BIOREBA

qPCR tomato brown rugose fruit virus (ToBRFV) set

User Guide

Table of contents

1. Introduction

1.1 General

The qPCR ToBRFV set enables the detection of Tomato brown rugose fruit virus (ToBRFV) with a triplex real-time RT-PCR. Viral RNA of ToBRFV, extracted from tomato or pepper samples, is amplified with two distinct primer pairs in a one-step RT-PCR reaction. The amplification of the cDNA can be monitored in real time, because the specific probes are labeled with fluorophores (ToBRFV: FAM and HEX). The target sequence for the FAM-probe and primer pair is located within the movement protein gene whereas the target sequence for the HEX-probe and primer pair is located within the coat protein gene of ToBRFV isolate Tom1-Jo. An internal positive control (IPC) is designed to detect a cytochrome oxidase (COX) gene sequence with a fluorophore-labeled (IC: ROX) specific probe. The control shows whether the extraction of tomato samples, the reverse transcription and the amplification of the RNA worked as intended.
The qPCR ToBRFV set was developed and validated by BIOREBA according to the recommendations of the PM 7/146 (1) Tomato brown rugose fruit virus European and Mediterranean Plant Protection Organization (EPPO) Bulletin (2021) 51 (1), 178-197.
Suitable tissue sources of viral RNA are tomato/pepper leaves, seeds and fruits. For the extraction of RNA, it is recommended to use standard RNA extraction protocols established for plant material. Upon request, BIOREBA can recommend an optimal extraction method.

Two sets are available for 96 or 192 reactions (see page 4 for details).

1.2 Information: ToBRFV

Tomato brown rugose fruit virus (ToBRFV), also known as „Jordan virus", has been first observed on tomatoes in 2014 and 2015 in Israel and Jordan (1, 2). In Europe, ToBRFV was first reported in Germany in 2018 (3). Since then, reports of infections have been accumulating across Europe but also on other continents (amongst others: Mexico, USA, China). ToBRFV belongs to the genus of *Tobamovirus* and was shown to have resistance-breaking properties against other *Tobamoviruses*, such as Tobacco mosaic virus (TMV) or Tomato mosaic virus (ToMV) (2). ToBRFV causes different symptoms in different varieties. Observed symptoms include yellow or brown wrinkled spots on fruits, narrowing and mosaic discolorations of leaves as well as deformed or irregularly ripened fruits. Host plants for ToBRFV mainly include tomatoes (*Solanum lycopersicum*) and peppers (*Capsicum spp.*). According to current knowledge, potato (*Solanum tuberosum*) is not a host plant of ToBRFV. In September 2020, the EPPO approved the addition of ToBRFV to the EPPO A2 list of pests recommended for regulation as quarantine pests.

1.3 Special handling instructions

Perform the tests in an RNase-free work environment. Always wear gloves when handling samples containing RNA and the components of the set. Do not touch any set components with an ungloved hand. Keep all components tightly sealed when not in use. Use appropriate laboratory disposable parts. In particular, use nuclease-free tubes and filter tips to avoid degradation and cross-contamination.
Do not use components from sets with different lot numbers in the same test procedure. In order to avoid cross-contamination and obtain reliable results, it is essential to strictly follow the protocol in this manual. Avoid unnecessary freeze-thaw cycles of the set components.

1.4 Warranty and liability

BIOREBA products are guaranteed to meet the specifications described on the product certificate of analysis and in the user guide, which is included with every shipment. No further warranties are given. If you have any questions about specifications or performance, please contact our administrative office (admin@bioreba.ch).

Our products are for laboratory use only and are not intended for human or animal applications. Should a product fail for reasons other than inappropriate handling or misuse, BIOREBA will replace the product free of charge or refund the purchase price.

BIOREBA shall not be liable for any direct or indirect, special or consequential damage of any kind resulting from the use of our products.

2. Intended use

The qPCR ToBRFV set is validated for the simultaneous detection (multiplex) of tomato brown rugose fruit virus (ToBRFV) and a cytochrome oxidase (COX) gene sequence which serves as internal positive control (IPC) by one-step real- time RT-PCR. Suitable tissues are tomato/pepper leaves, seeds and fruit samples. Samples of up to 1000 seeds can be pooled for RNA extraction and analyzed according to this qPCR protocol.

3. Format, content, storage condition and quality

3.1 Set format and contents

		Components		
Sales Part No.	**Product name**	**Colour of screw cap**	**Name**	**Volume**
889600 *	qPCR ToBRFV set 96	Blue	Taq Master Mix (2x) Art. No. 831412	1.2 ml
		Yellow	RT Master Mix (50x) Art. No. 830414	0.05 ml
		Clear	Primers/Probes Mix_ToBRFV/COX (10x) Art. No. 880100	0.2 ml
		–	Nuclease-free water Art. No. T143.4	1 ml
889200 *	qPCR ToBRFV set 192	Blue	Taq Master Mix (2x) Art. No. 831412	2 x 1.2 ml
		Yellow	RT Master Mix (50x) Art. No. 830414	2 x 0.05 ml
		Clear	Primers/Probes Mix_ToBRFV/COX (10x) Art. No. 880100	2 x 0.2 ml
		–	Nuclease-free water Art. No. T143.4	2 x 1 ml

*A ToBRFV RNA positive control (PC, Art. No. 880053, 30 µl) and a plant RNA negative control (NC, Art. No. 830043, 30 µl) is available separately upon request. It is mandatory to clarify before ordering, whether the ToBRFV PC may be imported into your country of destination.

3.2 Storage conditions

Store all qPCR components (Taq Master Mix, RT Master Mix, Primers/Probes Mix_ToBRFV/COX, Nuclease-free Water) at -30 °C to -10 °C.

3.3 Lot-to-Lot consistency

Quality control of the qPCR set is performed based on predetermined specifications to ensure consistent product quality. See lot-dependent certificate of analysis included with the shipment.

4. Specificity and Sensitivity information

4.1 Specificity

The two primer pairs used in this product specifically amplify two distinct fragments of the ToBRFV genome. The target sequence for the FAM-probe and primer pair is located within the movement protein gene whereas the target sequence for the HEX-probe and primer pair is located within the coat protein gene of ToBRFV.
The qPCR ToBRFV set is suitable for detecting ToBRFV in tomato/pepper leaves, seeds and fruit.
All isolates of ToBRFV tested so far can be detected (isolates from locations in Germany, Italy, Netherland, Switzerland and Israel).
No cross reactivity was observed with other *Tobamoviruses* tested so far, including Tobacco mosaic virus (TMV), Tomato mosaic virus (ToMV), Pepper mild mottle virus (PMMoV) and Cucumber green mottle mosaic virus (CGMMV). In addition, no cross reactivity was observed with other viruses within the same host range (TSWV, TBRV, TBSV, TYLCV, PepMV, ToRSV, CMV, INSV, TNV, ToANV and TSV) and no negative effect was observed from host matrix plant species (pepper, tomato, tobacco, quinoa and potato).

4.2 Sensitivity

The sensitivity of this product is very high and was tested with seed, fruit and leave samples from tomato and peppers.

The limit of detection for all tested samples and isolates ranged from 10^{-6} to 10^{-8}.

For seed lot routine testing, we mixed one ToBRFV-infected seed with different numbers of healthy tomato seeds. The sensitivity of the qPCR ToBRFV set for the distinct mixing ratios was as follows:

- Seed lot only infected seeds: Ct 15.9
- Seed lot mixed (1 infected in 250 healthy seeds): Ct 19.4
- Seed lot mixed (1 infected in 1'000 healthy seeds): Ct 23.7
- Seed lot mixed (1 infected in 3'000 halthy seeds): Ct 27.6

5. Materials and equipment (not provided)

- RNase-free filter tips and micropipettes
- Optical grade RNase-free tubes/plate
- Disposable latex or vinyl gloves
- Thermal cycler for real-time PCR

6. Protocol

Please pay attention to the following points:

- The protocol in this manual must be followed.
- Create an RNase-free environment by cleaning the bench with 1 % bleach followed by 70 % ethanol.
- Gloves must be worn at all times.
- Use nuclease-free tubes and filter tips.
- Use appropriate eye protection and wear protective clothing.
- To avoid cross-contamination, use separate rooms for
  a) nucleic acid extraction,
  b) preparation of the Master Mix and
  c) amplification.
- Avoid unnecessary freeze-thaw cycles of the qPCR components.

6.1 RNA extraction

For the extraction of RNA it is recommended to use standard RNA extraction protocols established for plant material. Upon request, BIOREBA can recommend an optimal extraction method.

6.2 Preparation of the qPCR samples

1. Slowly thaw the set components (with the exception of the RT Master Mix!) on ice or at 4 °C. Thereafter, the components should always be kept on ice. It is not necessary to thaw the RT Master Mix (the enzyme is in glycerol.) The RT Master Mix should be kept at -20 °C at all times.

2. Shake the tubes briefly, and spin down the liquid.

3. To prepare the reaction mix, first determine the number of reactions and then increase the number by 1 or 2.

4. Prepare the reaction mix (without RNA template) by combining the components of the set to reach a final volume of 20 µl per reaction (see Table 1).

Component	Volume
Taq Master Mix (2x) Blue	10 µl
RT Master Mix (50x) Yellow	0.4 µl
Primers/Probes Mix_ToBRFV/COX (10x) Clear	2 µl
RNA Template	2 – 5 µl
Nuclease-free water	to reach a final reaction volume of 20 µl

Table 1: Preparation of reaction mix

5. Add the reaction mix (without RNA template) to each PCR tube or well of an optical-grade PCR plate.

6. Add 2-5 µl RNA template to the reaction mix.

7. Seal the PCR tubes or PCR plates, centrifuge briefly to collect components at the bottom of the PCR tubes or wells. Protect from light before thermocycling.

6.3 Thermal cycling

Place the PCR tubes or PCR plate in a thermocycler. Start cycling according to the program below (Table 2).

Step	Cycles	Temperature	Time
Reverse Transcription	1	50 °C	20 min
RT inactivation	1	95 °C	5 min
Denaturation Annealing/Extension	40	95 °C 60 °C	15 sec 30 sec

Table 2: PCR cycling conditions

6.4 Monitoring the PCR amplification

To monitor PCR amplification in real-time, an appropriate thermocycler is required, which can measure the fluorescence of the following fluorophores:

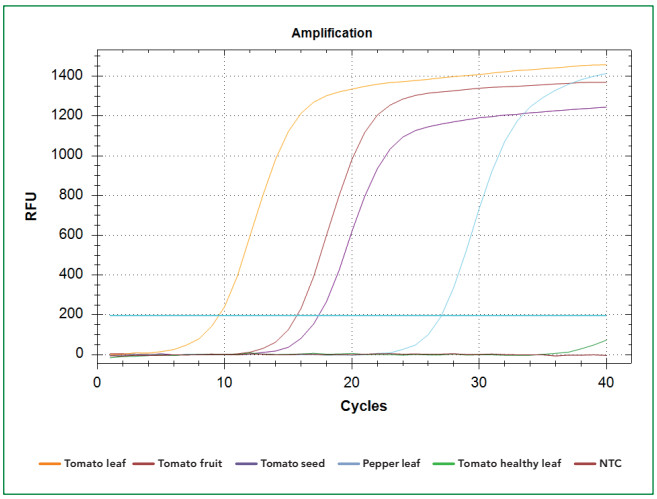
Dye	Virus/Control	Max. EX (nm)	Max. EM (nm)
FAM	ToBRFV	495	520
HEX	ToBRFV	535	556
ROX	COX (IPC)	576	601

Table 3: Fluorophores overview

Please refer to the manufacturer's manual for information on programming the thermocycler, monitoring and evaluation.

6.5 Amplification of typical samples

The graph below shows the amplification curves of different ToBRFV-infected samples (one tomato leaf, seed and fruit sample, and one pepper leaf sample). Healthy control samples and "no template control" (NTC) show no amplification.



Evaluation criteria

In order to distinguish positive from negative samples we recommend taking the following criteria into account:

A) The Ct value
B) The PCR efficiency
C) The delta RFU
(the difference between baseline and final RFU)

The range of values for each of the above can be determined for every channel by means of a dilution series of a known sample.

7. References

(1) Salem N., Mansour A., Ciuffo M., Falk B., Turina M. 2016. A new tobamovirus infecting tomato crops in Jordan. Arch Virol. 161(2):503–6.

(2) Luria, N., Smith, E., Reingold, V., Bekelman, I., Lapidot, M., Levin, I., Elad, N., Tam, Y., Sela, N., Abu-Ras, A. and Ezra, N., 2017. A new Israeli Tobamovirus isolate infects tomato plants harboring Tm-22 resistance genes. PloS one, 12(1), p.e0170429.

(3) Menzel W., Knierim D., Winter S., Hamacher J., Heupel M. 2019. First report of tomato brown rugose fruit virus infecting tomato in Germany. New Disease Reports 39:1.

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