Detection of tomato brown rugose fruit virus (ToBRFV) using an end-point RT-PCR

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1. Introduction

This work instruction describes a one-step reverse transcription-PCR for detection of Tomato Brown Rugose Fruit Virus (ToBRFV). This version is specifically prepared for the North American Plant Protection Organization (NAPPO) use.

1.1 **Pathogen information**: ToBRFV is an emerging tobamovirus with a global distribution. Since its first outbreak in Israel in 2015, the virus has been confirmed in in at least 25 countries (Salem et al., 2023). Important hosts of the virus include tomato (Solanum lycopersicum) and chilli pepper (Capsicum annuum). ToBRFV is mechanically transmissible and implicated to be transmitted by seeds which enable the virus to be dispersed in a wide geographic range.

1.2 **Assay Type**: uniplex reverse transcription-PCR

1.3 **Target** (s): 324 nt segment of the ToBRFV coat protein gene (Dey, K. et al 2021) from position 5856 to 6179 (NCBI acc. MT0022973.1).

1.4 **Internal control**: none

1.5 **Matrices**: total RNA extracted from *Solanaceous* seeds, specifically tomato and pepper. It can also be used with RNA extracted from hosts leaves or fruits.

1.6 **Instrument**: Any

1.7 **Validation**:

1.7.1 This assay has been validated to **Tier 3** through the NAPPO ToBRFV ring test (Mavrodieva et al., 2025).

1.7.2 **Limit of Detection** (LoD): comparable to the ISHI-Veg multiplex real-time RT-PCR and WI-B-T-NPO-1.

1.7.3 **Specificity**: Specificity was tested using over 16 tomato and pepper infecting viruses; no cross-reactivity was observed.

1.7.4 **Selectivity**: Selectivity was tested against tomato, pepper and eggplant seed extracts with no evidence of cross-reaction.

1.8 **Testing Details**: It is recommended to run each sample/subsample in duplicate wells.

2. Equipment, Materials and Reagents

2.1 **Equipment**

- Conventional PCR thermocycler (any vendor) PCR set-up workstation or dedicated PCR enclosure (any vendor)
- Micro centrifuge, bench-top, capable of >10,000 rpm (6,708 x g) (any vendor)
- Vortex (any vendor)
- Freezer, frost-free, capable of -20° C $\pm$ 2° C (any vendor)
- Dedicated, annually calibrated pipettes (P10, P50, P200, P1000, any vendor)

- Horizontal orbital shaker (any vendor)[a]
- Microwave (any vendor)[a]
- Analytical balance, capable of weighing 0.5 mg to 20.5 g (any vendor)[a]
- Gel Electrophoresis unit, capable of running a minimum of 14 samples (any vendor)[a]
- Power supply (any vendor)[a]
- Digital imaging system (any vendor)[a]
- 4200 TapeStation System (Agilent)[b]
- Vortex mixer IKA MS3 with adapter[b]

2.2 Materials

- Sterile filter (barrier) pipette tips (P10, P50, P200, P1000, any vendor)
- Microcentrifuge tubes, 1.5-1.7 mL (pre-sterilized, certified DNase & RNase free, any vendor)
- Thin-wall 0.2 mL PCR tubes (any vendor)
- Ice
- Gloves (any vendor)
- Paper mat or towels, absorbent (any vendor)
- Disposable, absorbent bench under pads (any vendor)
- Loading tips (Agilent 5067-5598, 1 pk or 5067-5599, 10 pk)[b]
- MicroAmp ™ Optical 8-tube strip, 0.2 mL (Catalog#4316567) and MicroAmp ™ optical 8- cap strips (Catalog#4323032)[b] or Optical tube 8X strip (Catalog#401428)[b] and Optical Cap 8X strip (Catalog#401425)[b]

2.3 Reagents

- One Step RT-PCR Kit (Qiagen Cat. No. 210212)
- Molecular Grade (MG) Water (any vendor)
- Primers, see Table 1 and 2
- TAE gel running buffer (or components for it)[a]
- Agarose (i.e. UltraPure, Invitrogen #15510-27)[a]
- 100 base pair DNA ladder (i.e. BioMarker® Low 50-1000bp/ Biomarker® EXT 50-2000bp (250 µL, CAT # M 1/CAT# MX, Bio-Ventures, Inc. or other vendor)[a]
- 6X Dye (i.e. BioTracker® Loading buffer (1 mL, CAT # BT-1, Bio-Ventures, Inc.) needed if many samples are processed. The DNA ladder (see #6) is provided with 6X dye[a].
- D1000 ScreenTape (Agilent Cat # 5067-5582)[b]
- D1000 Reagents (Agilent Cat# 5067-5583)[b]
- Biotium GelRed Nucleic Acid Gel 3X Stain (Fisher Scientific Cat#: NC9816614 or Biotium Cat#: 41001)[a]

[a] Equipment, materials and reagents needed if gel electrophoresis will be used to visualize PCR products
[b] Equipment, materials and reagents needed if Agilent TapeStation will be used to visualize PCR products.

3. Primer Preparation

Table 1: ToBRFV-Specific Primers (purification: standard desalting)

Primer name	Sequence
FL782	5'- TAG CGA AGT GTG GAA ACC TG-3'
FL783	5'- GGT GCA GAG GAC CAT TGT AA-3'

Note: Primer stock and working solutions should be prepared after receipt of new reagents. New reagents need to be tested using approved and validated positive and negative controls prior to

testing samples.

3.1 Tubes with lyophilized primers are centrifuged for 10-20 seconds at a minimum of 10,000 rpm (6,708 x g) before opening to ensure that the lyophilized material is on the bottom of the tube.

The following steps must be done in a decontaminated PCR hood/enclosure:

3.2 Concentrated freezer stock solutions (100 µM): primers are re-hydrated to a 100 µM stock solution with molecular grade water and store at -20° C.

Note: To make 100 µM stock solution; use the lyophilized oligo concentration in nmol, multiply by 10. This is the total amount (µl) of nuclease free water should be added to the lyophilized primer.

3.3 Working concentration of primer mixes: Follow Table 2 to prepare primer mix of 5 µM working concertation.

Table 2. Preparation of primer mix (ToBRFV)

Primer	Volume (µl)	Working Concentration
100 µM FL782 primer stock	50	5 µM
100 µM FL783 primer stock	50	5 µM
MG Water	900	N/A
Total	1000	N/A

Note: It is recommended to use rehydrated primers or probes within one year of the preparation date.

3.4 Vortex prepared primer mix for 10 seconds at setting 7-10, then centrifuge briefly (10-20 seconds) at 10,000 rpm (6,708 x g).

3.5 Aliquot the primer mix in 50-100 µL aliquots and store in -20 ±2°C freezer.

4. RT-PCR Parameters set up

4.1 Turn on the thermocycler and allow the machine to run through its self-testing procedures. Place the PCR tubes into the thermocycler.

4.2 Program the following settings for PCR conditions into the machine or select the correct saved program.

RT: 50°C for 30 minutes
Denaturation: 95°C for 15 minutes
PCR: 40 cycles of the following:
94°C for 30 seconds
55°C for 30 seconds
72°C for 60 seconds
Extension: 72°C for 10 minutes
Hold at 4°C

Note: Use maximum temperature ramping rate between steps

5. Controls

5.1 **Non-template control (NTC):** molecular grade water.

5.2 **Positive control:** ToBRFV total plant RNA, or an artificial template tested in advance of diagnostics

A synthetic DNA control of the target region has been engineered to contain an additional sequence with restrictions sites BamH1-Pst1-EcoR1: GGATCC-CTGCAG-GAATTC (EcoR1 3' terminal C is found in the virus sequence) for easy discrimination of the wild type. Digestion of the amplicon (324 bp) with one or more of the following restriction enzymes: BamH1, Pst1, and EcoR1, will yield about 160bp products. Sequencing of the amplicon product will also reveal the presence of the restriction site sequence.

TAGCGAAGTGTGGAAACCTGTCCCTCAAGTCACTGTTAGGTTTCCTGACAGTGGTTTTAAGGTGTATAGGTACAA
TGCGGTACTAGATCCTCTAGTTACTGCTTTGTTAGGAGCTTTCGATACTAGAAATAGGATTATAGAAGTCGAAAA
TCAGGCGAA**CGGATCCCTGCAGGAATT**CCCGACAACCGCCGAAACGTTAGACGCTACTCGTAGAGTAGATGA
CGCAACGGTGGCTATAAGGAGCGCTATAAATAATTTAGTAGTAGAATTGGTCAAAGGAACAGGTTTGTACAATC
AGAGCACATTTGAAAGTGCATCCGGTTTACAATGGTCCTCTGCACC

Fig. 1. Nucleic acid sequence of the ToBRFV synthetic control with BamH1-Pst1-EcoR1 restriction site sequence CGGATCCCTGCAGGAATT, not found in the natural virus sequence, highlighted.

6. RT-PCR Master Mix preparation

6.1 Master Mix preparation must be done in a decontaminated PCR workstation/ enclosure. Do not add controls or samples DNA while working inside the PCR workstation. Change gloves frequently. It is recommended that each sample and control are tested in duplicate.

6.2 Remove all reagents from -20 ±2°C freezer and thaw. Once frozen reagents are thawed, vortex briefly (5-10 seconds) at speed setting 7 and centrifuge for 10-20 seconds at a minimum of 10,000 rpm (6,708 x g) to settle the liquid to the bottom of the tube. Place tubes in ice.

6.3 Enzyme mix does not require thawing, place immediately on ice. To mix the solution, flick the tube several times, lightly vortex and spin briefly as described above.

6.4 In a separate ice bucket, thaw prepared RNAs (if frozen) including all controls. Vortex the tubes briefly (3-5 seconds) at a speed setting of 7 and centrifuge 10-20 seconds at a minimum of 10,000 rpm (6,708 x g) to settle the liquid to the bottom of the tube. If samples are on a plate, thaw and centrifuge briefly using a plate spinner. Place tubes or plate in ice.

6.5 Prepare the master mix described in Table 3 for the desired number of samples, plus 2 controls (one ToBRFV positive control and one NTC). To ensure adequate amount of master mix is made, calculate the mix for the number of samples plus 1 for each 10 samples (e.g. 11 samples master mix for 10 samples)

Table 3. PCR Master Mix

Component	Volume (µL)	Final Concentration
Water (Molecular Grade)	13.0	-
5x Qiagen OneStep RT-PCR Buffer	5.0	1×
Qiagen dNTPs (10mM)	1.0	0.4 mM
FL782 and FL783 primer mix (5 µM each)	3.0	0.6 µM (forward and reverse primers)
Qiagen OneStep RT-PCR Enzyme Mix	1.0	-
Template	2.0	-
Total	25.0	-

6.6 Mix master mix well by vortex or pipetting up and down several times, then pipette 23.0 µl into

each 0.2 ml PCR tube/strip. Lightly close the caps on the PCR tubes to prevent contamination during transportation to sample addition area.

6.7 Adding Test Samples and Controls

6.7.1 Take the PCR tubes/strips containing the Master Mix to an assigned lab bench and place on a new disposable lab mat. Do not add controls or samples RNA while working inside the PCR enclosure.

6.7.2 It is recommended to start loading the NTC (MG water) and then add samples. The positive controls should be at the end. Change gloves frequently when processing samples considering that each sample is a suspect of ToBRFV and the assay is highly sensitive for the detection of the pathogen.

6.7.3 Add 2.0 µL of RNA samples or controls to the corresponding well or tube. Mix by pipetting up and down 3-4 times. The total reaction volume is 25 µL.

NOTE: After automated sample extraction, the samples are often stored in a 96-well plate format sealed with an aluminum foil cover. Do NOT remove the aluminum foil cover. This could cause significant contamination issues. To add the sample to the real time RT-PCR-PCR reaction plate, pierce the aluminum foil with the pipette tip. Pipette sample up and down to mix before transferring it to the master mix tubes/plates.

6.7.4 After the addition of all samples and controls, close the caps and spin the PCR tubes/strips in a micro centrifuge for 10 seconds.

6.7.5 Place in the thermocycler and start the run.

7. Visualization of the PCR results

Either the Agilent TapeStation or Gel Electrophoresis may be used to visualize the PCR reaction.

7.1 Agilent TapeStation Procedures

7.1.1 Allow D1000 Reagents to come to room temperature for 30 minutes.

7.1.2 Launch the Agilent 4200 TapeStation Controller Software.

- Flick the D1000 ScreenTape device and load it into the 4200 TapeStation instrument.
- Place loading tips into the Agilent 4200 TapeStation instrument.

7.1.3 Prepare samples and controls

- Vortex reagents and spin down before use.
- Prepare ladder: For 1 – 15 samples: pipette 3 µL D1000 Sample Buffer and 1 µL D1000 Ladder at position A1 in a tube strip.
- For each sample, pipette 3 µL D1000 Sample Buffer and 1 µL DNA sample in a well plate (5042-8502) or a tube strip.
- Apply foil seal to sample well plate and caps to tube strips with ladder or sample.
- Mix liquids in sample and ladder vials using the IKA vortex at 2000 rpm for 1 min.
- Spin down to position the sample and ladder at the bottom of the well plate and tube strip.

7.1.4 Load samples into the Agilent 4200 TapeStation instrument. Carefully remove caps of tube strips.

- Place ladder in position A1 on tube strip holder in the 4200 TapeStation instrument.
- Select required sample positions on the 4200 TapeStation Controller Software.

7.1.5 Click Start.

7.1.6 The Agilent TapeStation Analysis Software opens after the run is complete and displays results (Figure 2).

7.2 Gel Electrophoresis

7.2.1 Prepare a 1.5% agarose gel(s) in 1X TAE buffer. Gels should be sufficient size to accommodate the number of samples, plus controls and a DNA ladder. Sample wells must be large enough to

accommodate at least 15 µL.

7.2.2 Prepare samples and controls
- Mix 10 µL of the 100 bp DNA ladder with 2 µL 6X loading buffer. Load 12 µL of ladder mixture on the left and right side of the sample PCR products.
- Mix 10 µL of the each 25 µL PCR reaction with 2 µL of 6X loading buffer. Mix by pipetting up and down and load each mixture into the corresponding wells. Load entire mixture into designated sample well.

7.2.3 Run the loaded gel in 1X TAE buffer for 1 hour at 100V (constant).

Note: The time to achieve good separation of the PCR products may vary depending on the equipment used, particularly the gel box and gel size. If necessary, adjust run time (not voltage) to achieve better separation.

7.2.4 Stain the gels for 10 minutes in 3X GelRed staining solution and de-stain 10 minutes in dH2O, using a horizontal orbital shaker for each stage

7.2.5 Document the gel results using a digital imaging system.

7.2.6 GelRed is considered hazardous waste. Please read the SDS prior to handling and use Dispose of the gel in a designated trash bin

8. Assessment of Conventional RT-PCR Results

Note: If using TapeStation: "Scale to Sample" tool on the TapeStation is not generally recommended. This feature can help to resolve low titer samples. Sample scaling should be used sparingly and considered alongside all other available data, including band size, concentration, and other comparable samples. Scale to sample can create noise in samples with non-specific banding.

8.1 Assessment of Quality Controls.

All controls must be valid to accept the sample results.

8.1.1 Ladders must be distinct and well resolved to be valid. If the ladders are not distinct and/or well resolved, determine the cause and correct, then rerun the gel with the remainder of the PCR reactions for the samples and controls.

8.1.2 Non-template control (NTC) should not contain any bands (other than primer dimer). If any bands are present, then the entire run is invalid. All samples and controls must be retested using this WI. This indicates contamination of the PCR run.

8.1.3 Positive control: The positive control should produce a band of 324bp. If such band is not present, the run is invalid, and all samples and controls must be retested using this WI. This indicates that the PCR reaction failed, typically a reagent was not added into the master mix or the control was not added to the reaction tube.

8.2 Assessment of Samples

All controls must be valid to accept the sample results

8.2.1 If a sample does not produce a band of 324 bp in both duplicates, then it is determined **negative** for ToBRFV in this assay.

8.2.2 If a sample produces a band of 324 bp in either of the duplicates, it is determined suspect **positive** for ToBRFV.

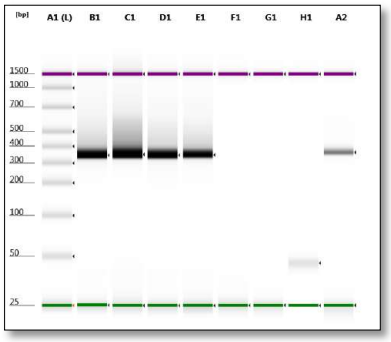

Fig. 2. TapeStation Electrophoresis. Lane A1: D1000 Ladder; Lanes B1, C1, D1 and E1: ToBRFV positive samples (expected band size 324 bp); Lane F1 and G1: Buffer Controls (no bands expected); Lane H1: NTC (no bands expected); Lane A2: ToBRFV Positive Control (expected band size 324 bp).
Sequencing of ToBRFV positive samples

9. Sequence analysis

The 324bp PCR amplicons can be sequenced in house or by commercial provider to confirm identity. Amplicons with concentration below 0.5ng/µl rarely produce useful sequence.

9.1 Once forward and reverse directions sequences are obtained and analyzed, a contig should be made resulting in a consensus sequence for each sample. The consensus sequence should be saved with the sample/control ID name.
9.2 The sequence is analyzed by BLAST Search using NCBI GenBank database.
9.3 Samples that produce sequences meeting the criteria listed below are determined **positive** for ToBRFV.
9.3.1 The query coverage must be at least 90% for the sequencing section (324 nt; minimum 291 nt).
9.3.2 The sequence must have more than 90% identity with ToBRFV.

Note: Pairwise alignments of ToBRFV to TMV, ToMV and ToMMV in the target region (324nt) is roughly calculated as ToBRFV vs TMV=82.7%; ToBRFV vs ToMV=77.5%; ToBRFV vs ToMMV=79.0%.

9.3.3 If using the artificial control, nucleotide sequence for the samples' PCR amplicons should **not** include CGGATCCCTGCAGGAATT sequence specific for the positive control (see 5.2 and Fig. 1)
9.3.4 Samples that produce sequences that do not meet the above criteria should be retested and/or re-sequenced using this work instruction.

Reference:

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
2. Salem, M.N., Jewehan, A., Aranda, M.A., Fox, A. 2023 Tomato Brown Rugose Fruit Virus Pandemic. Annual Review of Phytopathology 61:1, 137-164. DOI: https://doi.org/10.1146/annurev-phyto-021622-120703
3. Mavrodieva, V., Dennis, G., Xoconostle-Cazares, B., et al. 2025. Pilot for Harmonization of Diagnostic Protocols for Tomato Brown Rugose Fruit Virus (ToBRFV) in Tomato and Pepper Seeds PhytoFrontiers https://doi.org/10.1094/PHYTOFR-06-24-0070-FI
4. WI-B-T-1-89 Confirmatory Diagnostic of Tomato brown rugose fruit virus (ToBRFV) on

Solanaceous Seeds and Plant Material using a Conventional RT-PCR Assay

Document Revision History

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Author(s):
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