International Seed Federation
Seed is Life

Detection of Infectious *Tomato brown rugose fruit virus* (ToBRFV) in Tomato and Pepper Seed

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Developed by ISHI

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Detection of Infectious *Tomato brown rugose fruit virus* (ToBRFV) in Tomato and Pepper Seed

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

Pathogen(s): *Tomato brown rugose fruit virus* (ToBRFV)

Version: 1.6 (April 2024)

PRINCIPLE

Detection of *Tomato brown rugose fruit virus* (ToBRFV) in tomato and pepper seed by a seed extract reverse transcriptase (RT-)qPCR assay (SE-qPCR). If no virus is detected the seed lot is considered free from ToBRFV.

As the ToBRFV-specific SE-qPCR assay detects both infectious virions and non-infectious virus particles, a positive SE-qPCR is followed by a bioassay to confirm the absence of infectious ToBRFV. Leaves of indicator plants *Nicotiana tabacum* cv. Xanthi NN or *Nicotiana glutinosa* are inoculated with tomato or pepper seed extract. Infectious virions cause typical local lesions that demonstrate viability of the virus.

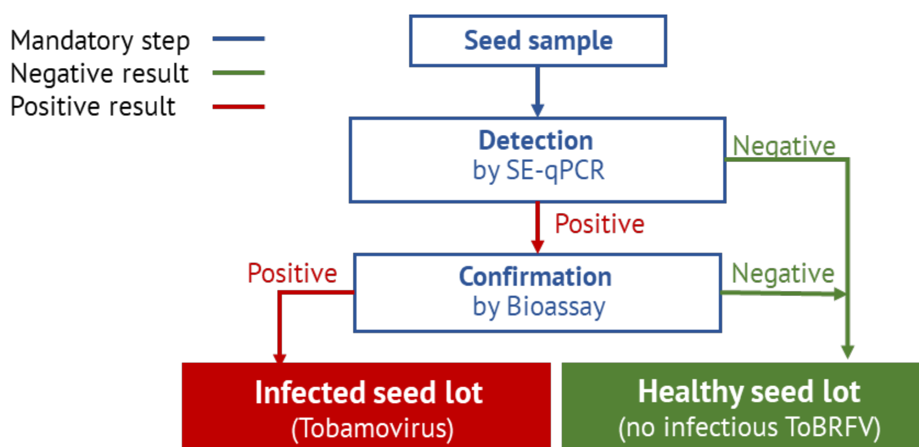

Figure 1. Method process flow.

METHOD VALIDATION

The SE-qPCR assay has been validated by ISHI (Berendsen *et al.*, 2020). Complementary data on reproducibility of the SE-qPCR is highlighted in an addendum to the validation report (Berendsen *et al.*, 2024).

The bioassay has been in use by seed companies for many years and is a seed industry standard. The method for detecting infectious *Tobacco mosaic virus* and *Tomato mosaic virus* in *Solanum lycopersicum* (tomato) seed by the bioassay (indexing) on *Nicotiana tabacum* plants is an ISTA Rule (7-028) since 2011 (www.seedtest.org). The US National Seed Health System (NSHS) (http://seedhealth.org) has approved the method as a Standard B.

In ISHI's method *Detection of Infectious Tobamoviruses in Tomato/Pepper Seed*, an ELISA pre-screen is used for the detection of tobamoviruses including ToBRFV, however it is known that antibodies typically react with several tobamoviruses without allowing for a specific identification.

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 (acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate, etc.) 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.

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 ToBRFV after a positive ELISA in ISHI's tests *Detection of Infectious Tobamoviruses in Tomato/Pepper Seed* and using the same (ELISA) seed extract.

In case of a positive result in the bioassay, the presence of infectious ToBRFV can be confirmed by a qPCR on the lesions.

SAMPLE AND SUBSAMPLE SIZE

The recommended minimum sample size is 3,000 seeds with a maximum subsample size of 250 seeds.

Note that extracts of four subsamples are combined for SE-qPCR (Section I, §3.1) consequently for SE-qPCR three datapoints are generated, each representing a subsample of 1,000 seeds.

Laboratories can also choose to perform directly the SE-qPCR on three subsamples of 1,000 seeds ground in 40 and 80 mL of PBS for tomato and pepper, respectively (Section I, § 2.2). From these extracts 100 µL is then taken from each subsample for RNA purification (Section I, §3.1) and further execution of the SE-qPCR steps. If extraction of the virus from the seed was performed directly on three subsamples of 1,000 seeds, a positive SE-qPCR result must be followed up with a bioassay on a new sample of 3,000 seeds in subsamples of 250 seeds, which is the maximum subsample size for the bioassay.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1	April 2019	
1.1	May 2019	In Table 4 nM corrected to µM.
1.2	May 2019	Note on primer name added in Table 2.
1.3	September 2019	Seed extraction buffer volume for pepper adjusted from 15-20 to 20 mL in § 2.2 of the Section on Seed Extract qPCR.
1.4	March 2020	Text in *Principle* adjusted for more clarity, paragraphs on ELISA pre-screen and the validation report added to *Method Validation* and note 1 rewritten for more clarity in *Method Execution*.
1.5	November 2020	Option for testing 3 x 1,000 seeds with the SE-qPCR more explicitly described.
1.6	April 2024	Protocol updated in accordance with ISHI protocol guidelines. Reference to validation report and addendum validation report added.

Protocol for detecting infectious ToBRFV 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)
- RNA purification kit and equipment
- RT-qPCR mix, primers (Table I.2) and equipment
- Controls (Table I.3)
- RNAse free water
- Centrifuge
- Laboratory disposables

Table I.1. Seed extraction buffer (phosphate buffered saline (PBS)) - pH 7.2 – 7.4 per liter[a].

Compound	Amount/L
NaCl	8.0 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g

[a] Use de-ionized water, and autoclave at 121 °C, 15 psi for 15 min.
If a different seed extraction buffer is used, it must be verified in a comparison using uniform positive control material that it does not lead to a reduction in the number of lesions of the bioassay obtained.

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

Name	Sequence (5'- 3')	Source
CaTa28 Fw	GGT GGT GTC AGT GTC TGT TT	ISHI-Veg, 2019
CaTa28 Pr	6FAM - AGA GAA TGG AGA GAG CGG ACG AGG - BHQ1	
CaTa28 Rv	GCG TCC TTG GTA GTG ATG TT	
CSP1325[a] Fw	CAT TTG AAA GTG CAT CCG GTT T	
CSP1325 Pr	VIC - ATG GTC CTC TGC ACC TGC ATC TTG AGA - BHQ1	
CSP1325 Rv	GTA CCA CGT GTG TTT GCA GAC A	
BaCV-F	CGA TGG GAA TTC ACT TTC GT	
BaCV-R	AAT CCA CAT CGC ACA CAA GA	
BaCV-P	TxR - CAA TCC TCA CAT GAT GAG ATG CCG - BHQ2	

[a] The name CSPtbrfv101 is also used for the primer sequence.

Table I.3. Types of controls used.

Control type	Description
Negative process control (NPC)	Tomato or pepper seed free of ToBRFV
Positive amplification control (PAC)	ToBRFV RNA aiming for a Cq (Cycle quantification) value between 28 and 32 *or*
	ToBRFV oligo DNA (oligonucleotide (single-stranded DNA) for all ToBRFV target sequences) aiming for a Cq value between 28 and 32 *or*
	ToBRFV cDNA aiming for a Cq value between 28 and 32.
Positive extraction control (PEC)	Spike solution[a] added to the sample aiming for a Cq value between 28 and 32. The PEC serves as an internal amplification control (IAC).
Inhibition control (IC)	Dilution of the PEC in a non-infected seed extract aiming for a Cq value between 28 and 32. Note: A non-infected seed extract is preferred over a seed extraction buffer, as a strongly diluted infected leaf extract may lead to a relatively high loss of RNA in the purification process (i.e., no carrier RNA present).
Negative template control (NTC)	Contains all PCR reagents but no target or spike DNA, RNA or PEC nucleic acids

[a] The spike solution is prepared by taking a leaf from a plant infected by *Bacopa chlorosis virus* (BaCV) and making an extract of it in PBS. The extract is diluted to obtain a suitable concentration and aliquots are stored at -80 °C. Other organisms such as *Dahlia latent viroid* (DLVd) and *Squash mosaic virus* (SqMV) may also be used and should be shown to be compatible with the ToBRFV primers in a multiplex PCR.

1. General Requirements

- Seed extracts and controls must be prepared at the same time, under the same laboratory conditions and stored under the same conditions.
- Seed extracts and all controls must be stored at 4 °C until the assay begins. It is strongly recommended to perform the bioassay within 24 hours following seed extraction.
- The final results of the bioassay must be validated through a comparison of the results given by both controls.

2. Extraction of the virus from the seed

2.1. Add the PEC (Table I.3) to the seed extraction buffer (Table I.1).

2.2. Grind seeds of each subsample and the NPC, 250 seeds each, in 10 mL of the seed extraction buffer for **tomato** seed, *or* in 20 mL seed extraction buffer for **pepper** seed.

Alternatively, grind three subsamples and the NPC of 1,000 seeds each directly in 40 and 80 mL of the seed extraction buffer for tomato and pepper, respectively.

Note: In case of a positive SE-qPCR result when using this option, a fresh seed extract from a new sample of 3,000 seeds in subsamples of 250 seeds is required for the bioassay.

2.3. Store seed extracts at 4 °C.

3. RNA extraction

3.1. In the case of 250 seed subsamples, combine 25 µL of four seed extract subsamples into a 100 µL combined sample. Use all three combined samples for further analysis.

In the case of 1,000 seed subsamples, use 100 µL from each subsample for further analysis.

3.2. Add RNA extraction buffer within 4 hours after grinding.

3.3. Use a commercial RNA extraction kit for RNA extraction. Process the three samples according to the supplier's instructions.

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

Note: The assay has been validated with the RNeasy kit (Qiagen, Hilden, Germany) and the sbeadex kit (LGC, London, UK). If a different RNA isolation kit is used, it is necessary to verify its performance.

4. Preparation of the RT-qPCR mix

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

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

4.2. Take 5 µL of the RNA sample as input for the PCR.

4.3. For each run, include an NTC and at least one PAC that give a Cq value between 28 and 32.

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

Table I.4. RT-qPCR ToBRFV mix.

Component	Target	Per reaction (in µL)	Final concentration
UltraPlex 1-Step ToughMix (4×)		6.25	1×
CaTa28 Fw (10 µM)	ToBRFV	0.75	0.3 µM
CaTa28 Pr (10 µM)		0.50	0.2 µM
CaTa28 Rv (10 µM)		0.75	0.3 µM
CSP1325 Fw (10 µM)	ToBRFV	0.75	0.3 µM
CSP1325 Pr (10 µM)		0.50	0.2 µM
CSP1325 Rv (10 µM)		0.75	0.3 µM
BaCV-F (10 µM)	BaCV	0.75	0.3 µM
BaCV-P (10 µM)		0.50	0.2 µM
BaCV-R (10 µM)		0.75	0.3 µM
RNAse free water		7.75	
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

5. Evaluation of the test results

5.1. 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.

5.2. Check for exponential amplification, indicated by an S-shaped amplification curve, for ToBRFV positive samples. Compare with the PAC results.

5.3. A negative test result for the SE-qPCR assay means that the sample does not contain ToBRFV.

6. Validity of test results

Test results are only valid when all included controls presented in Table I.3 give the expected results.

6.1. The PAC should give a clear signal < Cq 32.

6.2. The negative control samples (NPC and NTC) should give a Cq value above a cut off value defined by the laboratory during validation.

6.3. The PEC must give a Cq value within a three-cycle range of the Cq of the IC in all samples. If not, loss or inhibition of ToBRFV during amplification may have occurred. The Cq of the IC itself should be between 28 and 32.

Note: If DNA is used as a PAC for ToBRFV, the BaCV Cq is also a check of proper reverse transcription. A BaCV PAC is recommended to distinguish between RT-PCR failure (mix/program) and inhibition.

II. CONFIRMATION BY BIOASSAY

- For pepper seed, see the protocol for the Detection of Infectious Tobamoviruses in Pepper seed on the ISF website.
- For tomato seed, see the protocol for the Detection of Infectious Tobamoviruses in Tomato seed on the ISF website.

REFERENCES

Berendsen, S.M.H., Tavares, C., Hiddink, G. and Woudenberg, J.H.C. (2020). Detection of *Tomato brown rugose fruit virus* (ToBRFV) in Tomato and Pepper Seed by SE-qPCR. Validation report, International Seed Federation (ISF), Nyon, Switzerland. https://worldseed.org/our-work/seed-health/ishi-method-development-and-validation/.

Berendsen, S.M.H., Hiddink, G., Thomas, L. and Woudenberg, J.H.C. (2024). Detection of *Tomato brown rugose fruit virus* (ToBRFV) in Tomato and Pepper Seed by SE-qPCR. Addendum Validation report, International Seed Federation (ISF), Nyon, Switzerland. https://worldseed.org/our-work/seed-health/ishi-method-development-and-validation/.

ISHI-Veg (2019). *Tomato brown rugose fruit virus*. ISHI-Veg develops new, specific detection method. *Prophyta - The Annual*, **2019**, 10-15. https://www.prophyta.org/prophyta/Prophyta%20Annual%202019.pdf