REFERENCE PROTOCOL NAKTUINBOUW

Real-time RT-PCR (RT TaqMan PCR) for pospiviroids (CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd) on seeds of tomato (*Solanum lycopersicum)*

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Version:	2.6
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Validation:	Validated

1. Objective

To detect the absence or presence of potentially relevant pospiviroidae (CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd) in tomato seeds by isolation of RNA followed by RT TaqMan PCR.

2. Principle

RNA from seed extract of tomato is isolated and purified with a kit using KingFisher. The possible presence of viroid RNA is demonstrated by means of RT TaqMan PCR using selective sets of primers and labeled TaqMan probes. Each subsample is spiked with DLVd to monitor the performance of RNA extraction and RT TaqMan PCR.

3. Abbreviations

cDNA	Complementary DNA
CEVd	Citrus exocortis viroid
CLVd	Columnea latent viroid
Ct-value	“Cycle threshold” value (number of PCR cycli till reaching the threshold)
DLVd	Dahlia latent viroid (spike viroid)
DTT	Dithiothreitol
GH+ buffer	Guanidine–hydrochloride extraction buffer
KF	King Fisher
NAC	Negative amplification control (No template control)
NIC	Negative isolation control (tomato seeds process control)
PAC	Positive amplification control
PCFVd	Pepper chat fruit viroid
PIC	Positive isolation control (PSTVd contaminated tomato seed process control)
PSTVd	Potato spindle tuber viroid
RT TaqMan	Reverse transcriptase TaqMan
TASVd	Tomato apical stunt viroid
TCDVd	Tomato chlorotic dwarf viroid
TPMVd	Tomato planta macho viroid

4. Materials

Equipment

Biorad CFX 96 PCR apparatus
Interscience BagMixer 100
KingFisher Flex 96 (ThermoFisher Scientific)
Thermoshaker

Reagentia/buffers

DLVd stock (SPIKE)
DTT Sigma Aldrich
GH+ buffer (see appendix)
Primers and probes (see appendix)
Positive RNA controls
PC/NC seed (PIC/NIC)
Sbeadex Maxi Plant Kit, 960 samples (LCG genomics, Cat. No. 41620)
UltraPlex™ 1-Step ToughMix® (4x) (Quanta Biosciences)
RNase-free water

Consumables

Grinding bags 100 ml (Interscience BagPage)
Hard-Shell® Low-Profile, Thin-Wall, Skirted 96-Well PCR Plates, White Well from Biorad (or comparable)
KingFisher 96 tip comb for DW magnets, (ThermoFisher Scientific, Cat. No. 97002534)
KingFisher 96 KF plate, 200µL (ThermoFisher Scientific, Cat. No. 97002540)
KingFisher deepwell 96 plate, (ThermoFisher Scientific, Cat. No. 95040450)

5. Method

5.1 Safety and warnings

Viroids can be present in very high concentrations in plant tissue and cross-contamination is a possibility. Accuracy of operations is important to reduce the chance of cross-contamination. Wear gloves and use pipets with filter tips at all times.

5.2 Execution

5.2.1 Preparation of tomato seed samples

1. Weigh from each sample (3,000) 3 subsamples of 1,000 or appropriate number of subsamples of 400 seeds (conform request customer) and transfer each subsample to a grinding bag (Interscience BagPage 100ml).
2. Calculate the required amount of GH+ extraction buffer based on the total number of subsamples. Add the DLVd spike into the GH+ extraction buffer to monitor inhibition.
3. Include as PIC a PSTVd-contaminated subsample (preferably a PC tomato seed with a relatively high Ct value)) per serie. Also include NIC (NC tomato seed with a Ct > 35) per sample series.
4. Add to each bag with 1,000 or 400 seeds respectively 20 or 12 ml GH+ extraction buffer including the DLVd spike.
5. Soak the seeds for 30-60 minutes at room temperature.
6. Extract the subsamples for 90 seconds using the Interscience BagMixer (position 4).
7. Transfer 1.0 ml of seed extract per subsample gently into 1.5 ml tube.
8. Add immediately 30 µl DTT (5 M stock) to 1 ml of seed extract per subsample and vortex.
9. Preheat thermoshaker (setting: 65°C, 850 rpm).
10. Incubate the subsamples in thermoshaker for 15 minutes at 65 °C and 850 rpm.
11. Centrifuge the tubes for 10 minutes at 16,000 g at 4 ºC.
12. Continue further the RNA isolation.

5.2.2 RNA isolation

1. Use the "Sbeadex Maxi Plant Kit" for RNA isolation.
2. Code and fill in the plates as indicated in Table 1.
3. Transfer 250 µl of the supernatant (avoid pellet!) from the subsamples, the NIC seed and PIC seed into the binding plate.
4. Store the remainder of the extract and the extract controls at -20 °C until the test is completed.
5. Put all the plates in the correct position in the KF unit.
6. Select and start the KF7 program on the KingFisher Flex 96 (Table 2).
7. Cover the elution plate with foil sticker and store the elution plate on ice.
8. Continue directly with PCR or store purified RNA at -20 °C.

Table 1. Coding and composition of KF plates

Type of plate	Name	Composition		
KF deep well 96	Binding	600	µl	binding buffer PN (green)
		50	µl	Sbeadex particle suspension (white)
KF deep well 96	Wash 1	600	µl	Wash buffer PN1 (red)
KF deep well 96	Wash 2	600	µl	Wash buffer PN1 (red)
KF deep well 96	Wash 3	600	µl	Wash buffer PN2 (yellow)
KF 96 plate	Elution	100	µl	Elution buffer PN (black)

Table 2. King Fisher Flex 96 program KF7

Step	Plate	Steps per plate
Tip 1	Tipholder	1. Remove tip comb from this plate (96 DW tip comb) 2. Release tip comb in plate 2 “Wash 1”
Binding	Plate 1 “Cell lysate”	1. Fast mixing, 10 min. 2. Collect beads, 3 x 10 sec.
Wash 1	Plate 2 “Wash 1”	1. Release beads, bottom mix, 20 sec. 2. Fast mixing, 10 min. 3. Collect beads, 3 x 10 sec.
Wash 2	Plate 3 “Wash 2”	1. Release beads, bottom mix, 20 sec. 2. Fast mixing, 10 min. 3. Collect beads, 3 x 10 sec.
Wash 3	Plate 4 “Wash 3”	1. Release beads, bottom mix, 20 sec. 2. Fast mixing, 10 min. 3. Collect beads, 3 x 10 sec.
Elution	Plate 5 “Elution”	1. Release beads, bottom mix, 1 min. 2. Preheat and fast mixing at 65ºC, 10 min. 3. Collect beads, 5 x 10 sec.

5.2.3 RT TaqMan PCR

NB. *Work on ice as much as possible and prevent prolonged exposure of probes to light. Wear clean lab coat and gloves to minimize the risk of cross-contamination.*

1. Prepare RT TaqMan PCR mix (Table 3a, 3b, 3c and 3d).
2. Calculate the required amount of reaction mix.
3. Transfer 19 µl PCR mix into PCR plates with white bottom/green border.
4. Pipette 6 µl of the purified RNA sample in 19 µl mix.
5. In each run, include a NAC (no-template control) and PAC (positive RNA control).
6. Cover the plate after adding the RNA.
7. Spin the plate for 30 seconds.
8. Run the RT TaqMan PCR’s according to the following program (Table 4).

Tabel 3a. PCR mix PSTVd, TCDVd, TPMVd (FAM), PCFVd (VIC) and DLVd spike (Texas Red)

PCR mix	1 reaction	
RNase-free water	11,75	µl
UltraPlex™ 1-Step ToughMix® (4x) (Quanta Biosciences)	6,25	µl
Pospi A primer/probemix (#89)	1,0	µl
Subtotal	19,0	µl
Sample (RNA)	6,0	µl
Total	25,0	µl

Tabel 3b. PCR mix CEVd, CLVd (FAM) and DLVd (spike)(Texas Red)

PCR mix	1 reaction	
RNase-free water	11,75	µl
UltraPlex™ 1-Step ToughMix® (4x) (Quanta Biosciences)	6,25	µl
Pospi B primer/probemix (#90)	1,0	µl
Subtotal	19,0	µl
Sample (RNA)	6,0	µl
Total	25,0	µl

Tabel 3c. PCR mix TPMVd (FAM) and Nad5 (Texas Red)

PCR mix	1 reaction	
RNase-free water	11,75	µl
UltraPlex™ 1-Step ToughMix® (4x) (Quanta Biosciences)	6,25	µl
Pospi C primer/probemix (#91)	1,0	µl
Subtotal	19,0	µl
Sample (RNA)	6,0	µl
Total	25,0	µl

Tabel 3d. PCR mix TASVd (FAM)

PCR mix	1 reaction	
RNase-free water	11,75	µl
UltraPlex™ 1-Step ToughMix® (4x) (Quanta Biosciences)	6,25	µl
Pospi D primer/probemix (#92)	1,0	µl
Subtotal	19,0	µl
Sample (RNA)	6,0	µl
Total	25,0	µl

Tabel 4. RT-TaqMan program CFX96 (version 2 or higher) for mix A-D

	temperature	time
hold	50°C	10' 00"
hold	95°C	3' 00"
40 cycli	95°C	0' 10"
	60°C (+ plate read)	1' 00"

6. Evaluation and interpretation

6.1 Evaluation test result

- Turn on FAM, VIC and TR signal for all 96 wells in “view/edit plate”.
- Use single threshold setting (RFU 200).
- Check shape of curve (S-shape) for positive samples. Compare, if necessary, with PIC seed.
- Check atypical planar curves (not-shaped curve) and curves just above the threshold (considered as noise). In case of doubt always check with responsible person.
- Monitor the performance of PCs over time to maintain the reliability of the test.
- See decision matrix (Table 5a, b, c, and d) for interpretation of the results of samples. Based on the signals in different channels, an indication of the identity of a viroid can be obtained.

6.2 Validity of test result

- Result are valid when Ct value of spike DLVd ≤32, unless Ct PSTVd/TCDVd/TPMVd or CEVd/CLVd ≤32. Nad5 Ct >32 is acceptabel only if Ct value of spike DLVd ≤32. (Contact the responsible person).
- Results may only be issued if the positive controls give a clear signal (Ct PIC and PAC).
- Results may only be issued if Ct in the negative control samples > 35.
- Contact the responsible person when the results differ from the expectations.

6.3 Decision matrix (subsample level)

Table 5a. Decision matrix PCR mix 3a: PSTVd, TCDVd, TPMVd (FAM), PCFVd (VIC) and DLVd (spike-TR)

Ct FAM	Ct VIC	Ct TR	
>32	>32	≤32	PSTVd, TCDVd, TPMVd (part of strains) [a] and PCFVd not detected
>32	≤32	≤32	PSTVd, TCDVd and TPMVd not detected PCFVd detected
≤32	>32	≤32	PSTVd/TCDVd and/or TPMVd detected PCFVd not detected
≤32	≤32	≤32	PSTVd/TCDVd and/or TPMVd detected PCFVd detected[b]
>32	>32	>32	Not valid/repeat

Table 5b. Decision matrix PCR mix 3b: CEVd, CLVd (FAM) and DLVd (spike-TR)

Ct FAM	Ct TR	
>32	≤32	CEVd and CLVd not detected
≤32	n.a.	CEVd and/or CLVd detected
>32	>32	Not valid/repeat

Table 5c. Decision matrix PCR mix 3c: TPMVd (FAM) and Nad5 (TR)

Ct FAM	Ct TR	
>32	≤32	TPMVd (part of strains) not detected
≤32	n.a.	TPMVd detected
>32	>32	Not valid/repeat, contact responsible person.

Table 5d. Decision matrix PCR mix 3d: TASVd (FAM)

Ct FAM	
>32	TASVd not detected
≤32	TASVd detected

[a] TPMVd not detected when mix A and mix C are negative.
[b] Signal might leak from VIC to FAM channel when PCFVd load is high. Use singleplex Boonham to determine whether PSTVd/TCDVd and /or TPMVd are present.

7. Literature and instructions

Literature:

- Bakker, D., Bruinsma, M., Dekter, R.W., Toonen, M.A.J., Verhoeven, J. Th. J. and Koenraadt, H.M.S. 2015. Detection of PSTVd and TCDVd in seeds of tomato using real-time RT-PCR. EPPO Bulletin. Volume 45, Issue 1, pages 14–21,
- Boonham, N., L. González-Pérez, ,M.S. Mendez, E. Lilia Peralta, A. Blockley, K. Walsh, I. Barker & R.A. Mumford (2004). Development of a real-time RT-PCR assay for the detection of Potato spindle tuber viroid. Journal of Virological Methods 116:139-146.
- Botermans, M., van de Vossenberg, B. T. L. H., Verhoeven, J. Th. J.,Roenhorst, J. W., Hooftman, M., Dekter, R. & Meekes, E. T. M. (2013) Development and validation of a real-time RT-PCR assay for generic detection of pospiviroids. J. Virol. Methods 187, 43–50.
- Koenraadt, H., Jodlowska, A., van Vliet, A. & Verhoeven, K. (2009) Detection of TCDVd and PSTVd in seeds of tomato. Phytopathology 99, S66.
- Harrie Koenraadt, Agata Jodlowska, Maaike Bruinsma, Michel Ebskamp, Ko Verhoeven and Annelien Roenhorst. (2017) Biological relevance of the detection of pospiviroids on pepper and tomato seeds (poster at APS, San Antonio, Tx)
- Menzel, W., Jelkmann, W., and Maiss, E. (2002) Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. J. Virol. Methods 99: 81-92.
- Monger, W., Tomlinson, J., Boonham, N., Marn, M.V., Plesko, I.M., Molinero-Demilly,V., Tassus, X., Meekes, E., Toonen, M., Papayiannis, L., Perez-Egusquiza, Z., Mehle, N., Jansen, C., Nielsen, S.L., (2010) Development and inter-laboratory evaluation of real-time PCR assays for the detection of pospiviroids. J. Virol. Methods 169, 207–210.
- Mumford, R.A., A.L. Skelton, N. Boonham, K.I. Posthuma, M.J. Kirby, & A.N. Adams (2001) The Improved Detection of Strawberry Crinkle Virus Using Real-Time RT-PCR (TaqMan®). Acta Horticulturae 656: 81-86.

Manuals:

- Sbeadex maxi plant Kit (LCG genomics)
- UltraPlex™ 1-Step ToughMix® (4x)

8. History and revisions

- 20-12-2013 Version 1.0 - ~~Protocol based on ISO17025 accredited PSTVd /TCDVd seed assay (SPN-V003, version 3.1) for tomato~~. Overnight soaking of seeds was reduced to 30-60 minutes to limit break down of viroid. Introduction of DLVd spike to replace endogenous Nad5 target since amount of Nad5 RNA is highly variable (14-40) in the seed matrix. Use of GH+ extraction buffer instead of PN1 extraction buffer (LGC) since degradation in GH+ RNA extraction buffer is much less than in PN1 extraction buffer leading to a higher PSTVd sensitivity. Specification of method to purify RNA for NVWA. RNeasy purified RNA in general give better sequences in comparison with Sbeadex purified RNA although high Ct values samples are still problematic due to low sensitivity of sequencing method. Use of several new multiplex RT TaqMans to detect additional Pospiviroidae as CEVd, CLVD, PCFVd, TASVd and TPMVd.
- 03-02-2014 Version 1.1 - Correction numbering primer sets in appendices. 201d instead 201c, at 349 / mc instead of 249 at / c and 350a, 350b instead 250a, 250b.
- 21-04-2015 version 1.2 - 5.2.1: Specification of spike: “10 µl DLVd/100 ml GH+ buffer”.
- 15-02-2017 version 2.0 - Addition of DTT to seed extract, adaptations in Kingfisher program, replacement PCR mix, adaptation PCR program, MPVd replaced by TPMVd due to new taxonomic insights. Several textual changes. DLVd spike specification removed.
- 30-10-2017 v2.1 – Correction literature references in primer table in appendix.
- 05-03-2019 v2.2 – Several textual changes; introduction 1µl PCR mixes.
- 02-01-2020 v2.3 - In description of objective "possible" was removed due to change in Quarantine status.
- 15-05-2020 v2.4 - Table 5a: PSTVd/TCDVdb and/or TPMVd: “suspected” replaced with “detected”, “b PSTVd suspected extracts are send to the Dutch NPPO for sequence analysis” removed. Correction 5.2.1-3 limit NIC >35. 6.2 “Contact the responsible person when a seed sample is suspect” removed.
- 10-11-2020 v2.5 – Correction in History and Revision version 1.0; first sentence crossed out.
- 20-04-2021 v2.6 – Several textual changes.

9. Appendices

GH+ extraction buffer (6M)	Adjust to 1 liter demiwater	
guanidine-hydrochloride (harmful)	573	g
NaAC-buffer (4M)	50	ml
EDTA (di-natrium)	9.3	g
PVP-10	25	g

NaAc buffer (4M)	Adjust to 1 liter with demiwater	
NaAC (CH_3COONa)	328 g	g
Check/adjust pH to 5.2		

Primer	**No. Naktuinbouw primer collection**	**Sequence**	**Lit. ref.**	**End concentration (nM)**
PSTV-231F1	201a	GCCCCCTTTGCGCTGT	1	300
PSTV-296R	201b	AAGCGGTTCTCGGGAGCTT	1	300
PSTV-251T-	201d	6FAM-CAGTTGTTTCCACCGGGTAGTAGCCGA-BHQ1	1	200
DaVd1-FT	320a	GCTCCGCTCCTTGTAGCTTT	2	300
DaVd1-RT	320b	AGGAGGTGGAGACCTCTTGG	2	300
DaVd1-P	320c	Texas red-CTGACTCGAGGACGCGACCG-BHQ2	2	200
TPMVd-F1	350a	AAAAAAGAATTGCGGCCAAA	3	300
TPMVd-R	350b	GCGACTCCTTCGCCAGTTC	3	300
pUCCR2	307i	6FAM-CCGGGGAAACCTGGA-NFQ-MGB	4	200
CLVd-F	279a	GGTTCACACCTGACCCTGCAG	5	300
CLVd-F2	279b	AAACTCGTGGTTCCTGTGGTT	5	300
CLVd-R	279c	CGCTCGGTCTGAGTTGCC	5	300
CLVd-P	279d	6FAM-AGCGGTCTCAGGAGCCCCGG-BHQ1	5	200
CEVd-F2-	280a	CTCCACATCCGRTCGTCGCTGA	5	300
CEVd-R2-	280b	TGGGGTTGAAGCTTCAGTTGT	5	300
CEVd-P2-	280c	6FAM-CCCTCGCCCGGAGCTTCTCTCTG-BHQ1	5	200
TASVd-F2-	281a	CKGGTTTCCWTCCTCTCGC	5	300
TASVd-R2-	281b	CGGGTAGTCTCCAGAGAGAAG	5	300
TASVd-P2-	281c	6FAM-TCTTCGGCCCTCGCCCGR-BHQ	5	200
PCFVd-F	349a	TCTTCTAAGGGTGCCTGTGG	6	300
PCFVd-R	349b	GCTTGCTTCCCCTTTCTTTT	6	300
PCFVd-	349c	VIC-CTCCCCCGAAGCCCGCTTAG-BHQ1	6	200
nad5 F	151a	GATGCTTCTTGGGGCTTCTTGTT	7	300
nad5 R	151b	CTCCAGTCACCAACATTGGCATAA	7	300
nad5-Probe	151d	Texas red-AGGATCCGCATAGCCCTCGATTTATGTG-BHQ2	4	200

1: Boonham et al. (2004), 2: Naktuinbouw, 3: Naktuinbouw, 4: Botermans et al. (2013), 5: Monger et al. (2010), 6: Naktuinbouw, 7: Menzel et al (2002) and Monger et al. (2010).

Composition primer and probe mixes A, B, C and D		
Mix 89	Pospi primer/probe mix A	201a, 201b, 349a, 349b, 320a, 320b, 201d, 349c, 320c
Mix 90	Pospi primer/probe mix B	280a, 280b, 279a, 279b, 279c, 320a, 320b 280c, 279d, 320c
Mix 91	Pospi primer/probe mix C	350a, 350b, 151a, 151b, 307i, 151d
Mix 92	Pospi primer/probe mix D	281a, 281b, 281c