So 7.1 Method for testing Tomato brown rugose fruit virus (ToBRFV) on tomato (*Solanum lycopersicum*) and pepper (*Capsicum annuum*) seeds using TaqMan RT-PCR

VERSION: 2.2	**DATE:** 2/19/2024
PATHOGEN: Tomato brown rugose fruit virus (ToBRFV)	
HOST: tomato (Solanum lycopersicum; syn. Lycopersicon esculentum); and pepper (Capsicum annuum)	
COMMON NAME: Tomato brown rugose fruit virus (ToBRFV) - tomato - pepper	
METHOD: So 7.1 TaqMan RT-PCR Method, Ver 2.0 (National Seed Health System)	
METHOD CLASS: STANDARD (A); TEMPORARY STANDARD (B) applies to conventional PCR confirmation step	
SAMPLE: minimum 3,000 seeds; maximum subsamples of 500 or 1000 seeds for tomato / 500 seeds for pepper	
SUBMITTED BY: Chen & Thomas, Bayer; PCR confirmation step, NSHS Administration Unit	

REVISION HISTORY: Version 1.1: 11.6.2020 Editorial updates and change to denaturation time updated to 3 minutes. Version 2.0: 12.22.2022 Section 4.4.3 CT cutoff values revised for positive and inconclusive results. Temporary standard (B) confirmation step adds conventional PCR inconclusive testing as described in section 4.4.4. Version 2.1: Updated Conventional PCR heading. Version 2.2: Updated Submitted by information and method class editorial changes.

1. OBJECTIVE

To detect the presence or absence of Tomato brown rugose fruit virus (ToBRFV) in tomato and pepper seed by isolation of total RNA followed by Reverse Transcriptase (RT) quantitative PCR using TaqMan assays.

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 ToBRFV RNA can be detected by the specific set of primers and labelled TaqMan probes in a duplex RT-qPCR assay with an internal control (IC). An internal control is designed to detect the mitochondrial NADH dehydrogenase 5 (Nad5) from seed or an external spike of an RNA virus, Squash mosaic virus (SqMV), to monitor the quality of RNA extraction and potential inhibitory effects.

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
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, Qiagen RNeasy Plant Mini kit, LGC Sbeadex Plant Maxi Kit, MagMax Plant RNA isolation kit, or MagNA Pure LC Total Nucleic Acid kit TaqMan RT-PCR reagents, including primers and probes
Quanta qScript XLT One-Step RT-qPCR ToughMix, Low Rox (2X) or Ultraplex 1-Step ToughMix (4X)
MicroAmp™Fast Optical 96-Well Reaction Plate
MicroAmp™ Optical Adhesive Film
Real-time PCR system
Confirmation conventional RT PCR testing:
PCR thermal cycler
Agarose gel electrophoresis
Gel imaging system
Primer set – Dey et. al 2021 (DPI-782 5' -TAGCGAAGTGTGGAAACCTG -3' and DPI-783 5'-GGTGCAGAGGACCATTGTAA -3')

4. METHOD

4.1. **Sample preparation**

4.1.1. 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).

ii. Freeze sample tubes containing seeds and ball bearings at -80 or -20 °C overnight.
Option: Seed subsamples can be quickly frozen by placing tubes in liquid nitrogen

iii. For tomato seeds, grind seeds using geno grinder at 1400 - 1700 rpm, 2 minutes or until a fine powder is achieved (figure 1).

iv. For pepper seeds, grind seeds using a geno grinder at 1400-1700 rpm for 2 minutes twice, or until a fine powder is achieved (figure 1). Refreeze samples between grinding as needed via freezer, dry ice or liquid N2.

B. Option 2: IKA Mill (grinder)

i. Weigh subsamples of 500 or 1000 seeds for tomato or 500 seeds for pepper and place into 50 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 1. Demonstration of ground tomato (left) and pepper (right) seed flours comparing to unground seeds

4.1.2. If desired, prepare the internal control (IC) and add to subsamples prior to sampling for RNA extraction (see appendices).

4.1.3. 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 14,000 rcf for 5 minutes, transfer 200 ul of supernatant to a bead tube or a new 2.0 ml tube and follow appropriate steps according to preferred RNA extraction method (4.2.)

B. Option 2:

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

4.2. **RNA isolation**

Options: using Qiagen PowerPlant kit (13500-50), LGC Sbeadex Plant Maxi Kit, MagMax Plant RNA isolation kit, Promega RSC Plant RNA kit or MagNA Pure LC Total Nucleic Acid kit for RNA extraction or equivalent RNA extraction method upon validation.

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

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: Quanta qScript XLT One-Step RT-qPCR ToughMix (2X) or Ultraplex 1-Step ToughMix (4X). Fluorophores and quenchers of the probes may need to be adjusted depending on the thermocycler equipment used. With or without passive reference dye will depend on the PCR instrument used. Verify test performance by thorough in-lab validation.

4.3.2. Include internal control primers (Nad 5 or SqMV) and SqMV template (if necessary) 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

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

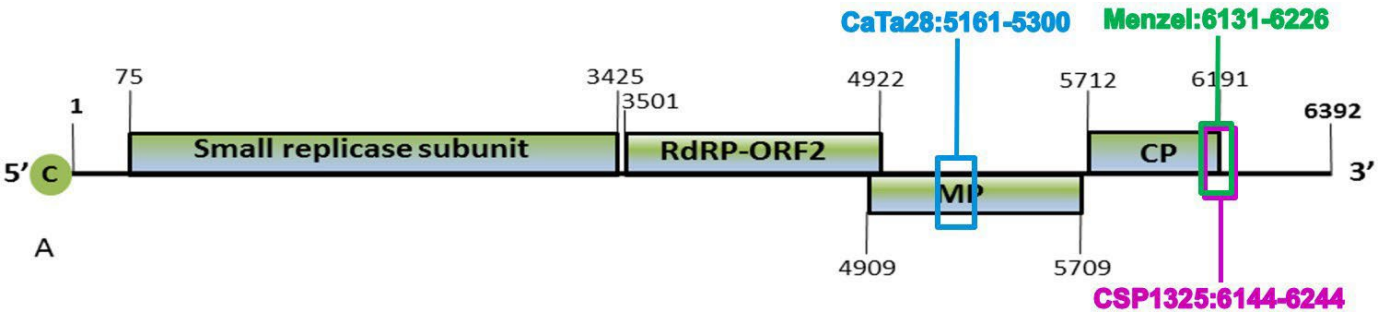
4.3.4. Prepare PCR mixes using the following template with 3 preferred ToBRFV primer sets:

A. One out of 2 primer sets targeting ToBRFV CP

B. CaTa28 primer set targeting movement protein

List of primers:

Primer/Probe	Target	Sequence (5' -> 3') (fluorophore FAM as an example)	Reference
CSP1325 Fw	ToBRFV CP and 3'UTR	CATTTGAAAGTGCATCCGGTTT	ISHI-Veg 2019
CSP1325 Pr		6FAM - ATGGTCCTCTGCACCTGCATCTTGAGA - BHQ1	
CSP1325 Rv		GTACCACGTGTGTTTGCAGACA	
Menzel Fw	ToBRFV CP and 3'UTR	CAATCAGAGCACATTTGAAAGTGCA	Menzel & Winter 2020
Menzel Pr		6FAM - ACAATGGTCCTCTGCACCTG - BHQ1	
Menzel Rv		CAGACACAATCTGTTATTTAAGCATC	
CaTa28 Fw	ToBRFV MP	GGTGGTGTCAGTGTCTGTTT	ISHI-Veg 2019
CaTa28 Pr		6FAM - AGAGAATGGAGAGAGCGGACGAGG - BHQ1	
CaTa28 Rv		GCGTCCTTGGTAGTGATGTT	



PCR mix example 1: triplex PCRs

Reagent	Final Conc.	Target
RNase-Free Water		
MasterMix	1x	
Primer set 1 -For	300 nM	ToBRFV
Primer set 1 -Rev	300 nM	
Primer set 1 -Probe 1	200 nM	
Primer set 2 -For	300 nM	ToBRFV
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 labeled with different fluorophores

PCR mix example 2: two sets of duplex PCRs

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

Probes 1 and 2 are labeled with different fluorophores

4.3.5. 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.

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

4.3.7. Run the assay using the following program:

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

4.4 Evaluation test result

4.4.1. Threshold setting has to be validated depending on the use of mastermix and thermal cycler

4.4.2. 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 SqMV spiked internal control.

4.4.3. Determine if ToBRFV was detected in each seed lot. If one or more PCR replicates with any of the selected primer sets has a Ct value ≤32, the seed lot is positive. If one or more PCR replicates with any of the selected primer sets has a Ct value >32 but ≤34 and all other data are in the negative range, the result is considered inconclusive.

4.4.4. Samples within the inconclusive range may be subjected to confirmatory testing.

4.4.4.1 Test using the Dey et al. primers. See PCR appendix for testing details.

4.4.4.2 The seed lot will be considered positive if a band at 324bp is observed in any replicates and the control reactions are as expected.

4.4.4.3 If no band is observed at 324bp in the replicate samples, the seed lot will be considered negative.

5. REFERENCES

5.1 Dey, K.K., Velez-Climent, M., Soria, P., Batuman, O., Mavrodieva, V., Wei, G., Zhou, J, Adkins, S., and J. McVey. 2021 First Report of Tomato brown rugose fruit virus infecting tomato in Florida, USA. https://doi.org/10.1002/ndr2.12028

5.2 ISHI-Veg, International Seed Federation. September 2019. Detection of infectious Tomato brown rugose fruit virus (ToBRFV) in tomato and pepper seed. Version 1.3 https://www.worldseed.org/wp-content/uploads/2020/03/Tomato-ToBRFV_2020.03.pdf

5.3 ISHI-Veg, International Seed Federation. 2019. ISHI-Veg develops new, specific detection method – Tomato brown rugose fruit virus. Prophyta Annual P10-15. http://www.prophyta.org/prophyta/Prophyta2019-ISHI-veg.pdf

5.4 ISHI-Veg, International Seed Federation. 2019. International Seed Health Initiative (ISHI) Best Practices for PCR Assays in Seed Health Tests. December 2019, Version 4. https://worldseed.org/wp-content/uploads/2021/11/ISHI-Veg_BestPractices_PCR_v4_Dec_2019_NEW-Template.pdf

5.5 Menzel, W & Winter S (2020) 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 (in press)

5.6 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.

5.7 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. https://seedhealth.org/so6-1/

5.8 Naktuinbouw reference protocol: Real-time RT-PCR (RT Taqman PCR) for pospiviroids (CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd) on seeds of tomato (Solanum lycopersicum). Protocol numner SPN-V043e, Version 2.4, Date: 15-05-2020. https://www.naktuinbouw.com/sites/default/files/SPN-V043e%20v2.4%20Pospiviroids%20tomatoseeds_0.pdf

6. APPENDICES

RNA extraction buffer

(reference from NSHS method for pospiviroid testing):

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

Alternate RNA extraction buffer – guanidine-HCl extraction buffer can also be used (from Naktuinbouw pospiviroid reference protocol)

	100 ml	1000 ml
Guanidine hydrochloride	57.30 g	573 g
Sodium acetate (4M)	5 ml	50 ml
EDTA-disodium	0.93 g	9.3 g
PVP-10	2.5 g	25 g
Deionized water	100 ml	1000 ml

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 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), spike into seed matrix with RNA extraction buffer and proceed with extraction procedure.
4. Run RT-qPCR to determine Ct values. Ct values of the IC should be standardized to 28.
5. After standardizing the IC to a Ct value of 28, aliquot into 2 ml tubes and freeze the tubes for future use.
6. Thaw the tubes and use 10 µl spike for each tube (sub-sample).
7. Discard the SqMV internal control standard after 1 or 2 times freeze and thaw.
8. Ct value of SqMV spike in unknown samples should be 28 ± 3.
9. Prepare new SqMV control if Ct values deviate from above

Optional Conventional PCR assay for Inconclusive qPCR Results

Follow best practices for PCR testing. Guidance can be found at ISHI's website in the document "Best Practices for PCR Assays in Seed Health Tests".

Dey et al. (2021) primers

DPI-782 5' -TAGCGAAGTGTGGAAACCTG -3'

DPI-783 5'-GGTGCAGAGGACCATTGTAA -3'

Dey et al. Primer PCR Cycle Settings

	Temperature	**Time**
Denaturation	50°C	30 minutes
	95°C	15 minutes
PCR cycling (40 cycles)	94°C	30 seconds
	55°C	30 seconds
	72°C	60 seconds
Final Extension	72°C	10 minutes
Hold	4°C	

Dey et al. PCR Mix

Reagent	**Final Conc.**	**Quantity**
Molecular biology grade water		13.0 µL
5x Qiagen OneStep RT-PCR buffer	1x	5.0 µL
Qiagen dNTPs (10mM)	0.4 mM	1.0 µL
FL782 and FL783 primer mix (5 µM each)	0.6 µM (forward and reverse primers)	3.0 µL
Qiagen One Step RT-PCR Enzyme Mix	1.0	1.0
cDNA		2.0
Total		**25.0**

Resulting PCR reactions should be run on a 1.5% agarose gel. The seed lot will be considered positive if a band at 324bp is observed in any replicates and the control reactions are as expected. If no band is observed at 324bp in the replicate samples, the seed lot will be considered negative.

This reaction has been validated using Qiagen One Step RT-PCR Enzyme mix and the listed thermocycler conditions. If you would like to use a different PCR mastermix or slightly modify the thermocycler conditions for use in your lab, data must be generated in your lab to provide evidence of equivalency.