PROJECT FULL TITLE:
TESTA—SEED HEALTH: DEVELOPMENT OF SEED TREATMENT METHODS, EVIDENCE FOR SEED TRANSMISSION AND ASSESSMENT OF SEED HEALTH
GRANT AGREEMENT NO.: *(311875)*

Workpackage:	5. Validation of detection methods
Deliverable:	D5.3 Validated methods for viruses and viroids
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With the increasing urge for quality, accredited and validated methods become more important. For newly developed assays it is necessary to objectively determine whether the new assay meets the requirements dictated in advance. Within the TESTA project, the methods were validated according to the EPPO guideline PM 7/98 (2) (EPPO, 2014), which describes guidelines for laboratories preparing for accreditation according to the ISO/IEC standard 17025. Whether the test is fit for its intended use was determined using several performance characteristics. For each test analytical sensitivity, analytical specificity, repeatability, reproducibility and if appropriate selectivity and trueness was determined. For deliverable 5.3, the assay for the detection of pospiviroids in seeds of tomato was validated according to this guideline.

Aim
Validated method for (a) RT Taqman for detection of pospiviroids in tomato seeds

Contents

5.3.a.

Task 5.1 Validation of a real-time RT-PCR (Reverse Transcriptase TaqMan PCR) for pospiviroids (CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd) in seeds of tomato (*Solanum lycopersicum)*

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Abstract

Pospiviroids are infectious, circular RNA, with a genome length of around 250-400 nucleotides. Among the genus Pospiviroid are some important plant pathogens of tomato. They can be detected with a molecular assay using six primer sets in four multiplex real-time PCRs. In addition, two Taqman RT-PCRs are used as a process control to monitor inhibition, the viroid DLVd and plant gene Nad5. These are incorporated in the four multiplex real-time PCRs. This validation report contains the results for the validation study of this real-time RT-PCRs for pospiviroids. The performance characteristics that were determined are: trueness, analytical sensitivity, analytical specificity, repeatability and reproducibility.

The assay was compared with the validated PSTVd and TCDVd assay (Bakker et al, 2015) for PSTVd and TCDVd samples and found to be more than ten times more sensitive than the assay by Bakker et al. Analytical sensitivity for this assay was good with detection of dilutions up to 1000x. The analytical specificity was good since no false-negatives were observed for all primer sets and none of the 29 non-target viroids and viruses reacted with the PCRs. However, cross-reactivity between pospiviroids was observed, TASVd isolates cross-reacted with the CEVd/CLVd primer mix. Together this makes the specificity acceptable. Repeatability and reproducibility of the assay were tested with mixed seed samples and were found to be good with a repeatability and reproducibility of 100%.

Using the real-time PCR assay as described in Naktuinbouw protocol SPN-V043 v2.0, CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd can be reliably detected in seeds of tomato. Based on this validation study the assay is suitable for its intended use.

Introduction

Potato spindle tuber viroid (PSTVd) is a quarantine pathogen and PSTVd-contaminated seeds of tomato are potentially a source of primary inoculum. Therefore, a high-throughput detection method (SPN-V003 version 1.0.) for PSTVd, *Tomato chlorotic dwarf viroid* (TCDVd) and *Mexican papita viroid* (MPVd) on tomato seeds based on RT Taqman PCR (Boonham et al. 2004) was previously developed and validated at Naktuinbouw (Bakker et al, 2015). Recently, the detection of several other pospiviroids, *Citrus exocortis viroid* CEVd), *Columnea latent viroid* (CLVd), *Tomato planta macho viroid* (TPMVd), *Pepper chat fruit viroid* (PCFVd), *Tomato apical stunt viroid* (TASVd) in the matrix tomato seeds became more urgent due to new international phytosanitary requirements. Therefore, a new broader pospiviroid assay was needed. From previous experiments it was known that a classic RT-PCR for all pospiviroids (Verhoeven et al., 2004) was not compatible with the high-throughput PSTVd detection method of Naktuinbouw (Koenraadt et al., 2013). Also the generic pospiviroid Taqman (Botermans et al., 2013), widely used for detection of pospiviroids in the matrix leaves, appeared not to be suitable for the matrix seeds (Koenraadt, unpublished). Research was initiated to develop a new pospiviroid assay for seeds of tomato with at least equal sensitivity as the current PSTVd seed assay and also relatively high sensitivity for several other relevant pospiviroids.

MPVd is a species that is recently reclassified as isolates of TPMVd (Verhoeven et al., 2011; Verhoeven and Owens, 2014; ICTV, 2014). Therefore, MPVd is included under TPMVd in this study. TPMVd is a genetically variable species and is detected with two primer mixes in the assay under validation (mix A and mix C).

The assay validated in this study, SPN-V043 v2.0 is generic pospiviroid assay for tomato seeds. The RNA extraction, Taqman PCRs and internal amplification control (IAC) were optimized to be able to reliably detect PSTVd, TCDVd, TPMVd, CLVd, CEVd, TASVd and PCFVd in the matrix tomato seed. The assay is validated according to the EPPO Guideline PM 7/98 (2) (EPPO, 2014). The performance characteristics that were determined are trueness, analytical sensitivity, analytical specificity, repeatability and reproducibility.

Materials

Naturally contaminated seeds were available for PSTVd, TCDVd, PCFVd, TASVd and CLVd (table 1). Not for all viroids naturally homogeneously contaminated seeds (for testing 1 contaminated seed in 999 negative seeds) were available. Therefore, artificially contaminated seeds were used where possible (TASVd and CLVd, provided by Steen Lykke Nielsen, Aarhus University) and if no alternative was available negative seed extract was spiked with viroid (CEVd and TPMVd) extracted from tomato leaves (89002600 and 3289954, respectively).

The pospiviroid assay is based on soaking and stomaching of the seeds followed by a Kingfisher RNA extraction and four (multiplex) Taqman PCRs. The protocol SPN-V043 v2.0 is described in appendix 5. The protocol was followed for all experiments, except that for the validation study more PCR cycles were run, 45 instead of 40, to have better insight in the shapes of the curves in case unexpected results would have been obtained.

After this validation study was started Quanta Biosciences announced that the production of qScript XLT Multiplex One-Step RT-qPCR ToughMix 2x would not be continued. Currently, the performance of alternative mixes such as the qScript XLT 1-Step RT-qPCR ToughMix 500 are under evaluation.

Table 1. Seed lots used for validation study.

Viroid	Seed lot	Remarks
PSTVd	ZZB453	Naturally contaminated seed lot, production year 2010
PSTVd	NAKT1	Seeds derived from naturally infested tomato plant used in seed treatment study, not HCl treated
TCDVd	NAKT2	Seeds from France, seed lot used in experiments in 2008, pectinase treatment, not HCl treated
CLVd	ZZB638	Greenhouse production of artificially infected seeds
TASVd	ZZB639	Greenhouse production of artificially infected seeds
PCFVd	ZZB104	Naturally contaminated seed lot, production year 1994
none	ZZB649	Seed lot negative for all pospiviroids, production year 2012

Trueness

Introduction

'The ability of a method to do what it 'says' (i.e. detection of pospiviroids in the matrix tomato seeds). In other words, the ability to detect the target organism in the matrix assessed with a second method' (Anonymous, 2010). In this study the validated assay for detection of PSTVd and TCDVd on tomato seeds (Naktuinbouw standard protocol SPN-V003 version 1.0) was used for this purpose.

The requirement is that the new method should be able to detect all positive samples that are also detected with the 'old' reference method.

Materials and methods

Three samples contaminated with PSTVd or TCDVd were composed (table 2) and analysed with two protocols, the validated protocol for detection of PSTVd and TCDVd SPN-V003 version 1.0 and the new generic pospiviroid protocol SPN-V0043 version 2.0. Eight replicates of each sample were analysed.

Table 2. Sample composition for trueness

Sample	PSTVd seeds	TCDVd seeds	Negative seed lot ZZB649
1	1 seed NAKT1	-	999
2	1000 seeds of ZZB453	-	-
3	-	1 seed NAKT2	999

Results

All samples with one seed of either PSTVd or TCDVd as well as the PSTVd-contaminated seed lot ZZB453 were detected with both methods (figure 1 and appendix 1). The new method resulted in significantly lower Ct-values (t-test, $p<0.01$) than the old method. The difference was on average 3.7 Ct. The internal controls, Nad5 for SPN-V003 and DLVd for SPN-V043, were good for all samples in both methods (appendix 1).

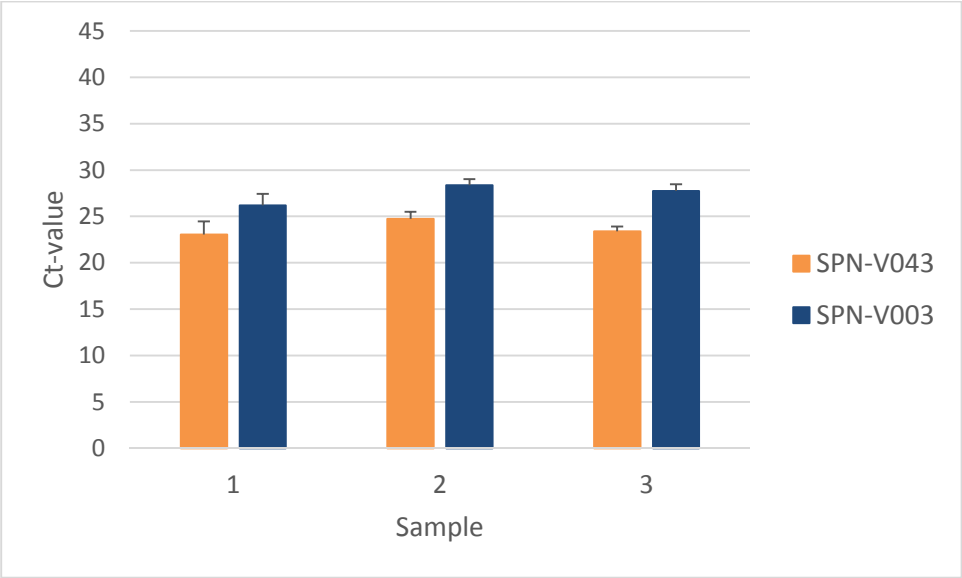

Figure 1. Comparison between the old reference method (SPN-V003 v1.0, blue) and new method (SPN-V043 v2.0, orange) for PSTVd- (sample 1 and 2) and TCDVd- (sample 3) contaminated seed samples. Average + standard deviation (n=8).

Discussion

The new method detected all samples that were detected with the old method. The new method is even more sensitive, the Ct-value was on average 3.7 Ct lower indicating that the new method is over 10 x more sensitive than the old method. The assay meets the requirement set for this performance characteristic.

A different internal control was used for the old reference method and the new method. In this experiment both internal controls showed good results. The internal control in the assay under validation is the Hostuviroid *Dahlia latent viroid* (DLVd). Therefore, this internal control is a better mimic of the target viroids than the old internal control Nad5, a plant gene.

Analytical sensitivity

Introduction

'The lowest value, in a laboratory sample, of the target pathogen or pest, which can still be determined with a certain degree of reliability' (Anonymous, 2010).

For viroids and viruses the absolute amount of pathogen cannot be easily quantified, therefore the requirement for sensitivity is set as a dilution factor. The requirement for each viroid is that it should still be detectable in at least a 100x dilution.

Materials and methods

When seed lots with a homogenous contamination were available, individual seeds were added to a negative background. For the viroids for which this was possible, PSTVd, CLVd, TCDVd and TASVd, two samples were prepared with 100, 10, or 1 contaminated seed in a total subsample of 1000 seeds (table 3 and 4). For the viroid PCFVd for which no individual contaminated seeds were available, seed extract was prepared and diluted (table 5). For two viroids (TPMVd and CEVd) no contaminated seeds were available. For these two viroids, spike was prepared from infected leaf material, this was

spiked in seed extract and diluted further in negative seed extract (table 5). Each dilution of each sample was prepared and tested in triplicate according to the protocol SPN-V043 v2.0.

Table 3. Sample 1: PSTVd and CLVd mixed in negative seed lot ZZB649

Dilution	**PSTVd seeds NAKT1**	**CLVd seeds ZZB638***	**Negative seed lot ZZB649**
10x	100		900
100x	10	10	980
1000x	1	1	998

* Only 100 seeds available in total, therefore 100 seeds (10x dilution) was not tested.

Table 4. Sample 2: PSTVd and CLVd mixed in negative seed lot ZZB649

Dilution	**TCDVd seeds NAKT2**	**TASVd seeds ZZB639***	**Negative seed lot ZZB649**
10x	100		900
100x	10	10	980
1000x	1	1	998

* Only 100 seeds available in total, therefore 100 seeds (10x dilution) was not tested.

Table 5. Sample 3 and 4: PCFVd+TPMVd and CEVd samples

Sample	**Seeds**	**Spike**
3	1000 seeds PCFVd (ZZB104)	+ TPMVd spike
4	1000 seeds negative seed lot ZZB649	+ CEVd spike

Results

For all pospiviroids dilutions up to 1000x were tested and for all pospiviroids in all replicates the expected viroid was detected, except for TPMVd (figure 2 and appendix 2). For TPMVd all 100x dilutions were detected, but 2 out of 3 1000x dilutions had Ct-values above the threshold of 32 (32.33 and 32.40). The internal controls, DLVd (high Ct) and nad5 (low Ct), were constant over all dilutions and the differences between 10 x dilutions were approximately 3 Ct as expected. Some cross-reaction was observed and some samples contained more viroids (appendix 2). However, this did not influence the sensitivity of the assay. Cross-reactivity is discussed under specificity of the assay.

Discussion

For all viroids at least the 100x dilution was detected and therefore the requirement detection of at least the 100x dilution was met. For most pospiviroids the 1000x dilutions were also detected in all replicates. Only for TPMVd not all 1000x dilutions were detected below the threshold of 32. The analytical sensitivity of this assay for CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd is good.

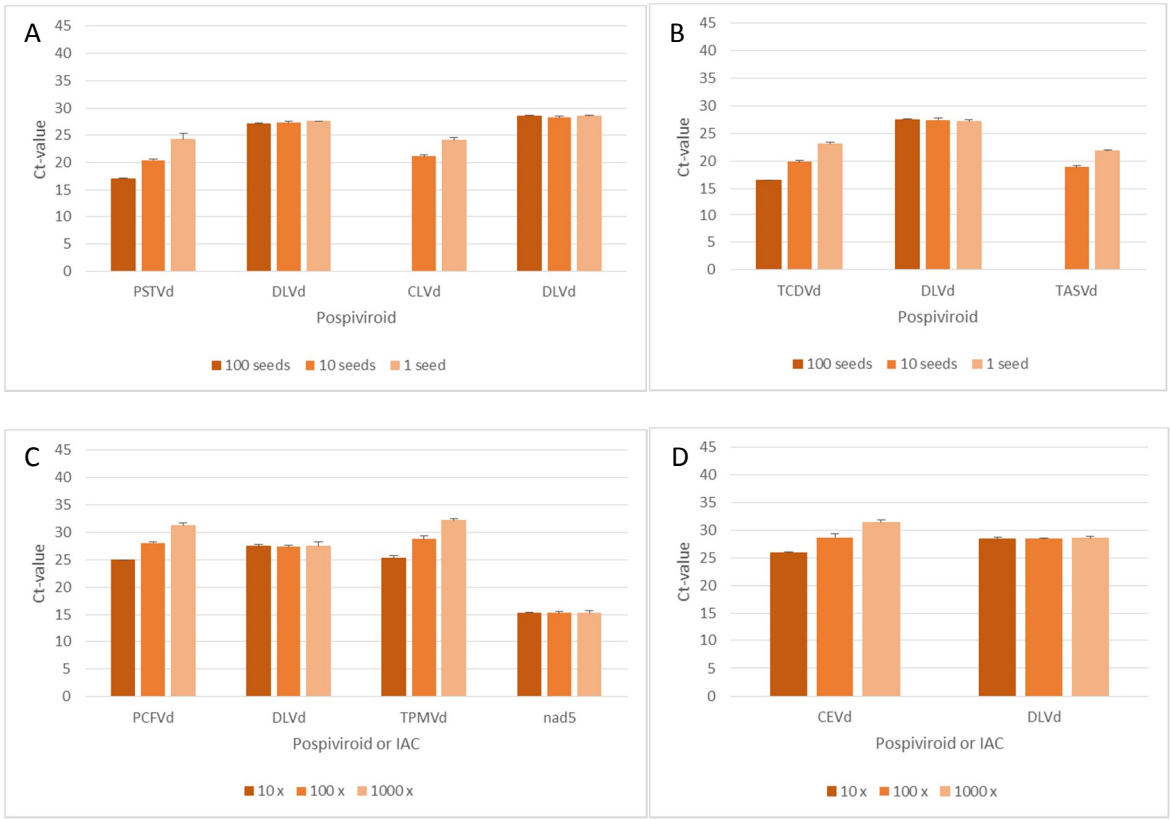

Figure 2. Detection of dilutions of pospiviroids for A) PSTVd and CLVd, individual seeds in a background of negative seeds, B) TCDVd and TASVd, individual seeds in a background of negative seeds, C) PCFVd (1000 seeds) and TPMVd (spike) dilution in negative seed extract, D) CEVd spike diluted in negative seed extract.

Analytical specificity

Introduction

'The ability of a method to distinguish the target organism (pospiviroid) from other organisms, whether related or not, and the extent to which the analysis can distinguish known variants of the organism' (Anonymous, 2010).

The requirement for this assay is that no pospiviroids are missed when all primer sets are used. Cross-reaction between pospiviroids is acceptable. Cross-reaction of unrelated viruses is acceptable up to 10%.

Materials and methods

All seven pospiviroids from the Naktuinbouw collection are tested in three-fold when available (appendix 3, table III). Besides pospiviroids, 14 other viroids and 15 viruses from Solanaceous plants are tested with all four primer mixes (appendix 3, table IV). As positive controls RNA of the composed samples of the repeatability and repeatability samples (for composition see table 8) was used. RNA was isolated using the protocol SPN-V043 v2.0.

Results

All pospiviroids available in the Naktuinbouw collection were detected with the proper primer mix (table 6). For the three TASVd isolates tested the CEVd/CLVd primer mix cross-reacted. None of the other viroids and viruses tested, cross-reacted with any of the primer mixes (table 7).

Discussion

All pospiviroids were detected with the proper primer mix. Cross-reactivity between pospiviroids was 17%, this is acceptable. Cross-reactivity between other viroids and viruses and the pospiviroid primer mixes was 0%. Therefore the analytical specificity requirements for this assay were met.

The cross-reactivity of TASVd with the CEVd/CLVd mix was observed for all three available isolates. In the analytical sensitivity experiment some cross-reactivity to the CEVd/CLVd set was observed for TASVd-contaminated seeds as well, however this was much weaker (Ct difference >9, see appendix 2) than in the specificity experiment (difference in Ct approximately 3-4, table 6).

Table 6. Specificity of pospiviroid primer mixes for the target viroids

		Mix A			Mix B		Mix C		Mix D
Sample	**Viroid**	**PSTVd/TCDVd**	**PCFVd**	**DLVd**	**CEVd/CLVd**	**DLVd**	**TPMVd**	**Nad5**	**TASVd**
1	CEVd	45	45	28.92	19.12	28.46	45	28.08	45
2	CEVd	45	45	28.54	18.60	27.96	45	27.29	45
3	CEVd	45	45	29.64	16.66	28.49	45	25.12	45
4	CLVd	45	45	28.16	17.11	27.35	45	25.93	45
5	CLVd	37.73	45	28.48	17.86	27.65	45	27.38	45
6	CLVd	45	45	28.43	21.65	28.20	45	26.08	45
7	TPMVd	17.95	45	27.79	45	27.75	45	26.77	45
8	PCFVd	38.33	20.43	28.12	35.07	28.64	45	28.46	45
9	PSTVd	19.09	45	29.68	45	29.91	45	29.37	45
10	PSTVd	21.42	45	27.74	36.34	28.09	45	22.04	45
11	PSTVd	17.11	45	28.44	45	28.66	45	26.45	45
12	TASVd	45	45	28.19	21.35	27.92	45	26.32	16.96
13	TASVd	45	45	29.40	21.19	28.81	45	24.44	18.22
14	TASVd	45	45	28.12	20.82	27.95	45	25.50	17.37
15	TCDVd	23.50	45	27.64	45	28.03	45	25.38	45
16	TCDVd	21.14	45	27.98	45	28.48	45	26.36	45
17	TCDVd	18.15	45	28.17	45	28.21	45	27.57	45
18	TPMVd	35.45	45	28.06	45	28.18	19.07	27.07	45
	GH+	45	45	29.53	45	29.15	45	29.15	45
	NTC	45	37.54	45	45	45	45	45	45
	PC RNA	25.18	25.99	29.12	24.10	28.56	24.10	29.41	22.94

Table 7. Specificity of pospiviroid primer mixes for non-target viroids and viruses

		Mix A			Mix B		Mix C		Mix D
Sample	**Species**	**PSTVd/TCDVd**	**PCFVd**	**DLVd**	**CLVd/CEVd**	**DLVd**	**TPMVd**	**Nad5**	**TASVd**
1	PLVd	45	45	30.49	45	30.61	45	21.47	45
2	CChMVd 1	45	45	29.11	45	29.74	45	26.13	45
3	CChMVd 2	45	45	29.02	45	29.76	45	25.36	45
4	CChMVd 3	45	45	29.20	45	29.72	45	25.15	45
5	CSVd 1	45	45	30.09	45	30.06	45	25.39	45
6	CSVd 2	45	45	30.27	45	30.11	45	26.35	45
7	CSVd 3	45	45	29.15	45	30.45	45	26.17	45
8	IrVD 1	45	45	28.10	45	28.79	45	25.00	45
9	IrVD 2	45	45	30.50	45	30.52	45	27.15	45
10	IrVD 3	45	45	30.00	45	30.86	45	24.28	45
11	HSVd 1	45	45	30.59	45	29.57	45	24.66	45
12	HSVd 2	45	45	28.90	45	29.15	45	22.17	45
13	CSVd	45	45	29.94	45	30.43	45	21.55	45
14	CSVd	45	45	29.31	45	29.37	45	21.34	45
15	TSV	45	45	29.21	45	29.46	45	22.31	45
16	TBRV/BRSV	45	45	29.95	45	30.01	45	19.42	45
17	ArMV	45	45	30.20	45	29.61	45	21.22	45
18	PVY	45	45	31.67	45	30.05	45	29.04	45
19	TRSV	45	45	31.00	45	30.21	45	23.42	45
20	ToMV/TMV	45	45	28.87	45	29.04	45	23.06	45
21	TYLCV	45	45	29.25	45	29.42	45	25.66	45
22	TRV	45	45	29.57	45	30.02	45	27.33	45
23	PMMV	45	45	29.15	45	29.29	45	25.30	45
24	PepMV	45	45	27.37	45	29.98	45	27.14	45
25	AMV 1	45	45	31.53	45	32.02	45	28.24	45
26	AMV 2	45	45	30.26	45	30.50	45	24.56	45
27	CMV1	45	45	30.03	45	29.82	45	20.35	45
28	CMV2	45	45	29.26	45	29.73	45	20.91	45
29	TSWV	45	45	29.71	45	30.06	45	27.15	45
	R&R 1	27.17	45	27.46	26.13	28.87	45	16.26	45
	R&R 2	28.61	24.04	27.40	24.15	28.57	45	15.98	23.01
	R&R 3	27.10	45	28.20	45	29.15	25.94	15.15	45
	NC seed	45	45	28.24	45	28.16	45	15.04	45

Repeatability and reproducibility

Introduction

The repeatability is 'the degree of correspondence between the results of successive measurements of the same measurand performed under equal conditions' (Anonymous, 2010).

The requirement for this characteristic was set at > 95% for both repeatability and reproducibility.

Materials and methods

For repeatability and reproducibility three samples were composed that together contain all pospiviroids, spread over the samples in such a way that all viroids in one sample are detected by a different primer mix (table 8). The seed extract was prepared for six replicates per sample and frozen. Three samples were analysed at one time by one operator (repeatability), the other three samples were analysed over several time points by another operator (reproducibility).

Table 8. Sample composition for repeatability and reproducibility

Sample	Pospiviroid positive seeds	Spike	Negative seed lot ZZB649	Signal in primer mix
1	100 seeds ZZB 453 (PSTVd)	CEVd	900	A, B
2	100 seeds ZZB104 (PCFVd and CLVd) + 1 artificially contaminated seed TASVd	-	899	A, B, D
3	1 TCDVd-contaminated seed	TPMVd	999	A, C
4	-	-	1000	-

Results

All pospiviroids were detected in all samples both in repeatability and reproducibility samples (table 9 and appendix 4). One difference that was observed was leakage of the VIC to the FAM channel in sample 2 in Mix A. This was stronger in the repeatability samples than in the reproducibility samples. Other than that the Ct-values were very similar and the standard deviations small. The maximum difference, observed in the reproducibility samples, was 1.05 Ct for a DLVd spike.

Table 9. Average and standard deviation of repeatability and reproducibility samples for Mix A, Mix B, Mix C, and Mix D (n=3).

Mix A

Sample	PSTVd/TCDVd (FAM)		PCFVd (VIC)		DLVd (Texas Red)	
	Repeatability	Reproducibility	Repeatability	Reproducibility	Repeatability	Reproducibility
1	27.94 ± 0.19	27.17 ± 0.27	45 ± 0	45 ± 0	28.91 ± 0.15	28.22 ± 0.53
2*	28.34 ± 0.20	42.06 ± 5.10	24.39 ± 0.19	23.80 ± 0.18	28.87 ± 0.22	27.84 ± 0.44
3	27.94 ± 0.24	27.17 ± 0.17	45 ± 0	45 ± 0	28.83 ± 0.08	28.21 ± 0.44
4	45 ± 0	45 ± 0	45 ± 0	45 ± 0	28.87 ± 0.11	28.08 ± 0.49

* Signal leakage from VIC to FAM channel.

Mix B

Sample	CEVd/CLVd (FAM)		DLVd (Texas Red)	
	Repeatability	Reproducibility	Repeatability	Reproducibility
1	26.73 ± 0.23	25.83 ± 0.42	28.73 ± 0.04	28.16 ± 0.19
2	24.60 ± 0.06	23.96 ± 0.32	28.77 ± 0.23	28.11 ± 0.22
3	45 ± 0	45 ± 0	29.19 ± 0.55	28.38 ± 0.13
4	45 ± 0	45 ± 0	28.72 ± 0.26	28.32 ± 0.15

Mix C

Sample	TPMVd (FAM)		Nad5 (Texas Red)	
	Repeatability	Reproducibility	Repeatability	Reproducibility
1	45 ± 0	45 ± 0	16.18 ± 0.02	16.08 ± 0.08
2	45 ± 0	45 ± 0	16.09 ± 0.04	16.00 ± 0.10
3	26.04 ± 0.10	25.32 ± 0.16	15.99 ± 0.07	15.41 ± 0.47
4	45 ± 0	45 ± 0	15.90 ± 0.04	15.31 ± 0.36

Mix D

Sample	TASVd (FAM) Repeatability	Reproducibility
1	45 ± 0	45 ± 0
2	23.16 ± 0.08	22.76 ± 0.10
3	45 ± 0	45 ± 0
4	45 ± 0	45 ± 0

Discussion
The repeatability and reproducibility were good and the requirements were met. All samples were detected and the differences in Ct-values were small.

Whether the observed signal in the FAM channel of Mix A in sample 2 was caused by signal leakage from VIC to FAM was checked by testing the RNA of sample 2 with a multiplex Taqman PCR with the Boonham pimers and DLVd primers (i.e. Mix A without PCFVd). All six samples of the reproducibility and repeatability gave Ct-values of 45 for Boonham and Ct-values around 28 for DLVd (table 10). Therefore, it was concluded that PSTVd or TCDVd was not present but signal leakage from the VIC to FAM channel caused this signal. It could be considered to change the FAM and VIC labels because of the quarantine status of PSTVd. However, it only influences the identification of the pospiviroids, and not the detection of the pospiviroids.

Table 10. RNA of sample 2 was tested with Mix A without PCFVd primers.

Sample	PSTVd/TCDVd (FAM) Repeatability	Reproducibility	DLVd (Texas Red) Repeatability	Reproducibility
2	45 ± 0	45 ± 0	28.54 ± 0.31	28.65 ± 0.04

General conclusion

A new generic protocol for the detection of seven pospiviroids in tomato seeds was developed. Compared to the 'old' reference method, the validated assay for PSTVd and TCDVd SPN-V003, the new assay has several advantages. The soaking time of the seeds was shorted from overnight soaking to 30-60 minutes. It was shown that this improved the assay (data not shown). Furthermore, the internal control DLVd was added, which is a better mimic of the target than the previous internal control Nad5. The biggest difference between the assays is in the broader scope of the new assay, expanding it to seven pospiviroids.

The new pospiviroid assay was validated for the reliable detection of CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd in tomato seeds. To this end, the performance characteristics trueness, analytical sensitivity, analytical specificity, repeatability and reproducibility were determined.

The assay was compared with the validated PSTVd and TCDVd assay (Bakker et al, 2015) for PSTVd and TCDVd samples and found to be more than ten times more sensitive than the assay by Bakker et al. Therefore the requirement for trueness was met. Analytical sensitivity for this assay was good with detection of dilutions up to 1000x. The analytical specificity was good since no false-negatives were observed for all primer sets and none of the non-target viroids and viruses reacted with the

PCRs. Some acceptable cross-reactivity of TASVd isolates with the CEVd/CLVd primer mix was observed. Objective of the seed assay is detect all relevant pospiviroids and identification of the pospiviroid is relatively less important. Anyway additional analysis is required to identify the pospiviroid at the species level. Together this makes the specificity acceptable. Repeatability and reproducibility of the assay were tested with mixed seed samples and were found to be good with a repeatability and reproducibility of 100%.

Using the real-time PCR assay as described in Naktuinbouw protocol SPN-V043 v2.0, CEVd, CLVd, PCFVd, PSTVd, TASVd, TCDVd and TPMVd can be reliably detected in seeds of tomato. Based on this validation study the assay is suitable for its intended use.

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Appendix 1 Trueness pospiviroids

Table I. Comparison between the old validated method for PSTVd and TCDVd SPN-V003 v1.0 and the new method SPN-V043 v2.0 for PSTVd- (sample 1 and 2) and TCDVd- (sample 3) contaminated samples (n=8)

	SPN-V043 (new)		SPN-V003 (old)	
	FAM	TR (DLVd)	FAM	VIC (Nad5)
1A	22.81	28.13	26.89	22.94
1B	21.73	28.10	26.19	22.64
1C	24.93	28.27	23.49	22.81
1D	23.71	28.13	25.28	21.53
1E	23.25	28.15	26.45	22.99
1F	23.45	28.20	27.31	22.40
1G	24.08	28.22	26.81	23.43
1H	20.47	28.19	27.04	23.31
2A	24.44	28.21	28.05	22.45
2B	24.52	28.38	27.95	22.68
2C	25.37	28.59	28.47	23.42
2D	25.29	28.45	27.51	22.35
2E	23.21	28.58	27.85	23.00
2F	24.37	27.42	29.46	22.32
2G	25.28	27.06	29.15	23.61
2H	25.38	26.69	28.43	23.03
3A	23.71	28.15	27.55	22.32
3B	22.60	27.84	27.61	22.36
3C	23.41	28.10	29.16	22.42
3D	23.36	28.09	28.04	22.24
3E	23.08	27.82	27.07	22.17
3F	23.14	27.49	26.82	21.57
3G	24.31	27.40	27.70	21.31
3H	23.62	27.11	28.09	22.73
NC seed	35.97	28.15	45	21.56
NTC	45	45	45	45
PC RNA	25.00	29.33	26.34	33.12

Appendix 2 Analytical sensitivity pospiviroids

Table II. All Ct-values for target and non-target viroids measured for analytical sensitivity

		Mix A			Mix B		Mix C		Mix D
	Sample composition*	**PSTVd/ TCDVd**	**PCFVd**	**DLVd**	**CEVd/ CLVd**	**DLVd**	**TPMVd**	**Nad5**	**TASVd**
1	100 PSTVd	17.03	45	27.3	45	28.60	45	16.24	45
1	100 PSTVd	17.17	45	27.00	45	28.51	45	16.20	45
1	100 PSTVd	17.20	45	27.14	45	28.65	45	16.12	45
1	10 PSTVd/10 CLVd	20.27	45	27.15	20.93	28.42	45	15.44	22.12
1	10 PSTVd/10 CLVd	20.64	45	27.41	21.26	28.29	45	15.19	23.24
1	10 PSTVd/10 CLVd	20.63	45	27.45	21.39	28.01	45	15.18	22.03
1	1 PSTVd/1 CLVd	25.51	45	27.60	24.57	28.54	45	15.25	25.31
1	1 PSTVd/1 CLVd	24.10	45	27.36	23.46	28.26	45	15.34	26.33
1	1 PSTVd/1 CLVd	23.38	45	27.57	24.16	28.54	45	16.02	25.13
2	100 TCDVd	16.10	45	27.35	43.5	28.46	45	16.14	45
2	100 TCDVd	16.42	45	27.65	45	28.40	45	16.03	45
2	100 TCDVd	16.85	45	27.39	40.97	28.09	45	16.09	45
2	10 TCDVd/10 TASVd	20.05	45	27.62	28.24	28.60	45	16.26	19.05
2	10 TCDVd/10 TASVd	19.83	45	27.63	28.13	28.31	45	16.23	18.54
2	10 TCDVd/10 TASVd	19.52	45	27.10	29.58	28.95	45	16.11	19.03
2	1 TCDVd/1 TASVd	23.10	45	27.07	40.73	28.17	45	16.17	21.80
2	1 TCDVd/1 TASVd	23.44	45	27.59	38.63	28.02	45	16.10	21.94
2	1 TCDVd/1 TASVd	22.67	45	26.83	30.45	28.18	45	16.13	21.90
3	1000 PCFVd/TPMVd 10x	45	25.01	27.83	24.29	28.40	25.70	15.12	45
3	1000 PCFVd/TPMVd 10x	45	24.76	27.40	24.41	28.14	24.84	15.39	45
3	1000 PCFVd/TPMVd 10x	45	24.98	27.08	25.07	28.09	25.37	15.28	45
3	1000 PCFVd/TPMVd 100x	45	28.11	27.43	28.00	28.24	29.37	15.45	45
3	1000 PCFVd/TPMVd 100x	45	27.83	27.59	28.18	28.45	28.18	15.13	45
3	1000 PCFVd/TPMVd 100x	45	28.22	26.96	28.87	28.57	28.85	15.51	45
3	1000 PCFVd/TPMVd 1000x	45	31.01	27.92	31.30	28.41	31.68	15.02	45
3	1000 PCFVd/TPMVd 1000x	45	31.67	27.90	31.14	28.50	32.33	15.25	45
3	1000 PCFVd/TPMVd 1000x	45	31.21	26.64	31.51	28.38	32.40	15.76	45
4	CEVd 10x	45	45	27.26	26.01	28.62	45	15.57	45
4	CEVd 10x	45	45	26.66	26.05	28.74	45	15.48	45
4	CEVd 10x	45	45	27.27	25.93	28.21	45	15.17	45
4	CEVd 100x	45	45	26.92	29.08	28.56	45	15.45	45
4	CEVd 100x	45	45	26.95	29.08	28.59	45	15.41	45
4	CEVd 100x	45	45	27.66	28.05	28.30	45	15.12	45
4	CEVd 1000x	45	45	26.85	30.88	28.99	45	15.45	45
4	CEVd 1000x	45	45	26.43	31.96	28.50	45	15.82	45
4	CEVd 1000x	45	45	27.22	31.41	28.55	45	15.16	45
	NC zaad	45	45	27.04	40.6	28.18	45	16.01	45
	NTC	45	45	45	45	45	45	45	45
	PC RNA	24.98	25.49	28.46	23.90	29.28	24.64	29.53	22.73

* Supplemented with negative seeds or seed extract of ZZB649

Appendix 3 Analytical specificity pospiviroids

Table III. Sample composition analytical specificity for target pospiviroids

Sample	Viroid	Matrix	Host	Original labno./code
1	CEVd	Leaf	*Solanum jasminoides*	3823889
2	CEVd	Leaf	*Solanum lycopersicum*	89002600
3	CEVd	Leaf	*Solanum tuberosum*	3823889
4	CLVd	Leaf	*Solanum lycopersicon*	93007481
5	CLVd	Leaf	*Solanum lycopersicum*	89001013
6	CLVd	Leaf	*Nemantanthus*	4812065
7	TPMVd	Leaf	*Solanum cardiophyllum*	OG1 (MPVd)
8	PCFVd	Leaf	*Solanum capsicum*	3264951
9	PSTVd	Leaf	*Solanum calibrachoa*	3289436
10	PSTVd	Leaf	*Brugmansia candida*	3241168-1A3
11	PSTVd	Leaf	*Solanum lycopersicum*	Howell
12	TASVd	Leaf	*Cestrum*	3153272
13	TASVd	Leaf	*Lycianthes rantonnetii*	3264933
14	TASVd	Leaf	*Solanum*	INS-11-09632
15	TCDVd	Leaf, stem	*Petunia*	no.21-23
16	TCDVd	Leaf, stem	*Petunia*	S20-1
17	TCDVd	Leaf	*Solanum lycopersicum*	22006456
18	TPMVd	Leaf	*Solanum lycopersicon*	3289954

Table IV. Sample composition analytical specificity for non-target viroids and viruses

Sample	Species	Matrix	Host	Original labnr./code
	Viroids			
1	PLVd	Fresh leaf	*Portulaca*	Vd 21 (2006)
2	CChMVd 1	Leaf	*Chrysanthemum*	Chr. 4608, PD: 11369-,,|| X41
3	CChMVd 2	Leaf	*Chrysanthemum*	Chr. 4610, PD: 20137-,,|| X41
4	CChMVd 3	Leaf	*Chrysanthemum*	Chr. 4609, PD: 36654-,,|| X41
5	CSVd 1	Leaf	*Solanum tuberosum*	4308774
6	CSVd 2	Leaf	*Solanum jasminoides*	2041332
7	CSVd 3	Leaf	*Chrysanthemum*	kas 2004-042
8	IrVd 1	Leaf	*Celosia*	Sequence analysis
9	IrVd 2	Leaf	*Verbena*	Pospi 1 primers/Naktuinbouw
10	IrVd 3	Leaf	*Celosia*	4416011
11	HSVd 1	Leaf	*Cucumis sativus*	YO9352
12	HSVd 2	Leaf	*Cucumis sativus*	4912349
13	CSVd	Fresh leaf	*Pericallis hybrida*	2009-02
14	CSVd	Fresh leaf	*Solanum jasminoides*	2006-004
	Viruses			
15	TSV	Fresh Leaf	*Impatiens*	2004-053
16	TBRV+BRSV	Fresh leaf	*Nepeta*	2008-001
17	ArMV	Fresh leaf	*Vinca major*	2004-081
18	PVY	Leaf	*Capsicum annuum*	03-F-14624
19	TRSV	Leaf	*Hemerocallis*	PD 3398797
20	ToMV+TMV	Fruit	*Solanum lycopersicum*	6762
21	TYLCV	Leaf	*Solanum lycopersicum*	NVWA 3181291
22	TRV	Leaf	*Ribes*	TCH: 7174
23	PMMV	Leaf	*Capsicum annuum*	PMMV 22-9-94 stam P.1,2
24	PepMV	Buds, leaf	*Solanum lycopersicum*	INS-15-14583-1,-3,-5
25	AMV	Leaf	*Chenopodium*	NVWA 5742564
26	AMV	Leaf	*Chenopodium*	NVWA Q300019
27	CMV	Leaf	*Dipladenia*	Naktuinbouw tray 65-385
28	CMV	Leaf	*Irisine*	Naktuinbouw tray 65-386
29	TSWV	Leaf	*Solanum lycopersicum*	INS-15-14295

Appendix 4 Repeatability and reproducibility pospiviroids

Table V. Results of all repeatability and reproducibility samples (Ct-values)

		Mix A			Mix B		Mix C		Mix D
Sample		PSTVd/ TCDVd	PCFVd	DLVd	CEVd/ CLVd	DLVd	TPMVd	Nad5	TASVd
1	Repeatability	27.94	45	28.75	26.46	28.77	45	16.18	45
1	Repeatability	28.13	45	29.04	26.85	28.70	45	16.17	45
1	Repeatability	27.76	45	28.95	26.88	28.71	45	16.20	45
1	Reproducibility	26.91	45	28.20	25.34	28.05	45	16.10	45
1	Reproducibility	27.16	45	27.71	26.08	28.04	45	15.99	45
1	Reproducibility	27.45	45	28.76	26.06	28.38	45	16.15	45
2	Repeatability	28.12*	24.17	28.75	24.67	28.54	45	16.12	23.09
2	Repeatability	28.39*	24.48	28.73	24.57	28.76	45	16.10	23.25
2	Repeatability	28.52*	24.52	29.12	24.57	29.00	45	16.05	23.14
2	Reproducibility	45	23.75	27.86	23.66	28.06	45	15.98	22.69
2	Reproducibility	45	23.64	27.39	23.92	27.92	45	15.91	22.88
2	Reproducibility	36.17*	24.00	28.27	24.30	28.36	45	16.10	22.71
3	Repeatability	28.15	45	28.75	45	28.93	25.92	15.91	45
3	Repeatability	27.99	45	28.82	45	28.82	26.12	16.01	45
3	Repeatability	27.67	45	28.91	45	29.82	26.07	16.04	45
3	Reproducibility	26.97	45	28.07	45	28.35	25.23	15.38	45
3	Reproducibility	27.28	45	27.85	45	28.27	25.23	14.96	45
3	Reproducibility	27.25	45	28.70	45	28.52	25.51	15.90	45
4	Repeatability	45	45	28.79	45	28.71	45	15.95	45
4	Repeatability	45	45	29.00	45	28.99	45	15.87	45
4	Repeatability	45	45	28.83	45	28.47	45	15.89	45
4	Reproducibility	45	45	28.26	45	28.50	45	15.18	45
4	Reproducibility	45	45	27.52	45	28.23	45	15.03	45
4	Reproducibility	45	45	28.45	45	28.24	45	15.72	45
	PC RNA	25.42	25.53	29.93	24.29	28.83	24.82	29.83	23.12

* Leakage from VIC to FAM channel, repeated without PCFVd nog signal in 45 cycles.