International Seed Federation
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Detection of *Tomato brown rugose fruit virus* (ToBRFV) in Tomato and Pepper Seed by SE-qPCR

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Introduction

The *Tobamovirus* genus comprises multiple economically important plant pathogenic viruses, including several that infect Solanaceous crops. They are considered to be the most stable and infectious viruses known and are readily transmitted mechanically by workers, tools and equipment during plant handling. Tobamoviruses can also be spread via fruits, insects and seeds. They survive in soil, water and infested debris from previous crops.

The ISHI-Veg method (https://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-protocols/) for detecting tobamoviruses in tomato seed, an industry standard, contains a direct test based on a local lesion assay that provides conclusive evidence of the presence of viable and infectious tobamoviruses. In the assay, leaves of indicator plant species *Nicotiana tabacum* cv. Xanthi NN and/or *Nicotiana glutinosa* are inoculated with an extract from tomato or pepper seed. If the seed extract contains infectious virions, it triggers a hypersensitive response in the host plant resulting in small, necrotic local lesions on the leaves that are typical for tobamoviruses. According to the current ISHI-Veg method and for higher throughput, an ELISA test can be used to pre-screen seed lots.

An ELISA test detects proteins that are specific to the target pathogen but does not confirm the presence of an infectious virus. An ELISA test, for instance, may also give a positive result when a disinfected seed is tested because inactivated virus fragments may still be present on or in the seed. ELISA is, therefore, considered to be an 'indirect' test and is used as a pre-screen; if the test result is positive the local lesion assay, a direct test, is required to confirm viability and pathogenicity of tobamoviruses (see ISF's Viewpoint on Indirect Seed Health Tests, http://www.worldseed.org/wp-content/uploads/2015/10/Indirect_Seed_Health_Tests_2013.pdf).

***Tomato brown rugose fruit virus* (ToBRFV)**

Tomato brown rugose fruit virus (ToBRFV) is a recently discovered species of tobamovirus that was first reported infecting resistant tomato cultivars grown in nethouses in 2014 in Southern Israel. This was followed by an outbreak of ToBRFV on tomatoes grown in greenhouses in Jordan in 2015 (Salem et al., 2016), and this isolate showed a high genomic sequence identity to the Israeli one. More recently, ToBRFV has been detected in tomato plants in production fields in Mexico, Germany, US and Italy according to EPPO (https://www.eppo.int/ACTIVITIES/plant_quarantine/alert_list_viruses/tomato_brown_rugose_fruit_virus) but a wider, possibly global, spread is likely.

Disease symptoms on tomato plants include chlorosis, mosaic and mottling accompanied occasionally by narrowing of leaves and yellow spotted rugose fruit, making fruit unmarketable (Figure 1). Fruit may also mature irregularly. On susceptible pepper (*Capsicum annuum* and *Capsicum chinense*) plants, EPPO describes symptoms including foliar deformation, with yellowing and mosaic, while fruits are deformed with yellow or brown areas, or green stripes.

Tobamovirus infection on tomato crops is of great concern in general but ToBRFV outbreaks are particularly worrisome because of its ability to overcome resistance rendered by the Tm-2^2 gene. Peppers carrying resistance (L)-genes withstand the disease, but it has been reported that a strong hypersensitive response of plants in heavily contaminated soils, especially at warm temperatures above 30˚C, can severely impact crop establishment (Luria et al., 2017). Therefore, good hygiene measures must be used throughout the production of tomato and pepper crops.

Figure 1. ToBRFV infection on tomato plants and fruits. A. Mild mosaic on leaves. B. Uneven ripening and necrotic lesions on fruit (Courtesy of David Levy, Hazera seed, Ltd)

Detection of ToBRFV

The ELISA assay pre-screen can be used for the detection of tobamoviruses including ToBRFV, however antibodies usually react with several tobamoviruses and it is not possible to identify a specific tobamovirus. ISHI-Veg has developed a protocol for the specific detection of ToBRFV in tomato and pepper seeds by a seed extract qPCR assay (SE-qPCR). The SE-qPCR specifically detects ToBRFV and can also be used to test for the presence of ToBRFV in ELISA-positive seed extracts. A negative SE-qPCR result confirms the absence of ToBRFV in the sample.

Because SE-qPCR assay detects RNA from both infectious and non-infectious virus particles, a positive SE-qPCR should be followed by the previously published local lesion assay to determine if infectious ToBRFV is present. ToBRFV causes the hyper-sensitive response typical for tobamoviruses on leaves of *Nicotiana tabacum* cv. Xanthi NN and *Nicotiana glutinosa* (Luria et al. 2017).

The new SE-qPCR method is based on the same seed extract as the ELISA (and the bioassay confirmation test) in order to allow for direct comparison of results. The method process flow is presented in Figure 2 and the protocol is in Annex A.

The goal of this report is to show the SE-qPCR assay is ft for purpose. The described experiments measure analytical specificity, analytical sensitivity, selectivity and repeatability of the method. According to the ISHI-Veg validation guidelines an interlaboratory comparative test would be required to validate method reproducibility. In this report, only two labs were able to run reproducibility experiments due to limits on the availability of infected material, rather than standard practice of minimum six laboratories. This report will be updated when a full comparative test is done.

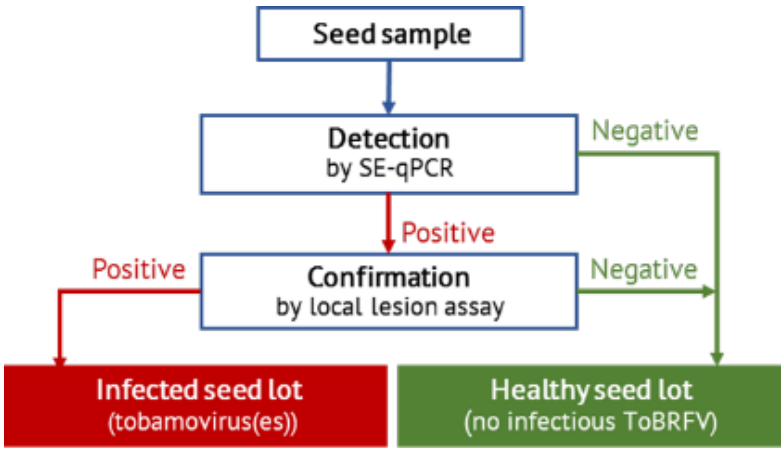

Figure 2. Method process flow

Experiments

The performance criteria and characteristcs used for validating the method are presented in Annex B.

Data for this validation review was compiled from different sources and experiments into one validation report. Analytical specificity data was generated in multiple laboratories and encompasses both *in-silico* and wet lab results. Analytical sensitivity, repeatability and reproducibility was obtained from 'Validation experiment 1'. Experiments set up to determine the limit of detection (LOD) for the ELISA, needed for the analytical sensitivity requirement of the SE-qPCR, were performed simultaneously on the same samples.

Additional information on selectivity in different backgrounds came from 'Validation experiment 2', and 'Validation experiment 3' provided information on the diagnostic performance. In the text we will refer to the different validation experiments.

Analytical specificity

Definition ISHI-Veg guidelines: *The ability of an assay to detect the target(s) pathogens (inclusivity) while excluding non-targets (exclusivity).*

The specificity requirements were: 1. *in silico* the primer/probe sets to have a 100% identity with all available ToBRFV sequences and less than 90% identity with sequences from closely related non-target organisms, and 2. all ToBRFV isolates to be detected by both primer/probe sets in the SE-qPCR assay (Cq <30) and all tested non target organisms to give a Cq-value > 35. The Cq-values were based on the assumption that a Cq-value of 37 represents 1 copy of the target RNA in the reaction (Life Technologies, 2012); the thresholds were chosen to facilitate a clear decision

about specificity. This threshold was set for the validation review only and not destined to be fixed in the protocol.

Experimental approach

The following sources were used to compile an overview of specificity data:

1- *In silico* data analysis. All sequences from ToBRFV isolates on GenBank NCBI and some sequences from closely related non-target organisms were aligned to the ToBRFV primer/probe sets used in the protocol.

2- Multiple company data from leaf and/or seed samples from various origins infected with the target virus ToBRFV or non-target virus/viroid were compiled.

The genetic information of ToBRFV is limited, and the number of available different strains is small. The requirement set, is to test at least one isolate from each of the following origins: Jordan, Israel, Germany, and Mexico. For non-target virus, a set of at least 20 different isolates, closely related to ToBRFV, is investigated.

Analytical sensitivity

Definition ISHI-Veg guidelines: *Smallest amount of the target pathogen that can be detected i.e. the limit of detection (LOD).*

The sensitivity requirements for the method was that the SE-qPCR should be at least as sensitive as the ELISA method.

It has been shown that with ELISA, using TMV Agdia antiserum, one seed infected with ToBRFV can be detected in subsamples of 250 seeds (Table 1; data presented by H. Koenraadt, Naktuinbouw, at ISHI-NL meeting 9th February 2019, Gouda). The cross-reactivity of the TMV antiserum has the same sensitivity for ToBRFV as for other tobamoviruses, such as ToMV (Table 2). This cross-reaction is also mentioned by the supplier on their website (https://orders.agdia.com/agdia-set-tmv-alkphos-sra-57400).

Table 1. Detection of ToBRFV with ELISA using 4 antisera sources and standard ELISA buffer.

Seeds/subsample		Average OD405 / 8 subsamples			
Infected	**healthy**	**TMV-antiserum[1]**	**ToMV-antiserum [1]**	**TMV- antiserum [2]**	**ToMV-antiserum [2]**
1	249	2.340	0.300	0.080	0.580
10	240	2.520	0.630	0.090	0.880

[1] Antibodies supplied by Agdia, USA.

[2] Antibodies supplied by Prime Diagnostics, the Netherlands.

Table 2. Determination of the cross reactivity of TMV Agdia antibodies.

	ToBRFV isolate		ToMV isolate	
Dilution	**Extinction**	**S/N ratio**	**Extinction**	**S/N ratio**
1 : 10	not tested	not tested	0.912	11.8
1 : 100	0.629	8.1	0.437	5.6
1 : 1,000	0.414	5.4	0.126	1.6
1 : 10,000	0.190	2.5	not tested	not tested

Experimental approach

Validation experiment 1: Two seed samples from different crop species (*Solanum lycopersicum* and *Capsicum annuum*) were used to prepare healthy seed extracts. The seed extracts were spiked with ToBRFV infected leaf material, and used to prepare three individual ten-fold dilution series up to six dilutions per seed sample. A total of 42 spiked samples (2 seed samples x 3 dilution series x 7 concentrations) were tested in triplicate. Each dilution was also tested in parallel to the SE-PCR, by ELISA with TMV Agdia (see Annex C for protocol) in triplicate. The lowest virus dilution in which the virus was still detected in all replicates was referred to as the LOD.

A pre-test was performed to determine the starting concentration of the ToBRFV infected leaf material used for spiking with the aim to reach the LOD of the ELISA at the second or third dilution step.

The aim of experiment 1 was to demonstrate that the LOD for SE-PCR is more sensitive than the ELISA and therefore is able to detect a single positive seed in a minimum of 250 seeds. (see Table 1.).

N.B. 10 ml PBS was used as extraction buffer for tomato seeds and 20 ml PBS was used as extraction buffer for pepper seeds. Seed samples were ground using a Silent Crusher M (Heidolph) at 15.000 RPM using an 22 G/M shaft. RNA extraction was performed with both the Qiagen RNeasy kit and the LGC sbeadex kit.

Repeatability

Definition ISHI-Veg guidelines: *Degree of similarity in results of replicates of the same seed lots when the method is performed with minimal variations in a single lab.*

The repeatability requirements for the SE-qPCR were that the ToBRFV SE-qPCR qualitative results of the three independent dilution series, just above the LOD, performed in triplicate, done in the same laboratory by the same technician using the same equipment, should be the same (positive/negative outcome).

Experimental approach

For repeatability the data generated in validation experiment 1 were used.

Reproducibility

Definition ISHI-Veg guidelines: *Degree of similarity in results when the method is performed across labs with replicates of the same subsamples.*

The reproducibility requirements for the method were that the ToBRFV SE-qPCR qualitative results of validation experiment 1 performed by two laboratories should be the same. This was a limited reproducibility experiment, a full comparative test involving more laboratories will be organized at a later date.

Experimental approach

A reproducibility experiment was organized in ISHI-Veg between two laboratories using the same ToBRFV source (experiment 1). ToBRFV infected *N. benthamina* leafs were freeze-dried and ground to a fine powder. Aliquots of 5 mg leaf powder were sent to the two participants. Each laboratory processed the seed extracts according to the described experiment using the Qiagen RNeasy kit, and the LGC sbeadex kit.

Selectivity

Definition ISHI-Veg guidelines: *The effect of different seed matrices on the ability of the method to detect target pathogen(s).*

The selectivity requirements for the SE-qPCR were that the leaf extract equivalent to at least 1 infected seed spiked in 250 healthy seeds from this validation study should be detected by SE-qPCR in all matrices (*S. lycopersicum*, *C. annuum*, *C. chinense* and rootstock tomato).

Experimental approach

Validation experiment 2: In addition to the data generated in validation experiment 1, two additional matrices were tested using the same spiking method. The dilution range tested in experiment 1 was also tested by SE-qPCR in the matrices *Capsicum chinense* and tomato rootstock in triplicate (2 matrices x 9 samples x 3 replicates = 54).

Any negative matrix effects on the PEC were also determined. All processed seed samples were tested according to the SE-qPCR protocol for ToBRFV and were spiked with BaCV infected leaf extract with an expected Cq-value of 30-32 based on a pretest to determine the appropriate concentration. Although BaCV is used in this validation, DLVd and SqMV are suitable as PEC as well (ISHI-Veg, Prophyta 2019). The PEC target should also be detected at a Ct-value within a 3-cycle range.

Diagnostic performance

Definition ISHI-Veg guidelines: *The ability of the method to detect target pathogens in known infected seed samples while excluding non-target organisms in known healthy seed samples.*

The diagnostic performance requirement for the SE-qPCR was that the ToBRFV SE-qPCR should give a positive result on ToBRFV infected seed, and give negative results on uninfected seed or on seed infected with other tobamoviruses .

Experimental approach

Validation experiment 3: Comparative data was generated by one laboratory in which two sub-samples from 10 different seed lots were tested using ToBRFV SE-qPCR as well as the Tobamovirus ELISA method (Agdia TMV). Depending on the antisera used, the ELISA can react with more than one Tobamovirus species, therefore all positive ELISA samples need to be confirmed by an identification method that can conclude if ToBRFV is present, i.e. the ISHI ToBRFV specific TaqMan PCR. Since it is expected for the SE-qPCR to be more sensitive than ELISA, the level of infection of the seed lot which was evaluated in experiment 3, was sufficiently high to avoid a bias in evaluating diagnostic performance.

Results

Analytical specificity

Primer and probe positions (in bp) are shown in Table 3. Both primer/probe sets were aligned to the six ToBRFV genomes currently available at NCBI (November 2019) and 32 genomes of nine closely related species (Annex D). This *in silico* analysis of both CaTa28 and CSP1325 primer/probe sets showed that both sets had a 100% match with the available ToBRFV sequences and less than 90% match with the nine closely related species. Note that some (e.g CaTa28 Pr with ToMV or CaTa28 Fw with TSAMV) primers or probes match at > 90%, but due to lower % matches in the primers/probe belonging to this primer/probe set, the overall match of the set is < 90%.

Multiple company data from leaf and/or seed samples from various origins infected with the target virus ToBRFV or non-target virus/viroid was compiled (see Annex E). All 17 available ToBRFV isolates, originating from eight different countries, were detected with both primer/probe sets and no non-ToBRFV strains reacted with either of the two sets. Seed sample 140902-Healthy-465 showed a Cq-value of 36 once, but no amplification was observed during repeated testing. Due to very low Cq-values in samples tested in the same assay, it was determined to be a cross-contamination during RNA extraction.

Table 3. The position of the primers and probes on ToBRFV genome sequence KX619418

	Fw primer	**Rv primer**	**Probe**	**Amplicon length (bp)**
CaTa28 ToBRFV	Location: MP protein (4908...5708 bp)			
CaTa28 Fw	5161-5180	5300-5281	5189-5212	139
CaTa28 Pr				
CaTa28 Rv				
CSPL ToBRFV	Location: At the end of CP gene (5711...6190 bp)			
CSP1325 Fw	6142-6163	6167-6193	6223-6242	100
CSP1325 Pr				
CSP1325 Rv				

The specificity requirements for the SE-qPCR were met, and for this validation criterion the detection method is fit for purpose.

Analytical sensitivity

Experiment 1, Table 4, demonstrated a LOD for the SE-qPCR at approximately dilution 10^{-8} (Cq < 35) and that the SE-qPCR for both tomato and pepper was at least 1,000-fold more sensitive than the ELISA, when extracted with the Qiagen RNeasy kit. See Table 4 for *S. lycopersicum* and Table 5 for *C. annuum* (see Annex F for raw data). This is in-line with expectations, since PCR includes a multiplication step, in contrast to ELISA, which leads to lower levels of detection.

According to data presented by Naktuinbouw (Table 1) a single ToBRFV infected tomato seed had an ELISA OD value of 2.340 with the Agdia TMV antibodies. The lowest ELISA OD value of a single ToBRFV infected seed is above the ELISA LOD found in this experiment of 0.122 in *S. lycopersicum* (Table 4) and 0.094 in *C. annuum* (Table 5).

Because the SE-qPCR was considerably more sensitive than the ELISA in this experiment, the minimum sensitivity requirements for the SE-qPCR were met, and this method is fit for purpose as a pre-screen for the detection of ToBRFV. However, the risk of false positive results previously

identified for the ELISA pre-screen (Grimault et al., 2012), is therefore likely to be even higher using this SE-qPCR pre-screen which reinforces the importance of applying an additional confirmation assay to identify samples that contain infectious virus.

Table 4. The LOD of ToBRFV in *S. lycopersicum* with two different methods: ELISA and SE-qPCR. The *average* extinctions values and Cq values including the standard deviation are displayed respectively.

	ELISA		SE-qPCR			
	S/N extinction ratio		Qiagen Rneasy		LGC sbeadex	
Dilution			CSP1325	CaTa28	CSP1325	CaTa28
Undil.	Not tested		Not tested	Not tested	Not tested	Not tested
10^{-1}	Not tested		Not tested	Not tested	Not tested	Not tested
10^{-2}	Not tested		Not tested	Not tested	Not tested	Not tested
10^{-3}	1.624	30.8	16.96 ± 0.15	16.50 ± 0.17	20.68 ± 0.43	20.15 ± 0.60
10^{-4}	0.670	12.7	20.18 ± 0.09	19.71 ± 0.14	23.30 ± 0..18	22.63 ± 0.28
10^{-5}	0.122	2.3	23.82 ± 0.23	23.38 ± 0.20	26.27 ± 0.25	25.61 ± 0.36
10^{-6}	0.058	1.1	27.03 ± 0.20	26.60 ± 0.21	29.09 ± 0.34	28.44 ± 0.43
10^{-7}	0.052	1.0	29.90 ± 0.13	29.52 ± 0.14	32.34 ± 0.46	31.80 ± 0.54
10^{-8}	0.051	1.0	33.05 ± 0.24	32.79 ± 0.22	37.18 ± 1.62	35.88 ± 1.11
10^{-9}	0.052	1.0	37.17[2] ± 1.35	35.94 ± 0.52	No amp.	38.08[2] ± 0.12

[1] Five mg dried leaf in buffer, used to spike seed
[2] includes multiple reactions without amplification
Red cell: The LOD of the method used
No amp.: No amplification

Table 5. The LOD of ToBRFV in *C. annuum* with two different methods: ELISA and SE-qPCR. The *average* extinctions values and Cq values are displayed respectively.

	ELISA		SE-qPCR			
	S/N extinction ratio		Qiagen Rneasy		LGC sbeadex	
Dilution			CSP1325	CaTa28	CSP1325	CaTa28
Undil.	Not tested		Not tested	Not tested	Not tested	Not tested
10^{-1}	Not tested		Not tested	Not tested	Not tested	Not tested
10^{-2}	Not tested		Not tested	Not tested	Not tested	Not tested
10^{-3}	1.721	39.4	17.44 ± 0.18	17.52 ± 0.16	19.49 ± 0.19	18.88 ± 0.20
10^{-4}	0.583	13.3	20.60 ± 0.11	20.67 ± 0.17	22.75 ± 0.18	22.05 ± 0.14
10^{-5}	0.094	2.2	24.15 ± 0.05	24.17 ± 0.08	26.06 ± 0.11	25.38 ± 0.11
10^{-6}	0.050	1.1	27.34 ± 0.09	27.41 ± 0.07	29.14 ± 0.17	28.47 ± 0.21
10^{-7}	0.045	1.0	30.28 ± 0.12	30.43 ± 0.19	32.56 ± 0.32	32.04 ± 0.27
10^{-8}	0.045	1.0	33.53 ± 0.44	33.79 ± 0.43	36.88 ± 1.56	35.45 ± 1.02
10^{-9}	0.046	1.1	37.44[2] ± 0.87	38.14[2] ± 0.97	No amp.	No amp.

[1] Five mg dried leaf in buffer, used to spike seed
[2] includes multiple reactions without amplification
Red cell: The LOD of the method used
No amp.: No amplification

Repeatability

The LOD for all three replicates for both *S. lycopersicum* and *C. annuum* using the Qiagen RNeasy kit is 10^{-8} with Cq-values of 33-34, (Table 6 and Annex F). These data demonstrate the 100%

repeatability of the method and validates the detection method as fit for purpose according to this criterion.

Table 6. Repeatability data around LOD (dilution 10^{-8}) from laboratory 2 using the Qiagen RNeasy kit. The Cq-values are displayed.

		S. lycopersicum			*C. annuum*		
Dilution series	**replicate**	**CSP1325**	**CaTa28**	**IAC**	**CSP1325**	**CaTa28**	**IAC**
1	first	33.35	33.16	30.27	33.85	34.27	30.21
	second	32.74	32.62	30.17	33.04	33.27	30.09
	third	33.01	32.60	30.33	33.30	33.94	30.20
2	first	32.91	32.62	30.31	34.46	34.53	30.50
	second	33.49	33.01	30.01	33.45	33.54	30.33
	third	32.98	32.58	30.05	33.38	33.41	30.65
3	first	33.07	32.83	29.94	33.27	33.45	30.42
	second	33.10	33.02	30.15	33.81	34.07	30.12
	third	32.81	32.65	30.16	33.23	33.60	30.41

Reproducibility

The LOD for all three replicates for *S. lycopersicum* and *C. annuum* in both labs was 10^{-8} with a Cq value of 34 when using the Qiagen RNeasy kit (Table 7 and Annex F). When using LGC sbeadex the LOD was approximately 10^{-7} (Table 8). This means that performing the SE-qPCR in multiple laboratories using multiple extraction kits on the sample samples gave the same result. These data demonstrate the 100% reproducibility of the method and validates the detection method as fit for purpose according to this criterion.

Table 7. Reproducibility data for *S. lycopersicum* dilution series 1 using the Qiagen RNeasy kit. The average Cq-values are displayed including the standard deviation. A red colored cell indicates a positive outcome, an orange cell indicates values around previous set LOD of 34 and a green cell a negative outcome. In case not all replicates give an amplification the data of all three replicates is shown (dilution 10^{-9})

		Laboratory 1			**Laboratory 2**		
Dilution		**CSP1325**	**CaTa28**	**IAC**	**CSP1325**	**CaTa28**	**IAC**
10^{-5}		23.32 ± 0.10	23.11 ± 0.35	24.88 ± 0.21	23.83 ± 0.25	23.42 ± 0.23	30.83 ± 0.25
10^{-6}		26.43 ± 0.07	26.18 ± 0.39	24.94 ± 0.14	26.87 ± 0.19	26.43 ± 0.17	30.40 ± 0.23
10^{-7}		29.63 ± 0.12	29.41 ± 0.26	24.86 ± 0.18	29.78 ± 0.16	29.43 ± 0.15	30.11 ± 0.10
10^{-8}		33.12 ± 0.15	32.34 ± 0.30	24.71 ± 0.12	33.03 ± 0.31	32.79 ± 0.32	30.26 ± 0.08
10^{-9}	rep 1	33.36	33.48 ± 1.06	24.74 ± 0.02	37.10 ± 0.56	36.15 ± 0.67	30.14 ± 0.14
	rep 2	No amp.					
	rep 3	No amp.					

No amp.: No amplification
rep: replicate

Table 8. Reproducibility data for *S. lycopersicum* dilution series 1 using the LGC sbeadex kit. The average Cq-values are displayed including the standard deviation. A red colored cell indicates a positive outcome, an orange cell indicates values around previous set LOD of 34 and a green cell a negative outcome. In case not all replicates give an amplification the data of all three replicates is shown (dilution 10^{-8} and 10^{-9})

		Laboratory 1			Laboratory 2		
Dilution		**CSP1325**	**CaTa28**	**IAC**	**CSP1325**	**CaTa28**	**IAC**
10^{-5}		26.09 ± 0.24	28.80 ± 0.40	26.30 ± 0.14	26.37 ± 0.27	25.77 ± 0.43	31.20 ± 0.16
10^{-6}		29.42 ± 0.17	32.02 ± 0.30	25.98 ± 0.37	29.46 ± 0.30	28.93 ± 0.42	30.63 ± 0.47
10^{-7}		32.69 ± 0.99	34.82 ± 0.61	26.25 ± 0.19	32.76 ± 0.60	32.26 ± 0.61	30.59 ± 0.31
10^{-8}	rep 1	No amp	39.20	25.77 ± 0.49	39.40	36.83 ± 1.24	31.33 ± 0.66
	rep 2		38.57		No amp.		
	rep 3		No amp.		38.02		
10^{-9}	rep 1	No amp	No amp	26.17 ± 0.12	No amp	38.03	31.37 ± 0.48
	rep 2					No amp.	
	rep 3					No amp.	

No amp.: No amplification
rep: replicate

Selectivity

All four matrices gave comparable Cq values with a standard deviation value below 1 in validation experiment 2 (Table 9 and Annex G). This demonstrates that there was no matrix effect and that the selectivity requirement for this method is met, therefore the method is fit for purpose based on this validation criterion.

Table 9. Selectivity data for 4 different matrices. The average Cq-values are displayed including the standard deviation over the 4 matrices. A red colored cell indicates a positive outcome, an orange cell indicates values around previous set LOD of 34 and a green cell a negative outcome.

	S. lycopersicum		*C. annuum*		*C. chinense*		Rootstock tomato		STD. DEV	
Dilution	**CSP1325**	**CaTa28**	**CSP132**	**CaTa28**	**CSP1325**	**CaTa28**	**CSP1325**	**CaTa28**	**CSP1325**	**CaTa28**
10^{-3}	17.64	16.92	17.83	17.21	18.31	18.18	17.42	17.53	0.38	0.54
10^{-4}	20.49	19.76	21.56	20.80	21.43	21.25	20.41	20.42	0.61	0.63
10^{-5}	23.93	23.03	24.45	23.64	24.55	24.37	24.41	24.48	0.28	0.68
10^{-6}	27.12	26.26	28.09	27.15	28.20	27.93	26.98	27.00	0.64	0.68
10^{-7}	30.20	29.44	31.00	30.32	31.06	30.87	30.81	31.11	0.39	0.74
10^{-8}	34.05	33.23	34.64	33.80	34.49	34.22	34.37	33.67	0.25	0.41
10^{-9}	37.46	36.86	37.24	36.86	37.89	36.80	38.18	37.27	0.42	0.22
10^{-10}	No amp.	No amp.	No amp.	37.84	No amp.	No amp.	No amp.	38.83	-	-

No amp.: No amplification

Diagnostic performance

Some naturally infected ToBRFV tomato seeds were obtained during the diagnostic performance experiments. The results of a tomato and a pepper subsample spiked with a single positive tomato seed (sample 21 and 22) were therefore included in this report. A summary of the results is displayed in Table 10 (see Annex H for raw data).

Tobamovirus ELISA positive tomato and pepper seed samples subjected to ToBRFV SE-qPCR were negative for other tobamoviruses as determined by Sanger sequencing. ToBRFV leaf extract spiked seed samples of tomato and pepper with a concentration just above ELISA LOD (extinction value of 0.100 OD) were ELISA and SE-qPCR positive for ToBRFV. The two subsamples spiked with a single naturally ToBRFV infected seed were ELISA positive (OD 0.139 and 0.262) as well as ToBRFV qPCR positive. Negative tobamo ELISA seed samples of *S. lycopersicum* and *C. annuum* were also negative with the ToBRFV SE-qPCR. The tobamovirus PCR (Letschert et al. 2002 and Kálmán 2003) was also negative.

The diagnostic performance requirements for the SE-qPCR were met, and the detection method is fit for purpose according to this validation criterion.

Table 10. Summary of the diagnostic performance data.

Total samples	Different lots	Seeds	DAS-ELISA	SE-qPCR	Sanger Sequence conclusion
8	4	Tomato	Positive	Negative	ToMV
4	2	Tomato	Negative	Negative	Not applicable
2*	1	Tomato	Positive	Positive	ToBRFV
1**	1	Tomato	Positive	Positive	ToBRFV
2	1	Pepper	Positive	Negative	PMMoV
5	3	Pepper	Negative	Negative	Not applicable
1*	1	Pepper	Positive	Positive	ToBRFV
1**	1	Pepper	Positive	Positive	ToBRFV

*Spiked with ToBRFV infected leaf material
** Single ToBRFV positive tomato seed added to 249 negative seeds

General conclusion

The SE-qPCR met all the validation criteria in this study.

Note, however, that the results for reproducibility in this report are from two labs due to limits on the availability of infected material. Therefore, this report is considered to be an interim validation report which will be complemented with a full comparative test at a later date.

The SE-qPCR can also be used to identify ToBRFV after a positive ELISA in ISHI-Veg's test *Detection of Infectious Tobamoviruses in Tomato / Pepper Seed* using the same (ELISA) seed extract. For positive results from the ELISA and TaqMan tests, the local lesion assay should be run to confirm viability and pathogenicity of tobamovirus in the seed lot.

Annexes

Annex A: Protocol "Detection of Infectious *Tomato brown rugose fruit virus* (ToBRFV) in Tomato and Pepper Seed", V1.2 May 2019

Annex B: ISHI-Veg Method Performance Criteria and Characteristics

Annex C: ELISA protocol used.

Annex D: Percent (%) identity of the Tobamovirus reference strains from NCBI with the ToBRFV Primers and Probes.

Annex E: Multiple company data from leaf and/or seed samples from various origins infected with the target virus ToBRFV or non-target virus/viroid.

Annex F: Results of validation experiment 1.

Annex G: Results of validation experiment 2.

Annex H: Results of validation experiment 3.

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Annex A: Protocol for detecting infectious ToBRFV in tomato and pepper seed by seed extract qPCR.

SEED EXTRACT qPCR

Material

Seed extraction buffer	RNA purification kit and equipment
TaqMan RT-qPCR mix, primers and equipment	1.5 ml RNase Free tube
Spike solution	RNAse free water
Controls	Centrifuge

Seed extraction buffer

Table A1. Phosphate Buffered Saline (PBS) - pH 7.2 – 7.4 per liter

Sodium chloride (NaCl)	8.0 g
Disodium hydrogen phosphate (Na_2HPO_4)	1.15 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g
Add de-ionized water up to 1 liter, adjust pH and autoclave buffer at 121 °C, 15 psi for 15 minutes.	

Note: If a different seed extraction buffer is used, it must be verified in a comparison using uniform positive control material that this does not lead to a reduction in the number of lesions obtained.

Primers

Table A2. Primers, their sequences and source

Name	Sequence	Source
CaTa28 Fw	5' - GGT GGT GTC AGT GTC TGT TT - 3'	Enza Zaden B.V. Netherlands
CaTa28 Pr	5' - 6FAM - AGA GAA TGG AGA GAG CGG ACG AGG - BHQ1 - 3'	
CaTa28 Rv	5' - GCG TCC TTG GTA GTG ATG TT - 3'	
CSP1325[1] Fw	5' - CAT TTG AAA GTG CAT CCG GTT T - 3'	CSP Labs USA
CSP1325 Pr	5' - VIC - ATG GTC CTC TGC ACC TGC ATC TTG AGA - BHQ1 - 3'	
CSP1325 Rv	5' - GTA CCA CGT GTG TTT GCA GAC A - 3'	
BaCV-F	5' - CGA TGG GAA TTC ACT TTC GT - 3'	Naktuinbouw Netherlands
BaCV-R	5' - AAT CCA CAT CGC ACA CAA GA - 3'	
BaCV-P	5' - TxR - CAA TCC TCA CAT GAT GAG ATG CCG - BHQ2 - 3'	

[1] The name CSPtbrfv101 is also used for the primer sequence

Spike solution

The spike solution is prepared by taking a leaf from a plant infected by *Bacopa chlorosis virus* (BaCV) and making an extract of it in PBS. The extract is diluted to obtain a suitable concentration and aliquots are stored at -80 °C.

Note: Other organisms such as *Dahlia latent viroid* (DLVd) and *Squash mosaic virus* (SqMV) may also be used and should be shown to be compatible with the ToBRFV primers in a multiplex PCR.

Controls

Table A3. Controls and their purpose

Negative Process Control (NPC)	Tomato or pepper seed free of ToBRFV
Positive Amplification Control (PAC)	ToBRFV RNA aiming for a Cq (Cycle quantification) value between 28 and 32
	ToBRFV oligo DNA (oligonucleotide (single-stranded DNA) for all ToBRFV target sequences) aiming for a Cq value between 28 and 32
	ToBRFV cDNA aiming for a Cq value between 28 and 32
Positive Extraction Control (PEC)	Spike solution added to the sample aiming for a Cq value between 28 and 32 The PEC serves as an Internal Amplification Control (IAC)
Inhibition Control (IC)	Dilution of the PEC in a non-infected seed extract aiming for a Cq value between 28 and 32 Note: a non-infected seed extract is preferred over a seed extraction buffer, as a strongly diluted infected leaf extract may lead to a relatively high loss of RNA in the purification process (i.e. no carrier RNA present)
Negative Template Control (NTC)	Contains all PCR reagents but no target or spike DNA, RNA or PEC nucleic acids

1. General Requirements

– Seed extracts and controls must be prepared at the same time, under the same laboratory conditions and stored under the same conditions.

– Seed extracts and all controls must be stored at 4°C until the assay begins. It is strongly recommended to perform the local lesion assay within 24 hours following seed extraction.

– The final results of the local lesion assay must be validated through a comparison of the results given by both controls.

2. Extraction of the virus from the seed

2.1. Add PEC to the extraction buffer.

2.2. Grind seeds of each sub-sample, 250 seeds, in 10 ml of the seed extraction buffer PBS for **tomato** seed, *or* in 15–20 ml of PBS buffer for **pepper** seed.

2.3. Store seed extracts at 4°C.

3. RNA extraction

3.1. Combine 25 µl of 4 seed extract sub-samples into a 100 µl combined sample. Use all three combined samples for further analysis.

3.2. Add RNA extraction buffer within 4 hours after grinding.

3.3. Use a commercial RNA extraction kit for RNA extraction. Process the three combined samples according to the supplier's instructions.

3.4. Use the eluted RNA for RT-qPCR using a commercial TaqMan RT-qPCR kit.

4. Preparation of the TaqMan RT-qPCR mix

4.1. Prepare the RT-qPCR mix with the components described in Table A4.

Note: Good results have been obtained by ISHI-Veg member laboratories with the TaqMan RT-qPCR mix: UltraPlex™ 1-Step ToughMix (QuantaBio). Any commercial PCR mix that is suitable for multiplex TaqMan RT-qPCR applications can be used as long as suitability and performance have been demonstrated in an in-lab validation study. The RT-qPCR parameters (Tables A4 and A5) may need to be adjusted when using a different commercial TaqMan RT-qPCR kit.

Table A4. RT-qPCR ToBRFV mix

ToBRFV/BaCV (PEC)	Target	Final concentration (µM)	Volume (µl)
UltraPlex 1-Step ToughMix (4x)			6.25
CaTa28 Fw (forward)	ToBRFV	0.3	
CaTa28 Pr (probe)		0.2	
CaTa28 Rv (reverse)		0.3	
CSP1325 Fw (forward)	ToBRFV	0.3	
CSP1325 Pr (probe)		0.2	
CSP1325 Rv (reverse)		0.3	
BaCV-F (forward)	BaCV	0.3	
BaCV-P (probe)		0.2	
BaCV-R (reverse)		0.3	
RNAse free water		adjust accordingly	
Subtotal PCR-mix			20.00
RNA			5.00
Total			25.00

4.2. Take 5 µl of the RNA sample as input for the PCR.

4.3. In each run, include a NTC and at least one PAC that give a Cq value between 28 and 32.

4.4. Run the TaqMan RT-qPCR according to the following program.

Table A5. RT-qPCR program

RT reaction	10 min 50°C
Denaturation	3 min 95°C
Cycling	10 sec 95°C 60 sec 60°C 40x

5. Evaluation of the test results

5.1. It is the responsibility of the user to determine a Cq threshold, positioned just above background fluorescence, for each of the fluorophores. A fixed threshold should be checked and modified if necessary to remain just above background fluorescence in each test.

5.2. Check for exponential amplification, indicated by an S-shaped amplification curve, for ToBRFV positive samples. Compare with the PAC.

5.3. A negative test result for the SE-qPCR assay means that the sample does not contain ToBRFV.

6. Validity of test results

6.1. Results are only valid if the positive amplification control (PAC) gives a clear signal < Cq 32.

6.2. Results are only valid if the Cq in the negative control samples (IC and NTC) is above a cut off value defined by the laboratory during validation.

6.3. The Cq of the PEC must be within a 3 cycle range of the Cq of the IC in all samples. If it isn't, loss of ToBRFV or inhibition during amplification may have occurred. The Cq of the IC itself should be between 28 and 32.

Note: If DNA is used as a PAC for ToBRFV, the BaCV Cq is also a check of proper reverse transcription. A BaCV PAC is recommended to distinguish between RT-PCR failure (mix/program) and inhibition.

Annex B: ISHI-Veg Method Performance Criteria and Characteristics

Performance Criteria	Characteristics
Analytical specificity of an assay	The ability of an assay to detect the target(s) pathogens (inclusivity) while excluding non-targets (exclusivity)
Analytical sensitivity	Smallest amount of the target pathogen that can be detected i.e. the limit of detection (LOD)
Selectivity	The effect of different seed matrices on the ability of the method to detect target pathogen(s)
Repeatability	Degree of similarity in results of replicates of the same seed lots when the method is performed with minimal variations in a single lab
Reproducibility	Degree of similarity in results when the method is performed across labs with replicates of the same subsamples
Diagnostic performance	The ability of the method to detect target pathogens in known infected seed samples while excluding non-target organisms in known healthy seed samples
Post-implementation surveillance	After a method has been shown to be fit for purpose evaluating its performance over time to ensure it is performing as intended

Source: ISHI-Veg Guidelines for the Validation of Seed Health Tests, Version 1, May 2018

Note: Version 2 dated May 2020 that is currently in force doesn't include Post Implementation Surveillance.

Annex C: ELISA protocol

Material

Seed extraction buffer	ELISA buffers
Antiserum	Controls
phosphatase substrate	

Seed extraction buffer

Table C1. Phosphate Buffered Saline (PBS) - pH 7.2 – 7.4 per liter

Sodium chloride (NaCl)	8.0 g
Disodium hydrogen phosphate (Na_2HPO_4)	1.15 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g
Add de-ionized water up to 1 liter, adjust pH and autoclave buffer at 121 °C, 15 psi for 15 minutes.	

Note: If a different seed extraction buffer is used, it must be verified in a comparison using uniform positive control material that it does not lead to a reduction in the number of lesions obtained.

ELISA buffers

Table C2. Coating buffer - pH 9.6 per liter

Sodium carbonate (Na_2CO_3)	1.59 gr
Sodium bicarbonate ($NaHCO_3$)	2.93 gr
Add de-ionized water up to 1 liter, adjust pH and autoclave buffer at 121 °C, 15 psi for 15 minutes.	

Table C3. Conjugate buffer - pH 7.2 – 7.4 per liter

Phosphate Buffered Saline (PBS) (Table B1.)	
Egg Albumine	2.00 gr

Table C4. Substrate buffer - pH 9.6

Diethanolamine	97 mL
Add de-ionized water up to 1 liter, adjust pH and autoclave buffer at 121 °C, 15 psi for 15 minutes.	

Controls

Table C5. Controls and their purpose

Buffer Control (BC)	The buffers and reagents used in the ELISA, with no seed/tissue matrix or target pathogen
Negative Process Control (NPC)	Tomato or pepper seed free of Tobamovirus
Positive Process Control (PPC)	Positive matrix that contains the target pathogen and is tested at the same time, using the same assay as the corresponding samples

1. General Requirements

- Seed extracts and controls must be prepared at the same time, under the same laboratory conditions and stored under the same conditions
- Seed extracts and all controls must be stored at 4°C until the assay begins. It is strongly recommended to perform the local lesion assay within 24 hours following seed extraction
- The final results of the local lesion assay must be validated through a comparison of the results given by both controls

2. Coating of the ELISA

2.1. Coat the ELISA plates with coating antibodies (TMV Agdia) in coating buffer

2.2. store the coated plate at ~7°C overnight to maximum 24 hrs before use

3. Extraction of the virus from the seed

3.1. Grind seeds of each sub-sample, 250 seeds, in 10 ml of the seed extraction buffer PBS for **tomato** seed, *or* in 20 ml of PBS buffer for **pepper** seed

3.2. Store seed extracts at 4°C

4. ELISA test

4.1. Clear out the coating buffer from the ELISA plate

4.2. Put the seed extract of the grinded samples in the plate

4.3. Store the ELISA plate with samples overnight at ~7°C

4.4. Wash out the samples from the ELISA plate

4.5. Conjugate the ELISA plates with the conjugate antibodies (e.g. TMV Agdia) in conjugate buffer

4.6. Store the conjugated plate at 28°C for 4 hrs

4.7. Dissolve 10 mg of phosphatase substrate per 10 ml substrate buffer

4.8. Wash out the conjugate buffer from the ELISA plate

4.9. Add the substrate buffer with phosphatase substrate to the ELISA plate

4.10. Measure after 2 hrs the ELISA plate extinction using a spectrophotometer at 405 nm and 620 nm

5. Evaluation of the test results

5.1. To calculate the S:N ratio for an ELISA, divide the average PPC OD value by the average NPC OD value

5.2. Cut-offf determination: use the vendor's recommended cut-off (e.g. an S:N ratio (TC: NPC) of 2). Alternatively, use the average NPC OD value plus 3 times the standard deviation of the NPC OD values. All samples with average OD values at or above the cut-off are considered positive

6. Validity of test results

6.1. It is the responsibility of the user that an ELISA have a S:N ratio of at least 10:1 to ensure sufficient separation between positive and negative results, see ELISA Development Guide (https://resources.rndsystems.com/pdfs/datasheets/edbapril02.pdf.)

Annex D. Percent (%) identity of the Tobamovirus reference strains from NCBI with the ToBRFV SE-qPCR primers and probes.

References ID	Species name	Isolate Name	Origin	Host	CSP1325			CaTa28		
					Fw	Pr	Rv	Fw	Pr	Rv
KT383474	ToBRFV	Tom1-Jo	Jordan	Tomato	100	100	100	100	100	100
MK133093	ToBRFV	TBRFV-P12-3G	Germany	Tomato	100	100	100	100	100	100
MK133095	ToBRFV	TBRFV-P12-3H	Germany	Tomato	100	100	100	100	100	100
KX619418	ToBRFV	TBRFV-IL	Israel	Tomato	100	100	100	100	100	100
MN167466	ToBRFV	ToB-SIC01/19	Italy	Tomato	100	100	100	100	100	100
MK648157	ToBRFV	ToBRFV-CaJO	Jordan	Pepper	100	100	100	100	100	100
AF332868	ToMV	Queensland	Australia	Tomato	72.73	81.48	77.27	85	91.67	65
AJ243571	ToMV	K1	Kazakhstan	Unknown	72.73	81.48	77.27	85	91.67	65
KY912162	ToMV	SL-1	Slovakia	Tomato	72.73	81.48	77.27	85	91.67	65
KR537870	ToMV	99-1	USA	Jasmine	72.73	81.48	72.73	85	91.67	65
KU321698	ToMV	AH4	Egypt	Tomato	72.73	81.48	77.27	85	91.67	65
KX711903	ToMV	Mutoko	Zimbabwe	Tomato	72.73	81.48	77.27	85	91.67	65
KP202857	ToMMV	10-100	USA	Tomato	68.18	66.67	72.73	85	79.17	65
KF477193	ToMMV	MX5	Mexico	Tomato	68.18	66.67	72.73	85	79.17	65
KR824951	ToMMV	TiLhaLJ	China	Chilli pepper	68.18	66.67	72.73	85	79.17	65
KX898033	ToMMV	SC13-05	USA	Tomato	68.18	66.67	72.73	85	79.17	65
KU594507	ToMMV	VLC-1	Spain	Tomato	77.27	70.37	72.73	80	79.17	65
MH128145	ToMMV	CpB1	Brazil	Tomato	72.73	66.67	72.73	85	79.17	65
MG515725	PMMoV	Huluado	China	Pepper	50	51.85	45.45	80	79.17	70
AB369276	TMV	IM	South Korea	*Nicotiana benthamiana*	68.18	77.78	81.82	75	83.33	70
NC001367	TMV	Vulgare	Unknown	Unknown	68.18	77.78	81.82	75	83.33	70
JX993906	TMV	SXFQ	China	Tomato	68.18	77.78	81.82	75	83.33	70
AF395129	TMV	TMV-152	China	Unknown	68.18	77.78	81.82	75	83.33	70
HE818420	TMV	Fengcheng	China	Tobacco	68.18	77.78	81.82	75	83.33	70
MG516107	TMV	Shenyang	China	Tobacco	68.18	77.78	81.82	75	83.33	70
FR878069	TMV	Ohio V	USA	Unknown	68.18	74.07	86.36	90	70.83	65
KU659022	TSAMV	Okeechobee	USA	Tropical soda apple	54.55	40.74	36.36	95	70.83	50
DQ355023	BpeMV	BpeMV	Netherlands	Eggplant	59.09	62.96	63.64	90	66.67	65
AB089381	PaMMV	Japanese	Japan	Pepper	54.55	55.56	50	85	54.17	55
KX187305	PaMMV	israeli	Israel	Pepper	45.45	55.56	50	85	54.17	55
NC001556	TMGMV	U2	Unknown	Unknown	54.55	51.85	27.27	90	62.5	60
AB078435	TMGMV	Japanese	Japan	Unknown	54.55	51.85	27.27	90	62.5	60
KU133476	RehMV	ES	South Korea	*Rehmannia glutinosa*	72.73	74.07	81.82	90	83.33	55
EF375551	RehMV	Henan	China	*Rehmannia glutinosa*	72.73	74.07	81.82	90	83.33	55
MG418836	RehMV	RG	South Korea	*Rehmannia glutinosa*	72.73	74.07	81.82	90	83.33	55
AB628188	RehMV	Japanese	Japan	Pepper	68.18	74.07	81.82	90	83.33	55
JX575184	RehMV	Shanxi	China	Rehmannia	72.73	74.07	86.36	85	83.33	55
MF348202	RehMV	NcaS	USA	*Nephila clavipes*	72.73	74.07	81.82	90	83.33	55

Annex E. Multiple company data from leaf and/or seed samples from various origins infected with the target virus ToBRFV or non-target virus/viroid

Strain	Original host plant	Matrix	ID / Provider	Country of origin	Collection date (Provider)	ToBRFV		
						CSP1325	CaTa28	IAC
ToBRFV-041/42	Tomato	Leaf	Enza Zaden	Israel	2015	18.82	19.96	29.56[d]
ToBRFV-054	Tomato	Leaf	Enza Zaden	Saudi-Arabia	2015	20.58	21.97	30.02[d]
ToBRFV-099	Tomato	Leaf	Enza Zaden	Mexico	2019	19.94	21.01	29.70[d]
ToBRFV-103	Tomato	Leaf	PV-1236 / DSMZ	Germany	2018	22.71	23.58	30.42[d]
ToBRFV-32607982	Tomato	Leaf	NVWA/Naktuinbouw	Egypt	2019	10.50	9.52	19.50[b]
ToBRFV-NVC 2191	Tomato	Leaf	Naktuinbouw	UK	2019	11.77	12.03	19.74[b]
ToBRFV-NVC 2189	Tomato	Leaf	Naktuinbouw	Jordan	2019	12.60	12.98	24.69[b]
ToBRFV-NVC 2190	Tomato	Leaf	Naktuinbouw	Saudi-Arabia	2019	13.20	12.93	18.01[b]
ToBRFV	Tomato	Leaf	Naktuinbouw	USA	2019	10.33	9.92	14.93[b]
ToBRFV 2015-406	Tomato	Leaf	2015-406/Rijk Zwaan	Jordan	2015	13.74	14.05	30.34[b]
ToBRFV 2015-558	Tomato	Leaf	2015-558/Rijk Zwaan	Jordan	2015	11.24	11.98	31.00[b]
ToBRFV 2015-560	Tomato	Leaf	2015-560/Rijk Zwaan	Saudi Arabia	2015	16.48	17.11	32.03[b]
ToBRFV 2016-29	Tomato	Leaf	2016-29/Rijk Zwaan	Jordan	2016	19.60	19.98	31.48[b]
ToBRFV 2018-1898	Tomato	Leaf	2018-1898/Rijk Zwaan	Mexico	2018	14.52	15.18	31.21[b]
ToBRFV 2018-2590	Tomato	Leaf	2018-2590/Rijk Zwaan	Israel	2018	16.43	17.22	30.98[b]
ToBRFV 2018-3034	Tomato	Leaf	2018-3034/Rijk Zwaan	Mexico	2018	11.34	11.21	31.02[b]
ToBRFV 2019-3310	Tomato	Leaf	2019-3310/Rijk Zwaan	Jordan	2019	8.16	7.70	31.93[b]
BepMV-030	Eggplant	Leaf	PV-0170 / DSMZ	Netherlands	1988	No amp.	No amp.	30.37[d]
TSaMV-031	Not known	Leaf	2010-039 / RZ	Netherlands	2014	No amp.	No amp.	30.22
PaMMV-052	*Capsicum* sp.	Leaf	PV-0606 / DSMZ	Greece	2000	No amp.	No amp.	30.34[d]
PaMMV-089	*Capsicum* sp.	Leaf	PV-1071 / DSMZ	Azerbaijan	2012	No amp.	No amp.	30.46[d]
PMMoV-029	*Capsicum annuum* cv. Frühzauber	Leaf	PV-0165 / DSMZ	unknown	1988	No amp.	No amp.	30.49[d]
PMMoV-056	Not known	Leaf	Enza Zaden	unknown	2016	No amp.	No amp.	30.96[d]
PMMoV-087	*Capsicum* sp.	Leaf	PV-0166 / DSMZ	USA	1988	No amp.	No amp.	30.79[d]
PMMoV-088	*Capsicum annuum*	Leaf	PV-0093 / DSMZ	Italy	1988	No amp.	No amp.	30.35[d]
Tm-058	Tomato	Leaf	Enza Zaden	unknown	2016	No amp.	No amp.	30.15[d]
ToMV-020	Tomato	Leaf	NH-69 / Prime Diagnostics	unknown	2013	No amp.	No amp.	30.51[d]
ToMV-021	Tomato	Leaf	GeRo TM2 / Prime Diagnostics	unknown	2013	No amp.	No amp.	30.38[d]

Strain	Original host plant	Matrix	ID / Provider	Country of origin	Collection date (Provider)	ToBRFV		
						CSP1325	CaTa28	IAC
ToMV-022	Tomato	Leaf	GM65 TM2 / Prime Diagnostics	unknown	2013	No amp.	No amp.	30.80[d]
ToMV-028	Not known	Leaf	PV-0135 / DSMZ	Germany	2014	No amp.	No amp.	30.75[d]
ToMV-080	Not known	Leaf	PV-0846 / DSMZ	Iran	1988	No amp.	No amp.	31.11[d]
ToMV-081	Eeggplant	Leaf	PV-1180 / DSMZ	India	2015	No amp.	No amp.	30.93[d]
ToMV-067	Tomato	Leaf	Enza Zaden	Peru	2016	No amp.	No amp.	30.34[d]
TMV-026	Not known	Leaf	PV-0055 / DSMZ	unknown	1987	No amp.	No amp.	30.16[d]
TMV-084	Not known	Leaf	PV-0107 / DSMZ	Germany	1988	No amp.	No amp.	30.50[d]
TMV-085	*Petunia hybrida*	Leaf	PV-1195 / DSMZ	Germany	2016	No amp.	No amp.	30.21[d]
TMGMV-027	*Nicotiana galuca*	Leaf	PV-0112 / DSMZ	USA	1988	No amp.	No amp.	30.14[d]
ToMMV-094	Tomato	Leaf	V13-07 / KS-Ling	USA	2018	No amp.	No amp.	30.28[d]
ToMMV-095	Tomato	Leaf	V16-07/ KS-Ling	USA	2018	No amp.	No amp.	30.39[d]
PepMV-036	Tomato	Leaf	37B EU / Prime Diagnostics	Europe	2015	No amp.	No amp.	30.31[d]
PepMV-037	Tomato	Leaf	US1 / Prime Diagnostics	USA	2015	No amp.	No amp.	29.97[d]
PepMV-038	Tomato	Leaf	37B US2 / Prime Diagnostics	USA	2015	No amp.	No amp.	30.11[d]
PepMV-039	Tomato	Leaf	WUR#48 CH2 / Prime Diagnostics	Chilli	2015	No amp.	No amp.	30.23[d]
KGMMV-024	*Cucumis* sp.	Leaf	Enza Zaden	unknown	2016	No amp.	No amp.	30.62[d]
CGMMV-053	*Cucumis* sp.	Leaf	PV-0375 / DSMZ	Germany	2015	No amp.	No amp.	30.60[d]
TCDVd-104	Not known	Leaf	Enza Zaden	unknown	2018	No amp.	No amp.	30.27[d]
CLVd -009	Not known	Leaf	Enza Zaden	unknown	2012	No amp.	No amp.	29.76[d]
TASVd-016	Pepper	Leaf	Enza Zaden	unknown	2012	No amp.	No amp.	29.93[d]
PCFVd-015	Tomato	Leaf	Enza Zaden	unknown	2012	No amp.	No amp.	29.99[d]
TPMVd-017	Tomato	Leaf	Enza Zaden	unknown	2012	No amp.	No amp.	30.35[d]
DLVd -032	Dahlia	Leaf	Enza Zaden	unknown	2014	No amp.	No amp.	28.42[d]
BaCV	Not known	Leaf	Enza Zaden	unknown	unknown	No amp.	No amp.	29.92[d]
TYLCV	Tomato	Leaf	Enza Zaden	unknown	unknown	No amp.	No amp.	30.32[d]
CMV-105	Cucumber	Leaf	Enza Zaden	unknown	2019	No amp.	No amp.	29.95[d]
Xv 796	-	Pure culture	Enza Zaden	USA	unknown	No amp.	No amp.	30.13[d]
Cmm 080	-	Pure culture	Enza Zaden	unknown	unknown	No amp.	No amp.	30.07[d]
Nc To. freeze dryer	Tomato	Leaf	Enza Zaden	Netherlands	2019	No amp.	No amp.	30.47[d]
140902-healthy-465	Tomato	Seed	Enza Zaden	Africa	2014	36.41*	36.77*	31.00[d]

Strain	Original host plant	Matrix	ID / Provider	Country of origin	Collection date (Provider)	ToBRFV		
						CSP1325	CaTa28	IAC
140815-healthy-122	Pepper	Seed	Enza Zaden	China	2014	No amp.	No amp.	32.47[d]
Nc Pepper routine	Pepper	Seed	Enza Zaden	unknown	unknown	No amp.	No amp.	32.92[d]
140902-healthy-9	Eggplant	Seed	Enza Zaden	Thailand	2014	No amp.	No amp.	30.71[d]
Nc Eggplant routine	Eggplant	Seed	Enza Zaden	unknown	unknown	No amp.	No amp.	31.30[d]
190612-healthy-600	Tomato	Seed	Enza Zaden	Netherlands	2016	No amp.	No amp.	26.69[d]
190612-healthy-601	Tomato	Seed	Enza Zaden	Netherlands	2017	No amp.	No amp.	27.59[d]
190612-healthy-602	Tomato	Seed	Enza Zaden	Netherlands	2018	No amp.	No amp.	26.66[d]
190612-healthy-603	Tomato	Seed	Enza Zaden	Netherlands	2018	No amp.	No amp.	25.23[d]
190612-healthy-604	Tomato	Seed	Enza Zaden	Netherlands	2013	No amp.	No amp.	25.92[d]
190612-healthy-605	Tomato	Seed	Enza Zaden	Netherlands	2014	No amp.	No amp.	27.15[d]
190612-healthy-606	Tomato	Seed	Enza Zaden	Netherlands	2015	No amp.	No amp.	26.48[d]
190612-healthy-607	Tomato	Seed	Enza Zaden	Netherlands	2017	No amp.	No amp.	27.41[d]
190612-healthy-608	Tomato	Seed	Enza Zaden	Netherlands	2017	No amp.	No amp.	27.26[d]
190612-healthy-609	Tomato	Seed	Enza Zaden	Netherlands	2013	No amp.	No amp.	26.02[d]
190612-healthy-610	Tomato	Seed	Enza Zaden	Netherlands	2014	No amp.	No amp.	26.44[d]
190612-healthy-611	Tomato	Seed	Enza Zaden	Netherlands	2012	No amp.	No amp.	26.87[d]
190612-healthy-612	Tomato	Seed	Enza Zaden	Netherlands	2015	No amp.	No amp.	26.09[d]
190612-healthy-613	Tomato	Seed	Enza Zaden	Netherlands	2013	No amp.	No amp.	27.02[d]
190612-healthy-614	Tomato	Seed	Enza Zaden	Netherlands	2012	No amp.	No amp.	27.02[d]
190612-healthy-615	Tomato	Seed	Enza Zaden	Netherlands	2018	No amp.	No amp.	27.45[d]
190612-healthy-616	Tomato	Seed	Enza Zaden	Netherlands	2006	No amp.	No amp.	26.76[d]
190612-healthy-617	Tomato	Seed	Enza Zaden	Netherlands	2017	No amp.	No amp.	26.47[d]
190612-healthy-618	Tomato	Seed	Enza Zaden	Netherlands	2015	No amp.	No amp.	27.01[d]
190612-healthy-619	Tomato	Seed	Enza Zaden	Netherlands	2014	No amp.	No amp.	26.76[d]

[b] Internal amplification control *Bacopa chlorosis virus*

[d] Internal amplification control *Dahlia latent viroid*

No amp.: No amplification

* Seed sample 140902-Healthy-465 gave an amplification in SE-qPCR once (Cq of 36), but this amplification was not observed during repeated testing. Due to very low Cq-values in samples tested in the same assay, it was determined to be a cross contamination during RNA extraction.

Annex F: Results of validation experiment 1.

TOMATO – LAB 1

Technical replicates		ELISA (Agdia TMV 1:400)						SE-qPCR (BaCV, Qiagen kit)								
		First		Second		Third		First			Second			Third		
DILUTIONS		Extinction	S/N ratio	Extinction	S/N ratio	Extinction	S/N ratio	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC
Dilution series 1	10^{-3}	0.459	10.4	0.498	11.3	0.500	11.4	16.89	16.27	26.43	16.78	16.64	24.87	16.74	16.6	25.75
	10^{-4}	0.286	6.5	0.284	6.5	0.288	6.5	20.07	19.46	25.17	20.12	20.05	25.16	20.00	19.9	24.85
	10^{-5}	0.096	2.2	0.091	2.1	0.094	2.1	23.27	22.72	24.77	23.43	23.4	25.12	23.26	23.21	24.76
	10^{-6}	0.052	1.2	0.050	1.1	0.055	1.3	26.35	25.73	24.92	26.49	26.4	24.82	26.44	26.42	25.09
	10^{-7}	0.044	1.0	0.047	1.1	0.048	1.1	29.75	29.11	25.06	29.52	29.52	24.79	29.62	29.59	24.72
	10^{-8}	0.044	1.0	0.050	1.1	0.048	1.1	32.94	32.00	24.73	33.19	32.48	24.59	33.22	32.54	24.82
	10^{-9}	0.050	1.1	0.046	1.0	0.047	1.1	33.36	34.69	24.76	no amp.	33.04	24.73	no amp.	32.72	24.72
Dilution series 2	10^{-3}	0.478	10.9	0.484	11.0	0.447	10.2	16.9	16.39	26.18	17.12	16.95	26.46	16.85	16.94	26.39
	10^{-4}	0.292	6.6	0.269	6.1	0.289	6.6	19.83	19.27	25.2	19.93	19.8	25.68	19.78	19.74	25.99
	10^{-5}	0.094	2.1	0.09	2.0	0.096	2.2	23.27	22.73	24.89	23.3	23.24	24.9	23.22	23.29	24.78
	10^{-6}	0.053	1.2	0.050	1.1	0.052	1.2	29.29	28.89	29.51	29.94	29.57	29.91	29.61	29.71	29.36
	10^{-7}	0.050	1.1	0.047	1.1	0.049	1.1	32.92	32.23	24.71	33.03	32.41	24.82	33.12	32.73	25.05
	10^{-8}	0.050	1.1	0.048	1.1	0.048	1.1	34.48	31.69	24.95	34.71	32.88	24.86	34.15	32.84	25.14
	10^{-9}	0.102	2.3	0.055	1.3	0.052	1.2	no amp.	no amp.	25.16	no amp.	no amp.	25.04	no amp.	no amp.	25.31
Dilution series 3	10^{-3}	0.466	10.6	0.406	9.2	0.455	10.3	16.78	16.19	27.1	16.95	16.8	26.24	16.69	16.63	26.03
	10^{-4}	0.243	5.5	0.254	5.8	0.266	6.0	20.06	19.52	25.15	20.06	20.04	24.84	20.06	20.08	24.87
	10^{-5}	0.091	2.1	0.091	2.1	0.092	2.1	23.43	22.94	25.01	23.25	23.27	24.89	23.25	23.24	24.83
	10^{-6}	0.074	1.7	0.051	1.2	0.050	1.1	26.32	25.86	25.17	26.6	26.51	25.39	26.27	26.31	25.04
	10^{-7}	0.050	1.1	0.049	1.1	0.046	1.0	29.83	29.3	25.21	29.49	29.56	25.1	29.36	29.36	25.01
	10^{-8}	0.048	1.1	0.045	1.0	0.047	1.1	33.48	32.59	25.16	33.58	32.89	25.11	32.82	32.59	25.12
	10^{-9}	0.047	1.1	0.049	1.1	0.048	1.1	no amp.	36.21	24.99	no amp.	35.9	25	no amp.	36.61	25.11

Contd.

TOMATO – LAB 1 contd.

Technical replicates		SE-qPCR (BaCV, Hamilton/Sbeadex)								
		First			Second			Third		
DILUTIONS		CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC
Dilution series 1	10^{-3}	21.03	23.26	29.35	20.28	22.5	29.05	20.29	22.52	28.84
	10^{-4}	26.28	29.53	26.1	26.38	29.95	26.1	26.55	30.32	27.04
	10^{-5}	26.00	28.58	26.2	25.91	28.57	26.25	26.36	29.26	26.46
	10^{-6}	29.46	32.33	25.68	29.56	31.99	26.4	29.23	31.73	25.86
	10^{-7}	32.4	34.52	26.46	31.88	34.43	26.08	33.79	35.52	26.21
	10^{-8}	no amp.	39.20	26.02	no amp.	38.57	25.2	no amp.	no amp.	26.09
	10^{-9}	no amp.	no amp.	26.26	no amp.	no amp.	26.22	no amp.	no amp.	26.04
Dilution series 2	10^{-3}	20.37	23.39	27.34	19.29	21.88	27.21	19.06	21.52	27.51
	10^{-4}	21.76	23.72	26.03	22.94	25.02	25.83	23.49	25.79	26.22
	10^{-5}	27.57	31.07	25.73	27.44	30.86	25.19	27.99	31.43	26.25
	10^{-6}	30.27	33.49	25.46	30.22	33.11	24.63	30.41	33.76	25.94
	10^{-7}	no amp.	39.61	25.48	no amp.	no amp.	25.68	35.27	36.22	25.24
	10^{-8}	no amp.	no amp.	26.01	no amp.	no amp.	26.42	no amp.	39.24	26.45
	10^{-9}	no amp.	no amp.	25.96	no amp.	no amp.	25.48	no amp.	no amp.	25.17
Dilution series 3	10^{-3}	22.41	26.79	28.95	22.52	26.22	28.38	22.52	27.03	30.26
	10^{-4}	24.75	27.04	25.82	25.4	27.74	26.32	25.43	27.75	26.21
	10^{-5}	28.59	31.35	26.14	28.47	31.41	26.15	28.58	31.65	26.19
	10^{-6}	30.27	33.17	25.57	29.89	32.48	25.23	29.83	32.98	25.57
	10^{-7}	34.65	37.1	25.59	33.15	35.05	26.13	no amp.	36.12	25.35
	10^{-8}	no amp.	no amp.	25.68	no amp.	no amp.	26.04	no amp.	no amp.	26.2
	10^{-9}	no amp.	no amp.	25.62	no amp.	no amp.	26.02	no amp.	no amp.	25.95

TOMATO – LAB 2

Technical replicates		ELISA (Agdia TMV 1:200)						SE-qPCR (BaCV, Qiagen kit)								
		First		Second		Third		First			Second			Third		
DILUTIONS		Extinction	S/N ratio	Extinction	S/N ratio	Extinction	S/N ratio	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC
Dilution series 1	10^{-3}	1.397	26.5	1.540	29.2	1.723	32.7	17.04	16.70	34.24	16.94	16.49	34.54	17.08	16.66	35.01
	10^{-4}	0.595	11.3	0.565	10.7	0.666	12.6	20.16	19.70	31.93	20.06	19.60	32.05	20.08	19.57	31.94
	10^{-5}	0.123	2.3	0.107	2.0	0.128	2.4	24.08	23.65	30.72	23.58	23.19	30.65	23.82	23.41	31.11
	10^{-6}	0.056	1.1	0.056	1.1	0.057	1.1	26.93	26.51	30.43	26.65	26.24	30.16	27.02	26.54	30.61
	10^{-7}	0.051	1.0	0.049	0.9	0.052	1.0	29.96	29.59	30.21	29.65	29.29	30.02	29.74	29.40	30.09
	10^{-8}	0.052	1.0	0.051	1.0	0.052	1.0	33.35	33.16	30.27	32.74	32.62	30.17	33.01	32.60	30.33
	10^{-9}	0.051	1.0	0.049	0.9	0.053	1.0	37.73	36.82	30.07	36.67	36.13	30.05	36.91	35.49	30.30
Dilution series 2	10^{-3}	1.808	34.3	1.709	32.4	1.645	31.2	16.87	16.36	34.59	16.67	16.22	34.90	16.88	16.40	34.58
	10^{-4}	0.791	15.0	0.706	13.4	0.673	12.8	20.15	19.63	31.64	20.16	19.68	31.66	20.14	19.58	31.71
	10^{-5}	0.119	2.3	0.120	2.3	0.127	2.4	23.79	23.29	31.15	24.08	23.61	31.17	23.55	23.14	31.63
	10^{-6}	0.058	1.1	0.059	1.1	0.058	1.1	26.92	26.43	30.13	27.26	26.83	30.59	26.94	26.53	30.51
	10^{-7}	0.051	1.0	0.054	1.0	0.051	1.0	29.90	29.49	30.29	30.05	29.68	30.47	29.99	29.51	30.45
	10^{-8}	0.050	0.9	0.051	1.0	0.049	0.9	32.91	32.62	30.31	33.49	33.01	30.01	32.98	32.58	30.05
	10^{-9}	0.053	1.0	0.052	1.0	0.051	1.0	no amp.	36.33	30.21	35.76	35.37	29.95	35.68	35.36	30.15
Dilution series 3	10^{-3}	1.685	32.0	1.679	31.9	1.427	27.1	17.00	16.48	35.15	16.94	16.42	34.57	17.18	16.75	34.50
	10^{-4}	0.691	13.1	0.679	12.9	0.667	12.7	20.31	19.90	32.13	20.24	19.79	32.35	20.33	19.93	32.10
	10^{-5}	0.137	2.6	0.138	2.6	0.102	1.9	23.98	23.49	31.02	23.51	23.14	30.87	24.00	23.54	31.20
	10^{-6}	0.055	1.0	0.063	1.2	0.056	1.1	27.15	26.72	30.38	27.11	26.65	30.67	27.32	26.93	30.48
	10^{-7}	0.053	1.0	0.057	1.1	0.051	1.0	29.94	29.44	30.27	29.93	29.60	30.18	29.94	29.71	30.26
	10^{-8}	0.048	0.9	0.053	1.0	0.050	0.9	33.07	32.83	29.94	33.10	33.02	30.15	32.81	32.65	30.16
	10^{-9}	0.050	0.9	0.057	1.1	0.050	0.9	36.95	36.30	30.17	36.51	35.50	30.19	38.32	36.13	30.14

Contd.

TOMATO – LAB 2 contd.

Technical replicates		SE-qPCR (BaCV, Hamilton/Sbeadex)								
		First			Second			Third		
DILUTIONS		CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC
Dilution series 1	10^{-3}	20.75	20.36	32.90	20.73	20.31	32.40	20.32	19.57	33.28
	10^{-4}	22.98	22.33	31.58	23.30	22.71	31.65	23.41	22.68	32.05
	10^{-5}	26.47	26.01	31.12	26.58	26.03	31.09	26.06	25.27	31.38
	10^{-6}	29.53	29.09	30.33	29.71	29.25	31.17	29.13	28.46	30.38
	10^{-7}	33.13	32.62	30.24	33.08	32.61	30.68	32.06	31.55	30.85
	10^{-8}	39.40	37.10	30.82	no amp.	37.91	32.08	38.02	35.47	31.09
	10^{-9}	no amp.	38.03	31.15	no amp.	no amp.	31.05	no amp.	no amp.	31.92
Dilution series 2	10^{-3}	21.22	20.81	32.42	21.21	20.84	32.35	21.15	20.81	32.32
	10^{-4}	23.59	23.10	30.67	23.36	22.88	31.13	23.38	22.81	31.20
	10^{-5}	26.34	25.76	31.05	26.38	25.81	30.75	26.51	25.90	30.94
	10^{-6}	28.73	28.06	29.98	28.87	28.19	30.22	28.75	28.08	30.37
	10^{-7}	31.95	31.24	29.92	32.15	31.40	31.03	31.88	31.16	30.73
	10^{-8}	36.99	35.90	31.48	36.14	34.92	31.26	36.42	36.51	30.66
	10^{-9}	no amp.	38.00	31.01	no amp.	38.26	30.24	no amp.	no amp.	30.63
Dilution series 3	10^{-3}	20.27	19.55	33.40	20.24	19.53	33.32	20.27	19.55	33.72
	10^{-4}	23.14	22.26	32.04	23.32	22.50	32.68	23.19	22.37	32.57
	10^{-5}	25.80	25.07	30.76	26.14	25.34	30.73	26.16	25.32	31.32
	10^{-6}	28.90	28.17	30.56	29.11	28.36	31.00	29.07	28.32	30.62
	10^{-7}	32.46	32.01	30.44	32.13	31.71	31.12	32.26	31.87	30.49
	10^{-8}	36.39	35.68	31.08	35.33	34.64	31.26	35.92	34.82	31.06
	10^{-9}	no amp.	38.09	30.77	no amp.	no amp.	31.23	no amp.	no amp.	31.31

PEPPER – LAB 1

Technical replicates		ELISA (Agdia TMV 1:400)						SE-qPCR (BaCV, Qiagen kit)								
		First		Second		Third		First			Second			Third		
DILUTIONS		Extinction	S/N ratio	Extinction	S/N ratio	Extinction	S/N ratio	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC
Dilution series 1	10^{-3}	0.288	6.3	0.319	7.0	0.318	7.0	21.2	20.47	31.02	20.92	20.56	32.71	21.31	20.49	27.17
	10^{-4}	0.161	3.5	0.144	3.2	0.162	3.5	24.42	23.67	31.57	24.17	23.91	31.97	24.59	23.72	26.56
	10^{-5}	0.060	1.3	0.061	1.3	0.064	1.4	27.5	26.87	31.13	27.2	26.84	30.2	27.62	26.74	26.94
	10^{-6}	0.041	0.9	0.046	1.0	0.048	1.1	30.78	29.71	31.58	30.28	29.55	31.31	30.68	29.48	24.74
	10^{-7}	0.042	0.9	0.046	1.0	0.048	1.1	33.95	31.7	30.09	34.45	31.70	30.33	33.24	32.09	26.49
	10^{-8}	0.041	0.9	0.042	0.9	0.049	1.1	no amp.	33.75	28.98	no amp.	34.78	30.63	no amp.	35.63	24.94
	10^{-9}	0.043	0.9	0.043	0.9	0.044	1.0	no amp.	36.22	30.23	no amp.	37.39	31.05	no amp.	no amp.	24.93
Dilution series 2	10^{-3}	0.276	6.0	0.306	6.7	0.285	6.2	21	20.28	29.99	20.72	20.34	31.58	21.14	20.27	26.61
	10^{-4}	0.137	3.0	0.13	2.8	0.135	3.0	24.15	23.43	31.42	23.9	23.63	31.59	24.37	23.55	25.22
	10^{-5}	0.059	1.3	0.06	1.3	0.057	1.2	27.2	26.42	30.07	27.03	26.62	32.67	27.52	26.66	27.81
	10^{-6}	0.049	1.1	0.044	1.0	0.043	0.9	30.54	29.69	29.59	30.35	29.78	31.95	30.83	29.68	27.45
	10^{-7}	0.049	1.1	0.043	0.9	0.042	0.9	35.4	32.01	28.26	no amp.	32.31	30.92	37.04	32.2	24.65
	10^{-8}	0.041	0.9	0.034	0.7	0.038	0.8	no amp.	34.82	28.82	no amp.	34.15	29.89	no amp.	34.53	26.28
	10^{-9}	0.045	1.0	0.049	1.1	0.044	1.0	no amp.	36.81	30.57	no amp.	36.58	31.52	no amp.	36.34	26.64
Dilution series 3	10^{-3}	0.249	5.5	0.262	5.7	0.25	5.5	21.01	20.33	30.61	20.88	20.55	30.3	21.16	20.3	26.94
	10^{-4}	0.124	2.7	0.115	2.5	0.112	2.5	24.31	23.61	31.38	24.1	23.85	30.92	24.42	23.6	24.71
	10^{-5}	0.052	1.1	0.053	1.2	0.052	1.1	27.63	27	30.54	27.43	27.14	31.06	27.72	26.91	26.74
	10^{-6}	0.044	1.0	0.045	1.0	0.046	1.0	30.7	30	30.05	30.66	30.13	30.66	30.6	29.56	26.5
	10^{-7}	0.043	0.9	0.042	0.9	0.043	0.9	33.19	32.64	29.19	33.33	32.62	31.11	33.29	32.91	29.14
	10^{-8}	0.046	1.0	0.043	0.9	0.044	1.0	no amp.	34.12	30.05	no amp.	34.60	31.05	no amp.	34.10	25.26
	10^{-9}	0.043	0.9	0.044	1.0	0.049	1.1	no amp.	no amp.	30.3	no amp.	38.65	31.18	no amp.	37.33	26.03

Contd.

PEPPER – LAB 1 contd.

Technical replicates		SE-qPCR (BaCV, Hamilton/Sbeadex)								
		First			Second			Third		
DILUTIONS		CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC
Dilution series 1	10^{-3}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-4}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-5}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-6}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-7}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-8}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-9}	nt	nt	nt	nt	nt	nt	nt	nt	nt
Dilution series 2	10^{-3}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-4}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-5}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-6}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-7}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-8}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-9}	nt	nt	nt	nt	nt	nt	nt	nt	nt
Dilution series 3	10^{-3}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-4}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-5}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-6}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-7}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-8}	nt	nt	nt	nt	nt	nt	nt	nt	nt
	10^{-9}	nt	nt	nt	nt	nt	nt	nt	nt	nt

PEPPER – LAB 2

Technical replicates		ELISA (Agdia TMV 1:200)						SE-qPCR (BaCV, Qiagen kit)								
		First		Second		Third		First			Second			Third		
DILUTIONS		Extinction	S/N ratio	Extinction	S/N ratio	Extinction	S/N ratio	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC
Dilution series 1	10^{-3}	1.641	37.6	1.766	40.4	1.758	40.3	17.27	17.39	35.16	17.29	17.43	34.48	17.32	17.39	34.98
	10^{-4}	0.538	12.3	0.579	13.3	0.569	13.0	20.72	20.93	33.98	20.73	20.87	34.49	20.66	20.75	33.8
	10^{-5}	0.089	2.0	0.091	2.1	0.094	2.2	24.15	24.21	32.32	24.18	24.23	32.37	24.18	24.21	32.53
	10^{-6}	0.048	1.1	0.050	1.1	0.050	1.1	27.34	27.44	31.00	27.28	27.41	31.58	27.21	27.29	30.55
	10^{-7}	0.044	1.0	0.045	1.0	0.045	1.0	30.34	30.57	30.64	30.37	30.65	30.62	30.39	30.63	30.60
	10^{-8}	0.044	1.0	0.045	1.0	0.046	1.1	33.85	34.27	30.21	33.04	33.27	30.09	33.30	33.94	30.20
	10^{-9}	0.044	1.0	0.044	1.0	0.047	1.1	no amp.	37.51	30.62	no amp.	no amp.	30.30	36.83	37.22	30.61
Dilution series 2	10^{-3}	1.751	40.1	1.711	39.2	1.744	39.9	17.76	17.81	35.23	17.55	17.59	34.16	17.66	17.72	34.22
	10^{-4}	0.572	13.1	0.571	13.1	0.596	13.6	20.49	20.57	34.19	20.42	20.46	33.74	20.59	20.66	33.53
	10^{-5}	0.095	2.2	0.093	2.1	0.096	2.2	24.06	24.08	31.20	24.08	24.07	31.22	24.13	24.17	31.31
	10^{-6}	0.051	1.2	0.050	1.1	0.050	1.1	27.34	27.45	30.30	27.36	27.45	30.59	27.24	27.33	30.68
	10^{-7}	0.045	1.0	0.046	1.1	0.045	1.0	30.18	30.29	30.79	30.09	30.24	30.35	30.15	30.37	30.69
	10^{-8}	0.046	1.1	0.046	1.1	0.043	1.0	34.46	34.53	30.50	33.45	33.54	30.33	33.38	33.41	30.65
	10^{-9}	0.047	1.1	0.046	1.1	0.046	1.1	no amp.	39.18	30.77	no amp.	no amp.	30.65	38.06	37.16	30.56
Dilution series 3	10^{-3}	1.737	39.8	1.684	38.6	1.696	38.8	17.39	17.55	34.68	17.39	17.42	34.91	17.33	17.38	34.67
	10^{-4}	0.616	14.1	0.606	13.9	0.597	13.7	20.53	20.42	33.26	20.70	20.75	33.1	20.55	20.63	32.63
	10^{-5}	0.098	2.2	0.096	2.2	0.097	2.2	24.22	24.08	31.12	24.21	24.27	31.15	24.16	24.22	30.59
	10^{-6}	0.050	1.1	0.049	1.1	0.052	1.2	27.48	27.36	31.25	27.42	27.54	30.73	27.38	27.43	31.01
	10^{-7}	0.046	1.1	0.046	1.1	0.047	1.1	30.25	30.09	30.50	30.37	30.47	30.66	30.38	30.53	30.38
	10^{-8}	0.046	1.1	0.046	1.1	0.047	1.1	33.27	33.45	30.42	33.81	34.07	30.12	33.23	33.60	30.41
	10^{-9}	0.047	1.1	0.047	1.1	0.047	1.1	no amp.	no amp.	29.59	no amp.	no amp.	29.71	no amp.	no amp.	29.46

Contd.

PEPPER – LAB 2 contd.

Technical replicates		SE-qPCR (BaCV, Hamilton/Sbeadex)								
		First			Second			Third		
DILUTIONS		CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC
Dilution series 1	10^{-3}	19.48	18.93	32.15	19.38	18.79	32.04	19.42	18.79	31.83
	10^{-4}	22.67	21.96	30.93	22.63	21.97	31.37	22.72	21.96	30.91
	10^{-5}	26.15	25.46	30.35	26.07	25.39	30.01	26.08	25.37	30.09
	10^{-6}	29.19	28.46	30.75	29.09	28.42	31.03	28.95	28.33	31.02
	10^{-7}	32.78	31.92	31.48	32.39	31.70	31.02	32.32	32.29	31.59
	10^{-8}	38.66	35.39	31.16	36.38	36.05	30.83	36.06	34.35	30.62
	10^{-9}	no amp.	37.09	31.06	no amp.	37.96	30.68	no amp.	no amp.	30.51
Dilution series 2	10^{-3}	19.54	18.94	33.84	19.81	19.15	33.53	19.65	19.08	33.01
	10^{-4}	22.68	22.06	31.24	23.18	22.37	32.17	22.80	22.10	30.97
	10^{-5}	25.92	25.21	30.77	25.89	25.27	31.27	26.01	25.30	31.57
	10^{-6}	29.37	28.78	32.76	29.11	28.43	30.79	29.41	28.82	31.47
	10^{-7}	32.37	31.76	30.49	32.28	31.86	31.27	32.52	32.19	31.55
	10^{-8}	34.45	34.25	30.83	35.65	35.46	31.05	36.02	35.43	30.73
	10^{-9}	no amp.	no amp.	31.22	no amp.	no amp.	30.30	no amp.	no amp.	30.57
Dilution series 3	10^{-3}	19.23	18.51	31.75	19.26	18.69	32.66	19.62	19.02	31.41
	10^{-4}	22.56	21.89	30.34	22.69	22.05	31.31	22.86	22.09	31.38
	10^{-5}	26.05	25.35	30.51	26.13	25.46	30.98	26.23	25.58	31.02
	10^{-6}	28.94	28.22	30.41	29.03	28.28	30.24	29.14	28.47	30.87
	10^{-7}	33.01	32.28	30.72	32.31	31.89	30.75	33.10	32.47	31.54
	10^{-8}	no amp.	35.44	31.44	37.45	37.72	31.21	39.03	35.00	31.09
	10^{-9}	no amp.	no amp.	30.15	no amp.	38.04	30.16	no amp.	36.74	30.93

CONTROLS – LAB 1

Technical replicates	ELISA (Agdia TMV 1:400)						SE-qPCR (BaCV, Qiagen kit)								
	First		Second		Third		First			Second			Third		
CONTROLS	Extinction	S/N ratio	Extinction	S/N ratio	Extinction	S/N ratio	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC
PC, ToBRFV 0x PBS	0.987	N/A	1.055	N/A	nt	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt
PC, ToBRFV 10x PBS	0.914	N/A	0.891	N/A	nt	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt
PC, TMV Agdia	nt	N/A	nt	N/A	nt	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt
NPC1-SL	0.045	N/A	0.043	N/A	0.05	N/A	no amp.	no amp.	26.16	no amp.	no amp.	26.58	no amp.	no amp.	27.15
NPC1-CA	0.044	N/A	0.044	N/A	0.05	N/A	no amp.	no amp.	34.52	no amp.	no amp.	28.93	no amp.	no amp.	27.26
NPC2-SL	0.043	N/A	0.042	N/A	0.047	N/A	no amp.	no amp.	23.24	no amp.	no amp.	27.35	no amp.	no amp.	27.22
NPC2-CA	0.047	N/A	0.043	N/A	0.047	N/A	no amp.	no amp.	24.55	no amp.	no amp.	29.42	no amp.	no amp.	28.68
SL-190612-hea-600	0.046	N/A	0.039	N/A	0.045	N/A	no amp.	no amp.	28.27	nt	nt	nt	nt	nt	nt
CA-190625-hea-245	0.044	N/A	0.042	N/A	0.046	N/A	no amp.	no amp.	29.79	nt	nt	nt	nt	nt	nt
BO-190627-HEA-159	0.040	N/A	0.065	N/A	0.057	N/A	no amp.	no amp.	29.28	nt	nt	nt	nt	nt	nt
BO-190627-HEA-165	0.040	N/A	0.053	N/A	0.043	N/A	no amp.	no amp.	22.9	nt	nt	nt	nt	nt	nt
LS-190101-HEA-158	0.060	N/A	0.065	N/A	0.067	N/A	no amp.	no amp.	29.28	nt	nt	nt	nt	nt	nt
PBS + BaCV	nt	N/A	nt	N/A	nt	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt
PBS	nt	N/A	nt	N/A	nt	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt
PPC-LL-assay BPMov	nt	N/A	nt	N/A	nt	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt

Contd.

CONTROLS – LAB 1 contd.

Technical replicates	SE-qPCR (BaCV, Hamilton/Sbeadex)								
	First			Second			Third		
CONTROLS	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC
PC, ToBRFV 0x PBS	nt	nt	nt	nt	nt	nt	nt	nt	nt
PC, ToBRFV 10x PBS	nt	nt	nt	nt	nt	nt	nt	nt	nt
PC, TMV Agdia	nt	nt	nt	nt	nt	nt	nt	nt	nt
NPC1-SL	nt	nt	nt	nt	nt	nt	nt	nt	nt
NPC1-CA	nt	nt	nt	nt	nt	nt	nt	nt	nt
NPC2-SL	nt	nt	nt	nt	nt	nt	nt	nt	nt
NPC2-CA	nt	nt	nt	nt	nt	nt	nt	nt	nt
SL-190612-hea-600	nt	nt	nt	nt	nt	nt	nt	nt	nt
CA-190625-hea-245	nt	nt	nt	nt	nt	nt	nt	nt	nt
BO-190627-HEA-159	nt	nt	nt	nt	nt	nt	nt	nt	nt
BO-190627-HEA-165	nt	nt	nt	nt	nt	nt	nt	nt	nt
LS-190101-HEA-158	nt	nt	nt	nt	nt	nt	nt	nt	nt
PBS + BaCV	nt	nt	nt	nt	nt	nt	nt	nt	nt
PBS	nt	nt	nt	nt	nt	nt	nt	nt	nt
PPC-LL-assay BPMov	nt	nt	nt	nt	nt	nt	nt	nt	nt

CONTROLS – LAB 2

Technical replicates	ELISA (Agdia TMV 1:200)						SE-qPCR (BaCV, Qiagen kit)								
	First		Second		Third		First			Second			Third		
CONTROLS	Extinction	S/N ratio	Extinction	S/N ratio	Extinction	S/N ratio	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC
PC, ToBRFV 0x PBS	nt	N/A	nt	N/A	nt	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt
PC, ToBRFV 10x PBS	2.982	N/A	3.035	N/A	3.130	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt
PC, TMV Agdia	3.501	N/A	3.558	N/A	nt	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt
NPC1-SL	nt	N/A	nt	N/A	nt	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt
NPC1-CA	nt	N/A	nt	N/A	nt	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt
NPC2-SL	0.050	1.0	0.055	1.0	0.053	1.0	no amp.	no amp.	30.44	no amp.	no amp.	30.31	no amp.	no amp.	30.29
NPC2-CA	0.044	1.0	0.043	1.0	0.044	1.0	no amp.	no amp.	31.20	no amp.	no amp.	31.17	no amp.	no amp.	31.02
SL-190612-hea-600	nt	N/A	nt	N/A	nt	N/A	Nt	Nt	nt	nt	nt	nt	nt	nt	nt
CA-190625-hea-245	nt	N/A	nt	N/A	nt	N/A	Nt	Nt	nt	nt	nt	nt	nt	nt	nt
BO-190627-HEA-159	nt	N/A	nt	N/A	nt	N/A	Nt	Nt	nt	nt	nt	nt	nt	nt	nt
BO-190627-HEA-165	nt	N/A	nt	N/A	nt	N/A	no amp.	no amp.	34.91	nt	nt	nt	nt	nt	nt
LS-190101-HEA-158	nt	N/A	nt	N/A	nt	N/A	no amp.	no amp.	33.86	nt	nt	nt	nt	nt	nt
PBS + BaCV	0.066	1.1	0.067	1.1	0.066	1.1	no amp.	no amp.	33.17	nt	nt	nt	nt	nt	nt
PBS	0.065	N/A	0.067	N/A	0.066	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt
PPC-LL-assay BPMov	nt	N/A	nt	N/A	nt	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt

Contd.

CONTROLS – LAB 2 contd.

Technical replicates	SE-qPCR (BaCV, Hamilton/Sbeadex)								
	First			Second			Third		
CONTROLS	**CSP1325**	**CaTa28**	**IAC**	**CSP1325**	**CaTa28**	**IAC**	**CSP1325**	**CaTa28**	**IAC**
PC, ToBRFV 0x PBS	nt	nt	nt	nt	nt	nt	nt	nt	nt
PC, ToBRFV 10x PBS	nt	nt	nt	nt	nt	nt	nt	nt	nt
PC, TMV Agdia	nt	nt	nt	nt	nt	nt	nt	nt	nt
NPC1-SL	nt	nt	nt	nt	nt	nt	nt	nt	nt
NPC1-CA	nt	nt	nt	nt	nt	nt	nt	nt	nt
NPC2-SL	no amp.	no amp.	31.64	no amp.	no amp.	30.10	no amp.	no amp.	31.05
NPC2-CA	no amp.	no amp.	30.35	no amp.	no amp.	31.12	no amp.	no amp.	29.78
SL-190612-hea-600	nt	nt	nt	nt	nt	nt	nt	nt	nt
CA-190625-hea-245	nt	nt	nt	nt	nt	nt	nt	nt	nt
BO-190627-HEA-159	nt	nt	nt	nt	nt	nt	nt	nt	nt
BO-190627-HEA-165	no amp.	no amp.	32.19	nt	nt	nt	nt	nt	nt
LS-190101-HEA-158	no amp.	no amp.	32.50	nt	nt	nt	nt	nt	nt
PBS + BaCV	no amp.	no amp.	31.29	nt	nt	nt	nt	nt	nt
PBS	nt	nt	nt	nt	nt	nt	nt	nt	nt
PPC-LL-assay BPMov	nt	nt	nt	nt	nt	nt	nt	nt	nt

Key to the tables in Annex F:

PC: Positive Control	PPC: Positive Process Control	NPC1: Negative Process Control (healthy seeds)	NPC2: Negative Process Control (healthy seeds + *N. benthamiana* leaf extract)
SL: *Solanum lycopersicum*	CA: *Capsicum annuum*	BO: *Brassica oleraceae*	No amp.: No amplification
LS: *Lactuca sativa*	nt: not tested	BPMoV - *Bell pepper mottle virus*	BaCV - *Bacopa chlorotic virus* (Internal Amplification Control)
N/A: not applicable	PBS - Phosphate Buffer Saline	Red cell: Positive result	Green cell: Negative result (for SE-qPCR: No amp.)
Orange cell: Amplification signal in SE-qPCR, but one of the replicates in the dilution series gave no amplification			

Annex G: Results of validation experiment 2.

		ELISA (Agdia TMV)						SE-qPCR (BaCV, Qiagen)								
	Dilution	First replicate		Second replicate		Third replicate		First replicate			Second replicate			Third replicate		
		Extinction	S/N ratio	Extinction	S/N ratio	Extinction	S/N ratio	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC
Solanum lycopersicum	10^{-3}	1.381	17.41	1.201	15.14	1.283	16.17	17.49	16.80	32.28	17.72	17.00	32.60	17.70	16.95	32.21
	10^{-4}	0.742	9.35	0.770	9.71	0.767	9.67	20.34	19.63	31.14	20.45	19.75	31.10	20.69	19.90	31.80
	10^{-5}	0.166	2.09	0.165	2.08	0.167	2.11	24.01	23.06	30.46	23.84	22.96	30.21	23.93	23.07	30.98
	10^{-6}	0.077	0.97	0.084	1.06	0.091	1.15	27.15	26.25	30.78	27.20	26.38	30.50	27.00	26.16	30.51
	10^{-7}	0.070	0.88	0.066	0.83	0.080	1.01	30.13	29.45	30.72	30.20	29.43	30.84	30.27	29.45	30.69
	10^{-8}	0.057	0.72	0.070	0.88	0.084	1.06	34.06	33.33	30.98	33.80	33.01	31.46	34.28	33.34	31.45
	10^{-9}	nt	N/A	nt	N/A	nt	N/A	38.78	37.99	32.10	36.55	36.16	31.42	37.05	36.43	31.57
	10^{-10}	nt	N/A	nt	N/A	nt	N/A	38.62	37.83	31.30	No amp.	No amp.	32.16	No amp.	38.87	31.76
Capsicum annuum	10^{-3}	1.478	28.79	1.410	27.47	1.502	29.26	17.71	17.15	31.50	17.71	17.13	31.57	18.06	17.34	32.16
	10^{-4}	0.626	12.19	0.574	11.18	0.595	11.59	21.37	20.44	30.60	22.23	21.70	31.36	21.07	20.26	31.01
	10^{-5}	0.106	2.06	0.106	2.06	0.108	2.10	24.42	23.70	30.71	24.39	23.58	31.07	24.55	23.63	31.21
	10^{-6}	0.052	1.01	0.055	1.07	0.058	1.13	28.06	27.21	30.82	28.07	27.12	31.02	28.13	27.11	30.94
	10^{-7}	0.050	0.97	0.051	0.99	0.053	1.03	31.00	30.25	30.82	30.79	30.21	31.07	31.20	30.50	31.08
	10^{-8}	0.049	0.95	0.051	0.99	0.052	1.01	34.97	34.21	31.82	34.36	33.58	32.11	34.59	33.60	31.66
	10^{-9}	nt	N/A	nt	N/A	nt	N/A	36.56	36.48	31.78	37.32	36.38	31.54	37.84	37.72	31.19
	10^{-10}	nt	N/A	nt	N/A	nt	N/A	N/A	37.69	31.40	36.92	38.14	31.87	37.00	37.68	31.55
Capsicum chinense	10^{-3}	1.452	23.29	1.439	23.09	1.421	22.80	18.14	18.07	32.93	18.48	18.27	33.18	18.32	18.20	33.20
	10^{-4}	0.569	9.13	0.576	9.24	0.335	5.37	21.55	21.31	32.36	21.36	21.21	31.94	21.38	21.24	32.25
	10^{-5}	0.112	1.80	0.108	1.73	0.095	1.52	24.60	24.41	31.98	24.53	24.33	31.21	24.53	24.36	31.78
	10^{-6}	0.069	1.11	0.062	0.99	0.061	0.98	28.31	28.08	31.84	28.16	27.91	31.27	28.14	27.81	31.28
	10^{-7}	0.067	1.07	0.059	0.95	0.061	0.98	31.17	31.01	31.53	30.93	30.73	31.10	31.08	30.88	31.18
	10^{-8}	0.087	1.40	0.056	0.90	0.063	1.01	34.46	34.32	31.77	34.59	34.12	31.66	34.43	34.22	31.46
	10^{-9}	nt	N/A	nt	N/A	nt	N/A	38.02	36.84	31.80	37.91	36.66	31.71	37.73	36.90	31.81
	10^{-10}	nt	N/A	nt	N/A	nt	N/A	No amp.	38.93	31.76	No amp.	No amp.	32.03	No amp.	No amp.	32.12

		ELISA (Agdia TMV)						SE-qPCR (BaCV, Qiagen)								
	Dilution	First replicate		Second replicate		Third replicate		First replicate			Second replicate			Third replicate		
		Extinction	S/N ratio	Extinction	S/N ratio	Extinction	S/N ratio	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC	CSP1325	CaTa28	IAC
Rootstock tomato	10^{-3}	1.485	18.96	1.227	15.66	1.318	16.83	17.42	17.50	33.23	17.45	17.55	33.15	17.39	17.54	33.23
	10^{-4}	0.647	8.26	0.709	9.05	0.677	8.64	20.30	20.38	31.24	20.44	20.41	31.71	20.48	20.48	31.43
	10^{-5}	0.150	1.91	0.154	1.97	0.150	1.91	24.56	24.52	31.48	24.28	24.42	30.83	24.39	24.49	31.30
	10^{-6}	0.089	1.14	0.094	1.20	0.085	1.09	26.89	26.98	30.10	27.02	26.96	30.51	27.04	27.07	30.35
	10^{-7}	0.081	1.03	0.076	0.97	0.078	1.00	30.74	31.03	30.95	30.98	31.21	30.75	30.71	31.09	30.75
	10^{-8}	0.075	0.96	0.081	1.03	0.082	1.05	34.12	33.53	31.03	34.87	33.74	31.08	34.12	33.73	31.44
	10^{-9}	nt	N/A	nt	N/A	nt	N/A	38.68	37.52	31.79	37.29	36.53	31.47	38.57	37.77	31.51
	10^{-10}	nt	N/A	nt	N/A	nt	N/A	39.26	38.40	31.75	37.57	39.22	32.33	40.00	38.86	31.55
Controls	NPC-SL	0.069	N/A	0.095	N/A	0.074	N/A	38.53	No amp.	31.47	No amp.	No amp.	31.49	39.00	39.10	31.76
	NPC-CA	0.048	N/A	0.055	N/A	0.051	N/A	No amp.	No amp.	31.52	No amp.	No amp.	31.61	No amp.	No amp.	32.28
	NPC-CC	0.068	N/A	0.056	N/A	0.063	N/A	38.68	38.86	32.42	38.45	38.89	32.67	No amp.	No amp.	32.84
	NPC-RS	0.076	N/A	0.078	N/A	0.081	N/A	N/A	38.75	31.99	38.68	N/A	31.85	No amp.	No amp.	31.54
	Bufffer	0.059	N/A	0.064	N/A	0.064	N/A	No amp.	No amp.	No amp.	No amp.	No amp.	No amp.	No amp.	No amp.	No amp.
	PC1	3.000	N/A	2.790	N/A	2.835	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt
	PC2	3.562	N/A	3.430	N/A	nt	N/A	nt	nt	nt	nt	nt	nt	nt	nt	nt

Key to the table Annex G

NPC: Negative Process Control (healthy seeds + *N. benthamina* leaf extract)	SL: *Solanum lycopersicum*	CA: *Capsicum annuum*	CC: *Capsicum chinense*
RS: Rootstock tomato	nt: not tested	N/A: not applicable	No amp.: No amplification
Green cell: Positive	Orange cell: around previously set LOD	Red cell: Negative, above LOD	

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Annex H: Results of validation experiment 3.

			ELISA				SE-qPCR				Tobamo virus PCR & Sequencing	
Sample no.	**Lot no.**	**Seeds**	**TMV[1]**	**ToMV[2]**	**PMMoV[2]**	**Result: Tobamo Pos/Neg**	**CSP1325**	**CaTa28**	**IAC**	**Result: ToBRFV Pos/Neg**	**gel based PCR**	**Result Sanger Seq**
1	1	Tomato	2.052	1.960	0.100	Positive	No amp.	No amp.	28.47	Negative	Positive	ToMV
2	4	Tomato	0.297	0.278	0.035	Positive	No amp.	No amp.	28.85	Negative	Positive	ToMV
3	2	Tomato	0.124	0.109	0.033	Positive	No amp.	No amp.	27.71	Negative	Positive	ToMV
4*	6	Tomato	0.243	0.329	0.039	Positive	25.08	25.61	30.96	Positive	Positive	ToBRFV
5	1	Tomato	2.082	1.960	0.118	Positive	No amp.	No amp.	28.82	Negative	Positive	ToMV
6	4	Tomato	0.385	0.411	0.035	Positive	No amp.	No amp.	28.23	Negative	Positive	ToMV
7	2	Tomato	0.144	0.107	0.043	Positive	No amp.	No amp.	29.64	Negative	Positive	ToMV
8	5	Tomato	0.036	0.064	0.036	Negative	No amp.	No amp.	28.72	Negative	Negative	not applicable
9	3	Tomato	0.124	0.129	0.044	Positive	No amp.	No amp.	29.31	Negative	Positive	ToMV
10	3	Tomato	0.123	0.119	0.043	Positive	No amp.	No amp.	27.81	Negative	Positive	ToMV
11	6	Tomato	0.066	0.095	0.031	Negative	No amp.	No amp.	29.56	Negative	Negative	not applicable
12	5	Tomato	0.031	0.057	0.029	Negative	No amp.	No amp.	29.11	Negative	Negative	not applicable
13*	6	Tomato	0.262	0.421	0.036	Positive	23.75	24.36	30.46	Positive	Positive	ToBRFV
21**	11	Tomato	0,229	0,302	0,034	Positive	25,01	25,15	30,19	Positive	Positive	ToBRFV
NPC	6	Tomato	0.046	0.067	0.034	Negative	No amp.	No amp.	28.98	Negative	Negative	not applicable
14	7	Pepper	0.035	0.088	0.041	Negative	No amp.	No amp.	28.78	Negative	Negative	not applicable
15	7	Pepper	0.037	0.090	0.042	Negative	No amp.	No amp.	30.11	Negative	Negative	not applicable
16	8	Pepper	0.066	0.650	3.251	Positive	No amp.	No amp.	30.01	Negative	Positive	PMMoV
17	8	Pepper	0.044	0.242	1.881	Positive	No amp.	No amp.	29.85	Negative	Positive	PMMoV
18	9	Pepper	0.033	0.058	0.033	Negative	No amp.	No amp.	28.79	Negative	Negative	not applicable
19*	10	Pepper	0.334	0.584	0.037	Positive	20.05	21.66	32.29	Positive	Positive	ToBRFV
20	9	Pepper	0.037	0.055	0.035	Negative	No amp.	No amp.	28.53	Negative	Negative	not applicable
22**	12	Pepper	0,139	0,335	0,036	Positive	25,24	26,52	28,29	Positive	Positive	ToBRFV
NPC	10	Pepper	0.048	0.086	0.039	Negative	No amp.	No amp.	29.44	Negative	Negative	not applicable

*Spiked with ToBRFV infected leaf material; **Single ToBRFV positive tomato seed added to 249 negative seeds

[1] supplier antiserum Agdia; [2] supplier antiserum Prime diagnostics

Green cell: Positive, Red cell: Negative