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EURL-Virology

(European Union Reference Laboratory for pests of plants on viruses, viroids and phytoplasmas)

TESTING OF TOMATO BROWN RUGOSE FRUIT VIRUS BY REAL-TIME RT-PCR (BERNABE-ORTS ET AL. 2021)

VALIDATION REPORT

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Contents

1 Scope of validation

Detection and identification of tomato brown rugose fruit virus.

Method	Real-time RT-PCR using primers and probe published in Bernabé-Orts et al. (2021) Test included in EPPO PM7/146(2), Appendix 7
Target	Tomato brown rugose fruit virus
Matrix (sample types)	Tomato and pepper: leaves and seeds

2 Description of methods

2.1 RNA extraction

RNA extraction was performed generally as described in EPPO PM7/146(2) Appendix 1.

A. Leaf

Total RNA was extracted from leaf material using RNeasy Plant mini kit (Qiagen, USA), following the manufacturer's recommendations, with minor modifications. For details, see below.

A1. CREA procedure

Total RNA was extracted from approximately 100 mg of leaf material using RNeasy Plant mini kit, with following modifications: plant tissue was put in an extraction bag and homogenized in phosphate buffer (1:10 (w/v); EPPO PM7/146(2) Appendix 1); 100 µL of the homogenate was added to 380 µL of RLT buffer from the RNeasy Plant mini kit without using 2-mercaptoethanol, and the final RNA elution was performed with two consecutive additions of 50 µL RNase-free water (total elution volume 100 µL).

A2. NIB procedure

Total RNA was extracted from approximately 200 mg of leaf material using RNeasy Plant mini kit with following modifications: extraction was performed without using 2-mercaptoethanol and the final RNA elution was performed with two consecutive additions of 50 µL of RNase-free water pre-warmed to 65°C (total elution volume 100 µL).

B. Seed

The RNeasy Plant Mini Kit (Qiagen) was used with the following modifications: the RLT buffer was replaced by GH+ buffer (EPPO PM7/146(2) Appendix 1) and the centrifugation temperature was decreased to 4°C at all steps to optimize RNA extraction from seeds. For details, see below.

B1. CREA procedure

Samples of 1000 seeds for tomato and 500 seeds for pepper were placed in a grinding bag (Interscience BagPage 100 mL extraction bag with synthetic interlayer) and 20 mL of GH + buffer was added to the seeds, after soaking at room temperature for 60 min, seeds were homogenized with an Interscience BagMixer on position 4 for 90 s (tomato) or 4 min (pepper).

For each sample, 1 mL of the seed homogenate was transferred into a 1.5 mL tube and 30 µL of dithiothreitol (DTT, 5 M) was added, followed by incubation in a thermoshaker at 850 rpm and 65°C for 15 min. After centrifugation at 16,000 g for 10 min, 750 µL of supernatant was loaded on the QIA shredder spin column and centrifuged. Thereafter the manufacturer's instructions of the RNeasy Plant Mini Kit (Qiagen) were followed (with centrifugation at 4 °C as stated above). RNA was eluted from the RNeasy Mini Spin columns by two consecutive additions of 50 µL of RNase-free water (total elution volume 100 µL).

B2. NIB procedure

Samples of 1000 seeds were placed in a grinding bag (Bioreba extraction bag with synthetic interlayer (universal long)) and ground with a hand Homex homogeniser or Homex 6 (Bioreba): firstly, seeds were crushed without buffer, then 5mL (tomato seeds)/10 mL (pepper seeds) GH+ buffer was added and seeds further crushed, and finally 15 mL (tomato seeds)/30 mL (pepper seeds) GH+ buffer was added and the homogenate mixed thoroughly.

For each sample, 1 mL of the seed homogenate was transferred into a 1.5 mL tube and 30 µL of dithiothreitol (DTT, 5 M) was added, followed by incubation in a thermoshaker at 850 rpm and 65°C for 15 min. After centrifugation at 16,000 g for 10 min, 750 µL of supernatant was loaded on the QIA shredder spin column and centrifuged. Thereafter the manufacturer's instructions of the RNeasy Plant Mini Kit (Qiagen) were followed (with centrifugation at 4 °C as stated above). RNA was eluted from the RNeasy Mini Spin columns by two consecutive additions of 50 µL of RNase-free water pre-warmed to 65°C (total elution volume 100 µL).

2.2 Real-time RT-PCR

Oligonucleotides:

Primers/probe	Sequence (5'-3')
AB- 620 Fw	CAGATGTGTCGTTGGTCAGAT
AB- 621 Rev	CATCACTACGGTGTA ATACTTC
AB- 622 Pr	FAM- CGTAGCTTTGTCAAGGCATACCCAAA- BHQ1

Preliminary studies found that weak cross-reactions with some non-target tobamoviruses may occur, so a Ct cut-off value was established (see below).

A. Real-time RT-PCR with TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit (Termo Fisher Scientific)

Master Mix:

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	-	6.3	-
AB- 620 Fw	10 µM	0.4	0.2 µM
AB- 621 Rev	10 µM	0.4	0.2 µM
AB- 622 Pr	10 µM	0.4	0.2 µM
TaqMan^{R} RT-PCR Mix	2x	10	1x
TaqMan^{R} RT Enzyme Mix	40x	0.5	1x
RNA	-	2	-
Total volume	-	20	-

Real-time RT-PCR cycling conditions:

Step	Temp (°C)	Time	No. of cycles
Reverse transcrition	48	15 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	40
Annealing	60	1 min	

Ct cut-off value determined with these reagents and cycling conditions: 35 (with equipment at CREA), and 34 (with equipment at NIB).

B. Real-time RT-PCR with AgPath-ID^{TM} One-Step RT-PCR Reagents (Termo Fisher Scientific)

Master Mix:

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	-	2	-
AB- 620 Fw	10 µM	0.2	0.2 µM
AB- 621 Rev	10 µM	0.2	0.2 µM
AB- 622 Pr	10 µM	0.2	0.2 µM
RT-PCR buffer (AgPath)	2x	5	1x
RT-PCR enzyme (AgPath)	25x	0.4	1x
RNA	-	2	-
Total volume	-	10	-

Real-time RT-PCR cycling conditions:

Step	Temp (°C)	Time	No. of cycles
Reverse transcrition	48	10 min	1
Initial denaturation	95	10 min	1
Denaturation	95	15 sec	45
Annealing	60	1 min	

Ct cut-off value determined with these reagents and cycling conditions: 35 (with equipment at CREA), and 33 (with equipment at NIB).

C. Real-time RT-PCR with KAPA PROBE FAST Universal One-Step qRT-PCR Kit (KAPA Biosystems)

Master Mix:

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	-	6.0	-
AB- 620 Fw	10 µM	0.4	0.2 µM
AB- 621 Rev	10 µM	0.4	0.2 µM
AB- 622 Pr	10 µM	0.4	0.2 µM
KAPA Probe Fast qPCR mix	2x	10	1x
ROX high reference dye	50x	0.4	1x
KAPA RT Mix	50x	0.4	1x
RNA	-	2	-
Total volume	-	20	-

Real-time RT-PCR cycling conditions:

Step	Temp (°C)	Time	No. of cycles
Reverse transcrition	42	5 min	1
Initial denaturation	95	3 min	1
Denaturation	95	3 sec	40
Annealing	60	30 sec	

Ct cut-off value determined with these reagents and cycling conditions: 35 (with equipment at CREA).

3 Validation procedure

Validation was performed according EPPO PM7/98 (5).

3.1 Analytical specificity

Inclusivity was evaluated with the following ToBRFV isolates:

- PV-1236 (DSMZ), tomato, leaf
- PV-1241 (DSMZ), tomato, leaf
- ToB-SIC 21/19 (CREA-DC), tomato, leaf
- ToB-SIC 22/19 (CREA-DC), tomato, leaf

- ToB-SIC 23/19 (CREA-DC), tomato, leaf
- ToB-SIC 24/19 (CREA-DC), tomato, leaf
- ToB-SIC 25/19 (CREA-DC), tomato, leaf

RNA from these ToBRFV isolates was extracted as described in section 2.1 A1, and real-time RT-PCR was performed with TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit.

Exclusivity was evaluated with the following non-target viruses:

- bell pepper mottle virus (PV-0170)
- cucumber green mottle mosaic virus (NIB V 271, NIB V 320)
- obuda pepper virus (PV-1176)
- odontoglossum ringspot virus (PV-1048)
- paprika mild mottle virus (PV-0606)
- ribgrass mosaic virus (PV-0145)
- streptocarpus flower break virus (PV-1058)
- sunn-hemp mosaic virus (PV-0156)
- tobacco mild green mosaic virus (PV-0124)
- tobacco mosaic virus (NIB V 037)
- tobacco mosaic virus (PV-0137, PV-0943)
- tomato mosaic virus (NIB V 036, NIB V 049, NIB V 072, NIB V 104)
- tomato mottle mosaic virus (PV-1267)
- youcai mosaic virus (PV-0527)

RNA from these non-target viruses was extracted as described in section 2.1 A2, and real-time RT-PCR was performed using the TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit and the AgPath-ID^{TM} One-Step RT-PCR reagents. Each isolate was analysed with each protocol in two replicates.

Note: isolates marked with "NIB" are from NIB collection, isolates marked with "ToB-SIC" are from CREA collection and isolates marked with "PV" are from DSMZ collection.

3.2 Analytical sensitivity

To determine the analytical sensitivity, a serial dilution was prepared from sap of infected tomato leaves diluted in sap from healthy leaves and tested at CREA by using the TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit, AgPath-ID^{TM} One-Step RT-PCR, and the KAPA PROBE FAST

Universal One-Step qRT-PCR Kit. RNA from the dilutions were extracted as described in section 2.1 A1.

3.3 Diagnostic sensitivity and diagnostic specificity

These two performance characteristics of the test were determined by CREA and NIB on seed samples of the Euphresco TPS (Giesbers et al., 2021). The panel of samples consisted of 37 blind samples and 3 controls (1000 seeds per sample) including:

- 6 non-target tomato seed samples
- 5 non-target pepper seed samples
- 4 tomato seed samples infected with a high concentration of ToBRFV
- 20 tomato seed samples infected with a medium concentration of ToBRFV
- 5 pepper seed samples infected with a medium concentration of ToBRFV

Procedure used at CREA: RNA extraction as described in section 2.1 B1, real-time RT-PCR using the TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit, and KAPA PROBE FAST Universal One-Step qRT-PCR Kit.

Procedure used at NIB: RNA extraction as described in 2.1 B2, real-time RT-PCR with TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit, and real-time RT-PCR with AgPath-ID^{TM} One-Step RT-PCR reagents.

The following calculations were used to evaluate the diagnostic sensitivity and diagnostic specificity:

- Diagnostic sensitivity = TP/ (TP + FN)
- Diagnostic specificity = TN/ (TN + FP)
- TP = true positive
- FN = false negative
- TN = true negative
- FP = false positive

Undetermined results were excluded from the calculation of these performance criteria.

3.4 Selectivity

Not evaluated.

3.5 Repeatability

Not evaluated.

3.6 Reproducibility

Two dilutions (10^{-5} and 10^{-6}) of the ToBRFV-positive RNA sample of isolate NIB V 329 were tested at NIB in five runs (on five different days). These were performed by real time RT-PCR using AgPath-IDTM One-Step RT-PCR reagents.

3.7 Robustness

Robustness was evaluated by analysis of the results of nine laboratories. These laboratories analysed the same set of seed samples as used for the proficiency test of the EURL-Virology, i.e., PT_2022_01_ToBRFV (Tiberini et al., 2022a and b). These sample sets were prepared at CREA, and included:

- 3 samples of healthy tomato seeds cv. RioGrande (1000 seeds per sample)
- 1 sample of healthy pepper seeds (500 seeds)
- 1 sample of healthy tomato seed spiked with freeze-dried plant material infected with tomato mosaic virus (ToMV) (1000 seeds)
- 1 sample of 3 ToBRFV-infected seeds in 997 healthy tomato seeds
- 1 sample of 5 ToBRFV-infected seeds in 995 healthy tomato seeds

RNA extraction methods used:

- RNeasy Plant Mini Kit (Qiagen) (7 laboratories)
- CTAB protocol with LiCl precipitation (1 laboratory)
- Maxwell RNA kit (Promega) for automatic extraction (1 laboratory)

Reagents for real-time RT-PCR used:

- Luna Universal Probe (NEB) (2 laboratories)
- TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit (Termo Fisher Scientific) (3 laboratories)
- AgPath-ID^{TM} One-Step RT-PCR Reagents (Termo Fisher Scientific) (2 laboratories)
- qScript XLT (Quantabio) (1 laboratory)
- KAPA PROBE FAST Universal One-Step qRT-PCR Kit (KAPA Biosystems) (1 laboratory)

Instruments used:

- Applied StepOne (1 laboratory)

- Applied QuantaStudio 5 (4 laboratories)
- Applie QuantaStudio7 (1 laboratory)
- Roche LightCycler (1 laboratory)
- Analytical Jena qTower (2 laboratories)

4 Results

4.1 Analytical specificity

The inclusivity assessed with seven ToBRFV isolates was 100%. The range of Ct values obtained at CREA for the ToBRFV isolates tested was between 6 and 22 (Table 1).

Table 1: Results of testing ToBRFV isolates

Target virus (isolate)	**Real-time RT-PCR with TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit (Ct)**	**Real-time RT-PCR with KAPA PROBE FAST Universal One-Step qRT-PCR Kit (Ct)**
PV-1236	6	11
PV-1241	11	14
ToB-SIC 21/19	17	21
ToB-SIC 22/19	19	22
ToB-SIC 23/19	12	13
ToB-SIC 24/19	14	15
ToB-SIC 25/19	8	14

Testing of 19 non-target viruses at NIB resulted in 100% exclusivity when a Ct cut-off was used (Ct cut-off 34 for the TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit; and Ct cut-off 33 for the AgPath-ID^{TM} One-Step RT-PCR reagents). No other relevant differences were observed between the two protocols) (Table 2).

Table 2: Results of testing non-target viruses with two protocols of real-time RT-PCR (parallel testing)

Non-target virus (isolate)	**Real-time RT-PCR with TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit (Ct)**	**Real-time RT-PCR with AgPath-ID^{TM} One-Step RT-PCR Reagents (Ct)**
bell pepper mottle virus (PV-0170)	undet, undet	undet, undet
cucumber green mottle mosaic virus (NIB V 271)	undet, undet	undet, undet
cucumber green mottle mosaic virus (NIB V 320)	undet, undet	undet, undet
obuda pepper virus (PV-1176)	undet, undet	undet, undet
odontoglossum ringspot virus (PV-1048)	undet, undet	undet, undet
paprika mild mottle virus (PV-0606)	36, 36	35, 34
ribgrass mosaic virus (PV-0145)	35, 36	35, 35
streptocarpus flower break virus (PV-1058)	undet, undet	undet, undet
sunn-hemp mosaic virus (PV-0156)	undet, undet	undet, undet
tobacco mild green mosaic virus (PV-0124)	undet, undet	undet, undet

tobacco mosaic virus (NIB V 037)	undet, undet	undet, undet
tobacco mosaic virus (PV-0137)	undet, undet	undet, 38
tobacco mosaic virus (PV-0943)	undet, undet	undet, undet
tomato mosaic virus (NIB V 036)	undet, undet	undet, undet
tomato mosaic virus (NIB V 049)	undet, undet	undet, undet
tomato mosaic virus (NIB V 072)	undet, undet	undet, undet
tomato mosaic virus (NIB V 104)	undet, undet	undet, undet
tomato mottle mosaic virus (PV-1267)	undet, undet	38, 38
youcai mosaic virus (PV-0527)	undet, undet	undet, undet

undet-undetermined (no signal)

4.2 Analytical sensitivity

The analytical sensitivity was 10^{-6}, based on the dilution series prepared from sap of infected tomato leaves diluted in sap from healthy leaves (Table 3).

Table 3: Results of testing the ToBRFV dilutions (parallel testing)

Target virus (dilution level)	**Real-time RT-PCR with TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit (Ct)**	**Real-time RT-PCR with AgPath-IDTM One-Step RT-PCR Reagents (Ct)**	**Real-time RT-PCR with KAPA PROBE FAST Universal One-Step qRT-PCR Kit (Ct)**
10^{-1}	19, 19, 19	not tested	not tested
10^{-2}	22, 22, 22	not tested	not tested
10^{-3}	25, 25, 25	not tested	not tested
10^{-4}	28, 28, 28	not tested	not tested
10^{-5}	31, 31, 31	29, 29, 29	32, 32, 32
10^{-6}	35, 35, 35	32, 32, 32	34, 34, 34
10^{-7}	undet*, undet, undet	34, 34, undet	37, undet, undet
10^{-8}	undet, undet, undet	35, 35, undet	undet, undet, undet

**undet-undetermined (no signal)*

4.3 Diagnostic sensitivity and diagnostic specificity

A summary of the results of testing seed samples from the Euphresco TPS (Giesbers et al., 2021) at CREA and NIB is given in Tables 4, 5, and 6. For details, see Giesbers et al. (2021).

Table 4: Overview of the results for tomato seeds

		Assigned value		
		Positive	Negative	Total
Result of test	Positive	TP = 45	FP = 0	TP+FP = 45
	Negative	FN = 2	TN = 12	FN+TN = 14
	Total	TP+FN = 47	FP+TN = 12	59*

*1 undetermined result is not included in the calculation

Table 5: Overview of the results for pepper seeds

		Assigned value		
		Positive	Negative	Total
Result of test	Positive	TP = 8	FP = 0	TP+FP = 8
	Negative	FN = 1	TN = 10	FN+TN = 11
	Total	TP+FN = 9	FP+TN = 10	19*

*1 undetermined result is not included in the calculation

Table 6: Calculated diagnostic sensitivity and diagnostic specificity

	Tomato seeds	Pepper seeds
Diagnostic sensitivity	95.7%	88.9%
Diagnostic specificity	100%	100%

**Note: At NIB, samples were analysed by two real-time RT-PCR protocols using different reagents, i.e. TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit, and AgPath-ID^{TM} One-Step RT-PCR. No relevant differences were found between the two protocols, so only the results of the real-time RT-PCR with TaqMan^{R} RNA-to-Ct^{TM} 1-Step Kit are included in the above analysis.*

4.4 Selectivity

No data.

4.5 Repeatability

No data.

4.6 Reproducibility

Two ToBRFV dilutions analysed in 5 runs of real-time RT-PCR at NIB from 12.1.2021 – 29.4.2021 showed that the reproducibility of the test was 100% (Table 7). The sample with a high amount of target RNA gave a mean Ct value of 25.62 with a standard deviation (STD) of 0.36. The mean Ct value for the sample with a low amount of target RNA was 29.41 with a standard deviation of 0.41.

Table 7: Ct values of multiple testing of RNA samples by real-time RT-PCR using AgPath-ID^{TM} One-Step RT-PCR reagents

Date of analysis	Dilution 10^{-5} of isolate NIB V 329	Dilution 10^{-6} of isolate NIB V 329
12.1.2021	25.94	29.58
14.1.2021	25.89	29.96
19.2.2021	25.04	29.51
19.4.2021	25.70	29.01
29.4.2021	25.55	29.01

4.7 Robustness

All nine laboratories that analysed the TPS sample set reported 100% accuracy. There were no discrepancies between the reports and the values assigned to the samples, despite the fact that the laboratories used different extraction methods and different reagents for the real-time RT-PCR than those evaluated at CREA and NIB. For details see Tiberini et al. (2022b).

5 Conclusions

Criteria		Results
Analytical sensitivity	Tested concentrations	Dilutions of ToBRFV infected tomato leaves in sap from healthy leaves
	LOD	10^{-6}
Analytical specificity	Number of tested samples	Number of targets tested: 7 (ToBRFV isolates) Number of non-targets tested: 19 (isolates of other tobamoviruses)
	Percentage of accurate results	100%
	Percentage of false positives	0%
	Percentage of false negatives	0%
Diagnostic sensitivity and specificity	Number of tested samples	Number of targets tested: 29 (seed samples) Number of non-targets tested: 11 (seed samples) Number of laboratories included in the evaluation of these performance characteristics: 2
	Diagnostic sensitivity	Tomato seeds: 95.7% Pepper seeds: 88.9%
	Diagnostic specificity	Tomato seeds: 100% Pepper seeds: 100%
Selectivity	Effect of sample on selectivity	Not evaluated
Repeatability	Number of parallels, percentage of identical results	Not evaluated
Reproducibility	Number of parallels, percentage of identical results	Percentage of identical results is 100% No. of target samples tested: 2 (RNA samples) No. of different days: 5
Robustness	Number of parallels, percentage of correct results	Percentage of correct results is 100% Number of targets tested: 2 (seed samples) Number of non-targets tested: 5 (seed samples) Number of laboratories included in the evaluation of these performance characteristics: 9 Number of different RNA extraction: 3 Number of different reagents for real-time RT-PCR: 5 Number of different instruments: 5

6 References

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