So 8.1 Method for testing Tomato mottle mosaic virus (ToMMV) on tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) seeds using TaqMan RT-PCR

VERSION: 1.0	**DATE:** 9/2025
PATHOGEN: Tomato mottle mosaic virus (ToMMV)	
HOST: tomato (*Solanum lycopersicum*); and pepper (*Capsicum annuum*)	
COMMON NAME: Tomato mottle mosaic virus (ToMMV)	
METHOD: So 8.1 TaqMan RT-PCR Method, Ver 1.0 (National Seed Health System)	
METHOD CLASS: STANDARD (A)	
SAMPLE: minimum 3,000 seeds; maximum subsamples of 1000 seeds for tomato / 500 seeds for pepper	

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REVISION HISTORY: Version 1.0: New method

1. OBJECTIVE

To detect the presence or absence of ToMMV in tomato and pepper seed by isolation of total RNA for Reverse Transcriptase (RT) quantitative PCR using TaqMan assays and follow by a conventional RT-PCR with a ToMMV-specific primer set as needed.

2. PRINCIPLE

Total RNA extracted from tomato/pepper seed is isolated and purified using Qiagen PowerPlant kit or other equivalent methods. The possible presence of ToMMV RNA can be detected by the specific sets of primers and labelled TaqMan probe in triplex or duplex RT-qPCR assays with an internal control (IC). An internal control is designed to detect the mitochondrial NADH dehydrogenase 5 (NAD 5) from seed or an external spike of an RNA virus-Squash mosaic virus (SqMV) to monitor the quality of RNA extraction and potential inhibitory effect.

3. MATERIALS AND EQUIPMENT

Geno/Grinder 2010
IKA Tube Mill 100 control
IKA Mills MT 40.100
50 ml conical shaped tubes
steel balls or zirconium beads
RNA extraction buffer (Guanidinium-based buffer, see appendices)
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 - Qiagen RNeasy PowerPlant Kit, ABI MagMax Plant RNA isolation kit, Promega RSC Plant RNA kit or other equivalent extraction method that is validated
Taqman RT-PCR reagents, including primers and probes
Mastermixes - Quanta Ultraplex 1-Step ToughMix (4X) Low Rox or other equivalent mastermix that is validated
Invitrogen SuperScript™ III One-Step RT-PCR System or other equivalent kit that is validated
MicroAmp™Fast Optical 96-Well Reaction Plate
MicroAmp™ Optical Adhesive Film
Real-time PCR system

4. SAFETY

Guanidinium-based buffer is harmful and take extra precaution when handling Guanidinium-based buffer. Follow SDS and safety guidelines as it requested.

5. METHOD

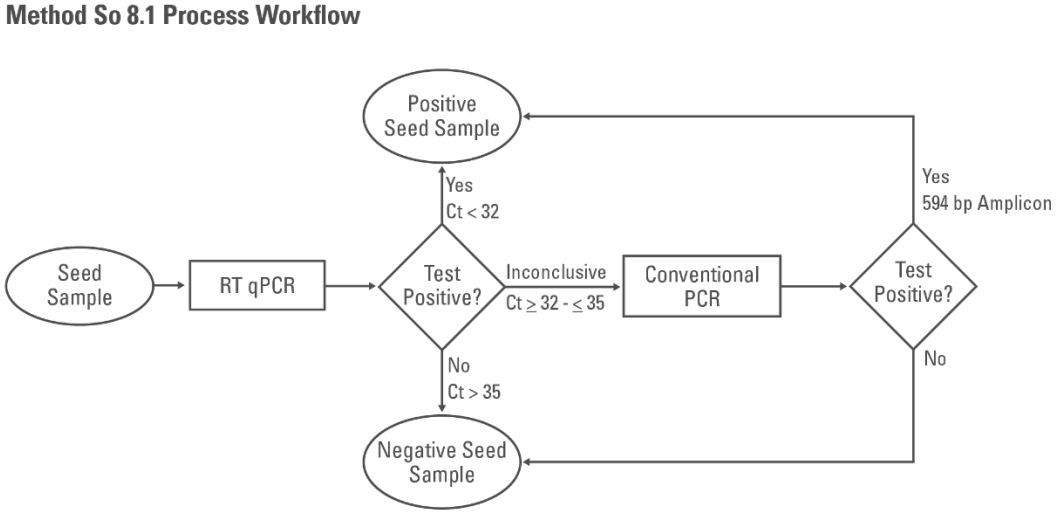

Figure 1. Flow chart of the method for detection of *Tomato mottle mosaic virus* in tomato and pepper seeds.

5.1. Sample preparation

5.1.1. Grinding

A. Option 1: Geno grinder

i. Weigh subsamples of 500 or 1000 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 or -20 °C overnight or seed subsamples can be quickly frozen by placing tubes in liquid nitrogen to enhance the grinding results

ii. For tomato seeds, grind seeds using geno grinder at 1400 - 1700 rpm, 2 minutes.
For pepper seeds, grind seeds using a geno grinder at 1400-1700 rpm for 2 minutes twice.
Option: Refreeze samples between grinding cycles as needed via freezer, dry ice or liquid N2 to enhance the grinding results

B. Option 2: IKA Mill (grinder)

i. Weigh subsamples of 500 or 1000 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 seconds and transfer ground seed flours to testing tubes for RNA isolation.

Figure 2. Demonstration of ground tomato (left) and pepper (right) seed flours comparing to unground seeds

5.1.2. Preparation for RNA isolation:

A. Option 1:

i. Add RNA extraction buffer to each ground subsample:

	500 seed	1000 seed
Tomato	6 ml	12 ml
Pepper	12 ml	--

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

iii. Centrifuge the sample tubes up to 29000 rcf for 5 minutes, transfer 200 ul 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 (5.2).

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

B. Option 2:

i. Weigh 70-80 mg per subsample from ground seed flours into a bead tube, a new 2.0 ml tube or a sample plate and follow appropriate steps according to preferred RNA extraction method (5.2).

5.1.3. If desired, prepare the internal control (IC) spike and add to each subsample aliquot (5.1.2). prior for RNA extraction (see appendices).

5. 2. RNA isolation

Options: using Qiagen PowerPlant kit (13500-50), ABI MagMax Plant RNA isolation kit, Promega RSC Plant RNA kit or equivalent RNA extraction method upon validation.

Follow Manufacturer's guidance. Use an elution volume of 100 ul.

5. 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.

5.3.1. Prepare the Taqman RT-PCR mixes according to the tables below and use the PCR mixes: Ultraplex 1-Step ToughMix (4X) or other equivalent mastermix that is validated. Fluorophores and quenchers of the probes also may need to be adjusted depending on the thermocycler equipment applied. With or without passive dye will depend on the use of PCR instrument. Verify test performance by thorough in-lab validation.

5.3.2. Ensure to add IC (Nad 5 or SqMV) in each PCR mix and calculate the required amount for reaction mixes

Internal control	Final Conc.	Target	Sequence 5'-3'
Nad 5-F	100 nM	Nad 5	GATGCTTCTTGGGGCTTCTTGTT
Nad 5-R	100 nM		CTCCAGTCACCAACATTGGCATAA
Nad 5-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-BHQ1

5.3.3. Ensure to include positive amplification controls for each PCR assay

5.3.4. Prepare PCR mixes using the following template with 2 preferred ToMMV primer sets:

A. One out of two primer sets (ToMMV2 or CSP1572) targeting ToMMV coat protein

B. Include ToMMV CaTa9 as the primer set targeting another region of viral genome

List of primers: Fluorophores and quencher are changeable upon in-lab validation

Primer/Probe	Target	Sequence (5' -> 3') (*fluorophore FAM as an example*)	Reference
CaTa9 Fw	ToMMV Rep	ATGTGGAGGAACCCTCTATGA	ISHI-Veg
CaTa9 Pr		6FAM-TCAATGGCCCGTGGTGAGTTACAA-BHQ1	
CaTa9 Rv		AATCTCCTCGCTCCTTGTAAAC	
ToMMV2-Fw	ToMMV CP and 3'UTR	GAAACATTGGATGCCACTCG	ISHI-Veg
ToMMV2-Pr		6FAM - CGATGCTACGGTTGCGATCAGGTC-BHQ1	
ToMMV2-Rv		CTCTGGTTGTAGAAACCTGTTCC	
CSP1572 Fw	ToMMV CP and 3'UTR	CCCGACTACAGCCGAAACAT	CSPL
CSP1572 Pr		6FAM - TGCCACTCGCAGAGTGGACGATGCTACG - BHQ1	
CSP1572 Rv		TTAACAGCGGACCTGATCGC	

5.3.5. Prepare PCR mixes:

Example 1: PCR mix for triplex PCRs

Reagent	Final Conc.	Target
RNase-Free Water		
MasterMix	1x	
Primer set 1 -For	300 nM	ToMMV CP
Primer set 1 -Rev	300 nM	
Primer set 1 -Probe 1	200 nM	
Primer set 2 -For	300 nM	ToMMV Rep
Primer set 2 -Rev	300 nM	
Primer set 2 -Probe 2	200 nM	
IC Forward	Reference above	Internal Control
IC Reverse		
IC Probe 3		
RNA extract	4 µl	
Total	20 µl	

Probes 1, 2, 3 are labelled with different fluorophores

Example 2: PCR mix for two sets of duplex PCR

Reagent	Final Conc.	Target
RNase-Free Water		
MasterMix	1x	
Primer set 1 -For	300 nM	ToMMV
Primer set 1 -Rev	300 nM	
Primer set 1 -Probe	200 nM	
IC Forward	Reference above	Internal Control
IC Reverse		
IC Probe		

RNA extract	4 µl	
Total	20 µl	

5.3.6. Transfer 16 µL of PCR mix into a 96-well reaction plate. Add 4 µL of RNA sample into 16 µL of PCR mix. Cover the plate with adhesive film.

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

5.3.8. Run the assay using the following program:

	Temperature	Time
cDNA synthesis	48-50 °C*	10-15 min*
Denaturation	95 °C	3-10 min*
PCR cycling (40 cycles)	95 °C	10 s
	60 °C	60 s

*The parameters can be optimized based on in-house instrument performance

5. 4. Evaluation RT-qPCR test result and interpretation

5.4.1. Threshold setting is critical and need to be validated in house depending on the use of mastermixes and thermal cycler

5.4.2. Results are valid only if positive controls give a clear signal with a Ct ≤ 30 and negative controls have a Ct of > 35. The amplification of an internal control should give a clear signal, preferably a Ct < 30 for endogenous control and Ct 28 ± 3 for spiked control.

5.4.3. Determine if ToMMV was detected in a seed sample:

A. A negative detection of ToMMV is all PCR replicates are Ct > 35.

B. A positive detection of ToMMV is any PCR replicate of target primer gives a Ct <32.

C. For Ct range between ≥ 32 to ≤ 35, the result is inconclusive and requires further confirmation steps as described at 5.5 (conventional RT-PCR).

5. 5 Confirmation assay using ToMMV-specific conventional RT-PCR

The following protocol is validated with Invitrogen SuperScript™ III One-Step RT-PCR System. Other RT-PCR systems need to be validated and shown equivalency by the user lab.

5.5.1. Primer:

Primer	Sequence (5' -> 3')	Size	Target
CSP594-F	5'-CGACCCTGTAGAATTAATAAATATTTGTACT	594 bp	ToMMV CP and 3' UTR
CSP594-R	5'-GAATCCCACGCATTATTACTTGT		
18S-Fw	ACGGATCGCACGGCCTTCGTG	300 bp	Housekeeping gene (optional)
18S-Rv	ACCAGACTTGCCCTCCAATGG		

5.5.2. RT-PCR:

A. RT-PCR parameters as followed or equivalent validated parameters

55 °C	30 mins	
94 °C	2 mins	

94 °C	15 sec	15 cycles
57 °C*	30 sec	
68 °C	45 sec	
94 °C	15 sec	25 cycles
52 °C	30 sec	
68 °C	45 sec	
68 °C	5 mins	
4 °C	∞	

* decrements 0.3 °C per cycle

B. Program the thermal cycler before setting up the reactions
C. Set up reaction mix using Invitrogen SuperScript™ III One-Step RT-PCR System as shown here or equivalent validated reagents.

Component	Volume
2x Reaction Mix	12.5 ul
Template RNA	4 ul
Forward primer (10 uM)	1 ul
Reverse primer (10uM)	1 ul
SuperScript III mix	1ul
RNase-free water	Up to total of 25 ul

D. Add SuperScript III as the last component into mastermixes then take aliquots to PCR plates on ice
E. Add RNA sample as the last component and transfer the PCR plate to cycler preheated to 55 °C and immediately start the RT-PCR program
F. Fractionate PCR products by agarose gel electrophoresis with 100 bp DNA ladder as marker

5.5.3. Data interpretation

A. Results are valid only if positive control gives a clear expected fragment size and negative control has no amplification.
B. A positive detection is PCR amplification size at 594 bp with the CSP594 primer set.
C. A negative detection is no amplification of a given sample indicating a negative result.

6. REFERENCE

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APPENDICES

RNA extraction buffer

1. Reference from NSHS method for pospiviroid testing protocol:

	100 ml	**1000 ml**
DI Water	35 ml	350 ml
PVP-40 (3%)	3 g	30 g
Guanidine (Iso)thiocynate (4M)*	47.30 g	473 g
Sodium acetate (0.2M)	1.6 g	16 g
0.5M EDTA (25mM)	5 ml	50 ml
Sodium sulfite (1%)	1 g	10 g
Adjust pH to 5.0 with 37% HCl		
Sodium metabisulfite	1 g	10 g

* Guanidine thiocynate can replace Guanidine isothiocynate

2. Guanidine-HCl extraction buffer can also be used (reference from Naktuinbouw pospiviroid reference protocol)

Preparation of SqMV as an Internal control for monitoring the quality of RNA extraction.

1. Take 0.1 gm 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 3 replications/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 that is close to Cq28
5. After confirming the selected dilution giving consistent Cq value of 28, aliquots can be prepared in to 2 ml tubes and freeze the aliquots for future use.
6. Thaw the tubes and use 10 µl spike for each extraction/subsample.
7. Discard the aliquot after 1 or 2 times freeze and thaw.
8. Cq value of SqMV spike in unknown samples should be 28 ± 3.
9. If Cq values deviate from expected values, prepare new SqMV control and validate before use.