Detection of tomato brown rugose fruit virus (ToBRFV) by real-time RT-PCR on ABI QuantStudio 5

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1. Introduction

This work instruction describes a multiplex, one-step reverse transcription real-time RT-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 chili 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:** duplex real-time reverse transcription-PCR

1.3 **Target (s):** a segment of the ToBRFV movement protein (Chanda, B., et al 2021) designed based on NCBI acc. #MT002973 and #MT002971 sequences.

1.4 **Internal control:** mitochondrial NADH dehydrogenase subunit 5 plant gene (*nad5*) primers (Menzel et al.2002) with modification (Chaudhary et al. 2015)

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:** ABI QuantStudio™ 5 Real-Time PCR system

1.7 **Validation:**

1.7.1 This assay has been validated to **Tier 3** through the NAPPO ToBRFV ring test (Mavrodieva et al., 2025).
Note: the following brands real-time thermocyclers were used during the ring test: Biorad CFX 96 and 384, Qiagen Rotor-Gene Q; ABI Quant Studio™ 5 and 6.

1.7.2 **Limit of Detection (LoD):** 67 copies of invitro transcript per reaction comparable to the ToBRFV ISHI-Veg multiplex real-time RT-PCR protocol.

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.

1.7.5 Recommended **Ct** cut-off for positive determination: 34.
Note: this cut off was established during validation at the PPCDL and during the ring-test. Each laboratory should additionally validate this assay in their specific conditions and establish a cut-off.

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

2. Equipment, Materials and Reagents

2.1 Equipment

- PCR clean station or set-up hood (any vendor)
- ABI QuantStudio™ 7 Flex Real-Time PCR System
- Labnet MPS 1000 Mini Plate Spinner
- Bench-top micro centrifuge, i.e. Eppendorf MiniSpin or MiniSpin Plus.
- Vortex (any vendor)
- Dedicated, annually calibrated pipettes (P10, P50, P200, multi-channel, any vendor)
- Freezer -20 ºC (non-frost-free, any vendor)

2.2 Materials

- 1.5 mL micro centrifuge tubes, clear and amber (pre-sterilized, certified DNase & RNase free, any vendor) and tube openers.
- ThermoFisher Scientific MicroAmp™ Optical 8-tube Strip, 0.2 mL (Cat #4316567); MicroAmp™ Optical 8-cap strips, 300/pc (Cat #4323032); OR MicroAmp™ 8-tube strip with attached optical caps (Cat. #A30588); OR MicroAmp™ Optical 96-Well Reaction Plate (Cat. #N8010560).
- MicroAmp™ Cap Installing Tool (Cat #4330015);
- MicroAmp™ Optical Adhesive Film (Cat. # 4311971);
- MicroAmp™ Adhesive Film Applicator (Cat. # 4333183); MicroAmp™ 96-Well Base (Cat. # N8010531)
- 10 mL Bulk Reservoirs, Sterile (Vista Labs Cat #3054-1012)
- Sterile filter (barrier) pipette tips for the corresponding pipettes (any vendor)
- Gloves (any vendor)
- Disposable bench tissue paper (any vendor)
- Absorbent disposable bench under pads (any vendor)
- Ice

2.3 Reagents

- Molecular Grade (MG) water (any vendor)
- TaqMan Fast Virus 1-Step Master Mix (ABI; Cat. #4444432)
- Primers and probes (Table 1)

3. Primer and Probe Preparation

Table 1. Primer and Probe Sequences

Target	Primer/Probe	Sequence 5'-3'
ToBRFV	KL 18-59 ToBRFV-F1	GCCCATGGAACTATCAGAAGAA
	KL 18-61 ToBRFV-R1	TTCCGGTCTTCGAACGAAAT
	KL 18-60 ToBRFV-P1	**56-FAM**-AGTCCCGATGTCTGTAAGGCTTGC-**QSY**
Nad5	Nad5f	GATGCTTCTTGGGGCTTCTTKTT
	Nad5RT1r	ACATAAATCGAGGGCTATGCGGATC
	Nad5p1	**VIC/Cy5**-CATAAGTAGCTTGGTCCATCTTTATTC CAT-**QSY**

Note 1: NAD probe could be labeled with Cy5 as well

Note 2: Primer and probe stock and working solutions should be prepared after receipt of new reagents. It is recommended that new reagents are tested using approved and validated positive and negative

controls prior to testing samples. When a PCR assay is being conducted, the analyst should use a 'working solution' of primers and probes.

3.1 Tubes with lyophilized primer or probe (Table 1) are centrifuged 60 seconds at minimum 10,000 rpm (6,708 x g) before opening to ensure that the lyophilized material is in 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) of the primers: primers are re-hydrated to 100 µM stock solution in molecular grade water and stored at -20 °C. Store freezer stocks of probes in amber-colored micro centrifuge tubes or conceal in clear tubes tin foil.

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.

Follow Table 2 A&B below to prepare the primer and probe mix.

Table 2A. ToBRFV Primers & Probes Mix

Primer & Probe	Volume (µL)	Working Conc.
100 µM ToBRFV-F1	80	8 µM
100 µM ToBRFV-R1	80	8 µM
100 µM ToBRFV-P1	40	4 µM
MG Water	800	N/A
Total	1000	N/A

Table 2B. Nad5 Primers & Probes Mix

Primer & Probe	Volume (µL)	Working Conc.
100 µM Nad5f	60	6µM
100 µM Nad5RT1r	60	6 µM
100 µM Nad5	30	3 µM
MG Water	850	N/A
Total	1000	N/A

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

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

3.4 Aliquot mixes in small volumes (e.g. 50 or 100µL) in amber centrifuge tubes.

3.5 Store the primer/probe mix at -20 °C. Use one tube at a time.

4. Real-Time PCR Instrument and Software Setup

4.1 Turn on the QuantStudio 5 Real-Time PCR instrument. Experiment can be set up on the instrument or using the QuantStudio™ Real-Time PCR System Software on the computer. Open a new experiment and set up the experiment. The experiment parameters can be saved as a template, which helps create new runs with the same setup information.

4.2 On the Properties screen on QuantStudio 5:
- Name the experiment
- Confirm the experiment settings:
- Instrument type: QuantStudio 5 System
- Block: 96-Well (0.2 mL)
- Type of experiment: Standard Curve
- Reagents: TaqMan® Reagents
- Run properties for the instrument: Standard

4.3 From Plate screen on QuantStudio 5, confirm/enter the Target Properties and Sample Names
- Targets:
  - Target Name: ToBRFV (ToBRFV FAM); Reporter: FAM; Quencher: QSY
  - Target Name: Nad5 (ToBRFV VIC); Reporter: VIC; Quencher: QSY
- Enter the Sample Name and the control name
- Select ROX as the Passive Reference dye
- On the Plate screen on the QuantStudio 5, select individual wells on the plate layout by highlighting the intended wells. Then, choose the targets and samples as defined above, according to the fluorescent probe targets and sample /control names.

4.4 Select Method on QuantStudio 5

4.4.1 20 (µL) in the Reaction Volume per Well

4.4.2 Thermal cycling conditions
- Hold Stage
  A. Instrument automatic ramp 2.552 ºC/s. AutoDelta disabled.
  B. Hold at 50.0 °C for 30 minutes with Data Collection OFF
  C. Hold at 95.0 °C for 20 seconds with Data Collection OFF
- PCR Stage
  A. Instrument automatic ramp 1.988 °C/s between cycling temperature
  B. 40 Cycles:
  C. 95.0 ºC for 3 seconds with Data Collection OFF
  D. 60.0 ºC for 30 seconds with Data Collection ON

5. Controls

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

5.2 **Extraction buffer control:** this is a buffer sample that has been extracted alongside diagnostics samples using the sample extraction protocol

5.3 **Positive control:** ToBRFV total plant RNA, or an artificial template tested in advance of diagnostics with known Cts for FAM and VIC

6. Real-time PCR set up

Each RNA sample and control are tested in duplicate (duplicate wells).

6.1 Remove the primer/probe mix from the freezer and thaw at room temperature. As soon as the primer/probe mix is thawed, vortex at setting 7-10 for 10 sec. Spin for 5-10 sec at maximum speed on a bench-top microcentrifuge and place on ice.

6.2 The TaqMan Fast- Virus 1-Step Master Mix should be kept on ice at all time. To mix the solution,

flick the tube several times, lightly vortex and spin briefly as described above.

6.3 If samples to be tested are frozen, remove the RNA samples from the -80 °C freezer and thaw. Once samples are thawed, lightly vortex for 5 seconds at speed setting 7 and centrifuge for 30 seconds at 10,000 rpm (6,708 x g) to collect the sample at the bottom of the tube. Place samples tubes/plate on ice.

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

6.4 Prepare the Master Mix described in Table 3 for the desired number of samples plus 3 controls (one ToBRFV positive control, one buffer extraction control and one NTC). Lightly vortex the reaction mix and follow by a quick spin down in a microcentrifuge. If preparing a full plate, pour the mix into the bulk reservoir after homogenization.

Note: Prepare an additional 10% of master mix either by multiplying all reagents by a factor of 1.1 or by preparing one additional sample per each 10. This will ensure enough master mix is prepared to test all samples.

Table 3. RT-PCR Master Mix

Reagent	1 Reaction (µL)	Final Concentration (µM)
Water (Molecular Grade)	11	---
4X TaqMan Fast Virus 1-Step Master Mix	5.0	1X
ToBRFV FRP Mix (8:8:4 µM)	1.0	0.4 µM (forward and reverse primers); 0.2 µM (probe)
Nad 5 FRP Mix (6:6:3 µM)	1.0	0.3 µM (forward and reverse primers); 0.15µM (probe)
Template (RNA)	2.0	---
Total Reaction	20.0	---

Note: ROX reference dye is incorporated into the TaqMan Fast Virus 1Step Master Mix.

6.5 Put a 96-Well Reaction Plate or 8-tube strips onto a 96-well base or a tube rack, arrange and record positions for samples and controls (positive and NTC), and aliquot 18.0 µL of the composed Master Mix into each well/tube.

6.6 Take the PCR plate/tube strips containing the Master Mix to an assigned lab bench and place all items on a new disposable lab mat. Add 2 µL of RNA samples and controls to the corresponding wells or tubes. The total reaction volume is 20 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 Seal appropriately:

6.7.1 Plate: Apply Optical Adhesive Film to the plate.

- Applying firm pressure, gradually move the Adhesive Film Applicator across the film horizontally and vertically several times to ensure a good final seal for all of the 96 wells.
- Hold the films short edge in place with the applicator edge, then grasp one end of the tab and sharply pull away. Repeat to remove the other tab.

- Finally, run the applicator edge along all four of the outer borders of the film.
- Place the film-sealed plate in a Labnet MPS 1000 Mini Plate Spinner (film-top to face centrally), spin for 1 minute. Visually check each PCR mixture is at the bottom of a well.

6.7.2 Tube Strip:

- Line up Optical 8-cap strips to the top of the tubes
- Using the MicroAmp™ Cap Installing Tool, press firmly down on the caps, rocking slowly back and forth to ensure a complete seal of the caps.
- Visually inspect the caps to ensure a complete seal.
- Place the tubes in a bench top centrifuge with the 8-tube strip adapter and spin for 1 minute. Visually check each PCR mixture is at the bottom of a well.

6.7.3 Place the PCR reaction plate/tube strips on a plate / tube adapter, load the reaction plate / tube strips into QuantStudio™ 5 Real-Time PCR instrument, start the PCR by clicking the START RUN button from Experiment Menu / Run group.

7. Results Analyses

7.1 Acquire the real-time PCR results

- In the Plate screen on QuantStudio 5highlight all the 96 wells on the Plate Layout
- Click the Analysis Settings button, open the Analysis Settings pop-up window, verify:
- Data Step Selection is on Stage 2, Step 2
- Algorithm Settings is on Baseline Threshold
- Verify Amplification Plot is on Δ Rn vs. Cycle; Plot Settings is on Log or Linear
- On the Options / Target drop-down menu, select one target at a time and verify Auto threshold is selected.
- Obtain the PCR results in Excel table format by clicking the Export button on the Export screen.

7.2 Quality Control PCR Reaction Assessment

All controls must be valid. If any control is determined to be invalid, all controls and samples must be retested.

7.2.1 NTC Control: For ALL channels (FAM and VIC), Ct values must be Undetermined/ /0.00

7.2.2 Extraction buffer Control: FAM Ct values must be Undetermined/0.00and VIC Ct >37.00

7.2.3 Positive Control: For BOTH channels (FAM and VIC), Ct values must be within the positive range (see 7.3.1. A and 7.3.2.B) and typically, ±3.5 Ct of the mean values established during preliminary testing.

7.3 Sample PCR Reactions Assessment

7.3.1 Test Sample Internal Control (VIC) Assessment

A. The Nad5 internal control of the sample duplicate wells must produce VIC Ct ≤ 30.
B. If the VIC Ct > 30 or VIC Ct =Undetermined/0.00 for one or both wells of each sample, then the sample must be retested.
C. If the sample retest again produces a VIC Ct > 30 or VIC Ct = Undetermined/0.00, it does not pass quality control. New RNA must be extracted and retested.

7.3.2 Test Sample for ToBRFV Reaction Assessment

A. If a sample produces FAM = Undetermined/0.00 or >34, AND VIC Ct ≤ 30 in both wells, then it has tested negative for ToBRFV.
B. If a sample produces FAM Ct value Ct ≤ 34.00 AND VIC Ct ≤ 30 in both wells, the sample is determined positive for ToBRFV.
C. If a sample produces one FAM Ct value Undetermined/0.00 and another < 39.99 then, the sample should be retested using this work instruction.

References

1. Chanda, B., Gilliard, A., Jaiswal, N., Ling, K. - S. 2021. Comparative analysis of host range, ability to infect tomato cultivars with Tm - 22 gene, and real - time reverse transcription PCR detection of tomato brown rugose fruit virus. Plant Disease, 105, 3643–3652. DOI:10.1094/PDIS-05-20-1070-RE
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. 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 J. Virol. Methods, 99 pp. 81-92
4. Chaudhary, N., Wei., G., Gavindarajulu, A., et al 2015. Detection of Citrus leprosis virus C using specific primers and TaqMan probe in one-step real-time reverse transcription polymerase chain reaction assays. Journal of Virological methods. 224: 105-109
5. 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
6. WI-B-T-1-75 Detection of Tomato brown rugose fruit virus (ToBRFV) on Solanaceous Seeds and Plant Material using a Multiplex Real-Time RT-PCR.

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