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Detection of Tomato mottle mosaic virus (ToMMV) in Tomato (*Solanum lycopersicum*) and Pepper (*Capsicum* spp.) by Seed Extract RT-qPCR (SE-qPCR)

Validation report, November 2025

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ISHI VALIDATION REPORTS

This ISHI validation study has been conducted to determine the fitness of the described method for its intended purpose according to the ISHI Guidelines for the Validation of Seed Health Methods[1] and followed by an independent review of its outcome.

DISCLAIMER

ISF cannot guarantee that laboratories following the protocol described herewith will obtain similar results. Many factors, such as staff skills, laboratory equipment and conditions, reagents and sampling methods can influence the results. Consequently, in case of any litigation ISF will not accept any liability on the use of these tests.

Published by:

International Seed Federation (ISF)

Reposoir 7, 1260 Nyon, Switzerland

Developed by ISHI

[1] Available at: https://www.worldseed.org/our-work/seed-health/ishi-method-development-and-validation/

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SUMMARY

Tomato mottle mosaic virus (ToMMV), now called *Tobamovirus maculatessellati*, is a member of the genus *Tobamovirus* first reported in 2013 from greenhouse tomato plants collected in 2009 in Mexico. Since then, ToMMV was also detected in other countries, including Australia, Brazil, China, Israel, Spain and USA. Symptoms caused by ToMMV are very similar to those of other tobamoviruses and make it impossible to identify species solely based on symptoms. Tomato and pepper are the main hosts, and some other experimental hosts and weeds were also identified. ELISA and the bioassay can detect but cannot distinguish ToMMV from other tobamoviruses. A seed extract (SE-) reverse transcriptase qPCR assay (SE-qPCR) for the specific detection of ToMMV is proposed as an (optional) pre-screening step, as an alternative to the ELISA pre-screening step used in the current *Tobamovirus* method. Three primer sets were evaluated, targeting either the replicase-associated or capsid protein region. The use of two primer sets specifically targeting ToMMV at different regions of genome is proposed for the robustness of the method in seed health testing.

In this validation study, the performance criteria analytical specificity, analytical sensitivity, selectivity, repeatability and reproducibility of the SE-qPCR assay were validated. In addition, the robustness of the method when changing reagents was evaluated.

The data presented in this validation reports shows that the SE-qPCR detection assay for ToMMV in samples of tomato and pepper seed meets the requirements set for the validation criteria listed above. Therefore, the ISHI ToMMV SE-qPCR assay is fit for its intended purpose to serve as a specific pre-screening assay for the detection of ToMMV.

1. INTRODUCTION

The *Tobamovirus* genus comprises multiple economically important plant pathogenic viruses, including several that infect solanaceous crops. They are among the most stable and infectious viruses known and are readily transmitted mechanically by workers, tools and equipment during plant handling. Tobamoviruses can also be spread via fruits, insects, and seeds. They survive in soil, water, and infested debris from previous crops.

The ISHI method for detecting tobamoviruses, an industry standard, contains a direct test based on a bioassay that provides conclusive evidence of the presence of viable and infectious tobamoviruses. In the assay, leaves of indicator plant species *Nicotiana tabacum* cv. Xanthi NN and/or *Nicotiana glutinosa* are inoculated with an extract from tomato or pepper seed. If the seed extract contains infectious virions, it triggers a hypersensitive response in the host plant resulting in small, necrotic local lesions on the leaves that are typical for tobamoviruses.

Tomato mottle mosaic virus (ToMMV)

Tomato mottle mosaic virus (ToMMV), now called *Tobamovirus maculatessellati*, is a species of the genus *Tobamovirus*, family *Virgaviridae*, which has a ~ 6.4 kb single-stranded positive-sense RNA genome with a typical tobamovirus genome organization. The genome consists of four open reading frames encoding two replication-associated proteins, a movement protein (MP) and a

capsid protein (CP), where MP and CP are expressed via sub-genomic RNAs (Li *et al*., 2013). ToMMV was first reported in 2013 from greenhouse tomato plants collected in 2009 in Mexico (Li *et al*., 2013). Since then, ToMMV was also detected in other countries, including Australia, Brazil, China, Israel, Spain and USA. ToMMV shares similar characteristics with other tobamoviruses, and genetically, the genome sequence is most closely related to Tomato mosaic virus (ToMV), now called *Tobamovirus tomatotessellati*. The stability of tobamoviruses and ease of spreading through survival in soil, dispersal via seeds, operational practices in nursery facilities/fields and global marketing increase the concern of ToMMV in solanaceous crops. Symptoms caused by ToMMV are very similar to those of other tobamoviruses, making it impossible to distinguish species solely based on symptoms, which can include leaves mosaic, interveinal yellowing and deformation, young leaves deformation and necrosis, young fruits discoloration, deformation and necrosis (Sui *et al*., 2017). Tomato and pepper are the main hosts, and some other experimental hosts and weeds were also identified (Li *et al*., 2017; Sui *et al*., 2017). ToMMV was also identified as mixed infection on eggplant with another tobamovirus - Tobacco mild green mosaic virus (TMGMV; Chai *et al*., 2018), now called *Tobamovirus mititessellati*.

Detection of ToMMV in seed

ELISA has been used widely to detect Tobacco mosaic virus (TMV), now called *Tobamovirus tabaci*, and ToMV as well as other tobamoviruses, including ToMMV. ELISA can be used to pre-screen seed lots in the ISHI bioassay method. However, ELISA and the bioassay cannot distinguish ToMMV from other tobamoviruses. Several PCR-based detection methods and primers are available in publications, however, very few studies demonstrate the method performance in seed testing.

In this study, three primer sets were evaluated, including two sets of ISHI primers and probes - CaTa9, targeting replicase-associated protein region (Hiddink *et al*., 2019) and ToMMV2 (Schoen *et al*., 2023), targeting capsid protein. The third set of primers, CSP1572, was developed by California Seed and Plant Lab (CSPL) and targets the capsid protein (Mehle *et al*., 2024). Here, the use of two primer sets specifically targeting ToMMV at different regions of the genome is proposed for the robustness of the method in seed health testing (Figure 1).

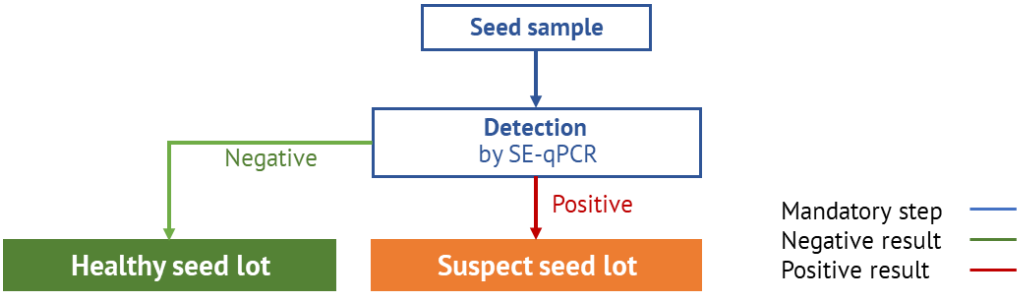

Figure 1. Method process flow.

2. OBJECTIVES

The goal of this validation overview is to confirm that the seed extract RT-qPCR (SE-qPCR) assay can be used as an alternative pre-screen to ELISA for the specific detection of ToMMV in tomato and pepper seed, described in the protocols "Detection of Infectious Tobamoviruses in Tomato Seed", v6.2 (July 2019) and "Detection of Infectious Tobamoviruses in Pepper Seed", v6.3 (September 2019), developed by ISHI. The described experiments measure analytical specificity, analytical sensitivity, selectivity, repeatability, and reproducibility of the method. In addition, the robustness of the method with respect to changes in reagents was evaluated.

3. METHOD VALIDATION

Data for this validation review was compiled from different sources and experiments into one validation report. The main part of this report was prepared by the ISHI working group based in the USA in collaboration with ISHI members from the Netherlands and France. Additional analytical specificity data was supplied by four ISHI members. The validated protocol indicated in Annex A corresponds to the ISF protocol for the detection of ToMMV.

3.1. Analytical specificity

Definition ISHI guidelines: *The ability of an assay to detect the target(s) pathogens (inclusivity) while excluding non-targets (exclusivity).*

The analytical specificity requirements will be met when no false negative results are obtained for the targets (inclusivity = 100%) and less than 5% false positive results for the non-targets (exclusivity > 95%). A false negative means that at least two ToMMV specific targets in the qPCR assay score Cq > 35 on a target isolate.

Experimental approach and results

The analytical specificity was tested by i) *in silico* data analysis of inclusivity, ii) *in vitro* analysis of inclusivity on isolates, and iii) *in vitro* analysis of exclusivity on isolates.

***In silico* data analysis of inclusivity**

All full-length sequences of ToMMV genome sequences that were available in GenBank in November 2024 were collected for *in silico* analysis of the inclusivity of each primer and probe. A consensus sequence was generated from sequence alignment of these 28 viral genomes from ten countries: Brazil, China, France, Japan, Mexico, Netherlands, Spain, Vietnam, United Kingdom, and USA.

The detailed information of each isolate is listed in Annex B, Table B.1. All primers and probes were then paired to the consensus sequence, and mismatches were identified (Annex B, Table B.2). The results indicated that two primers are 100% conserved with all viral sequences and the remaining six primers have one or two single nucleotide mismatches detected in one to seven of the 28 viral sequences. Similar results were obtained for the probes, which have one to two mismatches identified in two to five of the same viral sequences (Annex B, Table B.2). The most significant impact of a single mismatch was suggested at the last three to four positions from the primer 3' end (Wu *et al*., 2009) and none of the primers or probes were found to have mismatches at the 3' end (Annex B, Table B.2). Overall, these primers and probes evaluated via *in silico* analysis showed high degrees of homology for the available ToMMV isolates published in GenBank.

***In vitro* analysis of inclusivity on isolates**

In addition to the *in silico* analysis, for testing the inclusivity of the SE-qPCR, a total of six different ToMMV isolates were tested in three laboratories. This low number is due to the limited number of isolates accessible and the relatively limited genetic diversity. All primers and probes were tested on two isolates in laboratory 1 and laboratory 3. In addition, routine testing on seed was done with the CaTa9 and ToMMV2 PCR assays by laboratory 2. Analysis results of ToMMV isolates are presented in Table 1. All target isolates tested were detected with at least two assays.

Table 1. Inclusivity data for six ToMMV isolates.

Isolate ID	Laboratory	Cq values		
		CaTa9	ToMMV2	CSP1572
ToMMV HMC	1	20.65	24.49	22.34
MX5	2	12.42	12.94	Not tested
SHR0256	2	23.00	23.29	Not tested
SHR0297	2	29.45	29.72	Not tested
SHR4165	2	28.30	28.05	Not tested
PAS 3068	3	18.74	20.74	20.51

***In vitro* analysis of exclusivity on isolates**

For testing the exclusivity of the qPCR assays, data was collated from four laboratories with a total of 34 non-target isolates, representing 16 different *Tobamovirus* species (Annex B, Table B.3). All isolates were tested with CaTa9 and ToMMV2 assays, a total of 19 isolates were also tested with CSP1572. One ToMV isolate, out of ten tested, showed a Cq > 39 for the CaTa9 assay, and one PMMoV isolate, out of four tested, showed a Cq > 39 for the ToMMV2 and CSP1572 assays. All other isolates tested did not show any amplification (Annex B, Table B.3).

Conclusion

Overall, these primers and probes evaluated via *in silico* analysis showed high degrees of homology for the available ToMMV isolates published in GenBank. Inclusivity tested on six different ToMMV isolates was calculated to be 100%. This number of isolates tested for *in vitro* inclusivity is below the current recommended number of isolates to be tested for inclusivity, however the *in silico* data supports the conclusion that inclusivity is fit for purpose. After the application of a Cq cut-off at 35, the exclusivity on 34 non-target isolates was calculated to be 100% (34/34). Additionally, tomato and pepper seed lots came with other non-target organisms (e.g., pospiviroids, other tobamoviruses) were also used to demonstrate the method exclusivity, and these are shown below in Repeatability and Reproducibility experiments (Annex D and E). Therefore, for the validation criterium analytical specificity, the SE-qPCR detection method is deemed fit for purpose.

3.2. Analytical sensitivity

Definition ISHI guidelines: *Smallest amount of the target pathogen that can be detected i.e., the limit of detection (LOD).*

The analytical sensitivity requirements will be met when one contaminated seed will be detected in the seed subsample (being one seed in 1,000 for tomato and one seed in 500 for pepper and eggplant).

Experimental approach and results

An initial experiment was done using *in vitro* RNA transcripts. The analytical sensitivity was determined using naturally infected seeds.

Analytical sensitivity using *in vitro* RNA transcript

The analytical sensitivity of the PCR assay was determined using a serial dilution of *in vitro* RNA transcripts. The 362 and 1,162 nucleotide-long DNA templates targeting the replicase and the capsid protein/movement protein genes, respectively, were designed and synthesized based on a consensus sequence populated from 13 full-length ToMMV sequences from GenBank. The transcripts spanned nucleotide positions 1,881 to 2,180 and 5,300 to 6,399 and incorporated sequences of T7 and T3 promoters at 5' and 3' ends, respectively (Table 2). *In vitro* transcription was performed with MEGAshortscript kit (Ambion, AM1354, ThermoFisher Scientific, Waltham, MA) and MEGAscript kit (Ambion, AM1333, ThermoFisher Scientific, Waltham, MA) followed with DNase treatment. The yield and purity of RNA transcript were measured via Nanodrop spectrophotometer, Qubit fluorometer using the Qubit RNA BR assay kit (Q10210, ThermoFisher Scientific, Waltham, MA) and agarose gel visualization (data not shown). The two fragments (ToMMV-R and ToMMV-MPCP) were designed based on priming positions and target sequences of primers and probes. The fragment of ToMMV-R includes the CaTa9 targets at the replicase region. The fragment ToMMV-MPCP includes the CSP1572 and ToMMV2 targets.

Table 2. Nucleotide sequence of ToMMV (blue), promoters (red) and primer sequences (underlined).

RNA fragment	Sequence (5' - 3')
ToMMV-R	**AGCGCGCG**TAATACGACTCACTATAGGG**AGACCACC**AGTGGTCTTACTCTCACGTTTGAGCA GCCTACAGAAGCCAATGTTGCACTGGCACTTCAAGACTCTGAAAAAGCTTCGGAAGGCGCGT TGGTAGTTACCTCGCGTGATGTGGAGGAACCCTCTATGAAAGGTTCAATGGCCCGTGGTGAG TTACAATTGGCCGGATTAGCAGGTGACATTCCAGAATCTTCGTTTACAAGGAGCGAGGAGAT TGAGTCTTTAGAGCAGTTTCATATGGCAACAGCCAGTTCTCTGATTCAAAAGCAGATGTGTT CGATCGTGTACACAGGCACCATTAAA***GCTG***CCCTTTAGTGAGGGTTAATTGC
ToMMV-MPCP	**AGCGCGCG**TAATACGACTCACTATAGGG**AGACCACC**GATGCGGAAAAGAACATATGGCAAG TCCTAGTTAATATTAGGAATGTCAAGATGGCTGGGGGTTTCTGTCCCCTGTCGTTAGAATTT GTGTCTGTGTGTATAGTTTATAAAAATAATATAAAATTGGGTTTGAGGGAGAAGATTACAAG AGTGGATGACGCAGGTCCCATTGAACTTACCGAAGAAGTTGTTGATGAGTTCATGGAGAGTG TGCCTATGTCAGTCAGGCTTGCTAAATTTCGAACCAAATCCTCAAAAAGAGGTCCGAAACAT AATAGTAATAATACTAATGAAAGAAAAGGGCGGTCTAATTTCCGTAAGAAACAAGACCAGGA GAGTTATGGAGTTAGTGATAGTTTAGATAATTTGATTGAAGATGATACCGAGACGTCAGTCG CGGGATCTGATTCGTATTAAATATGTCTTACGCTATTACTTCTCCGTCACAATTCGTGTTTTT GTCATCAGCATGGGCCGACCCTGTAGAATTAATAAATATTTGTACTAATTCGTTAGGTAACC AGTTTCAAACACAACAAGCAAGGACTACTGTTCAACAGCAGTTCAGCGAGGTGTGGAAACCT TTCCCTCAAAGTACTGTCAGGTTCCCTGACAATGTATTTAAGGTGTATAGGTATAATGCGGT TATAGATCCTCTAATTACTGCATTGCTGGGAACTTTCGATACTAGAAATAGAATAATAGAGG TAGAAAATCAGCAAAGCCCGACTACAGCCGAAACATTGGATGCCACTCGCAGAGTGGACGAT GCTACGGTTGCGATCAGGTCCGCTGTTAATAATTTAGTTAATGAATTGGTAAGAGGAACAGG TTTCTACAACCAGAGTACTTTCGAAAGTATGTCTGGGTTGGCCTGGACTTCTGCGCCAGCGT CCTAAGTAATAAAGGACGAAAATTAAAGGAAGTGTATCCTAAAATACACGTGGTGCGTACGA TAACGTACAGTGTTTTTCCCTCCACTTAAATCGAAGGGTTTTGTCTTGGAACGCGCGGGTTA AATATACATGGTTCATGTATATCCGTAGACAAGTAATAATGCGTGGGATTCGAATTCCCCCG GAACCCCCGGTAGGGGCCCA***GCTG***CCCTTTAGTGAGGGTTAATTGC-3'

Based on the molecular weight of the RNA molecule, 1 fg of pure RNA transcript ToMMV-R and ToMMV-MPCP corresponds to 5,904 and 1,608 copies of molecules, respectively. RNA transcripts were prepared in ten-fold dilution series and used to determine analytical sensitivity of the qPCR assays. Results shown in Annex C, Table C.1 for ToMMV-MPCP, and Table C.2 for ToMMV-R suggested that the limit of detection (LOD) of qPCR assays is most likely between the levels of 1

fg – 100 ag for CSP1572, ToMMV2 and CaTa9 PCR assays and the Cq values fall in the range of 32 – 35. Based on the criteria described for LOD, the primers need to be able to detect 95% of replicates and to meet this criterion, copy numbers of ~1500 from each RNA transcript were used to evaluate the performance of respected qPCR assays. When 20 replicates are performed, 1 fg of ToMMV-MPCP and 250 ag of ToMMV-R gave a consistent detection at the Cq range of 32-34 (Annex C, Table C.3), in addition all No Template Control (NTC) reactions were negative (data not shown).

Analytical sensitivity using naturally infected seeds

Analytical sensitivity of the method was evaluated by spiking one infected seed in healthy tomato, pepper and eggplant seed subsamples. A high titre ToMMV-infected tomato seed batch (T2) was used as the ToMMV positive material for spiking in the study. To assess the overall infection rate and contamination level, individual tomato seeds from T2 were ground, total RNAs were extracted, and RNA extracts were analysed by triplex and duplex RT-qPCR with primer-probe sets targeting ToMMV and the *nad5* gene (internal amplification control (IAC); Menzel *et al*., 2002; Botermans *et al*., 2013). The RT-qPCR results showed 20 out of 20 seeds had Cq < 30, and among those, 15 out of 20 seeds had Cq <25 for ToMMV and the remaining five seeds gave Cq 25 – 30 for ToMMV. This showed that 100% of the seed batch was contaminated with ToMMV and among those ~75% of seeds had contamination levels of Cq <25 (Annex C, Table C.4). All three primer sets targeting ToMMV gave a consistent detection level and the IAC, *nad5*, was detected in all tested seeds. Note that a few higher Cq values (~27) for *nad5* were recorded in the triplex RT-qPCR, which were likely due to competition effect from the high titre of ToMMV in these seeds.

The sensitivity of the method to detect one ToMMV-contaminated seed in 1,000 tomato seed subsamples and 500 pepper and eggplant seed subsamples was evaluated at the method level where the complete procedure was followed as described in Annex A. A total of 20 subsamples of healthy seeds were prepared for tomato, pepper and eggplant, and each subsample had one seed from the ToMMV-positive tomato seed batch (T2) spiked into 999 ToMMV-free tomato seeds or 499 ToMMV-free pepper or eggplant seeds, respectively. Therefore, based on the characterization of T2 (Annex C, Table C.4), the results of one seed spike for tomato is expected to have ⩾ 75% detected below Cq 35; in contrast, those results for pepper and eggplant are expected to have ⩾ 75% detected below Cq 34, based on the seed dilution factors of 1:1000 and 1:500 respectively. The experiment was performed in three laboratories independently using the same ToMMV-contaminated seed lot but using laboratory specific ToMMV-free seed lots to spike into. Laboratory 1 performed a triplex RT-qPCR with the primers sets CaTa9, ToMMV2 and *nad5* as IAC and a duplex RT-qPCR with the primers set CSP1572 and *nad5* as IAC. Laboratory 2 performed two duplex RT-qPCRs with primer sets CaTa9 and CSP1572 in one duplex mix and ToMMV2 and SqMV as IAC in the second duplex mix and additionally ran 20 ToMMV-free subsamples without spike for their own comparative purposes. Laboratory 3 performed three duplex RT-qPCRs for tomato and pepper and two triplex RT-qPCR for eggplant with SqMV as IAC.

Results for tomato are summarised in Table 3 and raw data is shown in Annex C, Table C5 – C7. The result showed that, for laboratory 1, 19 subsamples obtained Cq ⩽ 35, and one subsample (subsample two) was at Cq 34-36, demonstrating ⩾ 95% detection rate. All target primer sets gave comparable and consistent detection as well as the internal amplification control, *nad5* (Annex C, Table C.5). Results from laboratory 2 showed that four out of 20 ToMMV-free subsamples had Cq 33-35 from one of three primer sets but not another two primer sets suggesting these might be non-specific amplification or background noise. In contrast, results from spiked subsamples

showed all 20 subsamples obtained Cq ⩽ 35 giving a 100% detection rate. The IAC, SqMV, gave consistent Cq values suggesting the equivalent recovery of RNA from all subsamples and that there was no inhibitory effect on those undetermined samples (Annex C, Table C.6). Laboratory 3 results were similar to other laboratories as all 20 subsamples gave Cq ⩽ 35 giving a 100% detection with all target primer sets giving a comparable and consistent detection and the IAC, SqMV, gave consistent detection level suggesting the equivalent processing control across all 20 subsamples on performing this method (Annex C, Table C.7).

Table 3. Qualitative RT-qPCR results for the evaluation of analytical sensitivity for tomato. One contaminated tomato seed (T2) was spiked in 20 subsamples of 999 healthy tomato seeds in three laboratories. Subsamples were tested by RT-qPCR in duplicate. Pos = average Cq value ⩽ 35. Neg = Average Cq value > 35.

Subsample	Laboratory 1			Laboratory 2			Laboratory 3		
	CaTa9	ToMMV2	CSP1572	CaTa9	ToMMV2	CSP1572	CaTa9	ToMMV2	CSP1572
1	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
2	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
3	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
4	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
5	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
6	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
7	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
8	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
9	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
10	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
11	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
12	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
13	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
14	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
15	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
16	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
17	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
18	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
19	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
20	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Regarding pepper, results are summarised in Table 4 and raw data is shown in Annex C, Table C8 – C10. Laboratory 1 evaluated only 18 subsamples due to an insufficient quantity of seeds. All 18 subsamples gave Cq ⩽ 34 showing 100% detection of a single seed spike in a pepper seed matrix. All target primer sets gave comparable and consistent detection as well as the IAC, *nad5* (Annex C, Table C.8). For laboratory 2, one or 2 primer sets were giving Cq ⩽ 34 in three out of 20 ToMMV-free subsamples suggesting that these might be non-specific amplification, background noise or potentially cross contamination (15% false positive rate in laboratory 2). Comparing to spiked 20 subsamples, 18 subsamples gave Cq ⩽ 34 with all primers giving an overall detection rate of 90%. Again, the IAC, SqMV, gave a consistent detection level across all 40 subsamples suggesting that there was no inhibitory effect in those no-amplification-subsamples (Annex C, Table C.9). All 20 spiked subsamples evaluated by laboratory 3 produced Cq ⩽ 34 with all target primer sets demonstrating 100% detection of the infected seed in the pepper seed matrix. The

IAC, SqMV, gave consistent detection level suggesting the equivalent processing control across all subsamples on performing this method (Annex C, Table C.10).

Table 4. Qualitative RT-qPCR results for the evaluation of analytical sensitivity for pepper. One contaminated tomato seed (T2) was spiked in 18 (laboratory 1) or 20 (laboratory 2 and 3) subsamples of 499 healthy pepper seeds. Subsamples were tested by RT-qPCR in duplicate. Pos = average Cq value ⩽ 34. Neg = Average Cq value > 34.

Subsample	Laboratory 1			Laboratory 2			Laboratory 3		
	CaTa9	ToMMV2	CSP1572	CaTa9	ToMMV2	CSP1572	CaTa9	ToMMV2	CSP1572
1	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
2	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
3	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
4	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
5	Pos	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Pos
6	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
7	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
8	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
9	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
10	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
11	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
12	Pos	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Pos
13	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
14	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
15	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
16	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
17	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
18	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
19				Pos	Pos	Pos	Pos	Pos	Pos
20				Pos	Pos	Pos	Pos	Pos	Pos

Regarding eggplant, results are summarised in Table 5 and raw data is shown in Annex C, Table C11 – C13. From laboratory 1, all 20 subsamples gave Cq ⩽ 34 showing 100% detection of a single seed spike in the eggplant seed matrix (Annex C, Table C.11). All target primer sets gave a comparable and consistent detection for each subsample as well as the IAC, *nad5* (Annex C, Table C.11). For laboratory 2, four out of 20 ToMMV-free subsamples had Cq ⩽ 34 from one or three primer sets suggesting that these might be non-specific amplification, background noise or potentially cross contamination. Comparing to spiked 20 subsamples, 19 subsamples gave Cq ⩽ 34 with all primers giving an overall detection rate of 95%, and the IAC, SqMV, gave consistent detection level across all 40 subsamples and indicating no inhibitory effect on those no amplification subsamples (Annex C, Table C.12). Laboratory 3 deviated from the protocol and used 6 mL rather than 12 mL of RNA extraction buffer. Results from laboratory three show 17 subsamples producing Cq values ⩽ 34 demonstrating 85% detection of a single seed spike. The IAC, SqMV, was used and gave consistent detection level suggesting the equivalent processing control across all subsamples on performing this method (Annex C, Table C.13).

Table 5. Qualitative RT-qPCR results for the evaluation of analytical sensitivity for eggplant. One contaminated tomato seed (T2) was spiked in 20 subsamples of 499 healthy eggplant seeds in three laboratories. Subsamples were tested by RT-qPCR in duplicate. Pos = average Cq value ⩽ 34. Neg = Average Cq value > 34.

Subsample	Laboratory 1			Laboratory 2			Laboratory 3		
	CaTa9	ToMMV2	CSP1572	CaTa9	ToMMV2	CSP1572	CaTa9	ToMMV2	CSP1572
1	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
2	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
3	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Neg	Neg
4	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
5	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
6	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
7	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
8	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
9	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Neg	Neg
10	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
11	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
12	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
13	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
14	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
15	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
16	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Neg	Neg
17	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
18	Pos	Pos	Pos	Pos	Neg	Pos	Pos	Pos	Pos
19	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
20	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Conclusion

Using *in vitro* RNA transcripts, the analytical LOD for qPCR assays was determined to be proximately equivalent to ~1500 copy of partial ToMMV RNA transcript molecules in RT-qPCR assays (Annex C Table C.1, C.2 and C.3).

While the virus load of the individual seeds from the T2 lot, used for the spiking experiments, was unknown, the expectations for detection were made based on the single seed analysis. For each single seed spiking experiment, the minimum expected detection rate was met and exceeded in all three laboratories. This shows that the method can consistently detect a single ToMMV-contaminated seed in a subsample of either 1,000 tomato seeds or 500 pepper or eggplants seeds. However, it is important to note that on healthy seed and in some NTC reactions, late positive Cq values were obtained (Annex C Tables C.1, C.6 and C.9) due to background signal or sensitivity to cross-contamination. It is important to consider this during test implementation when fixing a laboratory specific Cq cut-off. Cq values were obtained in up to 70% of negative seed samples (Table C.6), so without the use of a cutoff the method could lead to an unacceptably high false positive rate.

Regarding the analytical sensitivity criterium, the SE-qPCR method is deemed fit for purpose.

3.3. Selectivity

Definition ISHI guidelines: *The effect of different seed matrices on the ability of the method to detect target pathogen(s).*

The selectivity requirements will be met when in the three matrices tested, namely tomato, pepper and eggplant, one contaminated seed will be detected.

Experimental approach and results

To determine the selectivity of the assays the data from the sensitivity experiments spiking one contaminated tomato seed into healthy tomato, pepper and eggplant seeds (see Section 3.2) was used. A total of 20 subsamples of seeds was prepared for tomato, pepper and eggplant, and each subsample was spiked with one seed from the previously characterized ToMMV-positive tomato seed batch (T2) into 999 ToMMV-free tomato seeds or into 499 ToMMV-free pepper or eggplant seeds. The experiment was performed in three laboratories independently using the same ToMMV-contaminated seed lot but using laboratory specific ToMMV-free seed lots. Results are shown in Annex C, Tables C.5 to C.13. A summary of the results is depicted in Table 6.

Qualitatively, in all seed lots tested for the three crops ToMMV was detected. However, some variations in the average Cq values between the varieties within a crop for all qPCR assays were observed. Interestingly, the SqMV spike Cq values were similar in all three crops in two laboratories that used this spike (laboratories 2 and 3). These results show that one positive seed can be detected in the three different seed backgrounds for all three crops.

Table 6. SE-qPCR results for the evaluation of selectivity. One contaminated tomato seed (T2) was spiked in three different tomato, pepper and eggplant seed lots for each crop. The three different laboratories tested one of the spiked seed lots per crop. The average Cq values of 20 subsamples per crop per laboratory, including the standard deviation, are displayed.

Crop	Laboratory	CaTa9	ToMMV2	CSP1572	Qualitative result
Tomato	1	25.70 ± 3.73	26.62 ± 3.50	25.70 ± 3.54	Positive
	2	26.57 ± 5.09	26.81 ± 5.08	25.04 ± 4.86	Positive
	3	27.55 ± 3.80	27.40 ± 3.76	27.01 ± 4.21	Positive
Pepper	1	21.07 ± 2.23	21.43 ± 2.13	20.64 ± 2.22	Positive
	2	24.63 ± 5.82	25.00 ± 5.67	23.44 ± 5.39	Positive
	3	23.35 ± 2.52	23.40 ± 2.57	22.42 ± 2.52	Positive
Eggplant	1	21.07 ± 2.35	21.97 ± 2.45	21.37 ± 2.42	Positive
	2	25.41 ± 5.78	25.78 ± 5.65	24.07 ± 5.44	Positive
	3	27.70 ± 5.21	28.18 ± 3.64	27.82 ± 3.98	Positive

Conclusion

The one contaminated seed was detected in the three different seed backgrounds in the three crops. Therefore, the requirements for the selectivity criterium are met. Regarding selectivity, the SE-qPCR method is deemed fit for purpose.

The variation in Cq-values observed between the laboratories in all three crop species show that primer and method performance can be impacted by the overall laboratory processes in place and that a robust in-house validation process should be conducted and used to optimize primer and method performance. Also, the variable viral charges on each infected seed could have been

a factor influencing this variation. However, in this study final qualitative results were comparable between laboratories.

3.4. Repeatability

Definition ISHI guidelines: *Degree of similarity in results of replicates of the same seed lots when the method is performed with minimal variations in a single laboratory.*

The repeatability requirements will be met when accordance is > 90% and all subsamples yield consistent qualitative results between the two time points.

Experimental approach and results

The repeatability was evaluated at the method level for tomato and pepper seed lots following the complete procedure, as described in Annex A.

A total of 20 tomato seed lots including 10 ToMMV-free and 10 ToMMV-contaminated seed lots with various contamination levels (high, medium and low) were used; some of the seed lots were known to be contaminated with other tobamoviruses or pospiviroids. Two subsamples were prepared from each seed lot, one subsample was analysed on day one (Repetition I). The analysis was then repeated on the remaining subsample on the second day (Repetition II) by the same technician and using the same reagents and equipment. All PCRs for each subsample were conducted in a triplex reaction (with CaTa9, CSP1572 and *nad5*) and a duplex reaction (with ToMMV2 and *nad5*). The results of all subsamples from the ToMMV-free seed lots were either undetermined or with a Cq >35 (Annex D, Table D.1). Moreover, all subsamples from ToMMV-contaminated seed lots were detected in these two repeated analyses across three relative contamination levels: high (seed lots 11 and 12, Cq <20), medium (seed lots 13 and 14, Cq 20 - 26) and low (seed lots 15 - 20, Cq > 26) (Annex D, Table D.1). As seen with other targets, the variability in the Cq values between replicates increases as the infection level decreases, as it is expected that not every seed will be contaminated at the same rate. The IAC, *nad5* gave consistent Cq values in Repetition I and Repetition II experiments indicating equivalent recovery of RNA during extraction for all subsamples from the repeated experiments.

Ten different pepper seed lots were used for evaluating repeatability. While homogenously ToMMV-contaminated pepper seed lots were not available, all ToMMV-free pepper seed lots were tested with and without spiking with seed flour from a known homogenously ToMMV-contaminated tomato. A similar repeatability experimental design, as described above was also applied to these pepper seed lots using 500 seeds per subsample. Two subsamples in each experiment were prepared from each seed lot, one subsample was spiked with ToMMV-contaminated seed flour and the other one was not spiked. A total of 20 subsamples from 10 seed lots were analysed on day one (Repetition I). The analysis was repeated on a second set of subsamples on the next day (Repetition II) by the same technician and using the same reagents and equipment. The IAC, *nad5*, gave consistent Cq values in both experiments showing the equivalent recovery of RNA during the extraction and the consistency of method performance for all subsamples in these repeated experiments (Annex D, Table D.2). The results of all subsamples without a spike showed either no Cq or with a Cq > 35. On the contrary, the results of all subsamples that were spiked with ToMMV-contaminated seed flour gave consistent detection across all the seed lots in these two repeated analyses.

All tomato and pepper batches and their technical replicates gave the same consistent qualitative (positive or negative) results. The accordance for these batches was calculated as 100%.

Conclusion

These results demonstrated the repeatability of the method to differentiate subsamples with and without ToMMV. Additionally, these results show that different tomato and pepper seed matrices do not impact the detection accuracy of the method.

Regarding the repeatability criterium, the SE-qPCR method is deemed fit for purpose.

3.5. Reproducibility

Definition ISHI guidelines: *Degree of similarity in results when the method is performed across laboratories with replicates of the same subsamples.*

The reproducibility requirements will be met when concordance among participating laboratories in the comparative test is > 90%.

Experimental approach and results

A comparative test (CT) was executed using a variety of ToMMV-free and ToMMV naturally contaminated tomato and pepper seed lots. There were no naturally contaminated eggplant seed lots available, therefore, a ToMMV-free eggplant seed lot was spiked with either ToMMV-free or ToMMV-contaminated tomato seed flours, each eggplant subsample was spiked individually. Five laboratories across the US and one laboratory from the Netherlands participated in this experiment. The participating laboratories were selected based on their previous experience in performing the method, as described in Annex A. Seed lots were selected based on consistent homogeneous results from at least two independent assessments (three samples consisting of three subsamples each from tomato seed lots or six subsamples from pepper seed lots) (Data not shown). Identification of the ToMMV contamination level was performed using ToMMV specific primers in RT-qPCR assays.

Given that there were limited seed lots available, each laboratory received eight tomato, four pepper and two eggplant seed samples of 3,000 seeds each. Three ToMMV-free tomato seed batches and five naturally contaminated ToMMV tomato seed batches with low, medium or high levels were selected. A total of three pepper batches were used, including two ToMMV-free seed batches and one naturally contaminated but not homogeneous seed batch. One of two ToMMV-free pepper batch was further spiked with either ToMMV-free or ToMMV-positive seed flour (T2) to generate one negative and one positive-spiked pepper samples, respectively, to give rise to a total of four pepper seed samples. Additionally, one ToMMV-free eggplant seed batch was used and spiked with either ToMMV-free or ToMMV-positive tomato seed flour (T2) to generate one negative and one positive-spiked eggplant samples, respectively, to reach for the total of two eggplant seed samples.

All samples were coded, to blind CT participants for the infection status, and distributed to the CT participants. For the organising laboratory, the samples were coded again by non-participating personnel before the testing. All generated data and details of protocol used in each US laboratory were collected by an independent external laboratory. Table E. 1 in Annex E provides an overview of the method used by each laboratory including details of the subsample size, seed grinding method, RNA extraction method and RT-qPCR. All laboratories were using either IKA® mill (Staufen, Germany) or Geno/Grinder® (Cole-Parmer, Metuchen, NJ) to grind seeds, and RNA extraction buffer was used for homogenizing either the entire subsample or an aliquot of the ground seed flour subsample. The RT-qPCR assays were then performed in duplex or triplex PCR

reactions combined with either endogenous gene (*nad5*) or spike with unrelated pathogen (SqMV or DLVd) to serve as the IACs.

The raw data from all six CT participants is presented in Annex E, Table E.2. Note that although eight laboratories initially volunteered, participants 5 and 7 did not perform the test. Table 7 summarizes the qualitative testing results from the six CT participants for each sample.

The CT results of participants 1, 3, 4 and 6 were 100% in line with the expected results. Participant 2 detected two negative tomato seed samples (tomato-1 and -2) as positive but conducted a re-test on the two tomato samples and the results and their final conclusion was that the samples were negative (data not shown). It is suggested that the two false positive detections were due to a potential cross contamination as all previous data indicated that these lots gave negative results or Cq values > 35 (data not shown). Participant 8 detected one negative tomato seed sample (Tomato-3) as positive.

In general, the Cq values above 35 or undetermined were obtained in a total of six negative tomato, pepper and eggplant samples. The IACs, *nad5*, SqMV or DLVd spike, were detected in each sample.

The overall concordance of the comparative test was calculated to be 92.99% (positive pairings / total pairings = 5468/5880= 92.99%) using the method published by Langton *et al.* (2002), which is above the requirement of 90% fixed for the validation of this characteristic. The reduction in concordance was due to the positive results obtained by two laboratories on uninfected tomato seed samples.

Table 7. Qualitative results of the comparative test in which replicate samples were evaluated across laboratories. A total of fourteen samples (3,000 seed sample size), including eight tomato, four pepper and two eggplant samples. Contamination levels of ToMMV and other contaminants are indicated.

Sample	Known contaminant			Participant					
	ToMMV	ToMMV level[b]	Others	1	2	3	4	6	8
Tomato-1	Negative	Healthy	None?	Neg	Pos[c]	Neg	Neg	Neg	Neg
Tomato-2	Negative	Healthy	ToBRFV+	Neg	Pos[c]	Neg	Neg	Neg	Neg
Tomato-3	Negative	Healthy	None?	Neg	Neg	Neg	Neg	Neg	Pos
Tomato-4	Positive	High	None?	Pos	Pos	Pos	Pos	Pos	Pos
Tomato-5	Positive	Medium	ToMV+	Pos	Pos	Pos	Pos	Pos	Pos
Tomato-6	Positive	Low-medium	ToMV+	Pos	Pos	Pos	Pos	Pos	Pos
Tomato-7	Positive	Low	None?	Pos	Pos	Pos	Pos	Pos	Pos
Tomato-8	Positive	Low-medium	None?	Pos	Pos	Pos	Pos	Pos	Pos
Pepper-9	Negative	Healthy	PMMoV+	Neg	Neg	Neg	Neg	Neg	Neg
Pepper-10[a]	Negative	Healthy	PMMoV+	Neg	Neg	Neg	Neg	Neg	Neg
Pepper-11[a]	Positive	Low-medium	PMMoV+	Pos	Pos	Pos	Pos	Pos	Pos
Pepper-12	Positive	Low-medium	PMMoV+	Pos	Pos	Pos	Pos	Pos	Pos
Eggplant-13[a]	Negative	Healthy	None?	Neg	Neg	Neg	Neg	Neg	Neg
Eggplant-14[a]	Positive	Low-medium	None?	Pos	Pos	Pos	Pos	Pos	Pos

[a] Seed flour spikes were applied.

[b] ToMMV contamination level evaluated based on Cq values: High < 20; Medium 20-26, Low > 26.

[c] Two samples were re-tested in the same laboratory and two samples were negative.

Conclusion

Overall, the comparative test showed there was high reproducibility of the method across the participating laboratories. Molecular assays are generally sensitive to being compromised during the operation process, measures to prevent cross contamination are highly recommended.

3.5. Diagnostic performance

Definition ISHI guidelines: *An evaluation of the ability of the method to discriminate between positive and negative seed lots.*

The diagnostic performance requirements will be met when diagnostic sensitivity reaches 100% and the diagnostic specificity is ⩾ 90%.

Experimental approach and results

The diagnostic sensitivity and diagnostic specificity, the diagnostic performance parameters of the method in other words, were calculated according to the mathematical formulas detailed in Table 8. In the absence of a reference method for ToMMV, the qualitative data (detected/not detected) generated during the evaluation of reproducibility through the comparative test, *viz*. the expected and obtained results in all participating laboratories, were used in the analysis. The expected results were determined as described in Section 3.4, when selecting the seed lots for the comparative test.

Table 8. Calculations of the diagnostic sensitivity and diagnostic specificity based on the qualitative results of the comparative test.

		Expected results	
		Positive	**Negative**
Test outcome	**Positive**	True positive (TP) = 48	False positive (FP) = 3
	Negative	False negative (FN) = 0	True negative (TN) = 33
		DIAGNOSTIC SENSITIVITY = (TP / (TP + FN)) × 100 = 100	**DIAGNOSTIC SPECIFICITY = (TN / (FP + TN)) × 100 = 91.7**

The results of this method obtained 100% (48/48 samples) of true positive detection and 0% (0/48) of false negative detection of ToMMV (Table 8). On the negative samples 91.7% (33/36 of samples) were correctly identified, meaning 8.3% of false positives (Table 8). In terms of method diagnostic sensitivity, all laboratories detected ToMMV in all positive samples and showed comparable Cq values corresponding to high, medium and low level of ToMMV detection. Three false positive results were generated by two participants (2 and 8) in three tomato samples.

Conclusion

Results show a diagnostic sensitivity of 100% and diagnostic specificity of 91.7%. This means that both requirements for passing the diagnostic performance criteria are met.

Regarding the diagnostic performance criterium, the SE-qPCR method is fit for purpose.

3.6. Robustness

Definition ISHI guidelines:

Robustness is not defined in the ISHI guidelines as a characteristic for the validation of an ISHI method, but it is defined in this validation report as the capacity of a method to remain unaffected by small, deliberate variations in method parameters.

Experimental approach and results

The robustness was evaluated at the method level where the complete procedure was performed as described in Annex A. To demonstrate the ability of the method to remain unaffected by small but deliberate variations in method parameters and to indicate the reliability of the method during normal usage, the following four variables were evaluated: i) RNA extraction efficacy with different pre-lysis buffers, ii) two different RNA extraction methods, iii) two different one step RT-qPCR master mixes and iv) duplex *versus* triplex PCR assay performance.

RNA extraction efficacy with different pre-lysis buffers (RNA extraction buffer)

The robustness of the method was evaluated with different pre-lysis buffers at the RNA extraction step. The two guanidine-based buffers (GuHCl and Gu-isothiocyanate) from two reference protocols were listed in Annex A in which the main active ingredient is guanidine compound as the protein denaturant. A total of nine ToMMV-negative pepper seed lots were tested with or without the addition of a spike with ToMMV-positive seed flour. Each subsample of 500 seeds were ground by IKA® mill (Staufen, Germany) and seed flours were equally divided into two portions to minimize any potential variations among seeds in a given subsample. These two RNA extraction buffers were added separately to each sub-divided flour from each subsample and followed the procedure, as described in Annex A. The results of nine seed subsample comparison gave consistent and comparable results with all targets as well as the IAC, *nad5*, suggesting the equivalent recovery of RNA extraction for all subsamples with different RNA extraction buffers (Annex F, Table F.1).

Two different RNA extraction methods: MagMAX RNA isolation kit to Qiagen PowerPlant kit

The robustness of the method at RNA isolation options was also evaluated using the same seed extracts from the reproducibility study, including eight tomato samples (eight seed lots containing three ToMMV-negative seed lots and five ToMMV-positive seed lots with various contamination levels), four pepper samples (three seed lots, containing two ToMMV-negative seed lots and one ToMMV-positive seed lot) and two eggplant samples from one ToMMV negative seed lots. Among these samples, two of pepper and two of eggplant samples were spiked with ToMMV-negative and ToMMV-positive seed flours to generate a pair of positive and negative samples for pepper and eggplant, separately. All samples were processed as the complete procedure described in Annex A and an additional 200 µL of seed matrix from each subsample was taken to compare at the RNA isolation step between PowerPlant® kit (Qiagen, Hilden, Germany) and MagMAX™ RNA isolation kit (Applied Biosystems, ThermoFisher Scientific, Waltham, MA). The RT-qPCR results of the total 60 subsamples were consistent between both RNA isolation methods. ToMMV-negative samples remained undetermined or Cq > 35 with both extraction kits, while various levels of ToMMV-positive samples gave comparable Cq values with all target primers as well as the IAC with both RNA isolation methods (Annex F, Table F.2).

Two different one step RT-qPCR mastermixes: TaqPath MasterMix to Ultraplex ToughMix

Two different one step multiplex mastermixes were also evaluated for the robustness of the method. This comparison was conducted using previously identified nine ToMMV-negative

pepper seed lots using ToMMV-positive seed flour in which a spike was added or not. Two sets of nine pepper subsamples were prepared from nine seed lots. For the first set, no spike was added to the samples while in the second set, a spike was added using ToMMV-positive seed flour. A total of 18 subsamples were processed (complete procedure is described in Annex A) and the same RNA extracts were tested by PCR assays with either the TaqPath MasterMix (Applied Biosystems) and Ultraplex ToughMix (Quantabio), separately. The PCR results of the nine ToMMV-negative subsamples were undetermined or Cq > 35 from both mastermixes, while nine-spiked subsamples gave comparable and consistent Cq values with target primers. The IAC, *nad5*, also gave comparable Cq values suggesting the equivalent recovery of RNA extraction for all subsamples and that no inhibitory effect on those undetermined samples occurred (Annex F, Table F.3).

Duplex *vs*. triplex PCR assay performance

The robustness of the method also evaluated the PCR assay performance in duplex and triplex reactions. Three of ToMMV-positive subsamples from tomato, pepper and eggplant each were prepared separately, and each subsample was processed as per the procedure described in Annex A using SqMV as the IAC. Total RNA extract from each subsample was tested in combinations with one target primer set and IAC in duplex PCR assays and in combination with two target primer sets and IAC in triplex PCR assays. The results of PCR comparison gave consistent and comparable Cq values with all target primer sets as well as the IAC also gave comparable Cq values across PCR reactions (Annex F, Table F.4), suggesting the equivalent input of total RNA extracts was used in each PCR reaction and the format of duplex or triplex PCR assays gave equal and reliable performance.

Conclusion

These results supported the robustness of the method using alternative RNA extraction buffers, RNA isolation kits, mastermixes, and duplex/triplex assays.

Based on the results, the alternatives of RNA extraction buffer are included in the protocol (Annex A) and the options of using other equivalent mastermix than validated as described in the protocol (Annex A) is supported and alternative options for PCR assays are also included in the protocol (Annex A), which allows flexibility in PCR setting for testing efficiency.

4. CONCLUSION

The data presented in this validation reports shows that the SE-qPCR detection assay for ToMMV in samples of tomato and pepper seed meets the requirements set for the validation criteria listed above. Therefore, the ISHI ToMMV SE-qPCR assay is fit for its intended purpose to serve as a specific pre-screening assay for the detection of ToMMV followed by confirmation with the ISHI tobamovirus bioassay.

5. REFERENCES

Botermans, M., Vossenberg, B.T.L.H. van de., Verhoeven, J.Th.J. and Roenhorst, J.W., Hooftman, M., Dekter, R., and Meekes, E.T.M. (2013) Development and validation of a real-time RT-PCR assay for generic detection of pospiviroids, *Journal of Virological Methods*, **187** (1), 43-50. https://doi.org/10.1016/j.jviromet.2012.09.004.

Chai, A. L., Chen, L. D., Li, B. J., Xie, X. W., and Shi, Y. X. (2018) First report of a mixed infection of Tomato mottle mosaic virus and Tobacco mild green mosaic virus on eggplants in China. *Plant Disease,* **102**(12), 2668. https://doi.org/10.1094/PDIS-04-18-0686-PDN

Hiddink, G., Tavares, C., Beugelsdijk, D., Pannu, S., Geraats, B, Langens, M., and Ranganathan, R. (2019). Reliable detection and identification of Tomato brown rugose fruit virus (ToBRFV) and other tobamoviruses in seeds of Solanaceae. Poster presented at APS annual meeting.

ISHI-Veg, International Seed Federation. (2019). Detection of infectious Tomato brown rugose fruit virus (ToBRFV) in tomato and pepper seed. Version 1.3

ISHI-Veg, International Seed Federation. (2019). ISHI-Veg develops new, specific detection method – Tomato brown rugose fruit virus. *Prophyta Annual,* **2019**, 10-15 http://www.prophyta.org/prophyta/Prophyta2019-ISHI-veg.pdf

Letowski, J., Brousseau, R., and Masson, L. (2004) Designing better probes: effect of probe size, mismatch position and number on hybridization in DNA oligonucleotide microarrays. *Journal of Microbiological Methods*, **57**(2), 269-278.

Levitzky, N., Smith, E., Lachman, O., Luria, N., Mizrahi, Y., Bakelman, H., Sela, N., Laskar, O., Milrot, E., and 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*, **14**(1), p.e0210871.

Li, R., Gao, S., Fei, Z., and Ling, K-S. (2013). Complete genome sequence of a new tobamovirus naturally infecting tomatoes in Mexico. *Genome Announcements*, **1**(5), e00794-13. https://doi.org/10.1128/genomea.00794-13

Li, Y., Wang, Y., Hu, J., Xiao, L., Tan, G., Lan, P., Liu, Y., and Li, F. (2017). The complete genome sequence, occurrence and host range of Tomato mottle mosaic virus Chinese isolate. *Virology Journal*, **14**, 15.

Sui, X., Zheng, Y., Li, R., Padmanabhan, C., Tian, T., Groth-Helms, D., Keinath, A. P., Fei, Z., Wu, Z., and Lin, K.S. (2017). Molecular and biological characterization of Tomato mottle mosaic virus and development of RT-PCR detection. *Plant Disease*, **101**(5), 704-711.

Luria, N., Smith, E., Reingold, V., Bekelman, I., Lapidot, M., Levin, I., Elad, N., Tam, Y., Sela, N., Abu-Ras, A., Ezra, N., Haberman, A., Yitzhak, L., Lachman, O., and Dombrovsky, A. (2017). A new Israeli Tobamovirus isolate infects tomato plants harboring Tm-22 resistance genes. *PLoS ONE,* **12**(1), e0170429.

Mehle, N., Vučurović, A., Bajde, I., Brodarič, J., Grausgruber-Groeger, S., Baldwin, T., Ziebell, H., Fox, A., Abu-Ras, A., Zeidan, M., Gershon, T., Koenraadt, H., Barnhoorn, R., Delmiglio, C., Thompson, J., Shneyder, Y., Karimova, E., Kaiser, M., Frapolli, M., Amato, M., Rivera, Y., Padmanabhan, C., Tiberini, A., Manglli, A., Grant, N., Webster, W., Constable, F. (2024). ToMMV-detect 2022-A-394 Interlaboratory Test Performance Study. https://drop.euphresco.net/data/af730655-4022-4e87-a952-b94cfda3a971

Menzel, W., and Winter, S. (2021) Identification of novel and known tobamoviruses in tomato and other solanaceous crops using a new pair of generic primers and development of a specific RT-qPCR for ToBRFV. *Acta horticulturae*, **1316**, 143-148. https://doi.org/10.17660/ActaHortic.2021.1316.20

Menzel, W., Jelkmann, W., and Maiss, E. (2002). Detection of four apple viruses by multiplex RT-PCR assays with co-amplification of plant mRNA as internal control. Journal of Virological Methods, **99,** 81–92

National Seed Health System. (2020). Method for testing Pospiviroids (CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd) on tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) seeds using TaqMan RT-PCR.

Rodríguez-Mendoza, J., de García-Ávila, C., López-Buenfil, J. A., Araujo-Ruiz, K., Quezada-Salinas, A., Cambrón-Crisantos, J. M., and Ochoa-Martínex, D.L. (2019). Identification of *Tomato brown rugose fruit virus* by RT-PCR from a coding region or replicase (RdRP). *Revista mexicana de fitopatología*, **37**(2), Epub. https://doi.org/10.18781/r.mex.fit.1902-6.

Wu, J-H., Hong, P-Y., and Liu, W-T. (2009). Quantitative effects of position and type of single mismatch on single base primer extension. *Journal of Microbiological Methods*, **77**(3), 267-275. https://doi.org/10.1016/j.mimet.2009.03.001.

Schoen, R., de Koning, P., Oplaat, C., Roenhorst, A., Westenberg, M, van der Gaag, D.J., Barnhoorn, R., Koenraadt, H., van Dooijeweert, W., Lievers R., Woudt, B., Pinto Tavares, C. and Botermans, M. (2023) Identification of Tomato mottle mosaic virus in historic seed accessions originating from France, the Netherlands and Spain, indicates a wider presence before its first description. *European Journal of Plant Pathology*, **166**, 485–489.

6. ANNEXES

Annex A. ToMMV protocol.

Protocol for detecting Tomato mottle mosaic virus (ToMMV) on tomato (*Solanum lycopersicum*), pepper (*Capsicum spp.*) and eggplant (*Solanum melongena*) seeds using RT-qPCR

1. Objective

To detect the presence or absence of ToMMV in tomato, pepper and eggplant seed by isolation of total RNA for Reverse Transcriptase (RT) quantitative PCR using TaqMan assays. A SqMV or DLVd spike is used as a process control to monitor for inhibition. Alternatively endogenous targets such as *nad5* present in tomato, pepper and eggplant can be used for this purpose.

2. Materials and Equipment

- Geno/Grinder® 2010 (Cole-Parmer®, Vernon Hills, IL), or equivalent
- Tube Mill 100 control (Geno/Grinder 2010), or equivalent
- Mills MT 40.100 (IKA®, Staufen, Germany), or equivalent
- 50 mL conical shaped tubes
- Steel balls or zirconium beads
- RNA extraction buffer (Guanidinium-based buffer, see Table A.1)
- Thermal shaker or heat block
- Vortex mixer
- Centrifuges for 50 mL sample tubes and microcentrifuge tubes
- Vacuum manifolds (Optional)
- Positive RNA controls
- RNA extraction kit - RNeasy PowerPlant Kit (Qiagen, Hilden, Germany), MagMax Plant RNA kit (ABI, Foster City, CA, US), or other extraction kits with equivalent performance
- TaqMan RT-PCR reagents, including primers and probes
- Mastermixes - Ultraplex™ 1-Step ToughMix® (4×) Low Rox (Quantabio, Beverly, MA) or other mastermix with equivalent performance
- MicroAmp™Fast Optical 96-Well Reaction Plate (Thermofisher, Waltham, MA), or equivalent
- MicroAmp™ Optical Adhesive Film (Thermofisher), or equivalent
- Real-time PCR system

Table A.1. RNA extraction buffer[a].

Compound	Amount/L
DI Water	350 mL
PVP-40 (3% (w/v))	30 g
Guanidine (Iso)thiocynate (4 M)[b]	473 g
Sodium acetate (0.2 M)	16 g
0.5 M EDTA (25 mM)	50 mL
Sodium sulfite (1% (w/v))	10 g
Adjust pH to 5.0 with 37% (v/v) HCl	
Sodium metabisulfite	10 g

[a] Guanidine-HCl extraction buffer can also be used (Naktuinbouw Pospiviroid protocol SPN-V043e).

[b] Guanidine thiocyanate can replace Guanidine isothiocyanate.

Example of an internal amplification control (IAC): Preparation of SqMV spike for monitoring the quality of RNA extraction.

Other suitable IACs can be used (DLVd, BacV spikes, or endogenous *nad5* plant gene)

1. Take 0.1 mg of SqMV infected tissue, grind and add 50 mL of GenEx or PBS buffer.
2. From this 50 mL suspension, make 10-fold serial dilutions from 10^{-1} to 10^{-4}.
3. Take 10 µL from each dilution (at least three replications per dilution) and spike into seed matrix with RNA extraction buffer and proceed with extraction procedure.
4. Run RT-qPCR to identify Cq values of each dilution and make additional adjustment as needed to prepare a dilution to obtain Cq results close to 28.
5. After confirming the selected dilution giving consistent Cq value of 28, aliquots can be prepared into 2 mL tubes and freeze aliquots for future use.
6. Thaw the tubes and use 10 µL SqMV spike for each extraction/subsample.
7. Cq value of SqMV spike in unknown samples should be 28 ± 3.
8. If Cq values deviate from expected values, prepare new SqMV control and validate before use.

3. Safety

Guanidinium-based buffer is harmful and take extra precaution when handling guanidinium-based buffer. Follow SDS and safety guidelines, as recommended.

4. Method

4.1. Sample preparation

4.1.1. Grinding

Two examples of seed grinding are given below. Alternative grinding techniques should produce a seed powder visually similar to Figure A.1.

A. Example 1: Geno/Grinder

i. Weigh subsamples of 500 or 1,000 seeds for tomato or 500 seeds for pepper and place into 50 mL tubes. Add an appropriate ball bearing(s).

Option: Freeze sample tubes containing seeds and ball bearings at -80 °C or -20 °C overnight or seed subsamples can be snap frozen by placing tubes in liquid nitrogen to enhance grinding results.

ii. For tomato seeds, grind seeds using Geno/Grinder at 1,400 – 1,700 rpm for 2 min.

For pepper and eggplant seeds, grind seeds using a Geno/Grinder at 1,400-1,700 rpm for 2 min twice.

Option: Refreeze samples between grinding cycles as needed via freezer, dry ice or liquid N_2 to enhance grinding results.

B. Example 2: IKA Mill (grinder)

i. Weigh subsamples of 500 or 1,000 seeds for tomato or 500 seeds for pepper and place into 40 mL mill tube.

ii. Set the speed at 25,000 rpm for 20 sec and transfer ground seed flour to testing tube for RNA isolation (Figure A.1).

Figure A.1. Demonstration of ground tomato (A) and pepper (B) seed flours compared to unground seeds.

4.1.2. Preparation for RNA isolation

A. Option 1

i. Add RNA extraction buffer to each ground subsample according to Table A.2.

Table A.2. Buffer volumes to be added to subsamples.

Crop	500 seeds	1,000 seeds
Tomato		12-14 mL
Pepper/Eggplant	12-14 mL	--

ii. Mix samples with buffer vigorously by vortex or shaking and incubate samples at room temperature for 30-45 min.

iii. Centrifuge the sample tubes up to 29,000 × *g* for 5 min, transfer 200 µL of supernatant to a new 2.0 mL tube or a well of a sample plate and follow appropriate steps according to preferred RNA extraction method (step 4.2.)

Note: Various volume of supernatant may be used for the next RNA extraction step upon validation and demonstrated the equivalency to PowerPlant kit (Qiagen).

B. Option 2

i. Weigh 70-80 mg per subsample from ground seed flour into a bead tube, a new 2.0 mL tube or a sample plate and follow appropriate steps according to preferred RNA extraction method (step 4.2.)

4.1.3. If desired, prepare the SqMV spike and add to each subsample aliquot (step 4.1.2.) prior for RNA extraction. Alternatively, the endogenous *nad5* target can be used as a process control.

4.2. RNA isolation

The assay was validated with RNeasy PowerPlant kit (Qiagen), MagMax Plant RNA isolation kit (ABI), Sbeadex kit and Maxwell RSC Plant RNA kit (Promega). Other RNA extraction methods with equivalent performance can be used.

Follow Manufacturer's protocol. Use an elution volume of 100 µL.

4.3. TaqMan RT-PCR

Work on ice as much as possible and prevent prolonged exposure of probes to light. Wear clean lab coat and gloves to minimize the risk of cross-contamination.

4.3.1. Prepare the TaqMan RT-PCR mixes according to the tables below and use the PCR mixes: Ultraplex 1-Step ToughMix (4×) or other master mixes that have equivalent performance. Fluorophores, quenchers of the probes and passive dyes also may need to be adjusted depending on the equipment used. Verify test performance by thorough in-laboratory validation.

4.3.2. Add the appropriate primers and probe (*nad5* or SqMV) in each PCR mix and calculate the required amount for reaction mixes (Table A.3).

Table A.3. Examples of internal amplification control (IAC) primers and probe sequences.

Name	Final concentration	Target	Sequence (5' – 3')
Nad5 -F	100 nM	*nad5*	GATGCTTCTTGGGGCTTCTTGTT
Nad5 -R	100 nM		CTCCAGTCACCAACATTGGCATAA
Nad5 -Pr	50 nM		VIC-AGGATCCGCATAGCCCTCGATTTATGTG-NFQ-MGB
SqMV-F	200 nM	SqMV	TAGGAATTTCTGGGCAGAGT
SqMV-R	200 nM		GGGCTGTACTTTCTAAGGG
SqMV-Pr	100 nM		Texas Red-CAGCAGCTTGGAACTTATAATCCAAT-BHQ2

4.3.3. Include positive amplification controls (PAC) for each PCR assay.

4.3.4. Prepare PCR mixes using the following template with two of the three ToMMV primer sets (Table A.4):

A. Include one out primer set (ToMMV2 or CSP1572) targeting the ToMMV capsid protein (CP).
B. Include ToMMV CaTa9, as the primer set targeting another region of viral genome.

Table A.4. Primer and probe sequences and references.

Name	Target	Sequence (5' – 3') (*fluorophore FAM as an example*)	Source
CaTa9 Fw	ToMMV Replicase	ATGTGGAGGAACCCTCTATGA	Hiddink *et al.*, 2019
CaTa9 Pr		6FAM-TCAATGGCCCGTGGTGAGTTACAA-BHQ1	
CaTa9 Rv		AATCTCCTCGCTCCTTGTAAAC	
ToMMV2-Fw	ToMMV CP and 3'UTR	GAAACATTGGATGCCACTCG	Schoen *et al.*, 2023
ToMMV2-Pr		6FAM - CGATGCTACGGTTGCGATCAGGTC-BHQ1	
ToMMV2-Rv		CTCTGGTTGTAGAAACCTGTTCC	
CSP1572 Fw	ToMMV CP and 3'UTR	CCCGACTACAGCCGAAACAT	Mehle *et al.*, 2024
CSP1572 Pr		6FAM - TGCCACTCGCAGAGTGGACGATGCTACG - BHQ1	
CSP1572 Rv		TTAACAGCGGACCTGATCGC	

4.3.5. Prepare PCR mixes, examples are indicated in Table A.5 and Table A.6.

4.3.6. Include a positive RNA control and a no-template control in each run.

4.3.7. Run the assay using the program indicated in Table A.7.

Table A.5. Example 1: PCR mix for triplex PCRs.

Component	Final concentration	Target
RNase-Free Water		
MasterMix	1×	
Primer set 1 -For	300 nM	ToMMV CP
Primer set 1 -Rev	300 nM	
Primer set 1 -Probe 1[a]	200 nM	
Primer set 2 -For	300 nM	ToMMV Replicase
Primer set 2 -Rev	300 nM	
Primer set 2 -Probe 2[a]	200 nM	
IAC Forward	Reference above	Internal amplification control
IAC Reverse		
IAC Probe 3[a]		
RNA extract	4 µL	
Total	20 µL	

[a] Probes 1, 2, 3 are labelled with different fluorophores.

Table A.6. Example 2: PCR mix for two sets of duplex PCRs.

Reagent	Final concentration	Target
RNase-Free Water		
MasterMix	1×	
Primer set 1 -For	300 nM	ToMMV
Primer set 1 -Rev	300 nM	
Primer set 1 -Probe[a]	200 nM	
IAC Forward	Reference above	Internal amplification control
IAC Reverse		
IAC Probe[a]		
RNA extract	4 µL	
Total	20 µL	

[a] Probes 1 and 2 are labelled with different fluorophores.

Table A.7. RT-qPCR conditions.

Step	Temperature	Time
cDNA synthesis	48-50 °C[a]	10-15 min[a]
Denaturation	95 °C	3-10 min[a]
PCR cycling (40 cycles)	95 °C	10 sec
	60 °C	60 sec

[a] The parameters can be modified based on in-house instrument performance.

4.4. Evaluation RT-qPCR test result and interpretation

4.4.1. Threshold setting is critical and need to be fixed during method implementation depending on the use of master mixes and thermal cycler.

4.4.2. Results are valid only if positive controls give a clear signal with a Cq < 30 and negative controls have a Cq of > 35. The amplification of an IAC should give a clear signal, preferably a Cq < 30 for endogenous control and Cq 28 ± 3 for spiked control. *Nad5* Cq values can be variable between different seed lots due to heterogenous expression of this gene.

4.4.3. Determine if ToMMV was detected in each seed lot according to Table A.8.

Table A.8. Decision thresholds as used in the comparative test method validation.

ToMMV Primer 1	ToMMV Primer 2	RT-qPCR result
< 35	< 35	Positive
⩾ 35	< 35	Positive
< 35	⩾ 35	Positive
⩾ 35	⩾ 35	Negative

Annex B. Specificity.

Table B.1. Information of twenty eight full-length viral genome sequences of ToMMV collected from GenBank and used in inclusivity of primers and probes evaluation via *in silico* analysis. NA = not available.

Accession No.	Crop	Location	Collection Year	Isolate
KX898034.1	Tomato	USA	2016	CA16-01
MW582804.1	Tomato	USA	NA	NA
KF477193.1	Tomato	MX	2009	MX5
KX898033.1	Tomato	USA	2013	SC13-05
KR824951.1	Pepper	China	2013	TiLhaLJ
KR824950.1	Pepper	China	2013	YYMLJ
MH381817.1	Tomato	China	NA	HN
KT810183.1	Tomato	USA	2013	NY-13
KP202857.1	Tomato	USA	2010	FL 10-100
MG171192.1	Tomato	China	2016	Hainan
OK334224.1	Tomato	Vietnam	2020	ToMMV_83
MZ713256.1	Tomato	China	2020	NVWA3678860
MN853592.1	Tomato	China	2015	LN
MN654021.1	Pepper	Netherlands	2019	19-02305
MZ713257.1	Tomato	NL/UK	2021	NVWA41106813
KU594507.2	Tomato	Spain	2015	NA
MH128145.1	Tomato	Brazil	1992	CpB1
MW441234.1	Tomato	China	2020	SY11026
MZ713257.1	Tomato	NL/UK	2021	NVWA41106813
ON146334	Pisum sativum	China	2021	MZWD
MW373515	Tomato	China	2022	Shandong
OK180812	Pepper	China	2020	GD-2020
LC779003	Sweet pepper	Japan	2021	ToMMV-SP
PP894826	NA	China	2024	HLD
ON013925	Tomato	NA	2022	DSMZ PV-1342
ON987480	Pepper	Spain	2016	NPPO-NL 41979411
ON987481	Tomato	France	2011	NPPO-NL 41979438
ON987482	Tomato	Netherlands	2007	NPPO-NL 41979470

Table B.2. Inclusivity of primers and probes were evaluated via *in silico* analysis. Twenty-eight full-length viral genome sequences of ToMMV were collected from GenBank. All primers and probes were aligned to the sequences and mismatches (MM) were determined and are indicated in red.

Primer/Probe	Sequence (5' -> 3')	Sequence analysis result
CaTa9 Fw	ATGTGGAGGAACCCTCTATGA	1 nt MM in 3 out of 28 sequences
CaTa9 Pr	TCAATGGCCCGTGGTGAGTTACAA	1 nt MM in 2 out of 28 sequences
CaTa9 Rv	AATCTCCTCGCTCCTTGTAAAC	100% match
ToMMV2 Fw	GAAACATTGGATGCCACTCG	1 nt MM in 2 out of 28 sequences
ToMMV2 Pr	CGATGCTACGGTTGCGATCAGGTC	1 nt MM in 2 out of 28 sequences
ToMMV2 Rv	CTCTGGTTGTAGAAACCTGTTCC	1 nt MM in 2 out of 28 sequences
CSP1572 – Fw	CCCGACTACAGCCGAAACAT	100% match
CSP1572 – Pr	TGCCACTCGCAGAGTGGACGATGCTACG	2 nt MM in 5 out of 28 sequences
CSP1572 – Rv	TTAACAGCGGACCTGATCGC	2 nt MM in 7 out of 28 sequences

MM: Mismatch

Table B.3. Exclusivity data. N/A = No amplification.

Isolate ID	Virus	Laboratory	Cq values		
			CaTa9	ToMMV2	CSP1572
AMV	Alfalfa mosaic virus	2	N/A	N/A	Not tested
ArMV	Arabis mosaic virus	2	N/A	N/A	Not tested
BaCV	Bacopa chlorosis virus	2	N/A	N/A	Not tested
BPeMV	Bell pepper mosaic virus	1	N/A	N/A	N/A
CPMMoV	Chili pepper mild mottle virus	1	N/A	N/A	N/A
CMV	Cucumber mosaic virus	2	N/A	N/A	Not tested
DLVd	Dahlia latent viroid	2	N/A	N/A	Not tested
PepMV	Pepino mosaic virus	2	N/A	N/A	Not tested
PMMoV ISHI	Pepper mild mottle virus	1	N/A	N/A	N/A
PMMoV HMC		1	N/A	N/A	N/A
PMMoV PAS 2098		3	N/A	N/A	N/A
PMMoV PAS 486		3	N/A	39.22	39.75
TMGMV PV-0887	Tobacco mild green mosaic virus	4	N/A	N/A	Not tested
TMGMV PV-0124		4	N/A	N/A	Not tested
TMV HMC	Tobacco mosaic virus	1	N/A	N/A	N/A
TMV		2	N/A	N/A	Not tested
TMV PAS 601		3	N/A	N/A	N/A
TRSV	Tobacco ring spot virus	2	N/A	N/A	Not tested
TBRV	Tomato black ring virus	2	N/A	N/A	Not tested
ToBRFV HMC	Tomato brown rugose fruit virus	1	N/A	N/A	N/A
ToBRFV		2	N/A	N/A	Not tested
ToBRFV PAS 2609		3	N/A	N/A	N/A
ToMV HMC	Tomato mosaic virus	1	N/A	N/A	N/A
ToMV ISHI		1	N/A	N/A	N/A
ToMV		2	N/A	N/A	Not tested
ToMV PAS 598		3	39.75	N/A	N/A
ToMV PAS 599		3	N/A	N/A	N/A
ToMV PAS 603		3	N/A	N/A	N/A
ToMV PAS 600		3	N/A	N/A	N/A
ToMV PAS 1438		3	N/A	N/A	N/A
ToMV PAS 2178		3	N/A	N/A	N/A
ToMV PAS 2212		3	N/A	N/A	N/A
ToRSV	Tomato ring spot virus	2	N/A	N/A	Not tested
TSWV	Tomato spotted wilt virus	2	N/A	N/A	Not tested

Annex C. Analytical sensitivity.

Table C.1. Analytical sensitivity, determined by RT-qPCR performed on ten-fold serial dilutions of RNA transcripts for coat protein specific ToMMV primer sets: ToMMV2 and CSP1572. Two RNA transcripts were tested and copy numbers of input RNA transcripts were estimated. RT-qPCR were performed in duplicate; both Cq data points are listed below. N/A = No amplification.

Input RNA transcript	Copy number	PCR replicate	CSP1572		ToMMV2		*nad5*
			RNA 1	RNA 2	RNA 1	RNA 2	
100 pg	1.608×10^8	1	16.46	15.76	17.19	16.39	N/A
		2	16.26	15.68	16.73	16.33	N/A
10 pg	1.608×10^7	1	19.37	18.86	19.93	19.68	N/A
		2	19.32	18.92	20.17	19.67	N/A
1 pg	1.608×10^6	1	22.80	21.97	23.64	23.40	N/A
		2	22.80	22.07	23.68	23.62	N/A
100 fg	1.608×10^5	1	26.22	25.63	27.20	26.71	N/A
		2	26.19	25.54	27.12	26.77	N/A
10 fg	1.608×10^4	1	29.75	29.19	30.46	30.07	N/A
		2	29.75	29.04	30.89	30.18	N/A
1 fg	1608	1	33.02	32.82	34.04	33.95	N/A
		2	32.56	32.27	33.85	33.83	N/A
100 ag	160.8	1	36.38	36.16	38.59	36.95	N/A
		2	37.70	35.10	37.53	N/A	N/A
NTC	0	1	37.15	39.02	N/A	37.40	N/A
		2	N/A	N/A	N/A	N/A	N/A
Slope			-3.42	-3.36	-3.50	-3.46	--
R^2			0.999	1.000	0.999	1.000	--
PCR efficiency			95.91%	98.35%	92.94%	94.62%	--

Table C.2. Analytical sensitivity, determined by RT-qPCR performed on ten-fold serial dilutions of RNA transcripts for replicase specific ToMMV primer set: CaTa9. Copy numbers of input RNA transcripts were estimated. RT-qPCR were performed in duplicate; both Cq data points are listed below. N/A = No amplification.

Input RNA transcript	Copy number	PCR replicate	CaTa9	*nad5*
100 pg	5.904×10^8	1	13.71	N/A
		2	13.80	N/A
10 pg	5.904×10^7	1	17.11	N/A
		2	17.18	N/A
1 pg	5.904×10^6	1	20.32	N/A
		2	20.56	N/A
100 fg	5.904×10^5	1	23.75	N/A
		2	23.82	N/A
10 fg	5.904×10^4	1	27.05	N/A
		2	27.15	N/A
1 fg	5904	1	30.34	N/A
		2	30.54	N/A
100 ag	590.4	1	33.39	N/A
		2	34.13	N/A
10 ag	59.04	1	37.47	N/A
		2	N/A	N/A
NTC	0	1	N/A	N/A
		2	N/A	N/A
Slope			-3.32	--
R^2			0.999	--
PCR efficiency			99.60%	--

Table C.3. Data of the limit of detection using pure RNA transcripts. 250 ag and 1 fg, separately for ToMMV primer sets: CaTa9, CSP1572 and ToMMV2, was repeatedly detected in 20 replicates by each RT-qPCR assay. Copy numbers of input RNA transcripts were estimated. N/A = No amplification.

RNA input	**250 ag**		**1 fg**			
Copy number	**1477**		**1608**			
Replicate	**CaTa9**	***nad5***	**CSP1572**	***nad5***	**ToMMV2**	***nad5***
1	33.04	N/A	31.79	N/A	33.06	N/A
2	32.56	N/A	31.57	N/A	33.50	N/A
3	32.49	N/A	33.14	N/A	33.52	N/A
4	33.27	N/A	33.16	N/A	33.19	N/A
5	32.54	N/A	32.71	N/A	33.04	N/A
6	32.46	N/A	32.18	N/A	33.53	N/A
7	32.83	N/A	32.95	N/A	33.26	N/A
8	32.38	N/A	32.77	N/A	33.40	N/A
9	33.01	N/A	32.53	N/A	34.05	N/A
10	32.44	N/A	33.20	N/A	34.23	N/A
11	32.71	N/A	32.76	N/A	33.26	N/A
12	32.37	N/A	32.67	N/A	32.05	N/A
13	33.58	N/A	32.80	N/A	33.25	N/A
14	32.66	N/A	33.01	N/A	33.28	N/A
15	32.42	N/A	31.84	N/A	33.31	N/A
16	32.45	N/A	32.75	N/A	33.75	N/A
17	32.48	N/A	32.68	N/A	33.29	N/A
18	32.86	N/A	32.97	N/A	33.07	N/A
19	33.16	N/A	32.74	N/A	33.63	N/A
20	32.51	N/A	32.73	N/A	33.41	N/A

Table C.4. RT-qPCR results of 20 individual seeds of a ToMMV-positive seed batch (T2). Seeds were individually ground, and RNA extracted then analysed in either duplex (ToMMV2 and Nad5) or triplex (CSP1572, CaTa9, Nad5) reactions which included ToMMV and IAC targets. All 20 seed samples were RT-qPCR performed in duplicate.

Seed	PCR replicate	Triplex RT-qPCR			Duplex RT-qPCR	
		CSP1572	CaTa9	*nad5*	ToMMV2	*nad5*
1	1	16.60	15.63	21.74	16.22	21.30
	2	16.38	15.49	21.54	16.26	21.29
2	1	15.09	13.55	22.57	15.14	21.98
	2	15.30	13.62	23.07	15.23	22.01
3	1	19.30	18.33	22.08	19.30	22.04
	2	19.29	18.45	22.15	19.27	21.97
4	1	13.11	12.81	27.39	13.56	23.52
	2	12.97	12.83	27.58	13.65	23.52
5	1	28.23	28.21	24.33	28.52	24.06
	2	28.23	28.03	24.30	28.37	23.85
6	1	15.45	15.18	27.55	16.09	24.71
	2	15.45	15.23	26.63	16.21	24.83
7	1	21.31	20.82	22.17	21.15	21.87
	2	21.29	20.83	22.16	21.26	21.90
8	1	16.10	15.08	23.57	15.28	22.53
	2	16.11	15.07	23.64	15.38	22.59
9	1	27.40	26.51	24.01	27.29	23.56
	2	27.46	26.58	24.05	27.28	23.51
10	1	26.63	26.55	24.69	27.18	24.27
	2	26.81	26.53	24.66	27.03	24.28
11	1	26.46	26.02	24.41	26.60	23.90
	2	26.52	25.86	24.37	26.63	23.96
12	1	12.81	11.98	27.40	13.46	23.27
	2	12.71	12.07	27.77	13.31	23.11
13	1	12.94	11.81	27.93	13.26	24.37
	2	12.97	11.84	27.87	13.34	24.26
14	1	15.70	14.99	23.23	16.27	22.88
	2	15.78	14.99	23.70	16.27	22.81
15	1	15.72	14.60	20.87	15.92	20.69
	2	15.82	14.58	20.79	15.96	20.64
16	1	12.67	11.51	22.17	12.64	20.74
	2	12.68	11.52	22.55	12.54	20.45
17	1	14.74	14.50	25.07	14.96	23.40
	2	14.83	14.57	24.80	14.99	23.42
18	1	12.26	11.04	23.75	11.93	21.57
	2	12.30	11.08	24.27	11.96	21.37
19	1	11.98	10.94	23.02	12.21	20.95
	2	11.93	10.84	23.07	12.13	20.87
20	1	29.39	29.25	24.77	29.55	24.16
	2	29.50	29.52	24.66	29.76	24.22

Table C.5. Laboratory 1: RT-qPCR results of the analytical sensitivity of the method to detect one ToMMV-contaminated seed in 1,000 tomato seed. A total of 20 tomato subsamples were spiked with one contaminated seed from seed lot T2. All subsamples were analysed following the protocol described in Annex A and *nad5* was used as IAC. RT-qPCR were performed in duplicate.

Subsample	Negative seed	Positive seed	PCR replicate	Triplex RT-qPCR			Duplex RT-qPCR	
				CaTa9	ToMMV2	*nad5*	CSP1572	*nad5*
1	999	1	1	26.38	27.28	26.38	26.76	26.28
			2	26.59	27.29	26.56	26.92	26.49
2	999	1	1	35.66	35.66	26.73	34.18	26.59
			2	35.21	35.21	27.16	34.83	27.03
3	999	1	1	20.96	21.85	25.98	21.01	26.42
			2	21.13	21.88	26.68	21.06	26.09
4	999	1	1	27.82	29.00	26.05	28.62	26.29
			2	27.99	29.13	26.43	28.75	26.32
5	999	1	1	24.70	25.92	25.93	25.26	26.19
			2	24.86	26.14	26.23	25.42	26.32
6	999	1	1	31.61	31.61	26.94	29.82	24.89
			2	31.77	31.77	26.63	29.98	24.48
7	999	1	1	23.23	24.16	25.95	23.14	26.00
			2	23.47	24.27	26.33	23.24	25.94
8	999	1	1	25.13	26.14	27.24	24.98	26.98
			2	25.25	26.19	27.40	25.02	26.94
9	999	1	1	21.84	23.07	26.00	22.49	26.24
			2	22.12	23.22	26.17	22.59	26.53
10	999	1	1	27.89	29.10	26.85	27.61	26.47
			2	28.02	28.91	27.06	27.74	26.70
11	999	1	1	22.58	23.94	25.80	23.37	25.97
			2	22.67	24.07	26.05	23.43	25.93
12	999	1	1	23.98	24.59	26.12	23.86	26.29
			2	24.07	24.64	26.08	24.10	26.49
13	999	1	1	26.11	27.47	25.97	26.36	26.26
			2	26.24	27.61	26.31	26.57	26.54
14	999	1	1	27.00	27.62	26.13	26.84	26.29
			2	27.17	27.75	26.22	27.03	26.26
15	999	1	1	30.07	31.00	26.56	30.03	26.40
			2	30.38	30.82	26.55	30.18	26.41
16	999	1	1	22.89	23.98	27.36	23.38	27.02
			2	23.04	24.18	27.09	23.51	27.28
17	999	1	1	24.03	24.95	26.72	24.47	27.03
			2	24.23	25.20	26.90	24.72	27.32
18	999	1	1	28.13	29.74	26.97	28.45	27.15
			2	28.15	29.41	26.89	29.07	27.20
19	999	1	1	21.16	22.42	26.24	19.55	26.30
			2	21.26	22.39	26.42	19.64	26.70
20	999	1	1	21.64	22.59	27.35	21.91	27.23
			2	21.72	22.66	26.95	22.00	27.55

Table C.6. Laboratory 2: RT-qPCR results of the analytical sensitivity of the method to detect one ToMMV-contaminated seed in 1,000 tomato seed. A total of 20 tomato subsamples were spiked with one contaminated seed from seed lot T2. Results were compared to 20 unspiked subsamples. All subsamples were analysed following the protocol described in Annex A and *Squash mosaic virus* (SqMV) was used as IAC. RT-qPCR were analysed in duplicate. N/A = No amplification.

Subsample	Negative seed	positive seed	Duplex RT-qPCR		Duplex RT-qPCR	
			CaTa9	CSP1572	ToMMV2	SqMV
1	1000	0	N/A	N/A	N/A	29.21
			N/A	N/A	N/A	29.34
2	1000	0	36.72	35.30	N/A	29.08
			N/A	35.20	36.82	29.08
3	1000	0	N/A	35.67	37.26	28.89
			N/A	37.97	N/A	28.72
4	1000	0	36.17	34.31	N/A	28.44
			N/A	35.17	N/A	28.58
5	1000	0	N/A	33.97	35.15	28.88
			N/A	34.52	36.98	28.79
6	1000	0	N/A	N/A	N/A	29.34
			N/A	N/A	N/A	29.34
7	1000	0	N/A	N/A	N/A	29.50
			N/A	37.21	N/A	29.25
8	1000	0	N/A	N/A	N/A	28.94
			N/A	37.86	N/A	29.13
9	1000	0	N/A	37.21	N/A	29.83
			N/A	N/A	N/A	29.79
10	1000	0	N/A	N/A	N/A	29.61
			N/A	N/A	N/A	29.49
11	1000	0	36.19	35.81	N/A	29.50
			35.90	33.14	N/A	29.48
12	1000	0	N/A	N/A	N/A	29.72
			N/A	N/A	N/A	29.42
13	1000	0	36.44	N/A	N/A	29.59
			N/A	N/A	N/A	29.56
14	1000	0	N/A	N/A	N/A	29.18
			N/A	35.69	N/A	29.31
15	1000	0	N/A	35.64	36.96	29.66
			N/A	36.02	36.64	29.25
16	1000	0	N/A	35.56	N/A	29.24
			34.96	36.53	N/A	29.27
17	1000	0	N/A	N/A	N/A	29.36
			N/A	N/A	N/A	29.31
18	1000	0	N/A	N/A	N/A	30.26
			N/A	N/A	N/A	29.84
19	1000	0	N/A	N/A	N/A	29.61

			N/A	37.76	N/A	29.34
20	1000	0	N/A	N/A	N/A	29.36
			N/A	N/A	36.96	29.47
21	999	1	22.37	20.91	21.96	28.94
			22.46	20.68	21.85	29.01
22	999	1	22.33	21.42	22.69	28.90
			22.33	21.34	22.69	28.95
23	999	1	23.77	22.53	24.04	28.90
			23.79	22.51	24.24	28.94
24	999	1	22.26	21.04	22.47	28.16
			22.34	20.96	22.46	28.27
25	999	1	22.97	21.71	23.43	29.15
			23.05	21.72	23.61	29.23
26	999	1	32.28	31.53	32.51	29.00
			33.35	31.05	33.76	29.08
27	999	1	21.91	20.14	21.70	29.10
			21.94	20.11	21.76	29.08
28	999	1	27.56	25.84	27.66	29.10
			27.66	25.96	27.74	29.27
29	999	1	33.22	31.33	33.41	29.06
			33.22	31.33	33.46	29.26
30	999	1	22.43	21.43	22.99	29.27
			22.45	21.37	23.05	29.14
31	999	1	30.62	28.54	30.42	29.34
			30.61	28.55	30.48	29.36
32	999	1	31.93	31.06	33.18	29.06
			33.33	31.03	32.58	28.96
33	999	1	33.39	32.32	34.69	29.39
			34.51	32.29	34.35	29.38
34	999	1	32.74	30.81	33.23	29.48
			33.13	30.86	33.13	29.50
35	999	1	19.33	18.22	19.77	29.27
			19.39	18.22	19.91	29.22
36	999	1	20.75	19.85	21.55	29.32
			20.96	19.85	21.62	29.41
37	999	1	31.47	29.81	31.47	29.36
			31.88	29.98	31.77	29.16
38	999	1	21.33	19.79	21.34	29.51
			21.29	19.71	21.43	29.93
39	999	1	30.71	29.14	31.26	29.30
			31.10	29.12	31.26	29.18
40	999	1	25.36	23.72	25.64	29.38
			25.43	23.66	25.71	29.34

Table C.7. Laboratory 3: RT-qPCR results of the analytical sensitivity to detect one ToMMV-contaminated seed in 1,000 tomato seed (one positive seed in 999 healthy seeds). A total of 20 tomato subsamples were spiked with one contaminated seed from seed lot T2. All subsamples were analysed following the protocol described in Annex A and *Squash mosaic virus* (SqMV) was used as IAC. RT-qPCR were performed in duplicate.

Subsample	PCR replicate	Duplex RT-qPCR		Duplex RT-qPCR		Duplex RT-qPCR	
		CaTa9	SqMV	ToMMV2	SqMV	CSP1572	SqMV
1	1	33.03	28.84	33.01	29.57	34.88	28.95
	2	33.18	28.56	33.09	29.57	34.15	28.48
2	1	26.07	29.06	26.07	29.04	25.41	28.55
	2	26.11	28.89	26.28	29.32	25.49	28.58
3	1	24.49	29.23	24.54	29.64	23.85	29.00
	2	24.41	29.28	24.54	29.30	23.83	28.92
4	1	25.10	29.31	24.86	29.44	24.05	29.26
	2	25.14	29.37	25.22	30.05	24.24	29.72
5	1	30.91	29.89	29.83	30.23	28.71	29.82
	2	31.02	29.59	29.88	30.16	29.09	29.97
6	1	34.03	28.38	34.11	28.20	34.19	28.46
	2	33.52	28.11	31.06	28.54	33.69	28.04
7	1	23.93	28.66	23.84	29.69	23.84	28.88
	2	23.94	28.92	24.02	29.49	23.88	28.61
8	1	25.72	28.99	25.79	29.39	24.91	28.97
	2	25.54	29.13	25.60	29.17	24.97	28.57
9	1	25.25	29.08	25.65	29.36	25.13	29.02
	2	25.21	28.82	25.59	29.56	24.82	28.42
10	1	24.28	29.06	24.10	28.76	23.51	28.65
	2	24.34	29.16	24.28	29.12	23.57	28.90
11	1	32.87	29.48	31.94	29.61	32.56	29.69
	2	32.21	29.16	32.14	29.63	32.41	29.39
12	1	27.98	29.33	27.28	29.06	26.57	28.86
	2	27.95	28.92	27.38	29.17	26.53	28.83
13	1	24.83	28.51	24.75	28.73	24.14	28.33
	2	24.95	29.26	24.68	28.77	24.01	28.41
14	1	23.56	29.01	23.49	28.86	23.09	28.93
	2	23.59	29.04	23.52	29.13	22.70	28.59
15	1	27.81	28.40	27.59	28.70	26.84	28.17
	2	27.65	28.15	27.60	28.61	26.69	27.96
16	1	25.56	28.91	25.16	29.09	24.46	28.57
	2	25.48	28.86	25.08	28.85	24.24	28.89
17	1	33.21	29.03	33.71	29.76	33.53	29.23
	2	33.42	28.55	33.79	29.52	33.44	29.07
18	1	25.62	28.53	25.34	28.55	24.65	28.38
	2	25.57	28.36	25.34	28.64	24.64	28.23
19	1	23.29	28.56	23.29	28.76	22.36	28.47
	2	23.27	28.72	23.26	28.36	22.43	28.63
20	1	33.90	28.28	34.95	30.17	34.80	28.46
	2	34.22	28.27	34.52	29.55	34.06	28.37

Table C.8. Laboratory 1: RT-qPCR results of the analytical sensitivity of the method to detect one ToMMV-contaminated seed in 500 pepper seed. A total of 18 pepper subsamples were spiked with one contaminated tomato seed from seed lot T2. All subsamples were analysed following the protocol described in Annex A and *nad5* was used as IAC. RT-qPCR were performed in duplicate.

Subsample	Negative seed	Positive seed	PCR replicate	Triplex RT-qPCR			Duplex RT-qPCR	
				CaTa9	ToMMV2	*nad5*	CSP1572	*nad5*
1	499	1	1	20.14	20.39	21.65	19.55	21.78
			2	20.19	20.32	21.70	19.52	22.09
2	499	1	1	26.22	26.62	21.72	25.72	21.32
			2	25.97	26.36	21.60	25.32	21.31
3	499	1	1	22.91	23.34	21.93	22.14	21.49
			2	22.96	23.45	21.89	21.97	21.87
4	499	1	1	23.96	24.13	21.38	23.86	21.85
			2	24.12	24.26	21.48	23.65	21.38
5	499	1	1	19.77	19.95	21.80	19.35	21.93
			2	19.59	19.75	21.59	19.18	21.71
6	499	1	1	21.43	21.43	21.53	21.12	21.82
			2	21.46	21.48	21.59	20.97	21.61
7	499	1	1	20.17	20.54	21.76	19.90	21.93
			2	20.15	20.49	21.80	19.70	21.94
8	499	1	1	22.61	22.94	21.79	22.15	22.11
			2	22.60	22.87	21.79	22.30	22.12
9	499	1	1	18.83	19.21	21.51	18.66	21.58
			2	18.93	19.39	21.74	18.76	21.76
10	499	1	1	23.08	23.10	21.98	22.79	22.04
			2	23.10	23.08	22.05	22.47	21.83
11	499	1	1	20.60	20.79	21.96	20.48	22.08
			2	20.58	20.81	21.91	20.36	22.01
12	499	1	1	24.41	24.70	21.82	23.84	21.71
			2	24.32	24.55	21.93	23.91	21.51
13	499	1	1	19.74	20.10	21.59	19.42	21.88
			2	19.70	19.94	21.63	19.50	21.88
14	499	1	1	18.93	19.35	21.21	18.97	21.82
			2	19.04	19.55	21.36	19.16	21.89
15	499	1	1	19.38	19.79	21.63	19.23	21.95
			2	19.40	19.88	21.56	19.29	21.71
16	499	1	1	20.07	20.53	21.62	19.89	21.88
			2	20.19	20.59	21.73	19.63	21.58
17	499	1	1	18.44	18.80	21.42	17.98	21.64
			2	18.46	18.87	21.41	18.15	22.05
18	499	1	1	18.53	20.08	21.46	17.37	21.97
			2	18.52	20.04	21.45	16.90	21.49

Table C.9. Laboratory 2: RT-qPCR results of the analytical sensitivity of the method to detect one ToMMV-contaminated seed in 500 pepper seed. A total of 20 pepper subsamples were spiked with one contaminated tomato seed from seed lot T2 and compared with 20 unspiked subsamples. All subsamples were analysed following the protocol described in Annex A and *Squash mosaic virus* (SqMV) was used as IAC. RT-qPCR were performed in duplicate. N/A = No amplification.

Subsample	Negative seed	positive seed	Duplex RT-qPCR		Duplex RT-qPCR	
			CaTa9	CSP1572	ToMMV2	SqMV
1	500	0	36.31	N/A	37.20	29.96
			N/A	N/A	N/A	29.77
2	500	0	36.09	36.64	37.26	29.27
			N/A	35.68	N/A	29.18
3	500	0	34.77	34.67	35.86	28.82
			35.04	35.49	N/A	28.65
4	500	0	35.90	33.91	36.07	29.52
			35.86	34.20	35.71	29.47
5	500	0	N/A	N/A	N/A	29.25
			N/A	N/A	N/A	29.36
6	500	0	35.85	32.67	33.89	29.29
			35.31	33.48	36.20	29.44
7	500	0	N/A	N/A	N/A	29.93
			N/A	N/A	N/A	29.57
8	500	0	36.13	N/A	N/A	29.03
			N/A	N/A	36.90	28.93
9	500	0	N/A	N/A	N/A	29.08
			N/A	35.22	N/A	29.05
10	500	0	N/A	34.45	N/A	29.04
			N/A	34.27	35.80	28.99
11	500	0	N/A	35.47	35.16	29.49
			N/A	34.69	N/A	29.45
12	500	0	N/A	38.40	N/A	29.24
			35.96	N/A	37.63	29.46
13	500	0	33.34	32.69	34.47	30.41
			31.47	31.97	35.64	30.06
14	500	0	N/A	N/A	N/A	29.15
			N/A	36.67	N/A	29.09
15	500	0	36.13	N/A	N/A	29.07
			N/A	35.48	N/A	29.01
16	500	0	N/A	N/A	N/A	29.43
			N/A	35.41	N/A	29.20
17	500	0	N/A	36.38	N/A	29.30
			N/A	N/A	N/A	29.36
18	500	0	N/A	N/A	37.02	29.29
			N/A	N/A	N/A	29.38
19	500	0	N/A	N/A	36.93	29.49

			N/A	N/A	N/A	29.47
20	500	0	36.06	36.56	N/A	29.40
			36.10	35.76	N/A	29.46
21	499	1	21.19	20.58	21.35	29.28
			21.13	20.47	21.33	29.41
22	499	1	19.25	18.60	20.21	28.94
			19.30	18.67	20.15	28.75
23	499	1	18.98	18.01	19.43	28.93
			18.96	17.86	19.47	28.87
24	499	1	32.09	31.01	32.52	28.95
			32.32	30.84	32.41	29.03
25	499	1	33.91	32.05	33.97	29.49
			34.47	32.61	34.12	29.52
26	499	1	20.27	20.16	21.26	30.07
			20.22	20.08	21.47	29.83
27	499	1	18.78	17.61	18.80	29.03
			18.60	17.48	18.89	29.09
28	499	1	22.50	21.78	23.32	28.71
			22.60	21.86	23.29	28.64
29	499	1	28.48	26.24	28.01	28.77
			28.22	26.18	28.09	28.82
30	499	1	21.04	20.46	22.01	28.63
			21.09	20.49	22.03	28.62
31	499	1	24.69	23.21	24.93	29.29
			24.53	23.18	24.99	29.11
32	499	1	34.12	32.38	35.15	29.93
			34.27	32.41	35.07	30.01
33	499	1	20.40	19.68	21.01	28.92
			20.38	19.51	21.01	29.02
34	499	1	32.28	30.31	32.69	29.25
			32.72	30.44	32.68	29.13
35	499	1	20.39	19.35	21.10	29.03
			20.72	19.27	20.91	29.06
36	499	1	18.36	17.24	18.70	29.09
			18.30	17.23	18.83	29.11
37	499	1	32.65	30.76	32.49	29.01
			33.04	30.78	32.85	29.25
38	499	1	29.80	28.39	29.65	29.77
			29.68	28.29	29.87	29.98
39	499	1	23.60	22.93	23.62	29.11
			23.43	22.75	23.66	29.20
40	499	1	19.18	18.22	19.25	29.34
			19.13	18.03	19.21	29.32

Table C.10. Laboratory 3: RT-qPCR results of the analytical sensitivity of the method to detect one ToMMV-contaminated seed in 500 pepper seed (one positive seed in 499 healthy seed). A total of 20 pepper subsamples were spiked with one contaminated tomato seed from seed lot T2. All subsamples were analysed following the protocol described in Annex A and *Squash mosaic virus* (SqMV) was used as IAC. RT-qPCR were performed in duplicate.

Subsample	PCR replicate	Duplex RT-qPCR		Duplex RT-qPCR		Duplex RT-qPCR	
		CaTa9	SqMV	ToMMV2	SqMV	CSP1572	SqMV
1	1	31.56	28.40	31.92	29.12	30.40	27.74
	2	31.50	28.16	31.28	28.23	30.61	28.26
2	1	20.77	28.86	21.25	28.58	20.04	29.13
	2	21.13	29.52	21.26	29.10	19.95	28.41
3	1	22.58	27.50	23.06	27.69	22.06	27.20
	2	22.80	27.69	23.12	27.43	22.20	27.29
4	1	23.10	28.22	23.48	28.05	22.49	27.90
	2	23.21	28.04	23.64	28.29	22.65	27.96
5	1	24.12	28.39	24.85	28.52	24.18	28.24
	2	24.17	28.24	24.90	28.28	24.21	28.21
6	1	22.98	28.41	23.16	28.34	22.21	28.23
	2	22.87	28.46	23.08	28.16	22.10	28.07
7	1	25.13	28.13	24.68	28.14	23.67	27.80
	2	25.07	28.28	24.61	28.10	23.43	27.63
8	1	25.45	28.15	25.46	28.37	24.35	27.94
	2	25.56	29.02	25.53	28.35	24.41	28.22
9	1	23.29	28.37	22.55	27.97	21.73	28.20
	2	23.18	28.33	22.48	28.05	21.51	28.02
10	1	22.12	28.53	22.13	28.15	21.18	28.10
	2	22.05	28.20	22.10	28.15	21.11	27.82
11	1	23.00	27.74	23.04	27.63	22.03	27.39
	2	22.99	28.08	23.04	27.65	22.06	27.54
12	1	23.32	28.04	23.30	27.92	22.34	27.42
	2	23.34	27.66	23.33	27.97	22.42	27.80
13	1	26.04	28.24	26.03	28.34	24.61	28.43
	2	26.23	28.21	25.99	28.36	24.69	27.85
14	1	19.97	28.69	20.06	28.43	19.31	28.94
	2	19.88	28.54	20.06	28.54	19.29	29.10
15	1	23.49	28.28	23.60	27.81	22.86	27.64
	2	23.41	28.12	23.72	28.05	22.58	27.86
16	1	22.81	28.17	22.80	27.98	21.78	27.67
	2	22.78	28.24	22.73	27.59	21.76	27.67
17	1	23.43	28.13	23.56	27.81	22.65	27.99
	2	23.36	28.25	23.53	28.20	22.61	28.07
18	1	21.65	28.37	21.44	28.09	20.30	28.09
	2	21.30	28.40	21.52	28.16	20.38	28.38
19	1	22.99	27.45	23.21	27.77	22.64	27.49
	2	22.87	27.58	23.20	27.76	22.44	27.62
20	1	19.27	26.98	18.57	27.27	17.73	27.32
	2	19.24	27.21	18.56	27.42	17.72	28.04

Table C.11. Laboratory 1: RT-qPCR results of the analytical sensitivity of the method to detect one ToMMV-contaminated seed in 500 eggplant seed. A total of 20 eggplant subsamples were spiked with one contaminated tomato seed from seed lot T2 per subsample. All subsamples were analysed following the protocol described in Annex A; and *nad5* was used as IAC. RT-qPCR were performed in duplicate.

Subsample	Negative seed	Positive seed	Triplex RT-qPCR			Duplex RT-qPCR	
			CaTa9	CSP1572	*nad5*	ToMMV2	*nad5*
1	499	1	19.09	19.79	19.85	20.72	20.92
			19.34	19.74	19.84	20.62	20.71
2	499	1	22.41	23.01	20.58	23.11	19.82
			22.25	22.85	20.57	22.57	18.87
3	499	1	21.67	21.96	20.21	23.05	20.70
			21.71	21.86	20.23	22.91	20.82
4	499	1	21.00	21.03	20.50	21.50	20.42
			21.00	20.96	20.49	20.94	19.03
5	499	1	17.15	17.31	19.89	17.14	17.99
			17.26	17.30	19.92	18.35	19.93
6	499	1	20.94	21.19	20.31	22.09	20.07
			20.86	21.12	20.09	21.19	18.63
7	499	1	26.52	27.47	20.33	27.78	19.98
			26.41	27.07	19.99	27.90	20.03
8	499	1	23.51	23.65	20.99	23.76	19.07
			23.41	23.53	20.70	24.12	19.40
9	499	1	18.45	19.18	20.07	19.46	20.20
			18.98	19.45	20.60	19.27	19.54
10	499	1	18.38	18.50	20.01	19.46	20.62
			18.35	18.50	19.95	19.36	20.06
11	499	1	23.80	24.46	20.96	24.95	21.08
			23.81	24.40	20.69	24.95	20.73
12	499	1	19.89	19.91	20.51	21.24	21.58
			19.85	19.85	20.14	21.34	21.60
13	499	1	18.67	18.70	19.96	19.99	20.88
			18.82	18.68	20.00	19.40	19.29
14	499	1	21.79	21.86	20.41	23.20	21.02
			21.99	21.96	20.54	23.25	20.96
15	499	1	24.82	24.57	20.67	25.90	21.07
			24.80	24.65	20.73	25.79	20.72
16	499	1	19.42	20.20	20.16	20.53	20.62
			19.58	20.20	20.36	20.77	20.02
17	499	1	23.18	23.61	20.74	23.99	20.95
			23.34	23.92	20.82	24.13	21.03
18	499	1	20.60	20.69	20.80	21.10	21.07
			20.30	20.61	20.67	21.15	21.11
19	499	1	19.70	20.17	20.71	20.41	20.95
			19.72	20.10	20.46	20.44	20.79
20	499	1	19.95	20.44	20.63	20.51	20.84
			19.89	20.39	20.56	20.50	20.80

Table C.12. Laboratory 2: RT-qPCR results of the analytical sensitivity of the method to detect one ToMMV-contaminated seed in 500 eggplant seed. A total of 20 eggplant subsamples were spiked with one contaminated tomato seed from seed lot T2. Results were compared to unspiked samples. All subsamples were analysed following the protocol described in Annex A and *Squash mosaic virus* (SqMV) was used as IAC. RT-qPCR were performed in duplicate. N/A = No amplification.

Subsample	Negative seed	Positive seed	Duplex RT-qPCR		Duplex RT-qPCR	
			CaTa9	CSP1572	ToMMV2	SqMV
1	500	0	N/A	N/A	N/A	29.97
			N/A	N/A	N/A	29.58
2	500	0	35.00	33.29	34.42	29.81
			34.36	33.48	36.28	29.60
3	500	0	33.13	32.21	33.52	29.08
			33.32	31.73	33.72	29.05
4	500	0	N/A	36.37	35.75	29.01
			N/A	36.44	N/A	28.96
5	500	0	N/A	N/A	36.43	29.17
			N/A	N/A	N/A	29.50
6	500	0	N/A	41.30	36.68	29.61
			35.68	35.31	N/A	29.70
7	500	0	N/A	36.60	N/A	29.81
			N/A	35.33	36.48	29.47
8	500	0	N/A	N/A	37.01	29.50
			N/A	N/A	N/A	29.35
9	500	0	N/A	N/A	36.71	29.12
			N/A	36.39	N/A	28.84
10	500	0	N/A	36.35	36.78	29.13
			N/A	N/A	N/A	29.04
11	500	0	N/A	N/A	39.15	29.27
			N/A	N/A	N/A	29.11
12	500	0	35.73	N/A	N/A	29.40
			N/A	35.42	N/A	29.33
13	500	0	N/A	36.87	N/A	30.34
			36.28	N/A	N/A	30.04
14	500	0	N/A	N/A	N/A	29.16
			N/A	36.51	N/A	29.23
15	500	0	N/A	36.42	36.74	29.26
			34.35	33.86	35.54	29.31
16	500	0	36.10	35.45	34.67	29.76
			33.96	34.04	36.66	29.79
17	500	0	N/A	37.55	N/A	29.47
			N/A	N/A	N/A	29.48
18	500	0	N/A	N/A	N/A	29.70
			N/A	N/A	N/A	29.79
19	500	0	36.49	34.98	36.45	30.25

			N/A	36.08	36.59	30.02
20	500	0	N/A	36.54	N/A	29.71
			N/A	36.15	N/A	29.41
21	499	1	18.34	17.42	18.85	29.45
			18.29	17.38	18.99	29.42
22	499	1	19.92	19.10	20.43	29.22
			20.02	19.12	20.37	29.17
23	499	1	18.01	17.15	19.24	28.66
			18.11	17.12	18.79	28.43
24	499	1	30.38	28.51	29.93	28.82
			30.22	28.58	30.00	28.85
25	499	1	33.27	31.10	32.89	29.14
			32.05	31.23	32.87	29.11
26	499	1	20.01	18.65	20.30	29.33
			20.08	18.72	20.47	29.43
27	499	1	26.57	25.28	26.58	29.49
			26.70	25.12	26.45	29.17
28	499	1	18.27	17.39	18.95	28.63
			18.17	17.34	18.97	28.98
29	499	1	25.04	23.67	25.10	28.62
			24.99	23.67	25.13	28.58
30	499	1	28.68	27.38	29.21	29.21
			28.59	27.34	28.83	28.89
31	499	1	20.10	19.10	20.70	28.95
			20.07	19.08	20.52	28.76
32	499	1	33.04	31.34	33.21	29.48
			33.01	31.42	32.96	29.59
33	499	1	33.24	32.01	33.14	29.29
			33.48	31.85	33.61	29.05
34	499	1	30.52	29.48	32.12	29.53
			30.46	29.53	32.28	29.09
35	499	1	20.04	19.11	20.58	29.24
			20.06	19.12	20.71	29.30
36	499	1	30.76	29.16	30.40	28.98
			31.31	29.07	31.00	29.15
37	499	1	26.05	24.67	26.45	29.24
			26.22	24.67	26.47	29.29
38	499	1	33.93	31.19	35.14	30.17
			33.97	30.86	33.71	30.43
39	499	1	22.14	21.01	22.40	29.40
			22.22	21.00	22.42	29.28
40	499	1	20.12	19.07	20.30	29.00
			20.03	18.88	20.58	29.82

Table C.13. Laboratory 3 RT-qPCR results of the analytical sensitivity of the method to detect one ToMMV-contaminated seed in 500 eggplant seed. A total of 20 eggplant subsamples were spiked with one contaminated tomato seed from seed lot T2. All subsamples were analysed following the protocol described in Annex A except 6 mL of RNA extraction buffer was used rather than 12 mL in this experiment. *Squash mosaic virus* (SqMV) was used as IAC. RT-qPCR were performed in duplicate. N/A = no amplification.

Subsample	Negative seed	Positive seed	Triplex RT-qPCR			Triplex RT-qPCR		
			CaTa9	ToMMV2	SqMV	CaTa9	CSP1572	SqMV
1	499	1	26.30	27.15	30.61	26.36	26.41	29.39
			26.23	27.04	30.24	26.35	26.34	30.44
2	499	1	30.29	31.41	29.21	30.53	30.88	29.29
			30.29	31.41	29.22	30.61	30.85	29.50
3	499	1	38.34	34.28	29.97	N/A	34.85	29.36
			39.14	34.74	29.42	37.94	35.01	29.62
4	499	1	28.08	28.74	27.40	28.38	28.06	29.68
			28.22	28.84	27.39	28.36	28.16	29.91
5	499	1	30.30	30.74	27.21	30.48	30.83	29.98
			30.36	31.10	26.77	30.43	30.52	29.97
6	499	1	22.97	24.20	27.57	23.24	24.12	29.09
			22.83	24.09	27.63	23.17	24.09	28.99
7	499	1	22.56	23.88	29.20	22.76	23.52	27.33
			22.49	23.79	29.04	22.72	23.55	27.45
8	499	1	25.29	26.93	26.96	25.41	26.08	28.74
			25.30	26.95	26.41	25.42	26.17	28.51
9	499	1	N/A	34.17	26.74	39.14	34.58	28.06
			38.84	34.06	26.71	N/A	35.37	28.44
10	499	1	24.01	25.70	28.60	24.10	25.48	29.61
			24.06	25.79	28.76	23.99	25.53	28.09
11	499	1	23.47	24.40	27.65	23.56	24.12	28.01
			23.51	24.41	27.69	23.54	24.04	27.73
12	499	1	25.07	25.97	27.59	25.00	25.29	28.22
			24.92	25.87	27.62	25.02	25.33	28.60
13	499	1	33.71	33.42	26.99	33.51	33.50	28.12
			33.96	33.55	26.94	34.47	33.53	28.07
14	499	1	24.10	25.33	27.81	24.09	24.49	28.35
			24.11	25.42	28.05	24.03	24.40	30.13
15	499	1	26.18	26.97	26.80	26.32	26.60	28.10
			26.26	26.95	26.60	26.14	26.40	27.84
16	499	1	39.03	34.66	27.34	N/A	35.15	28.85
			39.03	34.16	27.23	38.99	34.55	29.51
17	499	1	22.67	24.08	27.45	22.74	23.43	25.22
			22.75	24.13	27.50	22.79	23.50	25.34
18	499	1	28.03	28.98	27.01	28.06	28.23	29.99
			28.05	29.10	26.98	28.00	28.28	28.26
19	499	1	24.81	26.21	27.97	24.77	25.28	29.71
			24.80	26.21	27.84	24.79	25.29	29.51
20	499	1	25.01	26.24	27.20	25.50	25.50	29.99
			25.05	26.32	27.32	25.55	25.57	30.11

Annex D. Repeatability.

Table D.1. RT-qPCR results from two independent repeated evaluations on ToMMV negative (lots 1-10) and positive (lots 11-20) tomato subsamples from 20 different seed lots with or without other non-target contaminant(s). All subsamples were analysed following the protocol described in Annex A and *nad5* was used as IAC. RT-qPCR were performed in duplicate. N/A = No Amplification.

Seed lot	Other contaminant	PCR replicate	Replicate I				Replicate II			
			CaTa9	CSP 1572	ToMMV 2	*nad5*	CaTa9	CSP 1572	ToMMV 2	*nad5*
1	ToMV	1	N/A	N/A	N/A	22.23	N/A	N/A	N/A	21.96
		2	N/A	37.86	N/A	22.20	N/A	37.91	N/A	21.90
2	ToMV	1	N/A	N/A	N/A	23.77	N/A	N/A	N/A	23.46
		2	N/A	N/A	N/A	23.75	N/A	N/A	N/A	23.42
3	Unknown	1	N/A	N/A	N/A	27.29	N/A	37.59	N/A	24.96
		2	N/A	N/A	N/A	27.23	N/A	N/A	N/A	24.98
4	ToMV? (Cq 34)	1	N/A	N/A	N/A	22.95	N/A	N/A	N/A	20.64
		2	N/A	N/A	N/A	22.82	N/A	N/A	N/A	20.63
5	ToBRFV	1	N/A	N/A	N/A	21.31	N/A	36.74	37.47	19.69
		2	N/A	37.92	N/A	21.32	N/A	N/A	37.67	19.76
6	ToMV	1	N/A	N/A	N/A	25.02	N/A	N/A	N/A	24.90
		2	N/A	N/A	N/A	25.06	N/A	N/A	N/A	24.84
7	ToBRFV	1	N/A	N/A	N/A	23.10	N/A	N/A	N/A	22.53
		2	N/A	N/A	N/A	23.29	N/A	N/A	N/A	22.63
8	CLVd/PCFVd	1	N/A	N/A	N/A	22.75	N/A	N/A	N/A	22.90
		2	N/A	N/A	N/A	22.89	N/A	N/A	N/A	23.10
9	ToMV	1	N/A	N/A	N/A	23.15	N/A	N/A	37.78	23.02
		2	N/A	N/A	N/A	23.18	N/A	N/A	37.59	23.05
10	Unknown	1	N/A	N/A	N/A	27.01	N/A	N/A	N/A	27.33
		2	N/A	N/A	N/A	26.84	N/A	N/A	N/A	27.29
11	ToMV	1	11.02	11.56	11.63	21.96	10.49	10.97	11.47	21.40
		2	11.09	11.47	11.55	22.21	10.58	10.98	11.32	21.33
12	ToMV	1	10.83	11.52	11.73	21.26	10.97	11.22	11.76	21.05
		2	10.85	11.71	11.96	21.23	10.98	11.20	11.60	20.59
13	ToMV	1	22.95	21.91	22.43	22.50	22.23	20.61	20.94	21.27
		2	22.97	21.88	22.48	22.52	22.31	20.57	20.91	21.16
14	ToMV	1	21.11	21.98	22.14	23.34	24.00	23.40	23.47	23.07
		2	21.15	22.24	22.18	23.34	24.00	23.46	23.45	23.03
15	ToMV	1	28.80	29.13	30.38	22.23	24.72	25.51	26.43	21.61
		2	28.82	29.32	30.17	22.22	24.72	25.56	26.34	21.66
16	ToMV	1	27.88	28.96	28.75	23.00	26.57	27.20	27.19	22.90
		2	27.93	28.93	28.64	22.98	26.83	27.26	27.20	22.72
17	ToMV	1	29.16	29.28	29.73	22.77	29.14	29.12	30.13	22.34
		2	29.20	29.12	29.95	22.63	29.14	29.09	30.25	22.34
18	ToMV	1	29.67	30.03	30.57	26.18	28.86	28.31	29.11	25.15
		2	30.01	30.22	30.84	26.15	28.98	28.33	29.13	25.21
19	ToMV	1	30.74	30.13	30.44	21.24	30.89	30.24	30.96	20.77
		2	30.93	30.13	30.51	21.32	31.05	30.21	30.90	20.90
20	ToMV	1	31.17	32.07	32.11	24.41	28.71	28.71	29.66	24.17
		2	31.31	31.95	31.83	24.45	28.77	28.80	29.61	24.19

Table D.2. RT-qPCR results from two independent repeated evaluations on un-spiked and spiked pepper subsamples from ten different healthy seed lots. Spiked subsamples were generated by adding flour from ten positive tomato seeds (~30 mg) to 500 ToMMV-free seeds. All subsamples were analysed following the protocol described in Annex A and *nad5* was used as IAC. RT-qPCR were performed in duplicate.

Seed lot	ToMMV Spike	PCR replicate	Replicate I				Replicate II			
			CaTa9	CSP1572	ToMMV2	*nad5*	CaTa9	CSP1572	ToMMV2	*nad5*
1	No	1	N/A	36.50	36.75	18.68	N/A	N/A	N/A	21.53
		2	N/A	35.74	N/A	18.76	N/A	37.89	N/A	22.01
	Yes	1	16.86	17.56	17.89	20.01	17.01	17.78	17.92	19.12
		2	16.81	17.46	17.85	19.93	17.16	17.95	18.00	19.47
2	No	1	N/A	N/A	37.66	21.81	N/A	37.95	N/A	19.33
		2	N/A	37.78	N/A	21.75	N/A	N/A	N/A	19.22
	Yes	1	16.19	16.92	17.19	19.69	16.43	17.27	17.36	20.30
		2	16.18	16.87	17.23	19.73	16.36	17.17	17.41	20.23
3	No	1	N/A	37.88	37.51	20.67	36.25	35.60	35.29	20.37
		2	N/A	36.96	N/A	20.70	N/A	35.07	35.24	20.20
	Yes	1	16.81	16.86	17.22	20.67	16.50	16.94	17.44	19.94
		2	16.76	16.74	17.37	20.81	16.55	17.03	17.40	19.96
4	No	1	N/A	36.41	36.69	20.46	N/A	37.91	N/A	21.37
		2	N/A	36.88	N/A	20.52	N/A	35.03	35.43	21.39
	Yes	1	17.26	17.12	17.18	20.91	17.83	17.05	17.30	20.93
		2	17.25	16.98	17.00	20.82	17.80	16.92	17.33	20.88
5	No	1	N/A	37.90	N/A	20.32	N/A	36.12	36.30	19.44
		2	N/A	N/A	N/A	20.51	N/A	35.72	37.41	19.55
	Yes	1	15.98	16.52	17.12	19.35	15.53	16.30	17.14	18.89
		2	16.01	16.53	17.09	19.36	15.57	16.36	17.12	18.94
6	No	1	N/A	N/A	38.73	18.29	N/A	37.05	N/A	20.15
		2	N/A	37.90	37.45	18.34	N/A	36.50	36.36	21.53
	Yes	1	16.33	16.55	16.73	18.15	16.78	17.16	17.36	22.01
		2	16.34	16.47	16.86	18.25	16.62	17.02	17.46	20.08
7	No	1	N/A	35.71	35.70	19.82	N/A	36.53	N/A	17.96
		2	N/A	36.90	35.50	19.80	N/A	35.67	35.79	17.77
	Yes	1	16.89	17.11	17.81	20.71	17.30	17.73	17.87	21.13
		2	16.93	17.20	17.90	20.49	17.20	17.63	18.02	21.19
8	No	1	N/A	36.97	N/A	19.97	N/A	36.95	N/A	20.56
		2	N/A	37.47	N/A	19.48	N/A	N/A	N/A	20.29
	Yes	1	17.69	18.10	18.83	21.09	16.21	17.05	17.58	20.86
		2	17.69	18.16	18.73	21.05	16.40	17.27	17.45	20.32
9	No	1	N/A	N/A	N/A	20.63	N/A	36.87	N/A	19.90
		2	N/A	38.86	N/A	20.55	N/A	36.30	N/A	19.99
	Yes	1	17.64	18.40	18.77	19.08	16.74	17.22	17.41	19.43
		2	17.47	18.14	18.74	18.98	16.73	17.26	17.46	19.37
10	No	1	N/A	N/A	N/A	19.16	N/A	37.07	N/A	19.19
		2	N/A	N/A	36.65	19.24	N/A	37.93	N/A	19.28
	Yes	1	16.64	17.23	17.81	19.13	16.44	16.74	17.06	20.99
		2	16.73	17.15	17.80	19.10	16.38	16.73	17.06	20.99

Annex E. Reproducibility.

Table E.1. Details of method execution from each participant of the comparative test. NA = Not applicable.

	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4	Laboratory 6	Laboratory 8
Seed Grinding						
Subsample handling prior to seed grinding	NA	Pre-freeze tubes with seed subsamples and zirconium beads at -20 ˚C overnight then grind	Pre-freeze tubes with seed subsamples and beads at -80 ˚C overnight then grind	NA	NA	NA
Instrument	IKA	Geno/Grinder	Geno/Grinder	IKA	Geno/Grinder	Geno/Grinder
Speed Duration-Tomato	25,000 rpm, 20 sec	1,300 rpm, 4 min	1,700 rpm, 2 min	25,000 rpm, 25 sec	25,000 rpm, 20 sec	1,300 rpm, 3 min
Speed Duration-Pepper	25,000 rpm, 20 sec	1,500 rpm, 4 min	1,700 rpm, 4 min	25,000 rpm, 25 sec	25,000 rpm, 20 sec	1,300 rpm, 6 min
Speed Duration-Eggplant	25,000 rpm, 20 sec	1,300 rpm, 4 min	1,700 rpm, 2 min	25,000 rpm, 25 sec	25,000 rpm, 20 sec	1,300 rpm, 6 min
RNA Extraction						
Amount Ground Seed used for RNA Extraction	Entire subsample	Entire subsample	Entire subsample	70-80 mg	Entire subsample	Entire subsample
Amount Buffer added to Ground Seed-Tomato	12 mL	14 mL	12 mL	750 µL	12 mL	13 mL
Amount Buffer added to Ground Seed-Pepper	12 mL	14 mL	12 mL	750 µL	12 mL	13 mL

	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4	Laboratory 6	Laboratory 8
Amount Buffer added to Ground Seed-Eggplant	12 mL	12 mL	12 mL	750 µL	12 mL	13 mL
Amount Seed Extract used for RNA Extraction	200 µL	200 µL	600 µL	200 µL	200 µL	200 µL
RNA Extraction Kit	Qiagen RNeasy PowerPlant kit	In-house Qiagen RNeasy equivalent kit	Promega Maxwell RSC Plant RNA kit	Qiagen RNeasy PowerPlant kit	Qiagen RNeasy PowerPlant kit	Sbeadex kit with KingFisher Machine
Elution volume	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
RT-qPCR						
Mastermix	Quantabio Ultraplex 1-step Toughmix	Quantabio Ultraplex 1-step Toughmix	Quantabio Ultraplex 1-step Toughmix	Quantabio Ultraplex 1-step Toughmix	ABI Fast Virus 1-Step MasterMix	Quantabio Ultraplex 1-step Toughmix
Instrument	ABI ViiA7	Bio Rad CFX-384	Bio Rad CFX96	ABI QuantStN/Aio 6 Flex	ABI Stepone plus	Bio Rad CFX-384
Amount of RNA used per Reaction	4 µL	5 µL	4 µL	4 µL	2 µL	5 µL
PCR mixes	Duplex and triplex	Duplex	Triplex	Duplex and triplex	Duplex and triplex	Triplex
Probes	FAM, VIC, Cy5	FAM, VIC	FAM, HEX, Texas Red	FAM, Joe, Cy5	FAM, VIC, Ned	FAM, YakimaYellow, Texas Red
Threshold: Manual or Automatic	Manual	Manual	Manual	Automatic	Manual	Manual
Ct cut-off	<32 positive	<32 positive	<32 positive	<32 positive	<32 positive	<32 positive
	32-35 2nd-tier positive	32-35 2nd-tier positive	32-35 2nd-tier positive	32-35 2nd-tier positive	32-35 2nd-tier positive	32-35 2nd-tier positive
	>35 negative	>35 negative	>35 negative	>35 negative	>35 negative	>35 negative
IAC	*nad5*	SqMV	SqMV	*nad5*	SqMV	DLVd

Table E.2. Raw data from five laboratories participating in the comparative test.

Table E.2 Raw data CT.xlsx

Annex F. Robustness.

Table F.1. RT-qPCR results of buffer comparison from nine pepper negative seed lots. One subsample from each seed lot was spiked with ToMMV positive seed flours (~30 mg/subsample). Seed flours were then equally divided into two tubes after grinding via IKA® mill (Staufen, Germany) at 25,000 rpm for 30 sec. Two extraction buffers were prepared as described in Annex A and 6 mL of each buffer was added accordingly into half portion of the ground sample flours separately. All samples were then processed as described in Annex A. RT-qPCR were performed in duplicate. N/A = No amplification.

Seed lot	ToMMV Spike	Gu-HCl				Gu-isothiocyanate			
		CaTa9	CSP1572	ToMMV2	*nad5*	CaTa9	CSP1572	ToMMV2	*nad5*
1	No	N/A	37.45	N/A	20.97	N/A	N/A	N/A	22.84
		N/A	37.10	N/A	20.93	N/A	37.89	N/A	23.01
	Yes	20.03	18.84	18.49	21.46	17.01	17.78	17.92	19.12
		19.88	18.71	18.44	21.41	17.16	17.95	18.00	19.47
2	No	N/A	N/A	N/A	24.34	N/A	37.95	N/A	19.33
		N/A	36.75	N/A	23.42	N/A	N/A	N/A	19.22
	Yes	18.75	18.97	18.72	23.11	16.43	17.27	17.36	20.30
		18.97	18.99	18.64	22.48	16.36	17.17	17.41	20.23
3	No	N/A	35.63	N/A	20.49	36.25	35.60	35.29	20.37
		N/A	36.46	N/A	20.73	N/A	35.07	35.24	20.20
	Yes	18.30	18.74	18.56	21.11	16.50	16.94	17.44	19.94
		18.26	18.77	18.64	21.13	16.55	17.03	17.40	19.96
4	No	N/A	N/A	36.58	20.74	N/A	36.12	35.22	19.44
		N/A	37.14	N/A	20.76	N/A	35.72	36.32	19.55
	Yes	17.47	17.95	17.70	21.09	15.53	16.30	17.14	18.89
		17.54	17.91	17.65	21.00	15.57	16.36	17.12	18.94
5	No	N/A	36.48	36.50	19.48	N/A	37.05	N/A	20.15
		N/A	38.28	35.85	19.71	N/A	36.50	36.36	20.08
	Yes	18.73	18.63	18.39	20.00	16.78	17.16	17.36	17.96
		18.73	18.48	18.42	20.11	16.62	17.02	17.46	17.77
6	No	N/A	N/A	36.41	21.89	N/A	36.53	N/A	21.13
		N/A	36.64	N/A	21.99	N/A	35.67	35.79	21.19
	Yes	18.64	19.27	18.97	20.89	17.30	17.73	17.87	20.56
		18.70	19.38	19.06	21.16	17.20	17.63	18.02	20.29
7	No	N/A	36.71	N/A	21.04	N/A	36.95	37.11	20.86
		N/A	36.36	N/A	21.36	N/A	N/A	36.44	20.32
	Yes	18.28	19.05	18.77	21.38	16.21	17.05	17.58	19.90
		18.18	18.92	18.77	21.33	16.40	17.27	17.45	19.99
8	No	N/A	N/A	36.43	20.06	N/A	36.87	N/A	19.43
		N/A	35.59	36.42	20.33	N/A	36.30	35.21	19.37
	Yes	18.61	19.02	18.73	20.12	16.74	17.22	17.41	19.19
		18.50	19.04	18.81	20.32	16.73	17.26	17.46	19.28
9	No	N/A	35.94	37.40	20.46	N/A	37.07	N/A	20.99
		N/A	N/A	36.49	20.43	N/A	37.93	N/A	20.99
	Yes	17.96	18.84	18.45	21.04	16.44	16.74	17.06	18.42
		17.84	18.70	18.43	21.14	16.38	16.73	17.06	18.54

Table F.2. RT-qPCR results from eight tomato (To) samples, four pepper (Pep) samples and two eggplant (EP) samples at 3,000 seeds from eight tomato, three pepper and one eggplant seed lots. All subsamples were prepared equally followed the complete procedure as described in Annex A and additional sample aliquot was taken to evaluate different RNA extraction methods: RNeasy PowerPlant kit (Qiagen) and MagMAX RNA isolation kit (Applied Biosystems). N/A = No amplification.

Seed Lot	Crop	ToMMV Spike	Sub	Qiagen PowerPlant kit				MagMax Plant RNA isolation kit			
				CaTa9	ToMMV2	CSP1572	*nad5*	CaTa9	ToMMV2	CSP1572	*nad5*
1	To	No	1	N/A	N/A	N/A	17.64	N/A	N/A	N/A	17.66
				N/A	N/A	N/A	19.18	N/A	N/A	N/A	17.27
			2	N/A	N/A	N/A	16.78	N/A	N/A	N/A	18.05
				N/A	N/A	N/A	19.32	N/A	N/A	N/A	17.05
			3	N/A	N/A	N/A	17.33	N/A	N/A	N/A	18.07
				N/A	N/A	N/A	19.22	N/A	N/A	N/A	17.27
2	To	No	1	19.80	20.19	19.11	17.37	21.10	20.75	20.43	20.43
				20.85	20.47	19.56	18.97	20.23	19.34	19.96	19.87
			2	20.63	20.69	21.16	18.97	23.11	21.59	21.51	20.56
				21.65	20.89	19.99	19.16	22.40	20.49	21.62	20.83
			3	20.12	20.41	20.75	19.20	22.07	21.49	21.28	19.97
				21.14	20.65	19.53	19.13	20.98	19.70	21.39	20.07
3	To	No	1	36.92	N/A	39.62	18.56	N/A	N/A	N/A	20.21
				38.17	N/A	37.86	18.39	N/A	N/A	N/A	20.44
			2	N/A	N/A	N/A	17.75	N/A	N/A	N/A	20.13
				N/A	N/A	N/A	18.00	N/A	N/A	N/A	20.53
			3	N/A	N/A	N/A	19.61	N/A	N/A	N/A	20.06
				N/A	N/A	N/A	20.14	N/A	N/A	N/A	20.49
4	To	No	1	26.81	29.33	29.74	19.29	28.63	28.91	29.98	19.15
				27.79	29.49	27.94	18.80	28.53	28.63	29.99	19.19
			2	26.23	28.35	28.51	18.71	27.49	28.19	28.98	19.18
				27.10	28.66	27.29	18.63	27.60	27.75	29.05	19.18
			3	27.01	29.46	29.64	19.25	27.19	27.64	28.99	18.80
				27.87	29.44	27.97	18.93	27.66	27.47	28.98	18.78
5	To	No	1	29.61	31.41	31.87	19.50	30.48	31.09	31.46	19.18
				30.76	31.37	30.04	19.19	30.43	30.95	31.60	19.26
			2	27.60	29.35	29.94	19.38	30.74	31.20	31.63	19.16
				28.55	29.72	28.33	19.17	30.67	30.88	31.51	19.19
			3	26.94	28.83	29.23	20.20	29.01	29.70	29.99	19.70
				28.10	29.26	27.84	19.99	28.82	29.42	29.97	19.63
6	To	No	1	N/A	N/A	N/A	16.39	N/A	N/A	N/A	15.70
				N/A	N/A	N/A	16.16	N/A	N/A	N/A	15.69
			2	N/A	N/A	N/A	16.35	N/A	N/A	N/A	15.38
				N/A	N/A	N/A	16.03	39.26	N/A	N/A	15.43
			3	N/A	N/A	N/A	16.33	N/A	N/A	N/A	15.95
				N/A	N/A	N/A	16.14	38.95	N/A	N/A	16.01
7	To	No	1	20.74	20.21	21.86	20.69	23.30	21.61	21.95	18.59

				20.36	18.97	19.33	19.34	23.11	21.20	21.92	18.62
			2	24.51	24.78	26.21	20.56	25.08	24.20	24.15	18.93
				24.59	24.18	24.47	20.01	24.96	23.73	24.16	18.88
			3	21.85	21.69	23.30	20.35	23.82	22.50	21.74	18.97
				22.11	21.12	21.93	20.26	24.04	22.15	21.71	18.96
8	To	No	1	8.48	10.81	11.28	26.84	10.44	10.88	11.09	14.58
				8.73	10.28	9.87	26.09	10.60	10.55	11.06	14.71
			2	9.63	11.08	11.77	15.09	10.75	11.00	11.20	13.85
				8.87	9.97	8.21	11.99	10.53	10.26	11.20	13.89
			3	8.81	10.89	11.16	14.94	10.78	10.78	11.08	14.06
				8.77	9.85	8.20	12.20	10.46	10.11	11.14	14.28
9	Pep	No	1	30.34	35.02	31.08	19.63	32.57	32.33	32.68	19.35
				30.98	N/A	31.28	21.11	32.47	32.55	32.46	18.50
			2	29.86	N/A	29.56	21.55	32.17	32.31	32.61	19.33
				30.57	N/A	29.69	22.80	32.09	32.40	32.42	18.59
			3	30.70	N/A	31.07	22.05	32.51	32.80	32.19	21.05
				31.77	N/A	31.04	23.09	31.87	32.22	31.98	20.34
			4	30.04	N/A	30.30	22.43	31.70	32.45	31.97	19.40
				31.11	N/A	30.28	21.91	31.81	32.10	31.77	18.60
			5	31.32	N/A	31.54	22.64	29.49	30.45	30.00	19.97
				32.54	N/A	31.84	23.86	29.51	29.65	29.63	19.27
			6	17.42	19.76	17.86	19.43	21.00	21.06	21.48	19.28
				19.42	20.86	18.15	21.16	21.06	20.47	20.94	18.66
10	Pep	No	1	N/A	N/A	N/A	21.13	N/A	N/A	N/A	17.92
				N/A	N/A	N/A	21.15	N/A	N/A	N/A	18.04
			2	N/A	N/A	N/A	18.95	37.56	N/A	N/A	18.61
				N/A	N/A	37.84	18.77	N/A	N/A	N/A	18.82
			3	N/A	N/A	39.52	20.16	N/A	N/A	N/A	17.95
				N/A	N/A	N/A	19.93	N/A	N/A	N/A	18.02
			4	N/A	N/A	N/A	20.72	N/A	N/A	38.85	17.42
				N/A	N/A	37.79	18.18	N/A	N/A	N/A	17.58
			5	N/A	N/A	N/A	19.19	N/A	N/A	N/A	18.36
				N/A	N/A	N/A	18.94	N/A	N/A	N/A	18.07
			6	N/A	N/A	N/A	20.40	N/A	N/A	N/A	18.38
				N/A	N/A	N/A	19.92	N/A	N/A	N/A	18.32
11	Pep	No	1	N/A	N/A	N/A	17.87	37.66	N/A	N/A	18.13
				N/A	N/A	N/A	16.00	N/A	N/A	N/A	18.00
			2	N/A	N/A	N/A	18.21	N/A	N/A	N/A	18.02
				N/A	N/A	N/A	18.36	N/A	N/A	N/A	17.98
			3	N/A	N/A	N/A	18.34	N/A	N/A	N/A	17.56
				N/A	N/A	N/A	16.20	N/A	N/A	N/A	17.97
			4	N/A	N/A	N/A	18.23	N/A	N/A	N/A	18.22
				36.52	N/A	N/A	16.19	N/A	N/A	N/A	18.16
			5	N/A	N/A	N/A	17.92	N/A	N/A	N/A	18.02

				N/A	N/A	N/A	16.08	N/A	N/A	N/A	18.24
			6	N/A	N/A	N/A	18.73	N/A	N/A	N/A	18.10
				N/A	N/A	N/A	16.34	N/A	N/A	N/A	18.15
	Pep	Yes	1	21.44	23.76	24.38	18.29	22.92	24.05	24.16	18.20
				21.43	23.01	21.23	16.13	22.66	23.33	24.02	18.19
			2	24.70	26.25	27.19	18.16	25.65	26.23	26.31	18.02
				24.64	25.29	24.18	15.75	25.50	25.58	26.17	17.87
			3	23.91	26.07	26.67	18.21	23.69	24.45	24.52	18.21
				23.83	25.25	23.69	16.14	23.71	23.92	24.47	18.21
			4	25.92	27.72	28.40	17.99	24.97	26.09	26.23	18.08
				25.91	27.08	25.34	16.20	25.06	25.68	26.17	18.20
			5	26.85	28.74	28.87	18.22	26.86	27.24	26.89	17.22
				27.90	28.96	27.72	18.36	26.78	26.68	27.21	18.15
			6	23.17	25.12	26.05	17.89	24.65	25.09	25.37	17.81
				24.09	25.29	24.24	17.42	24.54	24.60	25.51	17.99
12	EP	No	1	N/A	N/A	N/A	17.85	N/A	N/A	N/A	17.44
				N/A	N/A	N/A	17.99	N/A	N/A	N/A	17.46
			2	N/A	N/A	N/A	16.62	N/A	N/A	N/A	17.45
				N/A	39.95	N/A	15.59	N/A	N/A	N/A	17.58
			3	N/A	N/A	N/A	18.78	N/A	N/A	N/A	17.33
				N/A	N/A	35.18	16.58	N/A	N/A	N/A	17.45
			4	N/A	N/A	N/A	17.93	N/A	N/A	N/A	17.47
				N/A	N/A	N/A	14.66	N/A	N/A	39.52	17.58
			5	N/A	N/A	N/A	17.47	N/A	N/A	N/A	17.30
				N/A	N/A	N/A	15.57	N/A	N/A	N/A	17.40
			6	N/A	N/A	N/A	17.67	N/A	N/A	N/A	17.62
				N/A	N/A	N/A	14.81	N/A	N/A	N/A	17.76
	EP	Yes	1	25.62	27.37	28.20	17.98	26.25	26.67	26.89	17.43
				25.49	26.66	24.29	14.49	26.17	26.11	26.85	17.39
			2	24.85	27.20	27.82	17.63	26.78	27.37	27.37	17.46
				24.78	26.43	24.63	14.91	26.63	26.71	27.28	17.37
			3	26.42	28.66	29.38	17.70	26.60	26.89	26.98	17.43
				26.57	28.08	26.41	15.58	26.64	26.53	26.97	17.34
			4	25.96	28.26	28.69	17.40	28.25	28.37	28.47	17.51
				26.15	27.60	25.71	15.76	28.26	28.04	28.42	17.53
			5	23.90	25.86	26.62	17.20	25.73	26.39	26.54	17.69
				23.57	24.73	23.87	15.80	25.41	25.80	26.52	17.71
			6	26.19	28.26	28.45	17.01	23.33	23.56	23.97	17.76
				26.07	27.45	25.70	15.69	23.13	22.95	23.92	17.67

Table F.3. RT-qPCR results of total 18 pepper subsamples at 500 seeds from nine seed lots were followed the complete procedure as described in Annex A. Total RNA extract of each subsample was tested with two different one step RT-qPCR mastermixes. N/A = No amplification.

Seed lot	ToMMV Spike	Ultraplex			TaqPath		
		CaTa9	CSP1572	*nad5*	CaTa9	CSP1572	*nad5*
1	No	N/A	36.48	19.48	N/A	35.78	19.37
		N/A	38.28	19.71	N/A	N/A	19.27
	Yes	18.73	18.63	20.00	18.31	16.62	20.93
		18.73	18.48	20.11	18.37	16.62	21.34
2	No	N/A	N/A	20.74	N/A	36.22	20.74
		N/A	37.13	20.76	N/A	N/A	20.62
	Yes	17.47	17.95	21.09	16.86	15.88	20.34
		17.54	17.91	21.00	17.27	15.98	20.33
3	No	N/A	N/A	21.89	N/A	36.65	21.33
		N/A	36.64	21.99	N/A	37.13	21.97
	Yes	18.64	19.27	20.89	18.20	17.65	22.16
		18.70	19.38	21.16	18.06	17.03	22.10
4	No	N/A	36.71	21.04	N/A	36.00	20.64
		N/A	36.36	21.36	N/A	N/A	20.88
	Yes	18.28	19.05	21.38	17.64	17.19	23.00
		18.18	18.92	21.33	17.30	16.49	21.19
5	No	N/A	35.63	20.49	36.15	36.51	20.02
		N/A	36.46	20.73	37.06	36.97	20.37
	Yes	18.29	18.74	21.10	17.98	17.12	23.58
		18.25	18.77	21.13	17.97	16.90	24.70
6	No	N/A	37.45	20.97	N/A	38.16	19.41
		N/A	37.10	20.93	N/A	37.66	20.40
	Yes	20.02	18.84	21.46	18.48	16.24	21.69
		19.88	18.71	21.41	18.64	16.27	24.00
7	No	N/A	N/A	20.06	N/A	35.84	19.82
		N/A	35.59	20.33	N/A	N/A	19.87
	Yes	18.61	19.02	20.12	17.57	16.17	19.54
		18.50	19.03	20.32	17.93	16.74	20.05
8	No	N/A	35.94	20.46	36.72	36.14	19.61
		N/A	N/A	20.43	N/A	N/A	18.86
	Yes	17.96	18.84	21.04	17.26	16.08	20.05
		17.84	18.70	21.14	17.37	16.44	20.07
9	No	N/A	N/A	24.34	N/A	N/A	22.11
		N/A	36.75	23.42	N/A	38.65	23.10
	Yes	18.75	18.96	23.11	18.21	16.30	23.13
		18.96	18.99	22.48	18.24	16.67	23.26

Table F.4. RT-qPCR results of total three tomato (To), three pepper (Pep) and three eggplant (EP) subsamples at 1,000 or 500 seeds were used to evaluate the duplex vs. triplex PCR assay performance. All samples were processed as the complete procedure described in Annex A.

Sample	Crop	Duplex		Duplex		Duplex		Triplex			Triplex		
		CaTa9	SqMV	CSP1572	SqMV	ToMMV2	SqMV	CaTa9	CSP1572	SqMV	CaTa9	ToMMV2	SqMV
1	To	26.07	29.06	25.41	28.55	26.07	29.04	26.68	26.46	29.07	26.45	26.43	29.07
		26.11	28.89	25.49	28.58	26.28	29.32	26.75	26.62	29.09	26.57	26.53	29.05
2	To	24.49	29.23	23.85	29.00	24.54	29.64	25.14	24.89	29.61	25.02	24.95	29.57
		24.41	29.28	23.83	28.92	24.54	29.30	25.06	24.63	29.50	25.06	24.98	29.39
3	To	25.10	29.31	24.05	29.26	24.86	29.44	25.77	25.11	30.08	25.73	25.41	29.92
		25.14	29.37	24.24	29.72	25.22	30.05	25.70	25.09	29.50	25.71	25.41	30.00
4	Pep	20.77	28.86	20.04	29.13	21.25	28.58	21.50	21.00	30.26	21.38	21.55	28.69
		21.13	29.52	19.95	28.41	21.26	29.10	21.44	20.95	30.59	21.49	21.68	28.81
5	Pep	22.58	27.50	22.06	27.20	23.06	27.69	23.12	22.86	27.44	23.23	23.51	27.69
		22.80	27.69	22.20	27.29	23.12	27.43	23.22	23.00	27.76	23.30	23.59	27.61
6	Pep	23.10	28.22	22.49	27.90	23.48	28.05	23.80	23.61	28.16	23.66	23.96	28.30
		23.21	28.04	22.65	27.96	23.64	28.29	23.76	23.67	28.28	23.85	24.10	28.42
7	EP	24.83	29.67	25.39	29.66	25.76	29.21	24.40	24.82	28.27	24.25	25.10	28.52
		24.56	29.64	25.52	29.72	25.88	29.22	24.35	24.86	28.03	24.34	25.18	28.41
8	EP	27.33	29.40	28.53	29.88	29.01	29.97	27.19	28.04	28.18	27.04	28.19	28.72
		27.38	29.72	28.47	29.54	28.96	29.42	27.07	27.95	28.50	27.06	28.19	28.75
9	EP	23.86	27.86	24.86	28.00	25.18	27.40	23.49	24.25	26.53	23.42	24.42	26.96
		23.87	28.06	24.77	27.78	25.29	27.39	23.54	24.25	26.73	23.45	24.46	26.81