International Seed Federation
Seed is Life

Detection of Tomato mottle mosaic virus (ToMMV) in Tomato and Pepper Seed

NOVEMBER 2025

Developed by ISHI

Detection of Tomato mottle mosaic virus (ToMMV) in Tomato and Pepper Seed

Crop: Tomato (*Solanum lycopersicum*), Pepper (*Capsicum annuum*)

Pathogen(s): Tomato mottle mosaic virus (ToMMV, now *Tobamovirus maculatessellati*)

Version: 1.0 (November 2025)

PRINCIPLE

Detection of Tomato mottle mosaic virus (ToMMV, now *Tobamovirus maculatessellati*) in tomato and pepper seed is done by a seed extract reverse transcriptase (RT-)qPCR assay (SE-qPCR). If no virus is detected the seed lot is considered free from ToMMV. As the ToMMV-specific SE-qPCR assay detects both infectious virions and non-infectious virus particles, a positive SE-qPCR only demonstrates the presence of ToMMV RNA, and the seed lot is deemed suspect for the presence of ToMMV. See Figure 1 for the method process flow.

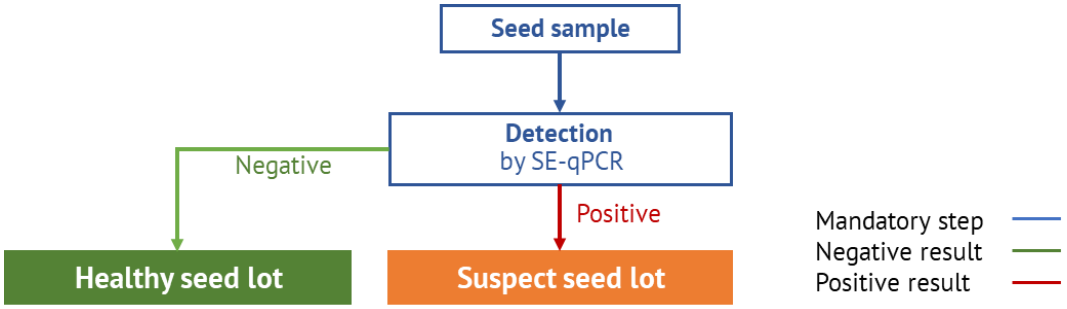

Figure 1. Method process flow.

METHOD VALIDATION

The SE-qPCR assay has been validated by ISHI (Chen *et al*., 2025).

RESTRICTIONS ON USE

Before using this protocol routinely, it is necessary to verify its performance, especially when material and consumables from different suppliers are used. Technical details on the reagents/material used in the validation study (e.g., supplier's information) are provided in the protocol and the validation report.

This method is suitable for testing untreated seed.

It is also suitable for testing seed that has been treated using physical (e.g., hot water) or chemical (e.g., acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate) processes with the aim of disinfestation/disinfection, provided that any residue, if present, does not influence the assay. It is the responsibility of the user to check for inhibition by experimental comparisons or other means.

This test method has not been validated for seed treated with protective chemicals or biological substances. If treated seed is tested using this method, it is the responsibility of the user to determine empirically (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals or biological substances have an effect on the method results.

Note that tobamoviruses, including ToMMV, are highly abundant and persistent in various environments. Environmental ToMMV, found in sources such as wastewater and dust within seed processing facilities, can easily cause false positive results when using this highly sensitive SE-qPCR assay (Rothman and Whiteson, 2022; Schoen *et al.*, 2023). To mitigate this risk, it is essential to incorporate adequate negative controls and perform routine dust swabbing in seed processing areas to monitor potential contamination.

METHOD EXECUTION

To ensure process standardization and valid results, it is strongly recommended to follow the Best Practices for Seed Health Tests developed by ISHI.

Note that this protocol can also be used to identify ToMMV after a positive ELISA in ISHI's tests *Detection of Infectious Tobamoviruses in Tomato/Pepper Seed*. Note that a new seed sample should be used in this case.

SAMPLE AND SUBSAMPLE SIZE

The recommended minimum sample size is 3,000 seeds with a maximum subsample size of 1,000 seeds for tomato and 500 for pepper.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1.0	November 2025	First version of the protocol

Protocol for Detection of ToMMV in Tomato and Pepper Seed

I. PRE-SCREEN BY SEED EXTRACT RT-qPCR

For PCR methods, in-house method optimization is often necessary, see Best Practices for PCR Assays in Seed Health Tests.

Materials

- Seed extraction buffer (Table I.1)
- Grinder (e.g., Geno/Grinder® 2010 (Cole-Parmer®, Vernon Hills, IL))
- 50 mL conical shaped tubes
- Vortex mixer
- RNA purification kit and equipment (e.g., RNeasy PowerPlant Kit (Qiagen, Hilden, Germany))
- RT-qPCR reagents, primers (Table I.2) and equipment
- Controls (Table I.3)
- Centrifuges
- Laboratory disposables

Table I.1. Seed extraction buffer[a] (Guanidine-based buffer (GH+)).

Compound	Amount/L
Guanidine-hydrochloride	573 g
NaOAc buffer (4 M)[b]	50 mL
EDTA	9.3 g
PVP-10	25 g

[a] Alternative buffers are described in the validation report of Chen *et al.*, 2024.

[b] NaOAc 4 M solution is prepared by dissolving 328 g NaOAc in distilled water to 1,000 mL adjusted to pH 5.2.

Table I.2. Primer and probe sequences and references.

Name	Target	Sequence (5' – 3') (*fluorophores 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		VIC – CGATGCTACGGTTGCGATCAGGTC - BHQ1	
ToMMV2-Rv		CTCTGGTTGTAGAAACCTGTTCC	
CSP1572 Fw	ToMMV CP and 3'UTR	CCCGACTACAGCCGAAACAT	Mehle *et al.*, 2024
CSP1572 Pr		VIC – TGCCACTCGCAGAGTGGACGATGCTACG - BHQ1	
CSP1572 Rv		TTAACAGCGGACCTGATCGC	
Nad5-F	*nad5* plant gene	GATGCTTCTTGGGGCTTCTTGTT	Menzel *et al.*, 2002
Nad5-R		CTCCAGTCACCAACATTGGCATAA	
Nad5-Pr		Texas Red – AGGATCCGCATAGCCCTCGATTTATGTG - BHQ2	Botermans *et al.*, 2013

Name	Target	Sequence (5' – 3') (*fluorophores as an example*)	Source
SqMV-F	SqMV	TAGGAATTTCTGGGCAGAGT	Ling *et al.*, 2011
SqMV-R		GGGCTGTACTTTCTAAGGG	
SqMV-Pr		Texas Red – CAGCAGCTTGGAACTTATAATCCAAT - BHQ2	

Table I.3. Types of controls used.

Control type	Description
Internal amplification control (IAC)	Squash mosaic virus (SqMV) spike[ab] *or*
	Endogenous *nad5* plant gene
Positive amplification control (PAC)	ToMMV RNA *or*
	ToMMV oligo DNA (oligonucleotide (single-stranded DNA) for all ToMMV target sequences) *or*
	ToMMV cDNA
Positive process control (PPC)	Tomato or pepper seed infected with ToMMV
Negative process control (NPC)	Tomato or pepper seed free of ToMMV
Non template control (NTC)	PCR mix free from any pathogen or seed

[a] The IAC also serves as inhibition control.

[b] The spike solution is prepared by grinding 0.1 mg of *Squash mosaic virus* (SqMV) infected tissue which is homogenised in 50 mL PBS buffer. The extract is diluted to obtain a suitable concentration, and aliquots are stored at -80 °C. Other organisms such as *Dahlia latent viroid* (DLVd) or *Bacopa chlorosis virus* (BaCV) may also be used, but compatible with the ToMMV primers in a multiplex qPCR should be verified.

1. Extraction of the virus from the seed

Seed extracts and controls must be prepared at the same time, under the same laboratory conditions, and stored at 4 °C until the assay begins.

1.1. Add SqMV spike solution to the seed extraction buffer (Table I.1) as internal amplification control (IAC, Table I.3).

1.2. Dry grind three subsamples of 1,000 tomato seeds or six subsamples of 500 pepper seeds using Geno/Grinder or equivalent equipment. The grinding should produce a seed powder visually similar to Figure I.1. Include a PPC and NPC.

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

1.2.1. Optional: Centrifuge at minimum of 5,000 × *g* for 5 min to reduce the risk of cross contamination when opening the tubes with grinded seed flour.

1.3. Add 20 mL of seed extraction buffer (Table I.1) to each subsample of ground seed powder.

Note: Good results have been obtained with 12 mL seed extraction buffer for tomato (1,000 seeds) and pepper (500 seeds). Experimental data shows that 12 and 20 mL provide similar results (ISHI internal data).

1.4. Vortex and incubate for 30 - 45 min at room temperature.

1.5. Centrifuge the tubes briefly to collect residual liquid from the lid to reduce the risk of cross contamination.

1.6. Continue with RNA extraction (Section 2).

2. RNA extraction

2.1. Use at least 100 µL supernatant from each subsample for the RNA extraction.

2.2. Use the commercial RNA isolation kit such as RNeasy (Power)Plant Mini kit (Qiagen, Hilden Germany) for RNA isolation. Process the subsamples according to the supplier's instructions.

2.3. Eluate the RNA in 100 µL elution buffer.

2.4. Use the eluted RNA for RT-qPCR using a commercial RT-qPCR kit.

Note: The RNA extraction has been validated with RNeasy (Power)Plant kit (Qiagen), MagMax Plant RNA isolation kit (ABI, Waltham MA, USA), Sbeadex kit (LGC, London, UK) and Maxwell RSC Plant RNA kit (Promega, Madison, WI). If a different RNA isolation kit is used, it is necessary to verify its performance.

3. Preparation of the RT-qPCR

3.1. Prepare the RT-qPCR mix with the components as described in Table I.4.

Note: Good results have been obtained by ISHI member laboratories with the RT-qPCR UltraPlex™ 1-Step ToughMix (QuantaBio, Beverly MA, USA). If different RT-qPCR mixtures and amplification programs are used, it is necessary to verify their performance.

3.2. Use 5 µL of the RNA sample as input for the qPCR.

3.3. For each run, include an NTC and at least one PAC (Table I.3) that gives a Cq value between 28 and 32.

3.4. Run the RT-qPCR according to the program presented in Table I.5.

Table I.4. RT-qPCR ToMMV mix.

Component	Target	Per reaction (in µL)	Final concentration
UltraPlex 1-Step ToughMix (4×)		6.25	1×
CaTa9 Fw (10 µM)	ToMMV - Replicase	0.75	0.3 µM
CaTa9 Pr (10 µM)		0.50	0.2 µM
CaTa9 Rv (10 µM)		0.75	0.3 µM
ToMMV2 *or* CSP1572 Fw (10 µM)	ToMMV CP and 3'UTR	0.75	0.3 µM
ToMMV2 *or* CSP1572 Pr (10 µM)		0.50	0.2 µM
ToMMV2 *or* CSP1572 Rv (10 µM)		0.75	0.3 µM
Nad5 *or* SqMV F (10 µM)	IAC	0.25 *or* 0.50	0.1 *or* 0.2 µM
Nad5 *or* SqMV P (10 µM)		0.25 *or* 0.50	0.05 *or* 0.1 µM
Nad5 *or* SqMV R (10 µM)		0.25 *or* 0.50	0.1 *or* 0.2 µM
PCR grade water		9.0 *or* 8.25	
Subtotal PCR-mix		20.00	
RNA extract		5.00	
Total		**25.00**	

Table I.5. RT-qPCR conditions SE-qPCR.

Step	Temperature	Duration
RT reaction	50 °C	10 min
Denaturation	95 °C	3 min
40 cycles	95 °C	10 sec
	60 °C	60 sec

4. Interpretation and decisions

Cut-off values must be established by each laboratory for their positive and internal amplification controls prior to the assay being used on routine samples. For recommendations on setting cut-off values, see Real-time PCR, an 'indirect' test used for pre-screening in seed health methods.

For interpretation and decision making, the results from all primer sets need to be taken into account, see Table I.6. Test results are only valid when all included controls presented in Table I.3 give the expected results.

Note: When using *nad5* as IAC, the Cq values can vary between different seed lots due to heterogenous expression of this gene.

Table I.6. Interpretation and decision table for the SE-qPCR.

CaTa9	ToMMV2 or CSP1572	IAC	SE-qPCR result	Follow-up
Positive	Positive	Positive or Negative	ToMMV suspect seed lot	Bioassay for confirmation on a new sample of seeds.
Positive	Negative	Positive or Negative		
Negative	Positive	Positive or Negative		
Negative	Negative	Positive	ToMMV negative seed lot	No follow up needed.
Negative	Negative	Negative	IAC failure	Repeat extraction and/or RT-qPCR. In case of repeatable results, no conclusion can be given for this sample by SE-qPCR. Continue with ELISA and/or Bioassay on a new seed sample.

REFERENCES

Botermans, M., van de Vossenberg, B.T.L.H., Verhoeven, J.Th.J., 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**, 43–50.

Chen, L.F., Pannu, S., Koenraadt, H., Hiddink, G., Berendsen, S., Relevante, S., Delisle, J., Reuven, S., and Baldwin, T. (2025). Detection of Tomato mottle mosaic virus (ToMMV) in Tomato *(Solanum lycopersicum*) and Pepper (*Capsicum* spp.) by Seed Extract RT-qPCR (SE-qPCR). Validation report, International Seed Federation (ISF), Nyon, Switzerland. https://worldseed.org/our-work/seed-health/ishi-method-development-and-validation/.

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.

Ling, K.-S., Wechter, W.P., Walcott, R., and Keinath, A. (2011).Development of a real-time RT-PCR assay for *Squash mosaic virus* useful for broad spectrum detection of various serotypes and its incorporation in a multiplex seed health assay. *Journal of Phytopathology*, **159**, 649–656.

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., and Constable, F. (2024). ToMMV-detect 2022-A-394 Interlaboratory Test Performance Study. https://drop.euphresco.net/data/af730655-4022-4e87-a952-b94cfda3a971

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

Rothman, J.A. and Whiteson, K.L. (2022). Sequencing and variant detection of eight abundant plant-infecting Tobamoviruses across Southern California wastewater. *Microbiology Spectrum*, **10**(6), e03040-22.

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.