Transcriptor RT reaction buffer (Roche)		5x	1	1.1	52.8
Transcriptor RT (Roche)		20 U/µL	0.25	0.275	13.2
Subtotal		/	20	22	1056
RNA		/	5	/	
Total		/	25	/	

LAMP amplification
amplification conditions: 65°C for 60 min
melting curve analysis: 98–80°C, Ramp rate 0.05°C per second

Interpretation of the results:
Positive test: Amplification curve is exponential. The expected Tm (melting temperature) on GenieII: 83.5 and 84.5°C. Read the time of positivity (Tp) in min.
Negative: no exponential amplification curve

Appendix 3: Results of homogeneity and stability testing

Results of homogeneity and stability testing for randomly selected aliquots of samples.
Samples were in two technical replicates. Legend: pos=positive; neg=negative; inc=inconclusive

		panel code:		L40									L17		L6			L9	
		storage condition:		-20 °C									-20 °C -> 1 week at room T -> '-20 °C		-20 °C -> 1 week at room T -> '-20 °C			-20 °C -> 3 days -> '-20 °C	
		date of testing:		February 2024									March 2024		April 2024			July 2024	
				RT-PCR			RT-qPCR[b]					LAMP	RT-qPCR[b]	RT-PCR	RT-qPCR[b]	RT-PCR	RPA	RT-qPCR[b]	RT-PCR
Virus	NIB ID	Dilution factor	Sample ID	Levitzky et al. (2019)[a]	Loewe (Cat. No. 09181)	Sui et al. (2017)	DAFF DEECA	Fowkes et al. (2022)	ISF[c]	Tiberini et al. (2022) singleplex	Tiberini et al. (2022) duplex[c]	Kimura et al. (2023)[d]	Tiberini et al. (2022) singleplex	Sui et al. (2017)	Tiberini et al. (2022) singleplex	Levitzky et al. (2019)[e]	Agdia RPA (XCS 22800)	Tiberini et al. (2022) singleplex	Levitzky et al. (2019)[e]
healthy tomato seed	D1977/23 + D93/23	25x	S-12	neg	neg	neg	inc (39)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
healthy tomato seed	D1977/23	25x	S-15	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
CGMMV	NIB V 403	25x	S-3	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
ObPV	NIB V 364	25x	S-19	pos (ObPV)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	inc	neg	neg	pos
ORSV	NIB V 365	25x	S-17	inc (ORSV)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
PaMMV	NIB V 366	25x	S-13	pos (PaMMV)	neg	neg	inc (34)	inc (36)	inc (33)	inc (35)	inc (37)	neg	inc (35)	neg	inc (35)	pos	pos	inc (36)	pos
PMMoV	NIB V 408	25x	S-11	pos (PMMoV)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	pos
TMGMV	NIB V 404	25x	S-5	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
TMV	NIB V 405	25x	S-8	pos (TMV)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	pos
TMV	NIB V 413	25x	S-22	pos (TMV)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	pos
ToBRFV	NIB V 331	25x	S-9	pos (ToBRFV)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	pos
ToMV	NIB V 410	25x	S-16	pos (ToMV)	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	pos
ToMV	NIB V 406	25x	S-18	pos (nd)	neg	neg	neg	neg	neg	neg/ 39[f]	neg	neg	neg	neg	neg	pos	neg	neg	pos
ToMMV	NIB V 373	2.5×10^{8}	S-21	neg	neg	neg	inc (37)	neg	inc (38)	neg/ 39[f]	inc (38)	neg	neg/ 38[f]	neg	neg/ 36[f]	neg	neg	inc (37)	neg
ToMMV	NIB V 373	2.5×10^{7}	S-6	neg	neg	neg	inc (34)	inc (36)	inc (33)	pos (34)	pos (34)	neg	inc (35)	neg	inc (36)	neg	pos	inc (36)	neg
ToMMV	NIB V 373	2.5×10^{6}	S-4	inc (nd)	neg	neg	pos (30)	pos (33)	pos (30)	pos (31)	pos (31)	pos (59)	pos (31)	neg	pos (30)	neg	pos	pos (31)	pos
ToMMV	NIB V 373	2.5×10^{5}	S-10	pos (nd)	neg	pos	pos (27)	pos (30)	pos (26)	pos (28)	pos (27)	pos (41)	pos (28)	pos	pos (28)	pos	pos	pos (28)	pos
ToMMV	NIB V 373	2.5×10^{4}	S-20	pos (ToMMV)	pos	pos	pos (24)	pos (27)	pos (23)	pos (24)	pos (24)	pos (31)	pos (24)	pos	pos (24)	pos	pos	pos (24)	pos
ToMMV	NIB V 373	2.5×10^{3}	S-2	pos (ToMMV)	pos	pos	pos (20)	pos (24)	pos (19)	pos (21)	pos (21)	pos (27)	pos (21)	pos	pos (21)	pos	pos	pos (21)	pos
ToMMV	NIB V 373	2.5×10^{2}	S-14	pos (ToMMV)	pos	pos	pos (17)	pos (20)	pos (16)	pos (18)	pos (18)	pos (24)	pos (18)	pos	pos (18)	pos	pos	pos (18)	pos
ToMMV	NIB V 414	$2x\ 10^{1}$	S-7	neg	neg	neg	pos (31)	pos (32)	pos (32)	pos (32)	pos (32)	pos (49)	pos (32)	neg	pos (32)	neg	pos	pos (32)	neg
ToMMV	NIB V 414	2x	S-1	neg	neg	pos	pos (28)	pos (30)	pos (28)	pos (29)	pos (29)	pos (41)	pos (29)	inc	pos (29)	neg	pos	pos (29)	inc
healthy tomato leaves	/	1x	neg tomato	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
ToMMV	NIB V 373	2.5×10^{1}	pos ToMMV	pos (ToMMV)	pos	pos	pos (14)	pos (17)	pos (12)	pos (15)	pos (15)	pos (21)	pos (15)	pos	pos (15)	pos	pos	pos (15)	pos

[a]In bracket, result of Sanger sequencing is given (nd – not determined due low sequence quality); [b]In bracket, Cq value is given for pos/ inc result; [c]Results for ToMMV only is given ; [d]In bracket, Tp in minutes is given for pos result;
[e]Sanger sequencing not performed; [f]each sample was tested in two parallels and only in case of different results the results of both parallels are given

Appendix 4: Rawdata submitted by participants

Legend and general notes:

Legend:

1	Positive
2	Inconclusive
0	Negative
nt	Test was not performed

FP	false positive
FN	false negative
INC	inconclusive final result

Notes:

One lab has tested some samples in two or even three runs of the same test. Only the results of the first run are considered, except in cases where it was obvious that there was a technical error in the first run.

The rules for sample termination in RT-qPCRs differ greatly from laboratory to laboratory. Therefore, the same rules were applied for all RT-qPCRs and all labs:

- all Cq values equal to or higher than the Cq values determined for PaMMV or healthy samples were considered inconclusive. In the absence of signal for PaMMV and healthy samples, all Cq values of 37 or higher were considered inconclusive results.

- deviations between the parallels were concluded as follows: positive + inconclusive = inconclusive, positive + negative = inconclusive; negative + inconclusive = negative

RT-PCR Levitzky et al. (2019)

		Tobamovirus detected*								
Sample description	Sample	Health status	L4	L10	L14	L16	L18	L19	L33	L34
healthy tomato seed	S-12	0	0	0	0	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	1	1	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	1	1	1	1	1	1	1	1	1
ORSV (NIB V 365) 25x	S-17	1	1	1	1	0	1	1	1	1
PaMMV (NIB V 366) 25x	S-13	1	1	1	1	1	1	1	1	1
PMMoV (NIB V 408) 25x	S-11	1	1	1	1	1	1	1	1	1
TMGMV (NIB V 404) 25x	S-5	1	0	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	1	1	1	1	1	1	1	1	1
TMV (NIB V 413) 25x	S-22	1	1	1	1	1	1	1	1	1
ToBRFV (NIB V 331) 25x	S-9	1	1	1	1	1	1	1	1	1
ToMV (NIB V 410) 25x	S-16	1	1	1	1	1	1	1	1	1
ToMV (NIB V 406) 25x	S-18	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	1	1	1	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	0	1	0	0	0	0	0
ToMMV (NIB V 414) 2x	S-1	1	1	1	1	0	0	1	0	0
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1	1	1

*Data not included in the further evaluation

		Sequencing result: ToMMV identified								
Sample description	Sample	Health status	L4	L10	L14	L16	L18	L19	L33	L34
healthy tomato seed	S-12	0	0	0	0	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	0	0	0
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	0	0	0	0
PaMMV (NIB V 366) 25x	S-13	0	0	0	0	0	0	0	0	0
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	0	0	0	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0	0	0
ToMV (NIB V 410) 25x	S-16	0	0	0	0	0	0	0	0	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	1	1	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	1	1	1	0	1	0	0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	0	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	0	0	0	0	0	0	0
ToMMV (NIB V 414) 2x	S-1	1	1	1	0	0	0	1	0	0
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1	1	1
		FP	0	0	0	0	0	0	0	0
		FN	2	2	4	4	5	3	5	5
		INC	0	0	0	0	0	0	0	0

Reported deviations from the protocol:

L4: Superscript III plus Platinum Taq (Invitrogen) was used instead of Qiagen OneStep RT-PCR

-> The results of this lab were not excluded from further analysis as no influence on the final ToMMV results was detected

Note: If sequencing was not performed because no or only a weak band was obtained in the PCR, the sample is considered to be ToMMV negative

		Other tobamovirus identified*								
Sample description	Sample	Health status	L4	L10	L14	L16	L18	L19	L33	L34
healthy tomato seed	S-12	0	0	0	0	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	CGMMV	0	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	ObPV	ObPV	ObPV	ObPV	ObPV/ PaMMV	ObPV	ObPV	ObPV	ObPV
ORSV (NIB V 365) 25x	S-17	ORSV	ORSV	ORSV	ORSV	0	ORSV	ORSV	ORSV	0
PaMMV (NIB V 366) 25x	S-13	PaMMV	PaMMV	PaMMV	PaMMV	PMMoV	PaMMV	PaMMV	PaMMV	PaMMV
PMMoV (NIB V 408) 25x	S-11	PMMoV	PMMoV	PMMoV	PMMoV	PMMoV	PMMoV	PMMoV	PMMoV	PMMoV
TMGMV (NIB V 404) 25x	S-5	TMGMV	0	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	TMV	TMV	TMV	TMV	TMV	TMV	TMV	TMV	TMV
TMV (NIB V 413) 25x	S-22	TMV	TMV	TMV	TMV	TMV	TMV	TMV	TMV	TMV
ToBRFV (NIB V 331) 25x	S-9	ToBRFV	ToBRFV	ToBRFV	ToBRFV	ToBRFV	ToBRFV	ToBRFV	ToBRFV	ToBRFV
ToMV (NIB V 410) 25x	S-16	ToMV	ToMV	ToMV	ToMV	ToMV	ToMV	ToMV	ToMV	ToMV
ToMV (NIB V 406) 25x	S-18	ToMV	ToMV	ToMV	ToMV	ToMV	ToMV	ToMV	ToMV	ToMV
ToMMV (NIB V 373) 2.5 x 10^8	S-21	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^6	S-4	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 414) 2 x 10^1	S-7	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 414) 2x	S-1	0	0	0	0	0	0	0	0	0
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	ToMMV	ToMMV	ToMMV	ToMMV	ToMMV	ToMMV	ToMMV	ToMMV	ToMMV

*Data not included in the further evaluation

RT-PCR Loewe (Cat no. 09181)

Sample description	Sample	Health status	L4	L10	L14	L16	L18	L19	L33	L34
healthy tomato seed	S-12	0	nt	0	0	0	nt	0	nt	0
healthy tomato seed	S-15	0	nt	0	0	0	nt	0	nt	0
CGMMV (NIB V 403) 25x	S-3	0	nt	0	0	0	nt	0	nt	0
ObPV (NIB V 364) 25x	S-19	0	nt	0	0	0	nt	0	nt	0
ORSV (NIB V 365) 25x	S-17	0	nt	0	0	0	nt	0	nt	0
PaMMV (NIB V 366) 25x	S-13	0	nt	0	0	0	nt	0	nt	0
PMMoV (NIB V 408) 25x	S-11	0	nt	0	0	0	nt	0	nt	0
TMGMV (NIB V 404) 25x	S-5	0	nt	0	0	0	nt	0	nt	0
TMV (NIB V 405) 25x	S-8	0	nt	0	0	0	nt	0	nt	0
TMV (NIB V 413) 25x	S-22	0	nt	0	0	0	nt	0	nt	0
ToBRFV (NIB V 331) 25x	S-9	0	nt	0	0	0	nt	0	nt	0
ToMV (NIB V 410) 25x	S-16	0	nt	0	0	0	nt	0	nt	0
ToMV (NIB V 406) 25x	S-18	0	nt	0	0	0	nt	0	nt	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	nt	0	0	0	nt	0	nt	0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	nt	0	0	0	nt	0	nt	0
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	nt	0	0	0	nt	0	nt	0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	nt	0	0	0	nt	2	nt	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	nt	1	1	0	nt	1	nt	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	nt	1	1	0	nt	1	nt	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	nt	1	1	1	nt	1	nt	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	nt	0	0	0	nt	0	nt	0
ToMMV (NIB V 414) 2x	S-1	1	nt	0	1	0	nt	0	nt	0
healthy tomato leaves	NC	0	nt	0	0	0	nt	0	nt	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	nt	1	1	1	nt	1	nt	1
		FP		0	0	0		0		0
		FN		6	5	8		5		5
		INC		0	0	0		1		0

Reported remark relevant for data evaluation:
L16: "Several containers were not properly sealed. The vessel with the DTT had crystallized and was also not properly sealed."
-> The results of this lab were excluded from further analysis as this could influence the results (this lab reported more false negative (FN) results compared to other labs)

Test was not performed by L4, L18 and L33 - reason:
Kit not received in time (2 labs) or no possibility to order the kit in the country where the lab is located (1 lab)

RT-PCR Sui et al. (2017)

sample description	sample	Health status	L4	L10	L14	L16	L18	L19	L33	L34
healthy tomato seed	S-12	0	0	0	0	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	0	0	0
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	0	0	0	0
PaMMV (NIB V 366) 25x	S-13	0	1	0	0	0	0	0	0	0
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	0	0	0	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0	0	0
ToMV (NIB V 410) 25x	S-16	0	0	0	0	0	0	0	0	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	1	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	1	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	0	1	0	0	2	0	0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	0	1	1	0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	0	1	0	0	2	0	0
ToMMV (NIB V 414) 2x	S-1	1	1	1	1	0	0	1	0	0
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1	1	1
		FP	1	0	0	0	0	0	0	0
		FN	0	4	2	5	6	2	5	6
		INC	0	0	0	0	0	2	0	0

Reported deviations from the protocol:

L4: Superscript III plus Platinum Taq (Invitrogen) was used.

-> The results of this lab were excluded from further analysis as this deviation from the protocol seems to have an impact on the sensitivity and specificity of the test

L34: RNaseOUT™ was omitted

-> The results of this lab were not excluded from further analysis as no influence on the final results was detected (false negative (FN) results in the same range as for other labs)

RT-qPCR DAFF DEECA

Results per well:

Cq values are presented if amplification curve was observed; und - no amplification

sample description	sample	rep.	L1	L2	L12	L20	L23	L25	L27	L28	L29	L30	L36	L37	L38
healthy tomato seed	S-12	1	und	und	und	und	und	und	und	und	und	und	und	und	und
healthy tomato seed	S-12	2	und	und	und	und	und	und	und	und	und	und	und	und	und
healthy tomato seed	S-15	1	und	38	und	und	und	und	und	und	38	und	und	und	und
healthy tomato seed	S-15	2	und	und	und	und	und	und	und	und	und	und	und	und	und
CGMMV (NIB V 403) 25x	S-3	1	und	und	und	und	und	und	und	und	und	und	und	und	und
CGMMV (NIB V 403) 25x	S-3	2	und	und	und	und	und	und	und	und	und	und	und	und	und
ObPV (NIB V 364) 25x	S-19	1	und	und	und	und	und	und	und	und	37	und	und	und	und
ObPV (NIB V 364) 25x	S-19	2	und	und	und	und	und	und	38	und	und	und	und	und	und
ORSV (NIB V 365) 25x	S-17	1	und	und	und	und	und	und	und	und	und	und	und	und	und
ORSV (NIB V 365) 25x	S-17	2	und	und	und	und	und	und	und	und	und	und	und	und	und
PaMMV (NIB V 366) 25x	S-13	1	35	33	und	32	33	31	33	35	31	34	34	33	34
PaMMV (NIB V 366) 25x	S-13	2	35	34	und	32	33	32	33	34	31	34	35	34	34
PMMoV (NIB V 408) 25x	S-11	1	und	und	und	und	und	und	und	und	39	und	und	und	und
PMMoV (NIB V 408) 25x	S-11	2	und	und	und	und	und	und	und	und	36	und	und	und	und
TMGMV (NIB V 404) 25x	S-5	1	und	und	und	und	und	und	und	und	und	und	und	und	und
TMGMV (NIB V 404) 25x	S-5	2	und	und	und	und	und	und	und	und	und	und	und	und	und
TMV (NIB V 405) 25x	S-8	1	und	und	und	und	und	und	und	und	und	und	und	und	und
TMV (NIB V 405) 25x	S-8	2	und	und	und	und	und	und	und	und	und	und	und	und	und
TMV (NIB V 413) 25x	S-22	1	und	und	und	und	und	und	und	und	und	und	und	und	und
TMV (NIB V 413) 25x	S-22	2	und	und	und	und	und	und	und	und	und	und	und	und	und
ToBRFV (NIB V 331) 25x	S-9	1	und	und	und	und	und	und	und	und	39	und	und	und	und
ToBRFV (NIB V 331) 25x	S-9	2	und	und	und	und	und	und	und	und	und	und	und	und	und
ToMV (NIB V 410) 25x	S-16	1	und	und	und	und	und	und	und	und	und	und	und	und	und
ToMV (NIB V 410) 25x	S-16	2	und	und	und	und	und	und	und	und	und	und	und	und	und
ToMV (NIB V 406) 25x	S-18	1	und	und	und	38	und	und	und	und	und	und	und	und	und
ToMV (NIB V 406) 25x	S-18	2	und	und	und	38	und	und	und	und	und	und	und	und	und
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	37	37	37	36	35	35	und	36	34	37	39	38	38
ToMMV (NIB V 373) 2.5 x 10^8	S-21	2	39	37	35	36	34	35	37	37	37	38	39	37	37

ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	34	34	und	32	33	31	33	35	32	35	35	34	34
ToMMV (NIB V 373) 2.5 x 10^7	S-6	2	34	34	39	33	33	32	33	34	31	36	35	34	34
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	31	30	38	29	29	28	30	31	29	31	31	31	31
ToMMV (NIB V 373) 2.5 x 10^6	S-4	2	31	30	34	29	29	28	30	30	29	32	31	31	31
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	28	27	31	26	25	25	26	27	25	28	28	27	27
ToMMV (NIB V 373) 2.5 x 10^5	S-10	2	28	27	31	26	25	25	26	27	25	28	28	27	27
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	24	23	24	23	21	22	24	24	21	25	24	24	24
ToMMV (NIB V 373) 2.5 x 10^4	S-20	2	24	24	21	23	22	22	24	24	21	25	25	24	24
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	23	20	27	19	18	18	20	20	19	22	21	21	21
ToMMV (NIB V 373) 2.5 x 10^3	S-2	2	23	20	27	19	18	18	20	20	19	22	21	21	21
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	18	17	21	16	14	15	16	17	15	18	18	17	17
ToMMV (NIB V 373) 2.5 x 10^2	S-14	2	18	17	20	16	15	15	16	17	15	18	18	17	17
ToMMV (NIB V 414) 2 x 10^1	S-7	1	32	32	38	31	30	30	31	33	30	32	32	31	32
ToMMV (NIB V 414) 2 x 10^1	S-7	2	32	31	36	31	30	29	31	33	29	33	33	31	32
ToMMV (NIB V 414) 2x	S-1	1	29	28	35	27	26	26	27	32	26	29	29	29	28
ToMMV (NIB V 414) 2x	S-1	2	29	28	35	27	27	26	27	32	27	29	29	28	28
healthy tomato leaves	NC	1	und	und	und	und	und	und	und	und	und	und	und	und	und
healthy tomato leaves	NC	2	und	und	und	und	und	und	und	und	und	und	und	und	nt
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	14	13	11	12	12	11	13	14	12	15	15	13	14
ToMMV (NIB V 373) 2.5 x 10^1	PC	2	14	13	11	12	12	12	13	14	12	15	14	14	14
Cq cut off value determined by participant			≤37	/	/	37	/	/	/	/	<37	35,36	<37	38	/
Cq values for inconclusive results			≥35	≥33	≥37	≥32	≥33	≥31	≥33	≥34	≥31	≥34	≥34	≥33	≥34

Reported deviations from the protocol:

L20: Modification in CSP1572-P (Yakima Yellow-TGCCACTCGCAGAGTGGACGATGCTACG-BHQ-1)

L28: OneTube RT-PCR TaqMan (Evrogen) was used instead of AgPath-ID one step RT-PCR reagents

L30: One step iTaq universal probe mastermix was used and consequently the following changes has been done:
Reverse transcription at 48 °C for 15 min; denaturation at 95 °C for 3 min; 40 cycles of denaturation at 95 °C for 15 s
and annealing and elongation at 60 °C for 60 s.

-> The results of these 3 labs were not excluded from further analysis as no influence on the final results was detected
(inconclusive (INC) results in the same range as for other labs)

Conclusions done by participants:

sample description	sample	Health status	L1	L2	L12	L20	L23	L25	L27	L28	L29	L30	L36	L37	L38
healthy tomato seed	S-12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
healthy tomato seed	S-15	0	0	2	0	0	0	0	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	0	2	0	0	0	0	0	0
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PaMMV (NIB V 366) 25x	S-13	0	1	1	0	1	1	1	1	1	1	1	1	1	1
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 410) 25x	S-16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	0	1	2	1	1	2	2	1	0	0	0	1	1
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	1	1	0	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	1	2	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	1	0	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2x	S-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Conclusions done by TPS organizer and used for further evaluation:

sample description	sample	Health status	L1	L2	L12	L20	L23	L25	L27	L28	L29	L30	L36	L37	L38
healthy tomato seed	S-12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PaMMV (NIB V 366) 25x	S-13	0	2	2	0	2	2	2	2	2	2	2	2	2	2
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0	0	0	2	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 410) 25x	S-16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	2	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	2	2	2	2	2	2	0	2	2	2	2	2	2
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	1	2	0	2	2	2	2	2	2	2	2	2	2
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	1	2	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	1	2	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2x	S-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		FP	0	0	0	0	0	0	0	0	0	0	0	0	0
		FN	0	0	1	0	0	0	1	0	0	0	0	0	0
		INC	2	3	3	4	3	3	2	3	4	3	3	3	3

RT-qPCR Fowkes et al. (2022)

Results per well:

Cq values are presented if amplification curve was observed; und - no amplification

sample description	sample	rep.	L1	L2	L12	L20	L23	L25	L27	L28	L29	L30	L36	L37	L38
healthy tomato seed	S-12	1	und	und	und	und	38	und	und	und	und	und	und	und	und
healthy tomato seed	S-12	2	und	und	und	und	36	und	und	und	und	und	und	und	und
healthy tomato seed	S-15	1	und	und	und	und	34	und	und	und	und	und	und	und	und
healthy tomato seed	S-15	2	und	und	und	und	36	und	und	und	und	und	und	und	und
CGMMV (NIB V 403) 25x	S-3	1	und	und	und	und	und	und	und	und	und	und	und	und	und
CGMMV (NIB V 403) 25x	S-3	2	und	und	und	und	und	und	und	und	37	und	und	und	und
ObPV (NIB V 364) 25x	S-19	1	und	und	und	und	und	und	und	und	und	und	und	und	und
ObPV (NIB V 364) 25x	S-19	2	und	und	und	und	und	und	und	und	und	und	und	und	und
ORSV (NIB V 365) 25x	S-17	1	und	und	und	und	und	und	und	und	und	und	und	und	und
ORSV (NIB V 365) 25x	S-17	2	und	und	und	und	und	und	und	und	und	und	und	und	und
PaMMV (NIB V 366) 25x	S-13	1	36	36	38	35	und	36	34	38	35	37	36	33	35
PaMMV (NIB V 366) 25x	S-13	2	35	36	37	35	und	36	34	36	36	36	36	34	34
PMMoV (NIB V 408) 25x	S-11	1	und	und	und	und	und	und	und	und	und	und	und	und	und
PMMoV (NIB V 408) 25x	S-11	2	und	und	und	und	und	und	und	und	und	und	und	und	und
TMGMV (NIB V 404) 25x	S-5	1	und	und	und	und	und	und	und	und	und	und	und	und	und
TMGMV (NIB V 404) 25x	S-5	2	und	und	und	und	und	und	und	und	37	und	und	und	und
TMV (NIB V 405) 25x	S-8	1	39	und	und	und	und	und	und	und	und	und	und	und	und
TMV (NIB V 405) 25x	S-8	2	und	und	und	und	und	und	und	und	und	und	und	und	und
TMV (NIB V 413) 25x	S-22	1	und	und	und	und	und	und	und	und	und	und	und	und	und
TMV (NIB V 413) 25x	S-22	2	und	und	und	und	und	und	und	und	37	und	und	und	und
ToBRFV (NIB V 331) 25x	S-9	1	und	und	und	und	und	und	und	und	und	und	und	und	und
ToBRFV (NIB V 331) 25x	S-9	2	und	und	und	und	und	und	und	und	und	und	und	und	und
ToMV (NIB V 410) 25x	S-16	1	und	und	und	und	und	und	und	und	und	und	und	und	und
ToMV (NIB V 410) 25x	S-16	2	und	und	und	und	und	und	und	und	und	und	und	und	und
ToMV (NIB V 406) 25x	S-18	1	und	und	und	und	und	und	und	und	und	und	und	und	und
ToMV (NIB V 406) 25x	S-18	2	und	und	und	und	und	und	und	und	und	und	und	und	und
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	39	und	und	und	und	38	und	39	38	und	und	38	und

ToMMV (NIB V 373) 2.5 x 10^8	S-21	2	39	und	und	und	und	38	und	und	37	und	und	37	37
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	36	35	und	36	34	36	35	37	36	36	37	35	36
ToMMV (NIB V 373) 2.5 x 10^7	S-6	2	38	35	und	36	34	36	35	36	36	37	37	35	34
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	33	32	34	32	32	34	32	33	32	34	34	31	32
ToMMV (NIB V 373) 2.5 x 10^6	S-4	2	33	33	33	33	33	34	33	33	32	34	34	31	32
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	31	29	30	29	27	30	29	30	29	30	30	28	29
ToMMV (NIB V 373) 2.5 x 10^5	S-10	2	31	29	30	30	27	30	29	31	29	30	31	28	29
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	26	26	27	26	25	27	26	27	26	27	27	25	25
ToMMV (NIB V 373) 2.5 x 10^4	S-20	2	27	26	27	26	25	27	26	29	25	27	27	25	25
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	24	23	und	24	21	23	23	24	23	24	24	21	22
ToMMV (NIB V 373) 2.5 x 10^3	S-2	2	24	23	und	23	22	24	23	23	23	24	24	21	22
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	20	19	21	19	21	20	20	20	19	20	20	18	18
ToMMV (NIB V 373) 2.5 x 10^2	S-14	2	20	19	21	19	17	20	19	20	19	20	21	18	18
ToMMV (NIB V 414) 2 x 10^1	S-7	1	32	32	33	32	31	32	32	33	32	34	33	31	31
ToMMV (NIB V 414) 2 x 10^1	S-7	2	33	32	33	32	31	33	32	33	31	34	33	31	31
ToMMV (NIB V 414) 2x	S-1	1	29	29	30	29	27	30	29	31	29	31	30	28	28
ToMMV (NIB V 414) 2x	S-1	2	29	29	30	29	27	30	29	30	28	31	30	28	28
healthy tomato leaves	NC	1	und	und	und	und	und	und	und	und	und	und	und	und	und
healthy tomato leaves	NC	2	und	und	und	und	und	und	und	und	und	und	und	und	und
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	16	16	17	16	15	17	16	17	16	17	17	14	15
ToMMV (NIB V 373) 2.5 x 10^1	PC	2	16	16	17	18	15	17	16	18	15	17	17	14	15
Cq cut off value determined by participant			≤37	/	/	/	/	/	/	/	<37,00	34,94	/	38	/
Cq values for inconclusive results			≥35	≥36	≥37	≥35	≥34	≥36	≥34	≥36	≥35	≥36	≥36	≥33	≥34

Reported deviations from the protocol:

L28: OneTube RT-PCR TaqMan (Evrogen) was used instead of iTaq Universal One-Step kit

L30: the following changes has been done: Reverse transcription at 48 °C for 15 min; denaturation at 95 °C for 3 min;
40 cycles of denaturation at 95 °C for 15 s and annealing and elongation at 60 °C for 60 s.

L37: AgPath-ID one step RT-PCR reagents were used instead of iTaq Universal One-Step kit

-> The results of these 3 labs were not excluded from further analysis as no influence on the final results was detected
(inconclusive (INC) and false negative (FN) results in the same range as for other labs)

Conclusions done by participants:

sample description	sample	Health status	L1	L2	L12	L20	L23	L25	L27	L28	L29	L30	L36	L37	L38
healthy tomato seed	S-12	0	0	0	0	0	2	0	0	0	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	1	0	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PaMMV (NIB V 366) 25x	S-13	0	1	1	2	1	0	1	1	1	1	0	1	1	1
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	0	0	0	0	0	0	1	0	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 410) 25x	S-16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	0	0	0	0	0	2	0	2	0	0	0	1	2
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	1	1	0	1	1	1	1	1	1	0	1	1	1
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	0	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2x	S-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Conclusions done by TPS organizer and used for further evaluation:

sample description	sample	Health status	L1	L2	L12	L20	L23	L25	L27	L28	L29	L30	L36	L37	L38
healthy tomato seed	S-12	0	0	0	0	0	2	0	0	0	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	2	0	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PaMMV (NIB V 366) 25x	S-13	0	2	2	2	2	0	2	2	2	2	2	2	2	2
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 410) 25x	S-16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	2	0	0	0	0	2	0	0	2	0	0	2	0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	2	1	0	2	2	2	2	2	2	2	2	2	2
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	0	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	1	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2x	S-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		FP	0	0	0	0	0	0	0	0	0	0	0	0	0
		FN	0	1	3	1	1	0	1	1	0	1	1	0	1
		INC	3	1	1	2	3	3	2	2	3	2	2	3	2

RT-qPCR ISF

Results per well:

Cq values are presented if amplification curve was observed; und - no amplification

sample description	sample	rep.	L1	L2	L12	L20 cata9	L20 ToMM V2*	L23	L25	L27	L28	L29	L30	L36	L37 CSP1572	L37 Cata9*	L38
healthy tomato seed	S-12	1	und	und	und	39	und	und	und	und	nt	und	und	und	und	und	und
healthy tomato seed	S-12	2	und	und	und	37	und	und	und	und	nt	und	und	und	und	und	und
healthy tomato seed	S-15	1	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
healthy tomato seed	S-15	2	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
CGMMV (NIB V 403) 25x	S-3	1	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	30
CGMMV (NIB V 403) 25x	S-3	2	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
ObPV (NIB V 364) 25x	S-19	1	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
ObPV (NIB V 364) 25x	S-19	2	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
ORSV (NIB V 365) 25x	S-17	1	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
ORSV (NIB V 365) 25x	S-17	2	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
PaMMV (NIB V 366) 25x	S-13	1	35	34	32	31	33	31	32	30	nt	28	33	34	33	32	31
PaMMV (NIB V 366) 25x	S-13	2	35	33	31	31	33	31	32	30	nt	29	33	34	33	32	31
PMMoV (NIB V 408) 25x	S-11	1	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
PMMoV (NIB V 408) 25x	S-11	2	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
TMGMV (NIB V 404) 25x	S-5	1	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
TMGMV (NIB V 404) 25x	S-5	2	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
TMV (NIB V 405) 25x	S-8	1	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
TMV (NIB V 405) 25x	S-8	2	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
TMV (NIB V 413) 25x	S-22	1	und	und	und	35	37	und	und	und	nt	und	und	und	und	und	und
TMV (NIB V 413) 25x	S-22	2	und	und	und	35	und	und	und	und	nt	und	und	und	und	und	und
ToBRFV (NIB V 331) 25x	S-9	1	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
ToBRFV (NIB V 331) 25x	S-9	2	und	und	und	und	und	und	und	38	nt	und	und	und	und	und	und
ToMV (NIB V 410) 25x	S-16	1	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
ToMV (NIB V 410) 25x	S-16	2	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
ToMV (NIB V 406) 25x	S-18	1	und	und	39	und	und	und	und	und	nt	und	und	und	und	und	und
ToMV (NIB V 406) 25x	S-18	2	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	38	37	35	35	39	34	36	33	nt	33	38	38	38	35	35

ToMMV (NIB V 373) 2.5 x 10^8	S-21	2	38	38	35	34	und	34	36	34	nt	32	38	38	37	36	35
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	36	34	32	32	34	31	34	31	nt	29	33	34	33	32	29
ToMMV (NIB V 373) 2.5 x 10^7	S-6	2	35	34	32	31	37	31	33	31	nt	29	34	34	33	32	29
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	32	31	28	28	32	27	28	28	nt	26	29	31	30	29	28
ToMMV (NIB V 373) 2.5 x 10^6	S-4	2	32	31	28	28	32	27	28	27	nt	26	29	31	30	29	28
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	29	27	25	25	29	24	24	23	nt	23	26	28	26	25	24
ToMMV (NIB V 373) 2.5 x 10^5	S-10	2	29	27	25	25	29	24	26	25	nt	23	26	28	26	26	24
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	26	24	22	21	25	20	22	21	nt	20	22	24	23	22	20
ToMMV (NIB V 373) 2.5 x 10^4	S-20	2	26	24	22	21	25	20	21	21	nt	20	22	24	23	22	20
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	22	21	19	18	22	17	19	17	nt	17	20	21	20	18	18
ToMMV (NIB V 373) 2.5 x 10^3	S-2	2	22	21	19	17	22	17	19	17	nt	17	20	21	20	19	18
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	19	17	15	14	18	14	16	14	nt	13	16	18	16	15	15
ToMMV (NIB V 373) 2.5 x 10^2	S-14	2	19	17	15	14	18	14	15	14	nt	13	16	18	16	16	13
ToMMV (NIB V 414) 2 x 10^1	S-7	1	33	33	30	30	31	29	32	29	nt	28	32	32	30	30	28
ToMMV (NIB V 414) 2 x 10^1	S-7	2	33	33	30	30	31	29	32	29	nt	27	32	33	31	31	28
ToMMV (NIB V 414) 2x	S-1	1	30	30	26	27	28	25	29	26	nt	25	28	30	28	27	25
ToMMV (NIB V 414) 2x	S-1	2	30	30	27	27	28	25	29	25	nt	24	28	30	27	27	26
healthy tomato leaves	NC	1	und	und	und	und	und	und	und	und	nt	und	und	und	und	und	14
healthy tomato leaves	NC	2	38	und	und	und	und	und	und	und	nt	und	und	und	und	und	und
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	14	14	12	11	14	11	13	11	nt	10	13	14	12	12	10
ToMMV (NIB V 373) 2.5 x 10^1	PC	2	14	11	12	11	15	20	13	11	nt	10	13	14	12	12	9
Cq cut off value determined by participant			≤35	/	/	35	35	/	/	/	nt	<30,0(	37,04	/	38	38	/
Cq values for inconclusive results			≥35	≥33	≥31	≥31	≥33	≥31	≥32	≥30		≥28	≥33	≥34	≥33	≥32	≥31

*Data not included in the further evaluation

Reported deviations from the protocol:

L20: Modification in ToMMV2 Pr (Yakima Yellow-CGA TGC TAC GGT TGC GAT CAG GTC-BHQ) and in Nad 5 (P-Texas red-AGG ATC CGC ATA GCC CTC GAT TTA TGT G-BHQ-2)

-> CaTa9 has not been changed and is only included in the evaluation

L30: One step iTaq universal probe mastermix was used and consequently the following changes has been done: Reverse transcription at 48 °C for 15 min; denaturation at 95 °C for 3 min; 40 cycles of denaturation at 95 °C for 15 s and annealing and elongation at 60 °C for 60 s.

L37: AgPath-ID one step RT-PCR reagents was used instead of Ultraplex 1-step ToughMix

L38: qScript XLT One-Step RT-qPCR ToughMix was used instead of Ultraplex 1-step ToughMix. Nad5 not included.

-> The results of these 3 labs (L30, L37, L38) were not excluded from further analysis as no influence on the final results was detected (inconclusive (INC) results in the same range as for other labs)

Conclusions done by participants:

sample description	sample	Health status	L1	L2	L12	L20	L23	L25	L27	L28	L29	L30	L36	L37	L38
healthy tomato seed	S-12	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0	0	nt	0	0	0	0	2
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
PaMMV (NIB V 366) 25x	S-13	0	0	1	1	1	1	1	1	nt	1	1	1	1	1
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0	2	nt	0	0	0	1	0
ToMV (NIB V 410) 25x	S-16	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	0	1	1	1	1	1	1	nt	0	0	0	1	1
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	0	1	1	1	1	1	1	nt	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	1	1	1	1	1	1	nt	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	1	1	1	nt	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1	1	nt	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	1	1	1	1	1	nt	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	1	1	1	1	1	1	1	nt	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	1	1	1	1	1	1	nt	1	1	1	0	1
ToMMV (NIB V 414) 2x	S-1	1	1	1	1	1	1	1	1	nt	1	1	1	1	1
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	nt	0	0	0	0	2
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1	1	nt	1	1	1	1	1

Conclusions done by TPS organizer and used for further evaluation:

sample description	sample	Health status	L1	L2	L12	L20	L23	L25	L27	L28	L29	L30	L36	L37	L38
healthy tomato seed	S-12	0	0	0	0	2	0	0	0	nt	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0	0	nt	0	0	0	0	2
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
PaMMV (NIB V 366) 25x	S-13	0	2	2	2	2	2	2	2	nt	2	2	2	2	2
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	0	2	0	0	0	nt	0	0	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
ToMV (NIB V 410) 25x	S-16	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	0	0	0	0	nt	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	2	2	2	2	2	2	2	nt	2	2	2	2	2
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	2	2	2	2	2	2	2	nt	2	2	2	2	1
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	1	1	1	1	1	1	nt	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	1	1	1	nt	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1	1	nt	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	1	1	1	1	1	nt	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	1	1	1	1	1	1	1	nt	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	2	1	1	1	2	1	nt	2	1	1	1	1
ToMMV (NIB V 414) 2x	S-1	1	1	1	1	1	1	1	1	nt	1	1	1	1	1
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	nt	0	0	0	0	2
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1	1	nt	1	1	1	1	1
		FP	0	0	0	0	0	0	0		0	0	0	0	0
		FN	0	0	0	0	0	0	0		0	0	0	0	0
		INC	3	4	3	5	3	4	3		4	3	3	3	3

RT-qPCR Tiberini et al. (2022) singleplex

Results per well:

Cq values are presented if amplification curve was observed; und - no amplification

sample description	sample	rep.	L2	L12	L23	L25	L27	L28	L30	L36	L37	L38
healthy tomato seed	S-12	1	und	und	und	und	und	und	und	und	und	und
healthy tomato seed	S-12	2	und	und	und	und	und	und	und	und	und	und
healthy tomato seed	S-15	1	und	und	und	und	und	und	und	und	und	und
healthy tomato seed	S-15	2	und	und	und	und	und	und	und	und	und	und
CGMMV (NIB V 403) 25x	S-3	1	und	und	und	und	und	und	und	und	und	und
CGMMV (NIB V 403) 25x	S-3	2	und	und	und	und	und	und	und	und	und	und
ObPV (NIB V 364) 25x	S-19	1	und	und	und	und	und	und	und	und	und	und
ObPV (NIB V 364) 25x	S-19	2	und	und	und	und	und	und	und	und	und	und
ORSV (NIB V 365) 25x	S-17	1	und	und	und	und	und	und	und	und	und	und
ORSV (NIB V 365) 25x	S-17	2	und	und	und	und	und	und	und	und	und	und
PaMMV (NIB V 366) 25x	S-13	1	und	33	und	35	und	37	37	34	36	33
PaMMV (NIB V 366) 25x	S-13	2	und	33	und	35	35	39	35	34	35	35
PMMoV (NIB V 408) 25x	S-11	1	und	und	und	und	und	und	und	und	und	und
PMMoV (NIB V 408) 25x	S-11	2	und	und	und	und	und	und	und	und	und	und
TMGMV (NIB V 404) 25x	S-5	1	und	und	und	und	und	und	und	und	und	und
TMGMV (NIB V 404) 25x	S-5	2	und	und	und	und	und	und	und	und	und	und
TMV (NIB V 405) 25x	S-8	1	und	und	und	und	und	und	und	und	und	und
TMV (NIB V 405) 25x	S-8	2	und	und	und	und	und	und	und	und	und	und
TMV (NIB V 413) 25x	S-22	1	und	und	und	und	und	und	und	und	und	und
TMV (NIB V 413) 25x	S-22	2	und	und	und	und	und	und	und	und	und	und
ToBRFV (NIB V 331) 25x	S-9	1	und	und	und	und	und	und	und	und	und	und
ToBRFV (NIB V 331) 25x	S-9	2	und	und	und	und	und	und	und	und	und	und
ToMV (NIB V 410) 25x	S-16	1	und	und	und	und	und	und	und	und	und	und
ToMV (NIB V 410) 25x	S-16	2	und	und	und	und	und	und	und	und	und	und
ToMV (NIB V 406) 25x	S-18	1	und	und	und	und	und	und	und	und	und	und
ToMV (NIB V 406) 25x	S-18	2	und	und	und	und	und	und	und	und	und	und
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	und	37	und	37	und	38	und	37	37	36
ToMMV (NIB V 373) 2.5 x 10^8	S-21	2	und	37	und	und	und	und	und	38	und	36

ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	und	33	35	35	35	und	35	34	35	33
ToMMV (NIB V 373) 2.5 x 10^7	S-6	2	und	34	und	34	35	37	35	34	35	34
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	31	31	32	31	31	33	32	30	32	30
ToMMV (NIB V 373) 2.5 x 10^6	S-4	2	30	30	30	31	31	33	32	31	32	30
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	27	27	26	28	28	29	28	27	29	28
ToMMV (NIB V 373) 2.5 x 10^5	S-10	2	27	27	26	28	28	29	29	27	29	27
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	23	23	23	25	24	25	26	24	26	24
ToMMV (NIB V 373) 2.5 x 10^4	S-20	2	23	23	23	25	24	26	26	24	26	24
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	21	20	21	21	21	23	23	20	23	21
ToMMV (NIB V 373) 2.5 x 10^3	S-2	2	21	19	19	21	21	23	22	20	23	20
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	17	16	17	18	18	19	19	17	18	17
ToMMV (NIB V 373) 2.5 x 10^2	S-14	2	17	16	16	18	18	19	19	17	19	17
ToMMV (NIB V 414) 2 x 10^1	S-7	1	30	32	31	32	31	33	34	32	32	32
ToMMV (NIB V 414) 2 x 10^1	S-7	2	30	32	30	32	31	33	33	32	32	32
ToMMV (NIB V 414) 2x	S-1	1	27	29	28	29	28	32	30	28	30	28
ToMMV (NIB V 414) 2x	S-1	2	27	29	27	29	28	31	30	29	29	29
healthy tomato leaves	NC	1	und	und	und	und	und	und	und	und	und	und
healthy tomato leaves	NC	2	und	und	und	und	und	und	und	und	und	nt
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	14	14	14	15	15	16	15	14	15	14
ToMMV (NIB V 373) 2.5 x 10^1	PC	2	14	14	13	15	15	16	15	14	15	15
Cq cut off value determined by participant			/	/	/	/	/	/	36,65	/	38	/
Cq values for inconclusive results			≥37	≥33	≥37	≥35	≥35	≥37	≥35	≥34	≥35	≥33

Reported deviations from the protocol:

L28: OneTube RT-PCR TaqMan (Evrogen) was used instead of TaqMan® RNA-to-Ct™ 1-Step Kit/ AgPath-ID One-Step RT-qPCR mix

L30: One step iTaq universal probe mastermix was used and consequently the following changes has been done:
Reverse transcription at 48 °C for 15 min;denaturation at 95 °C for 3 min; 40 cycles of denaturation at 95 °C for 15 s
and annealing and elongation at 60 °C for 60 s.

-> The results of these 2 labs were not excluded from further analysis as no influence on the final results was detected
(inconclusive (INC) and false negative (FN) results in the same range as for other labs)

Conclusions done by participants:

sample description	sample	Health status	L2	L12	L23	L25	L27	L28	L30	L36	L37	L38
healthy tomato seed	S-12	0	0	0	0	0	0	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	0	0	0	0	0
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	0	0	0	0	0	0
PaMMV (NIB V 366) 25x	S-13	0	0	1	0	1	2	0	1	1	1	1
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	0	0	0	0	0	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 410) 25x	S-16	0	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	0	1	0	2	0	2	0	1	2	1
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	0	1	2	1	1	2	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2x	S-1	1	1	1	1	1	1	1	1	1	1	1
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1	1	1	1	1

Conclusions done by TPS organizer and used for further evaluation:

sample description	sample	Health status	L2	L12	L23	L25	L27	L28	L30	L36	L37	L38
healthy tomato seed	S-12	0	0	0	0	0	0	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	0	0	0	0	0
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	0	0	0	0	0	0
PaMMV (NIB V 366) 25x	S-13	0	0	2	0	2	0	2	2	2	2	2
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	0	0	0	0	0	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 410) 25x	S-16	0	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	0	2	0	0	0	0	0	2	0	2
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	0	2	2	2	2	0	2	2	2	2
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2x	S-1	1	1	1	1	1	1	1	1	1	1	1
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1	1	1	1	1
		FP	0	0	0	0	0	0	0	0	0	0
		FN	2	0	1	1	1	2	1	0	1	0
		INC	0	3	1	2	1	1	2	3	2	3

RT-qPCR Tiberini et al. (2022) duplex*

Results per well:

Cq values are presented if amplification curve was observed; und - no amplification

sample description	sample	rep.	L1	L2	L12	L20	L23	L27	L29	L36	L37
healthy tomato seed	S-12	1	und	und	und	und	und	und	37	und	und
healthy tomato seed	S-12	2	und	und	und	und	und	und	und	und	und
healthy tomato seed	S-15	1	und	und	und	und	und	und	37	und	und
healthy tomato seed	S-15	2	und	und	und	und	und	und	und	und	und
CGMMV (NIB V 403) 25x	S-3	1	und	und	und	und	und	und	und	und	und
CGMMV (NIB V 403) 25x	S-3	2	und	und	und	und	und	und	und	und	und
ObPV (NIB V 364) 25x	S-19	1	und	und	und	und	und	und	38	und	und
ObPV (NIB V 364) 25x	S-19	2	und	und	und	und	und	und	und	und	und
ORSV (NIB V 365) 25x	S-17	1	und	und	und	und	und	und	und	und	und
ORSV (NIB V 365) 25x	S-17	2	und	und	und	und	und	und	und	und	und
PaMMV (NIB V 366) 25x	S-13	1	und	und	33	33	und	34	34	34	36
PaMMV (NIB V 366) 25x	S-13	2	und	und	33	32	und	34	33	33	35
PMMoV (NIB V 408) 25x	S-11	1	und	und	und	und	und	und	und	und	und
PMMoV (NIB V 408) 25x	S-11	2	und	und	und	und	und	und	37	und	und
TMGMV (NIB V 404) 25x	S-5	1	und	und	und	und	und	und	37	und	und
TMGMV (NIB V 404) 25x	S-5	2	und	und	und	und	und	und	und	und	und
TMV (NIB V 405) 25x	S-8	1	und	und	und	und	und	und	und	und	und
TMV (NIB V 405) 25x	S-8	2	und	und	und	und	und	und	und	und	und
TMV (NIB V 413) 25x	S-22	1	und	und	und	und	und	und	43	und	und
TMV (NIB V 413) 25x	S-22	2	und	und	und	und	und	und	und	und	und
ToBRFV (NIB V 331) 25x	S-9	1	und	und	und	und	und	und	und	und	und
ToBRFV (NIB V 331) 25x	S-9	2	und	und	und	und	und	und	und	und	und
ToMV (NIB V 410) 25x	S-16	1	26	und	und	und	und	und	37	und	und
ToMV (NIB V 410) 25x	S-16	2	26	und	und	und	und	und	und	und	und
ToMV (NIB V 406) 25x	S-18	1	und	und	und	und	und	und	und	und	und
ToMV (NIB V 406) 25x	S-18	2	und	und	und	und	und	und	und	und	und
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	38	und	36	37	und	und	37	39	39

ToMMV (NIB V 373) 2.5 x 10^8	S-21	2	39	und	38	36	und	und	37	39	und
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	36	und	33	32	und	34	33	34	36
ToMMV (NIB V 373) 2.5 x 10^7	S-6	2	35	und	34	33	und	35	33	34	36
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	33	30	30	29	34	32	31	30	33
ToMMV (NIB V 373) 2.5 x 10^6	S-4	2	33	30	30	29	31	32	31	31	33
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	29	27	26	26	27	28	27	28	29
ToMMV (NIB V 373) 2.5 x 10^5	S-10	2	29	27	26	25	27	28	27	28	29
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	26	23	23	23	24	24	23	24	27
ToMMV (NIB V 373) 2.5 x 10^4	S-20	2	26	23	23	23	24	25	23	24	27
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	22	21	19	19	22	21	20	20	23
ToMMV (NIB V 373) 2.5 x 10^3	S-2	2	22	21	19	19	20	21	20	20	23
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	und	17	16	15	17	18	16	17	19
ToMMV (NIB V 373) 2.5 x 10^2	S-14	2	und	17	16	15	17	18	17	17	19
ToMMV (NIB V 414) 2 x 10^1	S-7	1	34	30	32	31	31	31	32	33	33
ToMMV (NIB V 414) 2 x 10^1	S-7	2	34	30	32	30	31	31	31	33	33
ToMMV (NIB V 414) 2x	S-1	1	30	27	29	27	29	28	29	29	30
ToMMV (NIB V 414) 2x	S-1	2	30	27	29	28	28	28	28	29	30
healthy tomato leaves	NC	1	und	und	und	und	und	und	und	und	und
healthy tomato leaves	NC	2	und	und	und	und	und	und	36	und	und
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	15	14	22	13	13	15	14	14	18
ToMMV (NIB V 373) 2.5 x 10^1	PC	2	16	14	23	13	12	15	14	14	15
Cq cut off value determined by participant			≤40	/	/	/	/	/	<35,00	/	38
Cq values for inconclusive results			37	37	33	32	37	34	33	33	35

*Data for ToBRFV not included in the evaluation

Conclusions done by participants:

sample description	sample	Health status	L1	L2	L12	L20	L23	L27	L29	L36	L37
healthy tomato seed	S-12	0	0	0	0	0	0	1	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	0	0	0	0
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	0	0	0	0	0
PaMMV (NIB V 366) 25x	S-13	0	0	0	1	1	0	1	1	1	1
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	0	0	0	0	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 410) 25x	S-16	0	1	0	0	0	0	0	0	0	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	1	0	1	1	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	1	0	1	1	0	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	0	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2x	S-1	1	1	1	1	1	1	1	1	1	1
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1	1	1	1

Conclusions done by TPS organizer and used for further evaluation:

sample description	sample	Health status	L1	L2	L12	L20	L23	L27	L29	L36	L37
healthy tomato seed	S-12	0	0	0	0	0	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	0	0	0	0
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	0	0	0	0	0
PaMMV (NIB V 366) 25x	S-13	0	0	0	2	2	0	2	2	2	2
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	0	0	0	0	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0	0	0	0
ToMV (NIB V 410) 25x	S-16	0	1	0	0	0	0	0	0	0	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	2	0	2	2	0	0	2	2	0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	1	0	2	2	0	2	2	2	2
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	0	1	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	1	1	1	1	1	1	2	1
ToMMV (NIB V 414) 2x	S-1	1	1	1	1	1	1	1	1	1	1
healthy tomato leaves	NC	0	0	0	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1	1	1	1
		FP	1	0	0	0	0	0	0	0	0
		FN	1	2	0	0	2	1	0	0	1
		INC	1	0	3	3	0	2	3	4	2

RPA Agdia (XCS 22800)

sample description	sample	Health status	L5	L7	L11	L22	L31	L35
healthy tomato seed	S-12	0	0	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	0
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	0	0
PaMMV (NIB V 366) 25x	S-13	0	1	0	0	2	0	2
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	1	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0
ToMV (NIB V 410) 25x	S-16	0	0	0	0	0	0	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	2	0	2	0	0	0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	1	0	0	2	0	1
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	1	1	1	1	2	1
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	1	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	1	1	0	1	1	1
ToMMV (NIB V 414) 2x	S-1	1	1	1	1	1	1	1
healthy tomato leaves	NC	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1
	FP		1	0	1	0	0	0
	FN		0	2	2	1	2	1
	INC		1	0	1	2	1	1

Reported deviations from the protocol:
L31: QuantStudio3 was used for analysis
-> The results of this lab were not excluded from further analysis as no influence on the final results was detected (false negative (FN) results in the same range as for other labs)

LAMP Kimura et al. (2023)

sample description	sample	Health status	L3	L8	L13	L15	L24	L26
healthy tomato seed	S-12	0	0	0	0	0	0	0
healthy tomato seed	S-15	0	0	0	0	0	0	0
CGMMV (NIB V 403) 25x	S-3	0	0	0	0	0	0	0
ObPV (NIB V 364) 25x	S-19	0	0	0	0	0	0	2
ORSV (NIB V 365) 25x	S-17	0	0	0	0	0	2	0
PaMMV (NIB V 366) 25x	S-13	0	0	0	0	0	2	0
PMMoV (NIB V 408) 25x	S-11	0	0	0	0	0	0	0
TMGMV (NIB V 404) 25x	S-5	0	0	0	0	0	0	0
TMV (NIB V 405) 25x	S-8	0	0	0	0	0	0	0
TMV (NIB V 413) 25x	S-22	0	0	0	0	0	0	0
ToBRFV (NIB V 331) 25x	S-9	0	0	0	0	0	0	0
ToMV (NIB V 410) 25x	S-16	0	0	0	0	0	2	0
ToMV (NIB V 406) 25x	S-18	0	0	0	0	0	2	0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	1	0	0	0	0	2	0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	1	0	2	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1	0	1	0	0	2	1
ToMMV (NIB V 373) 2.5 x 10^5	S-10	1	0	1	0	1	1	1
ToMMV (NIB V 373) 2.5 x 10^4	S-20	1	0	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^3	S-2	1	2	1	1	1	1	1
ToMMV (NIB V 373) 2.5 x 10^2	S-14	1	1	1	1	1	1	1
ToMMV (NIB V 414) 2 x 10^1	S-7	1	0	0	0	0	2	0
ToMMV (NIB V 414) 2x	S-1	1	0	1	0	2	1	1
healthy tomato leaves	NC	0	0	0	0	0	0	0
ToMMV (NIB V 373) 2.5 x 10^1	PC	1	1	1	1	1	1	1
		FP	0	0	0	0	0	0
		FN	7	2	6	4	1	3
		INC	1	1	0	1	7	1

Reported deviations from the protocol:
L3: BioMaster kit for RT-LAMP with electrophoresis detection (Biolabmix) was used.
-> The results of this lab were excluded from further analysis as this could influence the results (this lab reported more false negative (FN) results compared to other labs)
L8: ISO001 LAMP mastermix was used
-> The results of this lab were not excluded from further analysis as no influence on the final results was detected (false negative (FN) results in the same range as for other labs)

Appendix 5: Comparison of the tests based on participants' unmodified qualitative results for the RT-qPCRs

It should be noted that for the RT-qPCRs the participants used very different approaches to determine the result of the sample and that the majority of participants were unable to perform the required analyses to determine the Cq cut-off value for each or some RT-qPCR tests, although they were aware that the Cq cut-off value is required for all RT-qPCRs tested, as cross-reactions with some other tobamoviruses are known to result in high Cq values.

Performance of RT-qPCR DAFF DEECA based on participants' unmodified qualitative results.
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

sample description	sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
healthy tomato seed	S-15	1	12	0			7,7	92,3	0,0	0,0	0,0	12	1	92,3	7,7
CGMMV (NIB V 403) 25x	S-3	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ObPV (NIB V 364) 25x	S-19	1	12	0			7,7	92,3	0,0	0,0	0,0	12	1	92,3	7,7
ORSV (NIB V 365) 25x	S-17	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	0	1	12			0,0	7,7	92,3	0,0	0,0	1	12	7,7	92,3
PMMoV (NIB V 408) 25x	S-11	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToBRFV (NIB V 331) 25x	S-9	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMV (NIB V 406) 25x	S-18	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	3			4	6	23,1	0,0	0,0	30,8	46,2	6	7	46,2	53,8
ToMMV (NIB V 373) 2.5 x 10^7	S-6	0			1	12	0,0	0,0	0,0	7,7	92,3	12	1	92,3	7,7
ToMMV (NIB V 373) 2.5 x 10^6	S-4	1			0	12	7,7	0,0	0,0	0,0	92,3	12	1	92,3	7,7
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 414) 2 x 10^1	S-7	0			1	12	0,0	0,0	0,0	7,7	92,3	12	1	92,3	7,7
ToMMV (NIB V 414) 2x	S-1	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
healthy tomato leaves	NC	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100	0
	Total	**6**	**168**	**12**	**6**	**120**	**1,9**	**53,8**	**3,8**	**1,9**	**38,5**	**288**	**24**	**92,3**	**7,7**

Performance of RT-qPCR Fowkes et al. (2022) based on participants' unmodified qualitative results.
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

sample description	sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	1	12	0			7,7	92,3	0,0	0,0	0,0	12	1	92,3	7,7
healthy tomato seed	S-15	0	12	1			0,0	92,3	7,7	0,0	0,0	12	1	92,3	7,7
CGMMV (NIB V 403) 25x	S-3	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ObPV (NIB V 364) 25x	S-19	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ORSV (NIB V 365) 25x	S-17	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	1	2	10			7,7	15,4	76,9	0,0	0,0	2	11	15,4	84,6
PMMoV (NIB V 408) 25x	S-11	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	12	1			0,0	92,3	7,7	0,0	0,0	12	1	92,3	7,7
ToBRFV (NIB V 331) 25x	S-9	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMV (NIB V 406) 25x	S-18	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	3			9	1	23,1	0,0	0,0	69,2	7,7	1	12	7,7	92,3
ToMMV (NIB V 373) 2.5 x 10^7	S-6	0			2	11	0,0	0,0	0,0	15,4	84,6	11	2	84,6	15,4
ToMMV (NIB V 373) 2.5 x 10^6	S-4	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			1	12	0,0	0,0	0,0	7,7	92,3	12	1	92,3	7,7
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 414) 2 x 10^1	S-7	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
ToMMV (NIB V 414) 2x	S-1	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
healthy tomato leaves	NC	0	13	0			0,0	100,0	0,0	0,0	0,0	13	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	13	0,0	0,0	0,0	0,0	100,0	13	0	100,0	0,0
	Total	**5**	**168**	**12**	**12**	**115**	**1,6**	**53,8**	**3,8**	**3,8**	**36,9**	**283**	**29**	**90,7**	**9,3**

Performance of RT-qPCR ISF based on participants' unmodified qualitative results.
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

sample description	sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
healthy tomato seed	S-15	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
CGMMV (NIB V 403) 25x	S-3	1	11	0			8,3	91,7	0,0	0,0	0,0	11	1	91,7	8,3
ObPV (NIB V 364) 25x	S-19	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
ORSV (NIB V 365) 25x	S-17	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	0	1	11			0,0	8,3	110,0	0,0	0,0	1	11	8,3	91,7
PMMoV (NIB V 408) 25x	S-11	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
ToBRFV (NIB V 331) 25x	S-9	1	10	1			8,3	83,3	10,0	0,0	0,0	10	2	83,3	16,7
ToMV (NIB V 410) 25x	S-16	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
ToMV (NIB V 406) 25x	S-18	0	12	0			0,0	100,0	0,0	0,0	0,0	12	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	0			4	8	0,0	0,0	0,0	33,3	66,7	8	4	66,7	33,3
ToMMV (NIB V 373) 2.5 x 10^7	S-6	0			1	11	0,0	0,0	0,0	8,3	91,7	11	1	91,7	8,3
ToMMV (NIB V 373) 2.5 x 10^6	S-4	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
ToMMV (NIB V 414) 2 x 10^1	S-7	0			1	11	0,0	0,0	0,0	8,3	91,7	11	1	91,7	8,3
ToMMV (NIB V 414) 2x	S-1	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
healthy tomato leaves	NC	1	11	0			8,3	91,7	0,0	0,0	0,0	11	1	91,7	8,3
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	12	0,0	0,0	0,0	0,0	100,0	12	0	100,0	0,0
	Total	**3**	**153**	**12**	**6**	**114**	**1,0**	**53,1**	**4,2**	**3,6**	**39,6**	**267**	**21**	**92,7**	**7,3**

Performance of RT-qPCR Tiberini et al. (2022) singleplex based on participants' unmodified qualitative results.
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

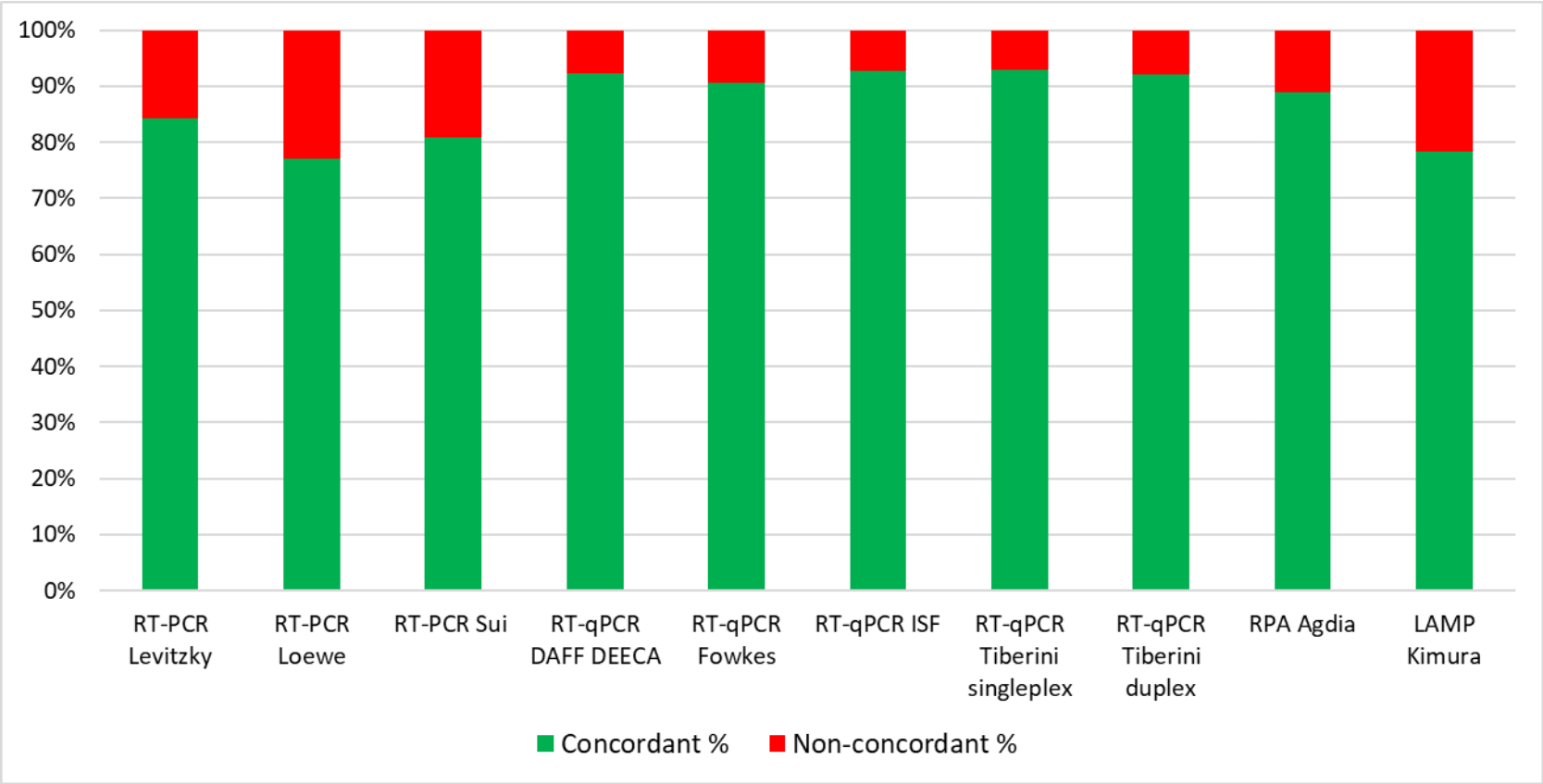
sample description	sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
healthy tomato seed	S-15	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
CGMMV (NIB V 403) 25x	S-3	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ObPV (NIB V 364) 25x	S-19	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ORSV (NIB V 365) 25x	S-17	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	1	3	6			10,0	30,0	60,0	0,0	0,0	3	7	30,0	70,0
PMMoV (NIB V 408) 25x	S-11	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ToBRFV (NIB V 331) 25x	S-9	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ToMV (NIB V 406) 25x	S-18	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	3			4	3	30,0	0,0	0,0	40,0	30,0	3	7	30,0	70,0
ToMMV (NIB V 373) 2.5 x 10^7	S-6	2			1	7	20,0	0,0	0,0	10,0	70,0	7	3	70,0	30,0
ToMMV (NIB V 373) 2.5 x 10^6	S-4	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
ToMMV (NIB V 414) 2 x 10^1	S-7	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
ToMMV (NIB V 414) 2x	S-1	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
healthy tomato leaves	NC	0	10	0			0,0	100,0	0,0	0,0	0,0	10	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	10	0,0	0,0	0,0	0,0	100,0	10	0	100,0	0,0
	Total	**6**	**133**	**6**	**5**	**90**	**2,5**	**55,4**	**2,5**	**2,1**	**37,5**	**223**	**17**	**92,9**	**7,1**

Performance of RT-qPCR Tiberini et al. (2022) duplex based on participants' unmodified qualitative results.
Legend: INC = inconclusive, TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

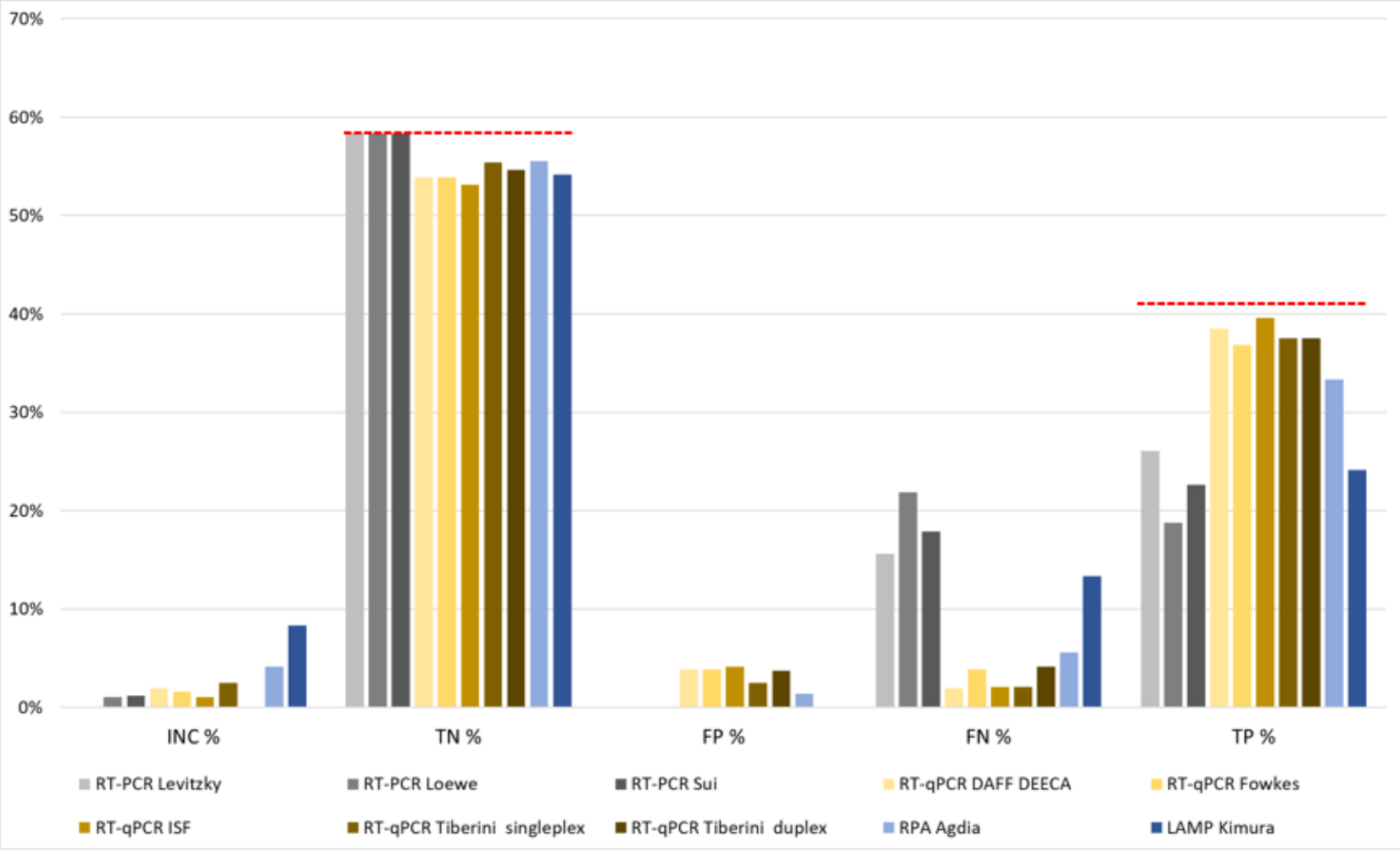
sample description	sample	INC	TN	FP	FN	TP	INC %	TN %	FP %	FN %	TP %	Concordant	Non-concordant	Concordant %	Non-concordant %
healthy tomato seed	S-12	0	8	1			0,0	88,9	11,1	0,0	0,0	8	1	88,9	11,1
healthy tomato seed	S-15	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
CGMMV (NIB V 403) 25x	S-3	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
ObPV (NIB V 364) 25x	S-19	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
ORSV (NIB V 365) 25x	S-17	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
PaMMV (NIB V 366) 25x	S-13	0	3	6			0,0	33,3	66,7	0,0	0,0	3	6	33,3	66,7
PMMoV (NIB V 408) 25x	S-11	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
TMGMV (NIB V 404) 25x	S-5	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
TMV (NIB V 405) 25x	S-8	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
TMV (NIB V 413) 25x	S-22	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
ToBRFV (NIB V 331) 25x	S-9	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
ToMV (NIB V 410) 25x	S-16	0	8	1			0,0	88,9	11,1	0,0	0,0	8	1	88,9	11,1
ToMV (NIB V 406) 25x	S-18	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^8	S-21	0			6	3	0,0	0,0	0,0	66,7	33,3	3	6	33,3	66,7
ToMMV (NIB V 373) 2.5 x 10^7	S-6	0			2	7	0,0	0,0	0,0	22,2	77,8	7	2	77,8	22,2
ToMMV (NIB V 373) 2.5 x 10^6	S-4	0			0	9	0,0	0,0	0,0	0,0	100,0	9	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^5	S-10	0			0	9	0,0	0,0	0,0	0,0	100,0	9	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^4	S-20	0			0	9	0,0	0,0	0,0	0,0	100,0	9	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^3	S-2	0			0	9	0,0	0,0	0,0	0,0	100,0	9	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^2	S-14	0			1	8	0,0	0,0	0,0	11,1	88,9	8	1	88,9	11,1
ToMMV (NIB V 414) 2 x 10^1	S-7	0			0	9	0,0	0,0	0,0	0,0	100,0	9	0	100,0	0,0
ToMMV (NIB V 414) 2x	S-1	0			0	9	0,0	0,0	0,0	0,0	100,0	9	0	100,0	0,0
healthy tomato leaves	NC	0	9	0			0,0	100,0	0,0	0,0	0,0	9	0	100,0	0,0
ToMMV (NIB V 373) 2.5 x 10^1	PC	0			0	9	0,0	0,0	0,0	0,0	100,0	9	0	100,0	0,0
	Total	**0**	**118**	**8**	**9**	**81**	**0,0**	**54,6**	**3,7**	**0,0**	**37,5**	**199**	**17**	**92,1**	**7,9**

Comparison of performance parameters determined for individual tests included in TPS over all submitted data sets. In this table, the performance of the RT-qPCRs is calculated based on participants' unmodified qualitative results. Legend: TN = true negative, FP = false positive, FN = false negative, TP = true positive, % = percentage.

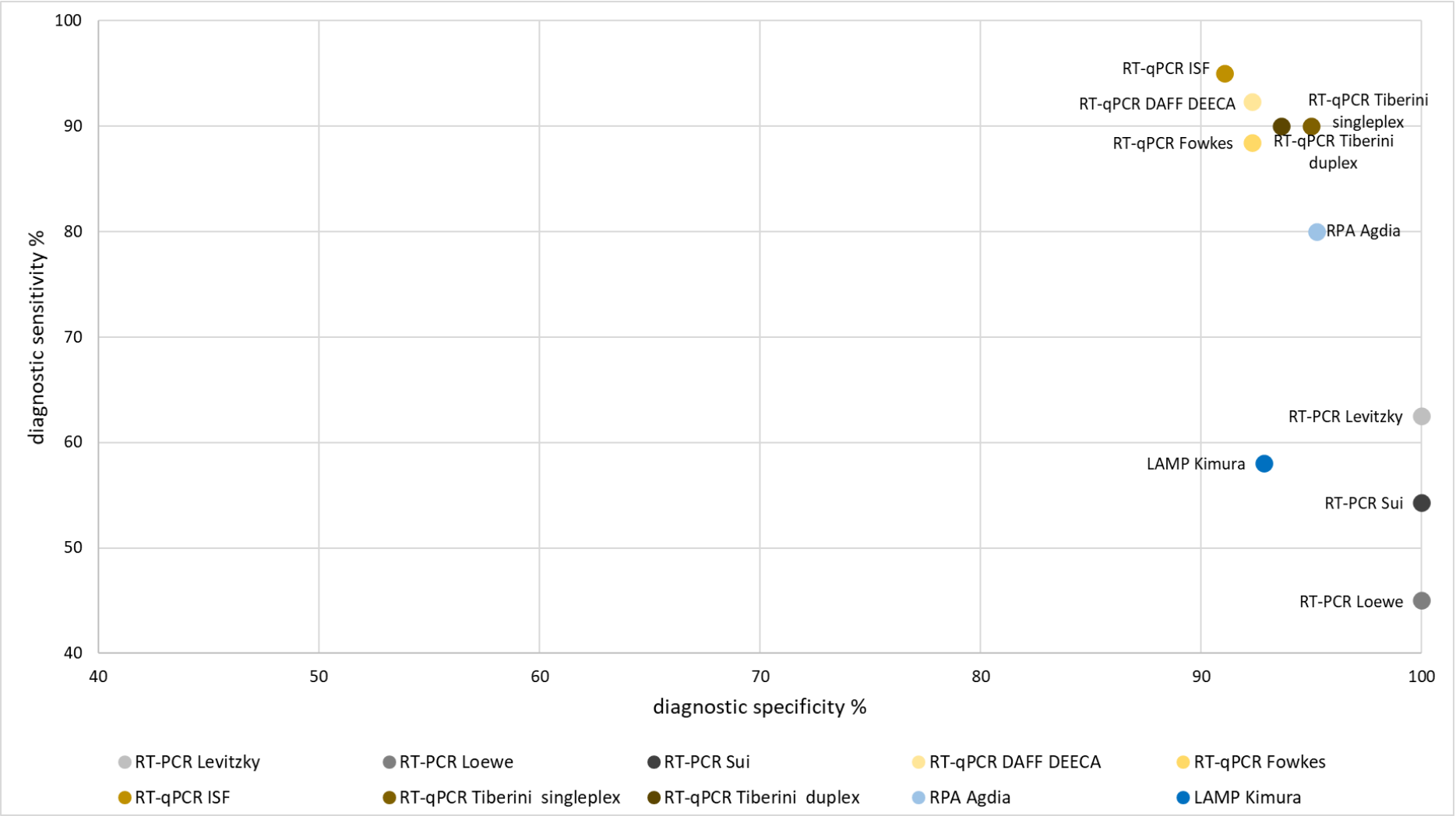
	RT-PCR			RT-qPCR					RPA	LAMP
Diagnostic parameter	Levitzky et al. (2019)	Loewe (Cat. No. 09181)	Sui et al. (2017)	DAFF DEECA	Fowkes et al. (2022)	ISF	Tiberini et al. (2022) singleplex	Tiberini et al. (2022) duplex	Agdia RPA (XCS 22800)	Kimura et al. (2023)
Total data sets	8	4	7	13	13	12	10	9	6	5
Expected positives	80	40	70	130	130	120	100	90	60	50
Expected negatives	112	56	98	182	182	168	140	126	84	70
Total data points	192	96	168	312	312	288	240	216	144	120
INC	0	1	2	6	5	3	6	0	6	10
TN	112	56	98	168	168	153	133	118	80	65
FP	0	0	0	12	12	12	6	8	2	0
FN	30	21	30	6	12	6	5	9	8	16
TP	50	18	38	120	115	114	90	81	48	29
INC %	0,0	1,0	1,2	1,9	1,6	1,0	2,5	0,0	4,2	8,3
TN %	58,3	58,3	58,3	53,8	53,8	53,1	55,4	54,6	55,6	54,2
FP %	0,0	0,0	0,0	3,8	3,8	4,2	2,5	3,7	1,4	0,0
FN %	15,6	21,9	17,9	1,9	3,8	2,1	2,1	4,2	5,6	13,3
TP %	26,0	18,8	22,6	38,5	36,9	39,6	37,5	37,5	33,3	24,2
Concordant	162	74	136	288	283	267	223	199	128	94
Non-concordant	30	22	32	24	29	21	17	17	16	26
Concordant %	84,4	77,1	81,0	92,3	90,7	92,7	92,9	92,1	88,9	78,3
Non-concordant %	15,6	22,9	19,0	7,7	9,3	7,3	7,1	7,9	11,1	21,7
diagnostic sensitivity %	62,5	45,0	54,3	92,3	88,5	95,0	90,0	90,0	80,0	58,0
diagnostic specificity %	100,0	100,0	100,0	92,3	92,3	91,1	95,0	93,7	95,2	92,9
false positive rate %	0,0	0,0	0,0	7,7	7,7	8,9	5,0	6,3	4,8	7,1
false negative rate %	37,5	55,0	45,7	7,7	11,5	5,0	10,0	10,0	20,0	42,0
relative accuracy %	84,4	77,1	81,0	92,3	90,7	92,7	92,9	92,1	88,9	78,3
positive predictive value %	100,0	100,0	100,0	90,9	90,6	90,5	93,8	91,0	96,0	100,0
negative predictive value %	78,9	72,7	76,6	96,6	93,3	96,2	96,4	92,9	90,9	80,2



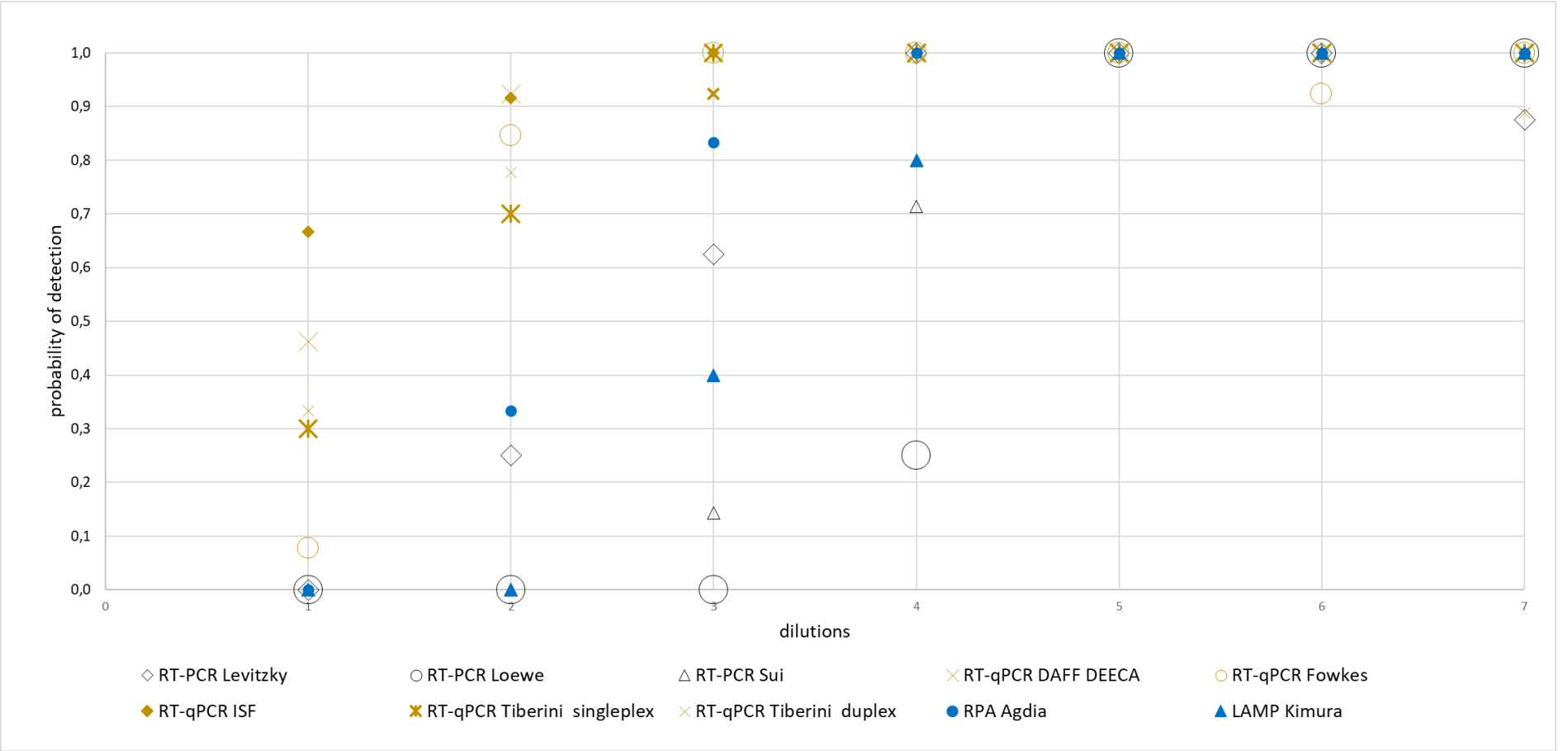
Graphical representation of concordance rates for studied tests. In this graph, the performance of the RT-qPCRs is calculated based on participants' unmodified qualitative results.



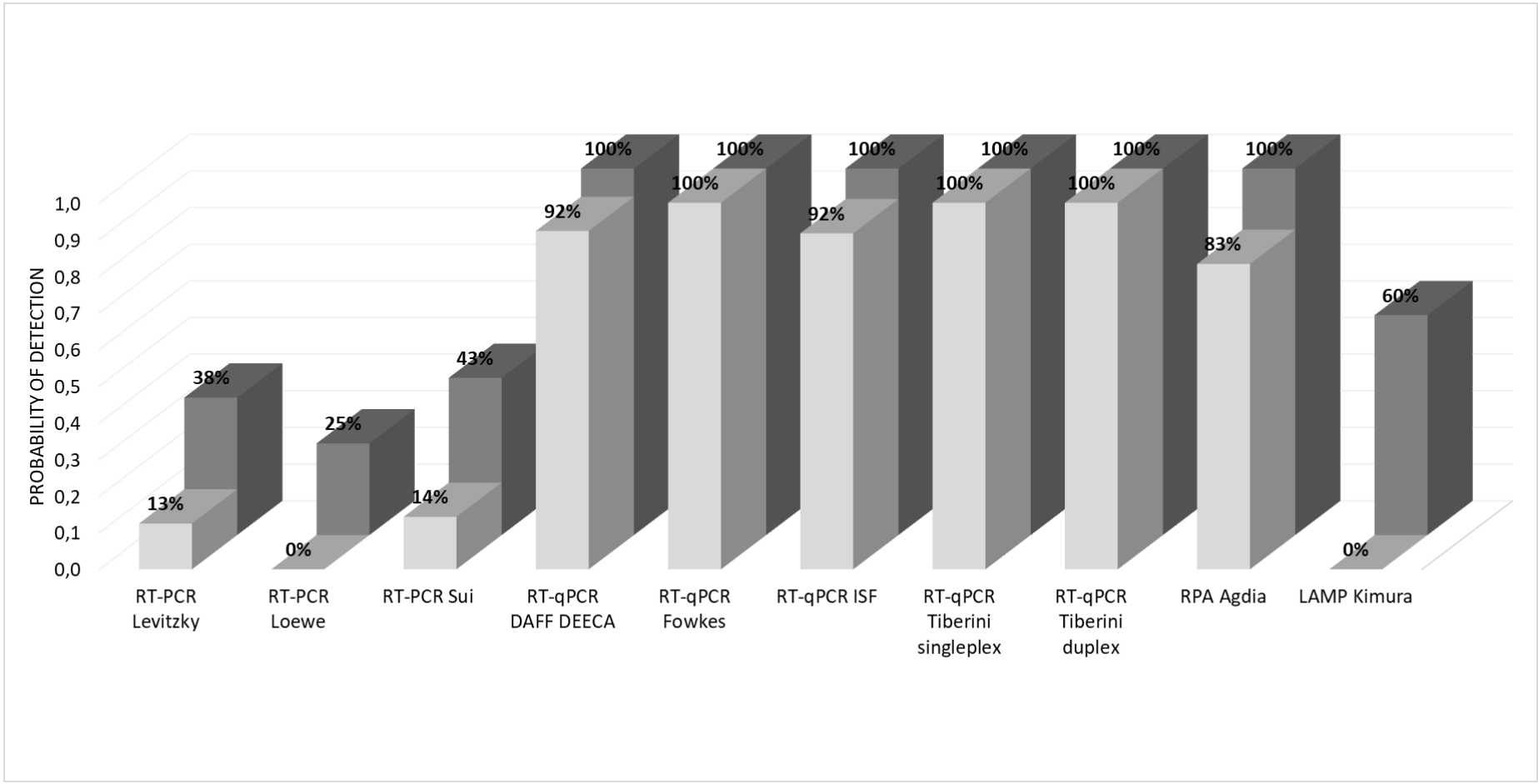
Graphical representation of the true-negative (TN), false-positive (FP), false-negative (FN), true-positive (TP) and inconclusive (INV) results of the tests examined. The red dotted line represents the expected percentage of TN and TP. In this graph, the performance of the RT-qPCRs is calculated based on participants' unmodified qualitative results.



Graphical representation of the value of diagnostic specificity and diagnostic sensitivity for tests examined. In this graph, the performance of the RT-qPCRs is calculated based on participants' unmodified qualitative results.



Graphical representation of the probability of detection for all tests examined based on the results of the dilution of the isolate ToMMV NIB V 373 in RNA from healthy tomato leaves (dilution factors are indicated on the x-axes). In this graph, the performance of the RT-qPCRs is calculated based on participants' unmodified qualitative results.



Graphical representation of the probability of detection for all tests examined based on the results of two RNA dilutions of the ToMMV isolate from seed (NIB V 414) (the results for the dilution factor 2x are shown in dark grey and the results for the dilution factor 20x in light grey). In this graph, the performance of the RT-qPCRs is calculated based on participants' unmodified qualitative results.

Summary of the reproducibility (%) of tests. In this table, the performance of the RT-qPCRs is calculated based on participants' unmodified qualitative results.

	RT-PCR			RT-qPCR					RPA	LAMP
sample description	Levitzky et al. (2019)	Loewe (Cat. No. 09181)	Sui et al. (2017)	DAFF DEECA	Fowkes et al. (2022)	ISF	Tiberini et al. (2022) singleplex	Tiberini et al. (2022) duplex	Agdia RPA (XCS 22800)	Kimura et al. (2023)
healthy tomato seed	100	100	100	100	92	100	100	89	100	100
healthy tomato seed	100	100	100	92	92	100	100	100	100	100
CGMMV (NIB V 403) 25x	100	100	100	100	100	92	100	100	100	100
ObPV (NIB V 364) 25x	100	100	100	92	100	100	100	100	100	80
ORSV (NIB V 365) 25x	100	100	100	100	100	100	100	100	100	80
PaMMV (NIB V 366) 25x	100	100	100	92	77	92	100	67	50	80
PMMoV (NIB V 408) 25x	100	100	100	100	100	100	100	100	100	100
TMGMV (NIB V 404) 25x	100	100	100	100	100	100	100	100	100	100
TMV (NIB V 405) 25x	100	100	100	100	100	100	100	100	100	100
TMV (NIB V 413) 25x	100	100	100	100	92	100	100	100	83	100
ToBRFV (NIB V 331) 25x	100	100	100	100	100	83	100	100	100	100
ToMV (NIB V 410) 25x	100	100	100	100	100	100	100	89	100	80
ToMV (NIB V 406) 25x	100	100	100	100	100	100	100	100	100	80
ToMMV (NIB V 373) 2.5 x 10^8	100	100	100	46	69	67	40	67	67	80
ToMMV (NIB V 373) 2.5 x 10^7	75	100	100	92	85	92	70	78	50	80
ToMMV (NIB V 373) 2.5 x 10^6	63	100	71	92	100	100	100	100	83	40
ToMMV (NIB V 373) 2.5 x 10^5	100	50	71	100	100	100	100	100	100	80
ToMMV (NIB V 373) 2.5 x 10^4	100	100	100	100	100	100	100	100	100	100
ToMMV (NIB V 373) 2.5 x 10^3	100	100	100	100	92	100	100	100	100	100
ToMMV (NIB V 373) 2.5 x 10^2	88	100	100	100	100	100	100	89	100	100
ToMMV (NIB V 414) 2 x 10^1	88	100	71	92	100	92	100	100	83	80
ToMMV (NIB V 414) 2x	63	75	57	100	100	100	100	100	100	60
Average:	**94**	**97**	**94**	**95**	**95**	**96**	**96**	**94**	**92**	**87**