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Detection of *Pepino mosaic virus* (PepMV) in Tomato Seed by Seed Extract RT-qPCR (SE-qPCR)

Validation report, June 2023

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ISHI VALIDATION REPORTS

This ISHI validation study has been conducted to determine the fitness of the described method for its intended purpose according to the ISHI Guidelines for the Validation of Seed Health Methods[1] and followed by an independent review of its outcome.

DISCLAIMER

ISF cannot guarantee that laboratories following the protocol described herewith will obtain similar results. Many factors, such as staff skills, laboratory equipment and conditions, reagents and sampling methods can influence the results. Consequently, in case of any litigation, ISF will not accept any liability on the use of these tests.

Published by:

International Seed Federation (ISF)

Reposoir 7, 1260 Nyon, Switzerland

Developed by ISHI

[1] Available at: https://www.worldseed.org/our-work/seed-health/ishi-method-development-and-validation/

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SUMMARY

Pepino mosaic virus (PepMV) is a Potexvirus known to cause a wide range of symptoms in cultivated tomato (*Solanum lycopersicum*) causing damage to fruit and crop. Seed is a proven pathway for the dispersal and the transmission of this virus. The ISHI initiated a project to develop and validate a seed extract (SE-) reverse transcriptase (RT) quantitative polymerase chain reaction (qPCR) assay for the detection of PepMV. The SE-qPCR assay is proposed as an (optional) pre-screening step, as an alternative to the ELISA pre-screening step used in the current PepMV method and is therefore performed using phosphate buffered saline (PBS) buffer as RNA seed extraction buffer. The PepMV SE-qPCR assay involves collecting virus particles by macerating tomato seeds and this seed extract is used in a total RNA extraction. Samples are analysed for the presence of PepMV RNA in a multiplex RT-qPCR reaction.

In this validation study, the performance criteria analytical specificity, analytical sensitivity, selectivity, repeatability, reproducibility and diagnostic performance were validated to determine if the PepMV SE-qPCR assay is fit for the intended purpose of serving as a pre-screen prior to a bioassay. In validating the analytical specificity of the PepMV detecting qPCR assays, using NAKT05 and Bejop primers, the alignment of 130 PepMV whole genome sequences show that 15 accessions (11.5%) have one or more mismatches with the NAKT05 and/or Bejop primers. Testing 25 PepMV isolates, representing the known five genotypes (CH2, EU, LP, PES and US1), and a synthetic ssDNA oligo containing the known mismatches show that the majority of the PepMV isolates will be detected by the two assays. The remaining isolates will at least be detected by one of the two PepMV assays. This showed a good inclusivity of the PCR primers. The tested 23 non-targets, consisting of 18 different virus/viroid species able to infect solanaceous crops, four virus-free plant material samples and nuclease free water, gave Cq values above the cut-off or no amplification with both PepMV primers, which showed that the exclusivity requirement is met for the assay. The analytical sensitivity of SE-qPCR was compared with ELISA and determined to be 1,000-fold more sensitive. The LOD with a 100% confidence level was equivalent for both qPCR assays, showing an ability to detect around 19 to 65 PepMV copies/5 µL eluate. Testing individual seeds from a PepMV infected seed lot in a background of healthy seeds has shown that the SE-qPCR assay is able to detect one PepMV infected seed in a subsample of 1,000 seeds. PepMV was detected in five matrices by SE-qPCR with comparable quantitative results, showing good selectivity of the assay. SE-qPCR results were repeatable, as shown with comparable detection of PepMV between replicate samples with 100% accordance of test results. A comparative test (CT) among eight ISHI laboratories showed a reproducibility of the SE-qPCR assay within the pre-set criterium. Six laboratories provided correctly scored data for the positive extraction control (PEC), negative process control (NPC) and positive process control (PPC) and were included in the concordance calculation. The two remaining laboratories obtained poor performance with the PEC resulting in no signal or reduced signal for the NPC. This failed control resulted in excluding these two laboratories from the concordance calculation. The concordance percentage for the six laboratories was with 98.6%, with a false negative percentage of 0.7%, above the required 90% indicating that the reproducibility criterium was met. For robustness, the seed sample set used in the CT was also tested with an alternative extraction method and compared with the extraction method described in the protocol. The samples processed with the

alternative extraction method, dry grinding, guanidine-based extraction buffer and RNA extraction kit suitable for KingFisher platform, showed an increased sensitivity in the detection of PepMV, but obtained the same qualitative positive/negative result. Although not fully validated, this alternative extraction method could be suitable for laboratories when increased throughput is needed. The SE-qPCR diagnostic performance in reference to ELISA is almost identical. All eight natural infected lots tested positive by ELISA were also tested positive by SE-qPCR. From the 13 ELISA negatives lots, one tested positive by SE-qPCR. This shows that diagnostic sensitivity is 100% and diagnostic specificity 92.86%, which is above the requirement of 90% for this criterium.

This report confirms that the PepMV SE-qPCR assay is a suitable pre-screen assay to the ISHI Bioassay, being able to detect a single PepMV contaminated seed in a background of 1,000 healthy seeds. The ISHI PepMV SE-qPCR assay is deemed fit for its intended purpose.

1. INTRODUCTION

Pepino mosaic virus (PepMV) is a Potexvirus, which is known to cause a wide range of symptoms in cultivated tomato (*Solanum lycopersicum*) causing damage to fruit and crop. Tomato seed is a proven pathway for PepMV (Hanssen *et al*., 2010), and PepMV is easily spread mechanically during crop handling, causing damage to fruit and crop such as fruit marbling, discoloration and the occurrence of 'open fruit' (Hanssen and Thomma, 2010). As stated in the ISF regulated pest list database (https://pestlist.worldseed.org/public/pestlist.jsp), the recommended management strategy is to evaluate seed productions by field inspection or seed testing of a representative sample of each seed lot. The current ISHI method for detecting PepMV in tomato seeds consists of i) inoculating *Nicotiana benthamiana* plants followed by a confirmatory ELISA assay or ii) pre-screening seed extracts using an ELISA assay followed by a bioassay for the ELISA positive seed lots to determine if infectious PepMV virions are present (ISF, 2021).

ISHI has been working on the development and optimisation of a pre-screen assay based on seed extract (SE) reverse transcriptase (RT) quantitative polymerase chain reaction (qPCR) assay (SE-qPCR) using phosphate buffered saline (PBS) buffer as RNA seed extraction buffer. This method may be useful in case of e.g., reduced availability of high-quality antibodies for the ELISA assays. The SE-qPCR assay described in the protocol in Annex A is proposed as an alternative to the ELISA assay used in the PepMV current method. The PepMV SE-qPCR can be used as a pre-screen assay to identify seed lots free from PepMV RNA. Remaining suspected seed lots can be tested by an indirect pre-screen ELISA assay and/or direct assay (bioassay) followed by an ELISA assay to detect and confirm the presence of infectious PepMV. Replacing the confirmation by ELISA with confirmation by RT-qPCR could be a next change in the method for the detection of *Pepino mosaic virus* on tomato seed (version 5, March 2021), but it was out of scope for this validation project. This is to reduce the complexity of the validation, e.g., including the matrix *Nicotiana benthamiana* leaf material.

The method process flow is shown in Figure 1. The pre-screen SE-qPCR assay is illustrated by the grey box and is the part of the method being validated in this project. If the SE-qPCR turns out to be negative for PepMV RNA, the seed lot can be considered free from PepMV. If the SE-qPCR is positive for PepMV RNA, the seed lot is considered suspect, and follow-up assays to detect and confirm the presence of infectious PepMV are required (marked in blue boxes).

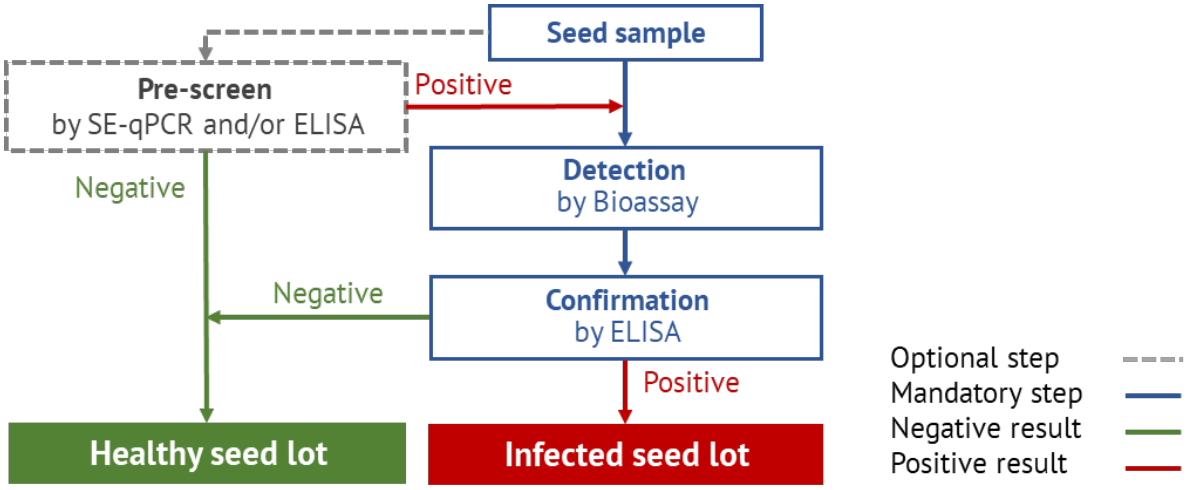

Figure 1. Method process flow.

2. OBJECTIVES

In this validation report, the experiments to investigate whether the SE-qPCR pre-screen assay developed by ISHI is fit for purpose, are described, together with their results. The bioassay and ELISA detection and confirmation steps to test if a suspected sample contains infectious virions have already been described (ISF, 2021) and are, therefore, out of the scope of this project. The described experiments assess the analytical specificity, analytical sensitivity, selectivity, repeatability, reproducibility and diagnostic performance of the SE-qPCR pre-screen assay and were designed according to the ISHI Guidelines for the Validation of Seed Health Test (ISHI, 2020).

3. METHOD VALIDATION

3.1. Analytical specificity

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

The analytical specificity requirements will be met when no false negative results are found for the targets and no false positive results for the non-targets. A false negative means that the two PepMV specific targets in the qPCR assay (Bejop and NAKT05) score Cq>35 to no amplification and that the sample has been proven to be PepMV positive by either Sanger sequencing or a positive bioassay result.

Bacopa chlorosis virus (BaCV) is used as a positive extraction control (PEC) and should give no reaction with any of the target primer sets.

Experimental approach

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

1- *In silico* data analysis: The evaluation of the primer/probe sets was included in the experimental approach. Due to the possibility of having unreliable PepMV sequences in Genbank, which would make the interpretation of the results complicated when deviant isolate(s) cannot be tested in wet laboratory experiments, only full genome accessions were included in these analyses. Accessions displaying a 100% match with either Bejop or NAKT05 assay were accepted.

In the case of accessions with mismatches in both target assays, these were either tested in a wet laboratory experiment or ordered as a representative synthetic ssDNA oligo containing the deviant mismatch to determine the effect of this mismatch on detection. Deviant isolates with mismatches that give Cq values comparable or were more sensitive than the positive/negative outcome of ELISA were accepted.

2- Data from leaf and/or seed samples: Data from leaf and/or seed samples from various origins infected with the target virus PepMV or non-target virus/viroid of which tomato is a host were compiled. At least 20 different isolates of PepMV and 20 non-targets, including virus free plant material, were investigated.

Statistical analysis

The data were analysed qualitatively (positive *vs*. negative).

Results

Primer and probe positions (in base pair (bp)) are shown in Table 1. Both primer/probe sets were aligned to the 130 PepMV genomes available at NCBI in June 2021 and the complete NCBI nucleotide collection for viruses.

Table 1. Sequence of PepMV-specific primers.

	Fw primer	**Probe**	**Rv primer**	**Amplicon length (bp)**
Bejop PepMV	Location : RNA dependent RNA polymerase (87...4406 bp)			
Bejop 134	195 - 214	232 - 259	261 - 278	84
Bejop 135				
Bejop 136				
Bejop 137				
Bejop 138				
Bejop 139				
Bejop 140				
NAKT05 PepMV	Location: Triple gene block 2 protein (5117...5488 bp)			
KL05_48	5129 - 5148	5184 - 5203	5216 - 5235	107
KL05_49				
NAKT05_50				
KL05_51				
KL05_52				

This *in silico* analysis of both Bejop and NAKT05 primer/probe sets showed that eight PepMV accessions have one or more mismatches with the NAKT05 assay, 14 PepMV accessions have only one mismatch with the Bejop assay and 15 PepMV accessions have one or more mismatches with both assays. A representation of these accessions is shown in Annex B. The 22 accessions with mismatches in the Bejop or NAKT05 assay have a 100% match with the complementary assay and are therefore expected to be detected by one of the primer/probe sets of this PCR method. The 15 accessions with mismatches in both assay targets were either validated by testing the isolate in wet lab experiments (e.g., Yur1.5 HG313807) or by testing and comparing representative synthetic ssDNA oligo with (PC_NAKT05m) and without (PC_NAKT05) the deviant mismatches in the NAKT05 assay. The design of these two ssDNA oligonucleotides is shown in Annex C. These ssDNAs, with a concentration of 100 pmol/µL, were separately diluted in IDTE (TE solution pH 8.0 solution (cat. n. 11-05-01-09, Integrated DNA Technologies, USA)) and tested

by RT-qPCR. The concentration is known; therefore, the number of target site copies per 5 µL of template used in a qPCR reaction was calculated. This experiment showed that the mismatches created in PC_NAKT5m ssDNA reduce the sensitivity of the assay by 10-fold, causing a detection limit of 300 copies/5 µL instead of the 30 copies/5 µL in the ssDNA without mismatches, as is shown in Table 2.

Table 2. Comparison of the PepMV NAKT05 assay on two synthetic ssDNA oligos. Rep.: replicate, No amp.: no amplification.

Concentration (copies/5 µL)	PC_NAKT05		PC_NAKT05m	
	Rep. 1 (Cq)	Rep. 2 (Cq)	Rep. 1 (Cq)	Rep. 2 (Cq)
3.01×10^{8}	11.70	11.57	14.56	14.39
3.01×10^{7}	15.17	14.88	18.20	18.00
3.01×10^{6}	18.43	18.26	21.53	21.28
3.01×10^{5}	21.50	21.58	24.94	24.76
3.01×10^{4}	25.29	25.24	28.40	28.29
3.01×10^{3}	28.64	28.47	31.52	31.71
3.01×10^{2}	32.35	31.58	34.47	34.74
3.01×10^{1}	36.43	35.68	39.51	No amp.
3.01×10^{0}	No amp.	37.95	No amp.	No amp.
3.01×10^{-1}	No amp.	No amp.	No amp.	No amp.
3.01×10^{-2}	No amp.	No amp.	No amp.	No amp.
3.01×10^{-3}	No amp.	No amp.	No amp.	No amp.

Company data from leaf and/or seed samples from various origins infected with the target virus PepMV or non-target virus/viroid was compiled (see Annex D, Table D.1 and Table D.2). All 25 PepMV isolates, originating from 14 different countries, were detected with the NAKT05 assay but two isolates were not detected with Bejop. This includes PepMV isolate Yur1.5 (accession HG313807) of which was shown in the *in silico* analyses that isolates Yur1.5, Tor9 and Chi2.9 had mismatches for both assays. RNA from YUR1.5 was provided by Dr. Fernando García-Arenal Rodríguez (Plant-Virus Interaction and Co-evolution Research Group at Centro de Biotecnología y Genómica de Plantas UPM-INIA). Here, it was shown that these mismatches do not affect the detection with NAKT05 assay but result in no amplification with Bejop assay. The genotype of the 25 isolates was determined by Sanger sequencing of the amplicon generated by primer set Pep5-2 TACYTAGCHAAACCAATGGC and Pep6-2 GCWCCATCTTGKGATTGRTC (personal communication B. Woudt Syngenta, 2003, adapted), located on nucleotide position 3,441 to 3,761 of PepMV reference sequence NC004067. Two samples showed a mixed infection of isolates from the Ch2 and LP strains. The Yur1.5 isolate did not give any amplicon with this primer set. The remaining 22 isolates were aligned and organised in a phylogenetic tree (Figure 2), using the UPGMA construction method and Jukes-Canto nucleotide distance method with a bootstrap analyses of 500 replicates in CLC Main Workbench version 20.0.3., to determine if the isolates were genetically unique. These analyses showed that the 22 PepMV isolates used in the analytical specificity are genetically unique and represent the five known genotypes of PepMV; US1, CH2, EU, LP and PES (Moreno-Pérez *et al*., 2014).

All 22 non-target samples, consisting of 18 different virus/viroid species and four virus free plant material samples, resulted in Cq >35 or no amplification with both PepMV assays. The BaCV assay

that was used as inhibition control (IC) and positive extraction control (PEC) only reacted with the *Bacopa chlorotic virus* isolate and not with the PepMV isolates or other non-targets.

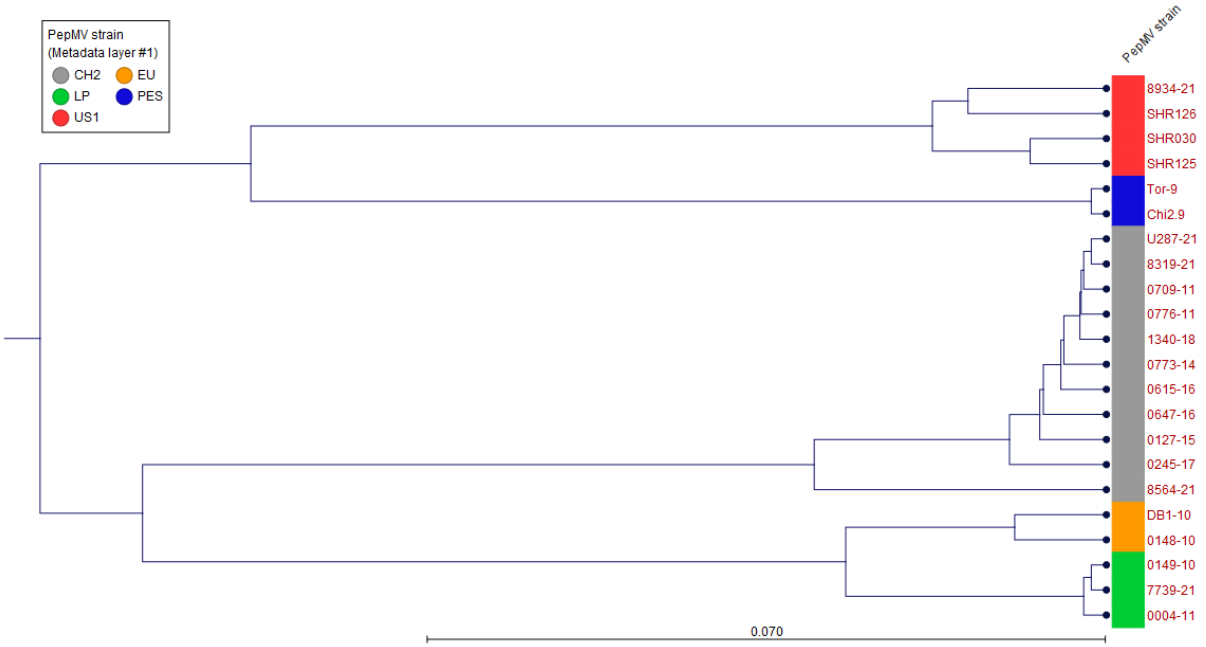

Figure 1. Phylogenetic tree of the 22 PepMV isolates used in the analytical specificity**.**

Conclusion

The *in silico* analyses showed that there are multiple PepMV accessions with mismatches on the target locations of the assays. The wet lab experiments with the ssDNA containing the observed mismatches have shown that this can reduce the detection limit of the assays. Together with three RNA samples of PepMV isolates Tor-9, Chi2.9 and Yur1.5 with relevant mismatches have proven that this does not result in an overall false negative outcome. The compiled data of the assays ran on 25 targets and 22 non-targets has shown that the specificity requirements for the SE-qPCR were met. Although in the validation plan it was decided that the non-targets should have a Cq above 35, it is noteworthy that all non-targets were above the later determined cut-off of Cq 37. For the validation criterium analytical specificity, the detection method is fit for purpose.

3.2. Analytical sensitivity

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

The analytical sensitivity requirements for the single seed detection will be met when a LOD equal to or lower than one infected seed in 1,000 seeds is obtained. The infection rate of the seed lot(s) was determined by ELISA.

Experimental approach

Sensitivity data consists of two data sets:

1- Limit of Detection (LOD) data: Three independent dilution series of PepMV infected leaf material were prepared in healthy seed extracts of tomato (*S. lycopersicum*) and diluted in serial steps of 10-fold. Each dilution was tested in triplicate by the SE-qPCR assay. The initial virus concentration was calculated using a dilution series of synthetic ssDNA of the PepMV assay

targets with a known number of copies as standard curve during the RT-qPCR. The lowest virus dilution in which PepMV can still be detected in all nine replicates is referred to as LOD and expressed as the number of copies per volume (copies/µL).

2- Single-seed detection data: Test samples of 1,000 seeds were prepared from three independent samples of a healthy seed lot spiked with 100, 10 and one single infected seed. The rate of infection and homogeneity of the PepMV positive seed lot used for spiking was determined by performing ELISA and SE-qPCR assays, in duplicate, on 30 individual seeds from the positive lot tested as 30 subsamples containing 249 healthy and one individual seed from the positive lot. The prepared seed samples of 1,000 seeds were tested in triplicate and the lowest seed dilution in which PepMV could still be detected in all nine replicates is referred to as LOD and expressed as the number of infected seeds per 1,000 seeds. Sensitivity of the test was performed with the highest expected seed subsample size of 1,000. Assuming that if one wants to test smaller subsample sizes in the same buffer ratio, it does not negatively affect the detection of the target.

Statistical analysis

The data were analysed qualitatively (positive *vs*. negative).

Results

The compiled results of the LOD experiment are shown in Table 3 (raw data available in Annex E). They demonstrated that the LOD for the SE-qPCR lies at approximately dilution 10^{-7}, which represents an average of 19 PepMV copies/5 µL (Cq<37). The SE-qPCR was at least 1,000-fold more sensitive than ELISA using the seed extraction buffer and the Qiagen RNeasy plant mini kit as described in the protocol. This is in-line with expectations since PCR includes a multiplication step, in contrast to ELISA, which leads to increased sensitivity.

Table 3. The LOD of PepMV in tomato seed extract with two different methods: ELISA and SE-qPCR. The average signal-to-noise (S/N) extinctions ratios and Cq values, including the standard deviation, are displayed, respectively. Red cell: the LOD of the method used. No amp.: no amplification.

Dilution[a]	ELISA		SE-qPCR (Cq)		PepMV (copies/5 µL)	
	Extinction (OD)	S/N ratio	NAKT05	Bejop	NAKT05	Bejop
Undiluted	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
10^{-1}	0.778	16.2	Not tested	Not tested	Not tested	Not tested
10^{-2}	0.309	6.6	18.92 ± 0.37	18.80 ± 0.10	1914444	2075556
10^{-3}	0.091[c]	2.0[c]	22.22 ± 0.07	22.13 ± 0.10	202667	260111
10^{-4}	0.049	1.1	25.60 ± 0.39	25.60 ± 0.16	21644	31800
10^{-5}	0.046	1.0	28.75 ± 0.18	28.61 ± 0.13	2560	4862
10^{-6}	0.043	0.9	33.00 ± 0.23	32.69 ± 0.10	150	406
10^{-7}	0.042	0.9	36.18 ± 0.64[d]	35.72 ± 0.24[d]	19	65
10^{-8}	0.041	0.9	38.45[b] ± 0.20	37.59[b] ± 0.00	4	22
10^{-9}	0.042	0.9	38.46[b] ± 0.00	No amp.	4	0
10^{-10}	0.043	0.9	No amp.	No amp.	0	0

[a] Five mg PepMV infected leaf tissue in buffer, used to spike seed extract.
[b] Includes six or more reactions without amplification.
[c] LOD ELISA: Twice the average of the healthy controls (S/N extinction ratio of 2.0).
[d] LOD SE-qPCR: The lowest virus dilution in which PepMV can still be detected in all nine replicates.

The single-seed detection experiment on 30 individual seeds from seed lot SHR218, which was determined ELISA positive for three subsamples in 2018 (data not shown) and one subsample in 2022 (Table 10, diagnostic performance) out of 12 subsamples of 250 seeds each, demonstrated that virus titter of individual seeds is below the detection limit of the ELISA (Table 4 and Annex E). The SE-qPCR was able to detect 29 out of the 30 samples in two repeats with both assays. The only exception was for sample 17, which gave a Cq of 35.68 with the Bejop primer/probe set and a Cq of 37.49 / no amplification with the NAKT05 assay.

Table 4. The comparison of the results of 30 individual seeds from mixing one single seed from a PepMV positive seed lot together with 249 healthy seeds, with two different methods: ELISA and SE-qPCR. The average signal-to-noise (S/N) extinctions ratios and Cq values, including the standard deviation, are displayed, respectively. No amp.: no amplification, NPC: negative process control.

Sample number	ELISA		SE-qPCR (Cq)		PepMV copies/5 µL[a]	
	Extinction (OD)	S/N ratio	NAKT05	Bejop	NAKT05	Bejop
1	0.043	0.94	31.23 ± 0.10	29.90 ± 0.12	545	1385
2	0.047	1.03	32.28 ± 0.06	31.13 ± 0.32	283	658
3	0.032	0.70	33.00 ± 0.05	31.31 ± 0.00	182	578
4	0.033	0.72	35.20 ± 0.10	33.80 ± 0.59	46	131
5	0.033	0.72	33.31 ± 0.01	31.16 ± 0.04	149	634
6	0.033	0.73	34.30 ± 0.68	31.31 ± 0.11	88	578
7	0.023	0.51	35.16 ± 0.18	33.16 ± 0.05	48	182
8	0.035	0.77	33.59 ± 0.06	31.34 ± 0.18	126	568
9	0.032	0.70	35.66 ± 0.05	32.63 ± 0.57	36	270
10	0.034	0.76	35.74 ± 1.07	34.26 ± 0.22	41	93
11	0.041	0.91	33.17 ± 0.11	31.46 ± 0.06	164	524
12	0.060	1.32	34.03 ± 0.27	32.03 ± 0.73	97	408
13	0.033	0.73	33.64 ± 053	31.06 ± 0.51	128	705
14	0.032	0.71	36.38 ± 1.08	33.96 ± 0.15	27	112
15	0.037	0.82	33.63 ± 0.44	31.64 ± 0.08	127	471
16	0.032	0.71	36.05 ± 0.05	34.83 ± 0.70	27	707
17	0.030	0.67	37.49/No amp.	35.68 ± 0.52	6	41
18	0.034	0.76	33.49 ± 0.70	31.83 ± 0.54	147	442
19	0.032	0.71	34.94 ± 0.12	35.63 ± 0.48	54	41
20	0.034	0.76	35.90 ± 0.56	33.64 ± 0.41	32	140
21	0.056	1.24	33.31 ± 0.58	31.16 ± 0.37	63	650
22	0.033	0.73	37.15 ± 0.39	33.99 ± 0.20	14	110
23	0.032	0.70	34.65 ± 0.09	33.57 ± 0.11	65	142
24	0.034	0.74	33.15 ± 0.38	31.17 ± 0.03	170	631
25	0.032	0.71	33.58 ± 0.45	32.16 ± 0.10	132	340
26	0.031	0.68	29.67 ± 0.06	28.54 ± 0.18	1435	3245
27	0.030	0.67	34.39 ± 0.35	32.52 ± 0.24	78	276
28	0.033	0.72	35.48 ± 0.23	33.15 ± 0.43	39	191
29	0.033	0.72	32.24 ± 0.27	31.45 ± 0.25	294	534
30	0.033	0.72	33.02 ± 0.05	32.25 ± 0.18	179	373
NPC	0.045	0.99	No amp.	No amp.	0	0

[a] The PepMV concentration (copies/5 µl) was calculated using a dilution series of synthetic ssDNA of the PepMV assay targets with a known number of copies as standard curve during the RT-qPCR.

Seed lot SHR218 was also used to create nine samples of 1,000 seeds of which three samples contained either one single seed, three samples with 10 positive seeds and three samples with 100 positive seeds from the positive lot, respectively, representing infection levels of 0.1%, 1.0% and 10%. The results, compiled in Table 5 (full data available in Annex E), demonstrate that the SE-qPCR is able to detect approximately 28 copies per reaction originating from a single infected seed.

Table 5. The average Cq values of three subsamples of 1,000 seeds with three different PepMV infection levels and a sample of 1,000 healthy seeds tested with SE-qPCR. The Cq values, including the standard deviation, are displayed. No amp.: no amplification.

Infection rate	SE-qPCR (Cq)			PepMV copies/5 µL[a]	
	NAKT05	Bejop	PEC	NAKT05	Bejop
0.1	35.68 ± 0.66	34.89 ± 0.92	32.30 ± 0.67	28	57
1.0	30.89 ± 0.57	29.68 ± 0.62	31.47 ± 1.39	626	1924
10.0	27.96 ± 0.87	26.95 ± 1.19	31.59 ± 1.71	4623	15847
0.0	No amp.	No amp.	31.71 ± 0.39	0	0

[a]The PepMV concentration (copies/5 µl) was calculated using a dilution series of synthetic ssDNA of the PepMV assay targets with a known number of copies as standard curve during the RT-qPCR.

Conclusion

The analytical sensitivity experiments have demonstrated that the SE-qPCR is 1,000-fold more sensitive than ELISA and is able to detect around 19 to 65 copies per reaction when testing independent PepMV dilutions series in a tomato seed background. When testing single infected seeds in a subsample of 1,000 seeds, 28 to 57 copies per reaction were detected. These results prove that the SE-qPCR is able to detect a single low infected PepMV seed in a sample of 1,000 seeds and is more sensitive than ELISA. In conclusion, regarding the analytical sensitivity validation criterium, the SE-qPCR detection method is fit for purpose.

3.3. Selectivity

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

The selectivity requirements will be met when:

- The Cq-values for both the spiked samples and the positive extraction control are within a range of 3 Cq between the different matrices,
- No positive results are obtained with the non-spiked samples, and
- No negative results are obtained with the spiked samples.

Experimental approach

The seed extract of a single healthy seed samples of 1,000 seeds from five varieties, each belonging to a different tomato segment (including rootstock), was spiked with PepMV leaf extract and diluted in serial steps of 10-fold in heathy seed extract until an equivalent of one infected seed, as determined by the analytical sensitivity criteria, was reached. All seed samples were processed according to the protocol described in Annex A. The different matrices consisted of three different tomato crops (beef tomato variety A produced in Chile in 2021, beef tomato variety T produced in Mexico in 2020 and truss tomato variety G produced in the Netherlands in 2021) and two rootstock varieties (rootstock variety X and Y, both produced in the Netherlands in 2020).

Statistical analysis

The analysis was done at a qualitative level per sample, viz. positive or negative result for PepMV.

Results

A dilution was determined positive when all three replicates for both of the PepMV qPCR assays obtained a Cq<37. The selectivity experiment average data (Table 6; full data in Annex E) demonstrated that the LOD for the SE-qPCR lies at approximately dilution 10^{-7} for all five tomato and rootstock varieties. The Cq-values of each dilution of the spiked samples, the positive extraction control (PEC) and the negative process control (NPC) for each variety are within a range of 3 Cq between the different matrices.

Table 6. The matrices effect on PepMV in the seed extract of five different tomato varieties using SE-qPCR method. The average Cq values, including the standard deviation, are displayed with the qualitative conclusion. Red cell: the LOD of the SE-qPCR, No amp.: no amplification, NPC: negative process control, NTC: negative template control, PEC: positive extraction control.

Crop	Variety code	Dilution[a]	SE-qPCR (Cq)			Qualitative conclusion
			NAKT05	Bejop	PEC	
Tomato	A	10^{-5}	28.31 ± 0.05	28.54 ± 0.13	29.39 ± 0.15	Positive
		10^{-6}	31.64 ± 0.13	32.15 ± 0.47	28.94 ± 0.17	Positive
		10^{-7}	34.63 ± 0.58	35.84 ± 0.73	27.57 ± 0.36	Positive
		10^{-8}	No amp.	37.83[b]	29.22 ± 0.27	Negative
Tomato	G	10^{-5}	27.96 ± 0.19	28.02 ± 0.25	28.51 ± 0.32	Positive
		10^{-6}	31.13 ± 0.11	31.50 ± 0.03	28.33 ± 0.04	Positive
		10^{-7}	34.79 ± 1.14	35.37 ± 0.79	28.49 ± 0.32	Positive
		10^{-8}	36.77 ± 0.01[b]	36.63[b]	28.25 ± 0.12	Negative
Tomato	T	10^{-5}	28.26 ± 0.12	28.20 ± 0.11	28.64 ± 0.12	Positive
		10^{-6}	31.31 ± 0.18	31.84 ± 0.31	27.06 ± 0.14	Positive
		10^{-7}	34.76 ± 1.68	35.00 ± 0.86	28.53 ± 0.07	Positive
		10^{-8}	36.46 ± 0.55[b]	36.02[b]	29.42 ± 0.15	Negative
Rootstock	X	10^{-5}	27.95 ± 0.33	28.49 ± 0.18	27.89 ± 0.12	Positive
		10^{-6}	31.14 ± 0.08	32.02 ± 0.36	28.50 ± 0.22	Positive
		10^{-7}	34.58 ± 0.64	34.91 ± 0.56	29.05 ± 0.15	Positive
		10^{-8}	36.96 ± 0.09[b]	No amp.	29.27 ± 0.04	Negative
Rootstock	Y	10^{-5}	28.12 ± 0.11	28.56 ± 0.21	29.54 ± 0.25	Positive
		10^{-6}	31.17 ± 0.54	31.38 ± 0.24	29.79 ± 0.28	Positive
		10^{-7}	34.87 ± 0.62	36.30 ± 0.17	30.02 ± 0.12	Positive
		10^{-8}	No amp.	35.93[b]	29.65 ± 0.07	Negative
Tomato	A	NPC	No amp.	No amp.	29.94 ± 0.65	Negative
Tomato	G	NPC	No amp.	No amp.	28.44 ± 0.10	Negative
Tomato	T	NPC	No amp.	No amp.	30.16 ± 0.26	Negative
Rootstock	X	NPC	37.26[b]	No amp.	29.64 ± 0.06	Negative
Rootstock	Y	NPC	No amp.	No amp.	29.48 ± 0.13	Negative
Water		NTC	No amp.	No amp.	No amp.	Negative

[a] Five mg PepMV infected leaf in buffer, used to spike seed.

[b] Includes six or more reactions without amplification.

Conclusion

No matrices effect was observed when using the seed extraction buffer and the Qiagen RNeasy plant mini kit as described in the protocol. For the selectivity validation criterium, the SE-qPCR detection method is fit for purpose.

3.4. Repeatability

Definition ISHI 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 will be met when accordance is >90% and all subsamples yield consistent qualitative results between the three time points.

Experimental approach

One technician using the same batch of material and equipment performed the SE-qPCR as described in Annex A at three different time points with three replicates of four different seed lots with a sample size of 1,000 seeds. The four lots consisted of a healthy seed lot (tomato seed lot A, Chile 2021), low-infected (0.5%), medium-infected (1%) and highly-infected (10%) PepMV seed lot. The low and medium seed lot was made by adding five or ten seeds from naturally infected seed lot SHR218, to 995 or 990 seeds of the healthy seed lot, respectively. The highly infected seed lot was prepared by adding 100 seeds of a second naturally infected seed lot SHR203 to 900 seeds of the healthy seed lot. The samples were tested on the following time points, day 1, day 8 and day 9.

Statistical analysis

The analysis was conducted on a qualitative level per sample, viz. positive or negative results for PepMV. The method of Langton *et al.* (2002) was used to evaluate the accordance (repeatability of data) of the SE-qPCR.

Results

The results of the four different batches tested with three replicates on three different days are compiled in Table 7 and available in Annex E. The healthy batch resulted in no amplification for all nine replicates for the PepMV assays but did give amplification with the PEC. Concluding that this healthy batch is indeed repeatably negative. The three batches with a low, medium or high infection level of PepMV did give for all replicates an amplification for both PepMV assays and both qPCR reaction repetitions. Although there is some natural variation in Cq values between replicates of the same batch, this did not result in a different outcome. All replicates of the three infected batches were repeatable and determined PepMV positive. All batches and their repeats gave the same consistent qualitative (positive or negative) result. The accordance for these batches was calculated as 100%.

Table 7. The repeatability results of four different seed lots with different PepMV infection levels tested on three different days in triplicate. No amp.: no amplification, Rep: replicate.

Infection rate	Time point	SE-qPCR (Cq)						Conclusion
		NAKT05		Bejop		PEC		
		Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	
Healthy	Day 1	No amp.	No amp.	No amp.	No amp.	30.39	30.27	Negative
		No amp.	No amp.	No amp.	No amp.	31.11	30.96	Negative
		No amp.	No amp.	No amp.	No amp.	30.88	31.04	Negative
	Day 8	No amp.	No amp.	No amp.	No amp.	30.59	30.53	Negative
		No amp.	No amp.	No amp.	No amp.	27.15	27.26	Negative
		No amp.	No amp.	No amp.	No amp.	30.68	30.67	Negative
	Day 9	No amp.	No amp.	No amp.	No amp.	30.71	30.50	Negative
		No amp.	No amp.	No amp.	No amp.	30.80	30.63	Negative
		No amp.	No amp.	No amp.	No amp.	30.30	30.14	Negative
0.5	Day 1	29.01	28.73	27.42	27.31	27.42	27.41	Positive
		29.09	29.00	27.56	27.36	28.87	28.77	Positive
		32.65	32.34	30.77	30.57	29.46	29.54	Positive
	Day 8	33.18	33.41	32.81	33.12	30.02	30.25	Positive
		26.40	26.33	26.48	26.32	30.14	30.17	Positive
		31.61	31.91	30.76	30.55	27.48	27.77	Positive
	Day 9	33.20	32.79	32.49	32.47	31.25	31.13	Positive
		26.36	26.32	26.31	26.22	30.18	29.97	Positive
		32.42	32.37	31.90	32.18	30.49	31.06	Positive
1.0	Day 1	30.16	29.81	28.38	28.66	29.77	29.32	Positive
		27.87	27.79	26.59	26.38	30.70	30.96	Positive
		28.90	29.17	27.13	27.17	30.14	30.14	Positive
	Day 8	30.50	30.50	30.13	30.17	31.31	31.03	Positive
		30.78	30.71	29.93	29.62	31.42	31.49	Positive
		31.09	31.02	30.34	30.43	31.59	31.31	Positive
	Day 9	27.31	27.36	26.69	26.62	29.07	29.40	Positive
		30.50	30.38	29.63	29.57	30.59	30.79	Positive
		27.49	27.64	26.10	26.32	31.48	31.89	Positive
10	Day 1	22.84	22.63	21.05	20.96	31.65	31.62	Positive
		26.13	25.76	24.04	23.86	27.20	26.94	Positive
		27.15	27.09	25.15	25.17	31.61	31.30	Positive
	Day 8	22.43	22.28	20.74	20.58	29.79	29.40	Positive
		26.07	25.64	24.20	23.93	31.12	30.05	Positive
		26.03	25.96	24.12	24.13	30.13	30.10	Positive
	Day 9	26.92	26.86	24.99	24.94	31.28	31.33	Positive
		26.09	26.14	24.16	24.13	30.64	31.02	Positive
		26.67	26.52	24.75	24.66	30.61	30.27	Positive

Conclusion

Testing these four batches with different PepMV infection levels by the same technician at three different time points resulted in consistent qualitative results and an accordance of 100%. This is above the 90% that was set as the minimum requirement for this validation criterium. For the repeatability validation criterium, the SE-qPCR detection method is fit for purpose.

3.5. Reproducibility

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

The reproducibility requirements will be met when there are at least six participating laboratories, and the concordance is >90%. In addition, the false negative rate should be 5% or less.

Experimental approach

A comparative test (CT) was organized in ISHI with eight participating laboratories. Each participant received a total of 24 samples of 1,000 seeds each. The 24 samples consisted of four healthy samples from previously described tomato seed lot variety A (chapter 3.3.), six low-infected (0.5%) samples, six medium-infected (1%) samples and eight highly-infected (10%) samples. These samples were artificially created, as described in chapter 3.4. One healthy sample was coded as negative process control (NPC), and one highly infected sample was coded as positive process control (PPC). The remaining 22 samples were randomized and coded "A to V". All participants processed the seed samples according to the protocol described in Annex A.

In addition to the work described for reproducibility, the organizing laboratory of the CT had tested prior to the CT nine samples of 1,000 seeds of each of the seed lots with different infection levels used in the CT for homogeneity testing (the repeatability data in Table 7 of section 3.4 was used) and one CT set of 24 samples after receiving the results from all participants for stability testing. These data provided stability and homogeneity data of the samples.

Statistical analysis

The analysis was done on a qualitative level per sample, viz., positive or negative results for PepMV. The method of Langton *et al.* (2002) was used to evaluate the concordance (reproducibility of data) of the SE-qPCR.

Homogeneity results

To determine homogeneity of the samples used for the CT the organizing laboratory tested nine samples of 1,000 seeds of each of the four different PepMV infection rates, healthy (0%), 0.5%, 1% and 10% infected seeds, following the protocol as described in Annex A. The results of these 36 samples are shown in Figure 3. The raw data is shown in Table 7, Section 3.4.

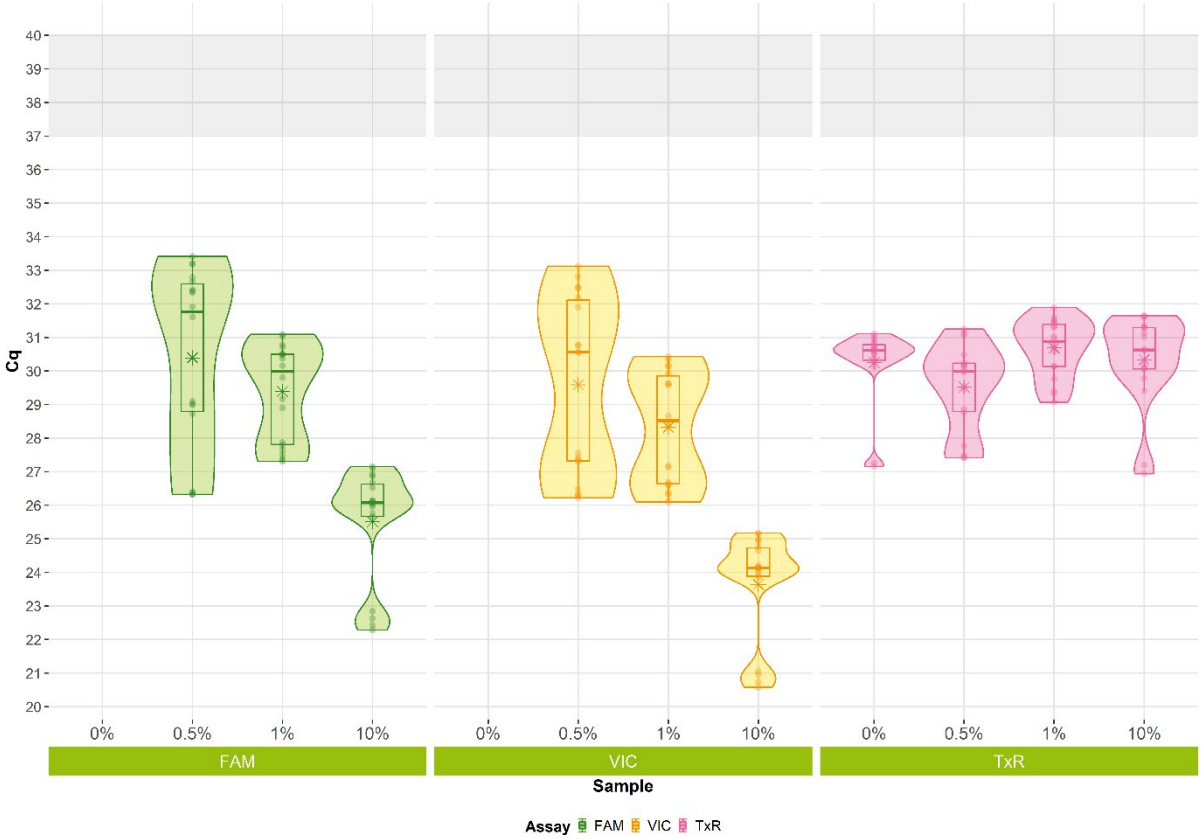

Figure 2. Combined boxplot and violin plot (kernel density distributions) of Cq values obtained by the PepMV triplex SE-qPCR using BaCV as positive extraction control. FAM: NAKT05 assay, VIC: Bejop assay, TxR: BaCV (PEC) assay. *: Average Cq value.

Comparative test results

The data of the eight participating laboratories were combined and qualitatively shown in Table 8, and the quantitative data of each laboratory is presented in Annex E.

The CT results of participants 1, 2, 5 and 6 are 100% in line with the expected results.

Participant 4 did not detect one sample with an infection rate of 0.5% (sample S). The question is if this participant missed the PepMV in this sample or that they received a sample from this lot without PepMV positive seeds, because the infection rate of the used lot was not 100% but 96.67%. According to random distribution, the probability of selecting five uninfected seeds from the used seed lot could lead to some of the 0.5% samples not containing any infected seeds. For the concordance calculations, the outcome of this sample will be seen as a missed PepMV detection (false negative).

Table 8. The qualitative results of all participating laboratories in random anonymous order. NPC: negative process control, PPC: positive process control.

Infection rate	Sample name		Lab 1	Lab 2	Lab 3a/3b	Lab 4	Lab 5	Lab 6	Lab 7[a]	Lab 8[a]
10%	1	A	Pos	Pos	Pos/Pos	Pos	Pos	Pos[b]	Pos[b]	Pos
	4	D	Pos	Pos	Pos/Pos	Pos[b]	Pos	Pos	Pos[b]	Pos
	7	G	Pos	Pos	Pos/Pos	Pos	Pos	Pos	Pos[b]	Pos
	12	L	Pos	Pos	Pos/Pos	Pos	Pos	Pos[b]	Pos[b]	Pos[b]
	14	N	Pos[b]	Pos	Pos/Pos	Pos[b]	Pos	Pos[b]	Pos[b]	Pos[b]
	20	T	Pos	Pos	Pos/Pos	Pos	Pos	Pos[b]	Pos[b]	Pos[b]
	21	U	Pos	Pos	Pos/Pos	Pos	Pos	Pos[b]	Pos[b]	Pos
	24	PPC	Pos	Pos	Pos/Pos	Pos	Pos	Pos[b]	Pos[b]	Pos[b]
1.0%	2	B	Pos	Pos	Pos/Pos	Pos	Pos	Pos	Pos[b]	Pos[b]
	6	F	Pos	Pos	Pos/Pos	Pos	Pos	Pos	Pos[b]	Pos
	10	J	Pos	Pos	Pos/Pos	Pos	Pos	Pos	Pos[b]	Pos[b]
	13	M	Pos	Pos	Pos/Pos	Pos	Pos	Pos	Pos[b]	Pos[b]
	18	R	Pos	Pos	Pos/Pos	Pos	Pos	Pos	Failed	Pos[b]
	22	V	Pos	Pos	Pos/Pos	Pos	Pos	Pos	Pos[b]	Pos[b]
0.5%	5	E	Pos	Pos	Pos/Pos	Pos	Pos	Pos	Pos[b]	Pos
	8	H	Pos	Pos	Pos/Pos	Pos	Pos	Pos	Pos[b]	Neg
	11	K	Pos	Pos	Pos/Pos	Pos	Pos	Pos	Failed	Pos[b]
	15	O	Pos	Pos	Pos/Pos	Pos	Pos	Pos	Failed	Failed
	16	P	Pos	Pos	Neg/Pos	Pos	Pos	Pos	Failed	Failed
	19	S	Pos	Pos	Pos/Pos	Neg	Pos	Pos	Failed	Failed
0%	3	C	Neg	Neg	Neg/Neg	Neg	Neg	Neg	Failed	Neg
	9	I	Neg	Neg	Neg/Neg	Neg	Neg	Neg	Failed	Neg
	17	Q	Neg	Neg	Neg/Neg	Neg	Neg	Neg	Failed	Failed
	23	NPC	Neg	Neg	Neg/Neg	Neg	Neg	Neg	Failed	Failed

[a] Excluded from further analysis

[b] Would be a failed test result (no signal or above Cq ⩾33 for PEC) if it had not been concluded PepMV positive.

Participant 3(a) also missed one sample with an infection rate of 0.5% (sample P), but it was also noticed that the Cq values for all samples for both PepMV assays (and not the PEC) were, on average, 6 Cq higher than the results of the other participants. The organizer of the CT contacted participant 3 to determine the cause of this Cq difference in PepMV. After several tests, participant 3 concluded that the results were caused by a specific batch of PepMV primers. After replacing the primers with a new batch and processing the RNA samples again, all their new results were 100% in line with the organizers expected results (Annex E, 3.5 Reproducibility Data, lab 3). All Cq-values were lowered with ~6 Cq, which caused seed sample P to be detected as positive while it was detected negative in the first run. The change of primer batch did not result in false positive results of the negative seed lot samples C, I, Q and the NPC. Since the cause of the difference in Cq values between participant 3 and the other participants was identified and the problems solved, the results of the repeated experiment from participant 3 (3b) were used for the concordance calculation.

Due to the fact that all samples of participant 7 gave a failed test result, including no signal for PEC and the NPC, they contacted the organizer of the CT. After consultation between the organizer and participant 7, an additional test on their RNA samples was conducted. A selection of the RNA samples was additionally tested with the NAD5 assay (Menzel *et al*., 2002), which can detect endogenous tomato RNA. The organizing laboratory was able to detect NAD5 with Cq values between 25 and 28 on their own extracted RNA, while participant 7 did not obtain any amplification signal with their RNA. This is an indication that the RNA-extraction of the samples of participant 7 failed and not specifically the qPCR. Although the exact reason for the failure could not be explained, it was decided to exclude participant 7 for further analysis.

Participant 8 also had some issues with the PEC. Although all samples gave a PEC amplification signal, the average PEC value was 33.17, with some extreme outgroup values of 26.70 and 37.82. The standard deviation value for participant 8 was 2.44, while for the other participants (1 to 6), this value was below 1. Meaning the other laboratories obtained reproducible PEC results with their samples. As participant 8 detected an important number of failed samples, including the NPC, the participant questioned their results and contacted the organizer of the CT. The PepMV Cq values were also 3 to 6 Cq higher as expected. To investigate whether the problem was the RNA-extraction or the qPCR, participant 8 and 2 exchanged RNA of their CT material and repeated the qPCR experiment on this RNA. Participant 2 obtained comparable results on the RNA received from participant 8 as the original results of participant 8. The PEC values were higher when tested by participant 2. The average value of the PEC was 37.25 instead of the 33.17, but the standard deviation of all the PEC values was 0.95, which is a better reproducibility than the original value of 2.44 of participant 8. When participant 8 tested again the RNA provided by participant 2, their qPCR results for PepMV were as expected. The PEC-Cq values were 2 to 3 Cq higher than the original data from participant 2, inducing some FAILED results (PEC > Cq 33) for some of the negative samples and the NPC. The fact that participant 2 also had some increased PEC results in this additional experiment suggests that shipment on dry ice negatively affected PEC RNA, while this was not the case for the PepMV RNA. The experiment showed that the qPCR test of participant 8 was not the cause of their failed results for their samples but rather suggested an issue with the extraction of the samples. Therefore, it was decided to exclude participant 8 from the concordance calculations.

Based on the results of participants 1 to 6 (six participants was also the minimum stated in the validation requirements), the concordance of this CT was calculated to be 98.6%. The percentage of false negatives obtained in the 10% and 1.0% infection level samples was 0%. The percentage of false negatives in the 0.5% infection level samples was 2.8%. The percentage of false negatives detected in all expected positive samples was 0.7%. No false positives were found.

Conclusion

It was stated that the reproducibility requirements will be met when performance criteria (concordance) are >90%, while the false negative rate remains below 5%. Our results show that the concordance percentage is 98.6% and the false negative rate is 0.7%, meaning that both requirements for passing the reproducibility criterium were met.

3.6. Diagnostic performance

Definition ISHI guidelines: *An evaluation of the ability of the method to discriminate between positive and negative seed lots*.

As the SE-qPCR assay precedes the bioassay with ELISA confirmation in the method workflow, the diagnostic sensitivity (no false negatives) is more important than the diagnostic specificity (no false positives), as false positives will be detected by the bioassay.

The diagnostic performance requirements will be met when positive samples with the ELISA pre-screen assay or the bioassay with ELISA confirmation method (ISF 2021) also yield a positive result with the SE-qPCR. In case of a negative result by bioassay with ELISA confirmation method, a positive result with SE-qPCR is still possible due to non-infectious PepMV presence. The diagnostic performance requirements will therefore be met when diagnostic sensitivity reaches 100% and the diagnostic specificity is ⩾ 90% when comparing the two indirect assays, SE-qPCR and ELISA.

Experimental approach

Comparative data was generated by one laboratory in which 21 seed lots from different origins, infection levels and production years (Table 10) were tested by SE-qPCR as well as by ELISA assay (Annex F). All suspected lots identified as positive by either SE-qPCR or by ELISA assay were also tested by bioassay (Annex G). The SE-qPCR results of the diagnostic performance will be based on the SE-qPCR data obtained from an alternative described protocol, meaning pooling 25 µL of four different 250-seed subsamples resulting in a 100 µL sample for extraction, as described in Annex A paragraph 1.2. By doing so, a comparison of the same seed subsamples between the three methods was possible. The previously described non-infected beef tomato variety A produced in Chile 2021 was used as NPC. The PPC was prepared by grinding naturally infected tomato leaf material in 1 mL of the NPC seed extract.

Statistical analysis

The analysis was carried out on a qualitative level per sample, viz. positive or negative results for PepMV. The diagnostic sensitivity and diagnostic specificity were calculated according to the formulas presented in Table 9.

Table 9. The formulas used for diagnostic sensitivity and diagnostic specificity calculation.

		Validated method result / independent assessment	
		Positive	**Negative**
Test outcome	**Positive**	True positive (TP)	False positive (FP)
	Negative	False negative (FN)	True negative (TN)
		DIAGNOSTIC SENSITIVITY = (TP / (TP + FN)) × 100%	**DIAGNOSTIC SPECIFICITY = (TN / (FP + TN)) × 100%**

Results

The 21 seed lots were tested by ELISA and SE-qPCR and the suspect samples as detected by either ELISA or SE-qPCR were further processed by bioassay. The results of these 21 seed lots are shown in Table 10 and the raw data is provided in Annex E. From the 21 seed lots tested, eight were ELISA positive, nine qPCR positive, and none of the seed lots were bioassay positive. The PPC was ELISA, SE-qPCR and bioassay positive, while the NPC was negative for all three tests. The reason for negative bioassay results for all ELISA and/or qPCR positive seed lots may be the absence of infectious virus particles. Based on these results, including the PPC and NPC, the diagnostic sensitivity and diagnostic specificity were calculated for the SE-qPCR in comparison to the ELISA results. The diagnostic sensitivity was 100% and the diagnostic specificity was 92.86%.

Table 10. Diagnostic performance data of the ELISA, qPCR and Bioassay. NPC: negative process control, nt: not tested, PPC: positive process control.

Crop	Sample code	Production		Positive subsamples/total subsamples			Qualitative conclusion		
		Country	Year	ELISA	SE-qPCR	Bioassay	ELISA	qPCR	Bioassay
Rootstock	SHR039	Chile	2015	0/12	3/3	0/12	Neg	Pos	Neg
Tomato	SHR126	Chile	2003	5/12	3/3	0/12	Pos	Pos	Neg
Tomato	SHR202	Tanzania	2005	12/12	3/3	0/12	Pos	Pos	Neg
Rootstock	SHR203	Chile	2006	12/12	3/3	0/12	Pos	Pos	Neg
Tomato	SHR218	Spain	2018	1/12	3/3	0/12	Pos	Pos	Neg
Tomato	SHR219	Spain	2018	10/12	3/3	0/12	Pos	Pos	Neg
Tomato	SHR302[a]	Netherlands	2022	7/7	2/2	0/7	Pos	Pos	Neg
Tomato	SHR303[a]	Netherlands	2022	10/10	3/3	0/10	Pos	Pos	Neg
Tomato	SHR304[a]	Netherlands	2022	7/7	2/2	0/7	Pos	Pos	Neg
Rootstock	SHR601	Chile	2022	0/12	0/3	0/12	Neg	Neg	nt
Rootstock	SHR602	Chile	2022	0/12	0/3[b]	0/12	Neg	Neg	nt
Tomato	SHR603	Netherlands	2020	0/12	0/3	0/12	Neg	Neg	nt
Tomato	SHR604	Peru	2019	0/12	0/3	0/12	Neg	Neg	nt
Tomato	SHR605	Mexico	2020	0/12	0/3	0/12	Neg	Neg	nt
Tomato	SHR606	Peru	2020	0/12	0/3	0/12	Neg	Neg	nt
Tomato	SHR607	Peru	2019	0/12	0/3	0/12	Neg	Neg	nt
Tomato	SHR608	Netherlands	2021	0/12	0/3	0/12	Neg	Neg	nt
Tomato	SHR609	Chile	2021	0/12	0/3	0/12	Neg	Neg	nt
Tomato	SHR610	Netherlands	2022	0/12	0/3	0/12	Neg	Neg	nt
Tomato	SHR611	Mexico	2022	0/12	0/3	0/12	Neg	Neg	nt
Tomato	SHR612	Peru	2021	0/12	0/3	0/12	Neg	Neg	nt
Tomato	PPC			4/4	4/4	4/4	Pos	Pos	Pos
Tomato	NPC			0/4	0/4	0/4	Neg	Neg	Neg

[a] The total amount of available seeds was below 3,000.

[b] PEC was above Cq 33, namely >37 for all three subsamples; therefore, no conclusion can be made for this seed sample.

Conclusion

It was stated that the diagnostic performance requirements will be met when diagnostic sensitivity is 100% and the diagnostic specificity is ⩾ 90%. Our results show that the diagnostic sensitivity reached 100% and the diagnostic specificity was 92.86% for qPCR *vs.* ELISA. This means that both requirements for passing the diagnostic performance criteria are met.

3.7. Robustness

Definition ISHI guidelines:

Robustness is not defined in the ISHI guidelines but was defined in this validation report as the capacity of a method to remain unaffected by small, deliberate variations in method parameters. This allows for the comparison of data in the case of an alternative extraction method as the one described in the protocol validated in this report.

Experimental approach

Next to the standard protocol using PBS for RNA extraction that allows inoculation of the seed extract on an indicator plant, an additional set of 24 samples were tested using guanidine-based lysis buffer. The same set of samples as used for the CT as described in the reproducibility Section 3.5., was extracted using an alternative extraction method and compared to the quantitative and qualitative CT results of the laboratory performing this experiment. Samples were dry ground in a 50 mL conical tube with a 14 mm bullet inside using a GenoGrinder 2000 at 1300 rpm for 3 min. A volume of 20 mL of a guanidine-based lysis buffer spiked with BaCV was added to each tube of ground seeds and homogenized by vortexing. The seed extracts were incubated for 30 min at room temperature (~20 °C) and afterwards centrifuged at 1,000 × *g* for 1 min. From each of the tubes, 1 mL seed extract was mixed with 50 µL of 500 mM TCEP solution (catalogue number C4706, Sigma-Aldrich), which was used as an alternative for β-mercaptoethanol, and incubated for 15 min at 65 °C and shaken at 850 rpm in a thermomixer. After incubation, 200 µL of this extract was used for RNA extraction using a KingFisher platform following the supplier's protocol for the Sbeadex maxi plant kit. The RNA was eluted in a final volume of 100 µL elution buffer. The RNA samples were tested in two repetitions with the same qPCR mixture and using the protocol as described in Annex A.

Statistical analysis

The analysis were done on a quantitative and qualitative level per sample*, viz*. positive or negative result for PepMV.

Results

The results of the two extraction methods executed on two different comparative test sample sets were compared and the average Cq values of the replicates are shown in Table 11. Both methods were able to detect all samples with infected PepMV seeds. Both methods did not result in false positive results.

The samples with the same infection level contain natural variation; therefore, it is expected to observe variation between methods with the same sample name. For the comparison, the average Cq value of all the samples with the same infection rate was calculated and compared. The guanidine KingFisher method reached an average Cq value of 19.22, 23.06 and 24.32 with NAKT05 and 17.85, 23.09 and 24.56 with Bejop assay for the 10%, 1% and 0.5% infection level, respectively. The method validated in this report using the described seed extraction buffer and Qiagen kit as presented in Annex A obtained an average Cq value of 25.01, 29.26 and 30.63 with NAKT05 and 22.87, 28.61 and 29.52 with Bejop assay for the 10%, 1% and 0.5% infection level, respectively. This means that the Cq values of the guanidine KingFisher method are 5 to 6 Cq lower with this test set, which correlates to increased sensitivity by 32 to 64 times. The four samples with no infected PepMV seeds obtained for both methods Cq values >37 (cut-off value for positive/negative outcome) or a no amplification and are determined PepMV negative.

Table 11. Average PepMV quantitative results of two extraction methods on two comparative test sample sets tested by the same participant. NPC: negative process control, No amp: no amplification, PPC: positive process control.

Infection rate	Sample name	Average Cq values					
		Guanidine KingFisher			Seed Extraction Buffer Qiagen		
		NAKT05	Bejop	PEC/IAC	NAKT05	Bejop	PEC/IAC
10%	A	22.08	20.75	28.62	25.25	23.29	31.20
	D	21.68	20.47	28.95	26.61	24.64	30.40
	G	16.49	15.35	29.37	26.67	24.32	31.04
	L	15.93	14.40	29.49	22.20	20.24	32.16
	N	17.96	16.56	29.16	25.29	23.12	31.19
	T	15.81	14.41	29.29	25.86	23.55	30.94
	U	21.67	20.31	29.00	23.96	21.62	31.37
	PPC	22.14	20.59	28.54	24.25	22.18	31.62
1%	B	24.78	25.07	28.73	27.60	26.74	29.54
	F	24.79	25.09	28.95	30.06	28.61	32.07
	J	23.38	23.37	28.71	28.75	27.79	31.27
	M	22.46	22.17	29.02	29.93	29.50	30.89
	R	21.90	21.91	29.04	26.50	26.18	30.43
	V	21.06	20.96	28.88	32.71	32.85	31.34
0.50%	E	23.74	23.55	27.59	27.90	26.89	30.76
	H	20.40	20.58	29.02	34.95	32.72	31.65
	K	26.94	27.41	29.09	30.28	28.99	30.88
	O	27.45	27.44	28.89	29.27	28.26	30.40
	P	25.53	25.94	28.18	29.51	28.69	31.20
	S	21.87	22.43	28.18	31.89	31.59	30.55
0%	C	38.56	37.50	29.06	No amp.	No amp.	30.23
	I	No amp.	No amp.	27.89	No amp.	No amp.	30.87
	Q	No amp.	No amp.	29.57	No amp.	No amp.	30.53
	NPC	No amp.	No amp.	29.09	No amp.	No amp.	30.25

Conclusion

The two compared extraction methods show differences in the quantitative results for the PepMV infected samples in which the guanidine extraction method was ~64 times more sensitive. The healthy samples raised a negative outcome for both extraction methods. This shows that the increased sensitivity does not lead to an increase in false positive results. Both methods obtained the same qualitative results. All samples expected to be positive were positive and the negative samples negative. The diagnostic performance criterium showed that the SE-qPCR with seed extraction buffer was already more sensitive than ELISA. Therefore, despite the improved sensitivity, there is no substantiated necessity to use the guanidine-based extraction method to prevent false negative results.

4. CONCLUSION

The data presented in this validation report shows that the SE-qPCR detection assay for PepMV in samples of tomato seed meets the requirements set for the validation criteria analytical specificity, analytical sensitivity, selectivity, repeatability, reproducibility, and diagnostic performance. It is concluded that the ISHI PepMV SE-qPCR assay is fit for its intended purpose to serve as an optional pre-screening assay for the ISHI PepMV bioassay.

5. REFERENCES

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6. ANNEXES

Annex A. Protocol for detecting *Pepino mosaic virus* (PepMV) in tomato seed by SE-qPCR

PRE-SCREEN BY SEED EXTRACT RT-qPCR

For PCR methods, in-house method optimization is often necessary, see Best Practices for PCR Assays in Seed Health Tests for information.

Materials

- Seed extraction buffer (Table A.1)
- Grinder
- RT-qPCR mix, primers (Table A.2) and equipment
- Controls (Table A.3)
- Spike solution (see description below)
- RNA purification kit and equipment
- 1.5 mL RNase Free tube
- RNAse free water
- Centrifuge

Sample Size

The recommended minimum sample size is 3,000 seeds with a maximum subsample size of 1,000 seeds.

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 seed extraction buffer (Table A1). The extract is diluted to obtain a suitable concentration usable as positive extraction control (PEC) 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 PepMV primers in a multiplex PCR.

Table A.1. Seed extraction buffer.

Compound	Amount/L
NaCl	8.0 g
$Na_2HPO_4.7H_2O$	2.17 g
KH_2PO_4	0.2 g
KCl	0.2 g
Na_2SO_3[a]	1.0 g

[a] Add after autoclaving. Use within 24 hours of preparation.

Note: If a different seed extraction buffer is used, it must be verified in a comparison using uniform positive control material. The use of a different seed extraction buffer can lead to a different limit of detection and could result in increased number of false positive or false negative outcomes.

Table A.2. Primer and probe sequences and references.

Name	Sequence (5' – 3')	Source
Bejop 133	ATC AAT TGT CCT TAT GCG CT	Bejo Zaden B.V., Netherlands
Bejop 134	ATC AAT TGT CCT TAC GCG CT	
Bejop 135	ATC AAT TGT CCC TAT GCG CT	
Bejop 136	ATC AAC TGT CCT TAT GCG CT	
Bejop 137	VIC – ACA CCC TTG AGA ATC TTG GTG TCA CAA T – BHQ1	
Bejop 138	VIC – ACA CCC TTG AGA ATT TAG GTG TCA CAA T – BHQ1	
Bejop 139	GTT TGA ATT GCA TGA GGG TT	
Bejop 140	TTG GAT TGC ATG GGG RTT	
KL05_48	ACT CCT AGA GCT GAC CTC AC	Ling *et al.,* 2007
KL05_49	ACT CCT AGA GCT GAT CTT AC	
KL05_51	TCT CCA GCA ACA GGT TGG TA	
KL05_52	TCA CCT GCA ACT GGT TGA TA	
NAKT05_50	6FAM – TGT CAG CTT GCA TTT ACT TC – MGB NFQ	Naktuinbouw[a], Netherlands
NEC BaCV F	CGA TGG GAA TTC ACT TTC GT	Naktuinbouw, Netherlands
NEC BaCV R	AAT CCA CAT CGC ACA CAA GA	
NEC BaCV-P	TxR – CAA TCC TCA CAT GAT GAG ATG CCG – BHQ2	

[a] Adapted from Ling *et al.*, 2007.

Table A.3. Types of controls used.

Control type	Description
Negative process control (NPC)	Tomato seed-free of PepMV
Positive process control (PPC)	Tomato seed with infectious PepMV
Positive amplification control (PAC)	PepMV RNA aiming for a Cq (Cycle quantification) value between 28 and 32 *or*
	PepMV oligo DNA (oligonucleotide (single-stranded DNA) for all PepMV target sequences) aiming for a Cq value between 28 and 32 *or*
	PepMV cDNA aiming for a Cq value between 28 and 32
Positive extraction control (PEC)	Spike solution added to the simple 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 buffers, as a strong 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. Seed extraction

1.1. Add the positive extraction control (PEC) to the seed extraction buffer (Table A.1).

1.2. Soak the 1,000 seeds of each subsample, the positive process control (PPC), and the negative process control (NPC) in 40 mL seed extraction buffer containing the PEC for 30 to 60 min.

Macerate the samples for 90 sec using an Interscience BagMixer or equivalent equipment. See Figure A.1 for before and after macerating.

Notes: In case of a positive result when using this option, a fresh seed extract from a new sample of 3,000 seeds in subsamples of 250 seeds is required for the bioassay.

Alternatively, grind 12 subsamples of 250 seeds directly in 10 mL seed extraction buffer containing the PEC.

1.3. Process extracts immediately after grinding, place on ice for up to 1 hour or store at -20 °C for one day. Do not freeze if the extracts are to be used for the bioassay after RT-qPCR, but store at 4 to 7 °C. The bioassay must be completed within 20 hours after extraction. Store the PPC and NPC (Table A.3) under similar conditions as the samples to validate the results.

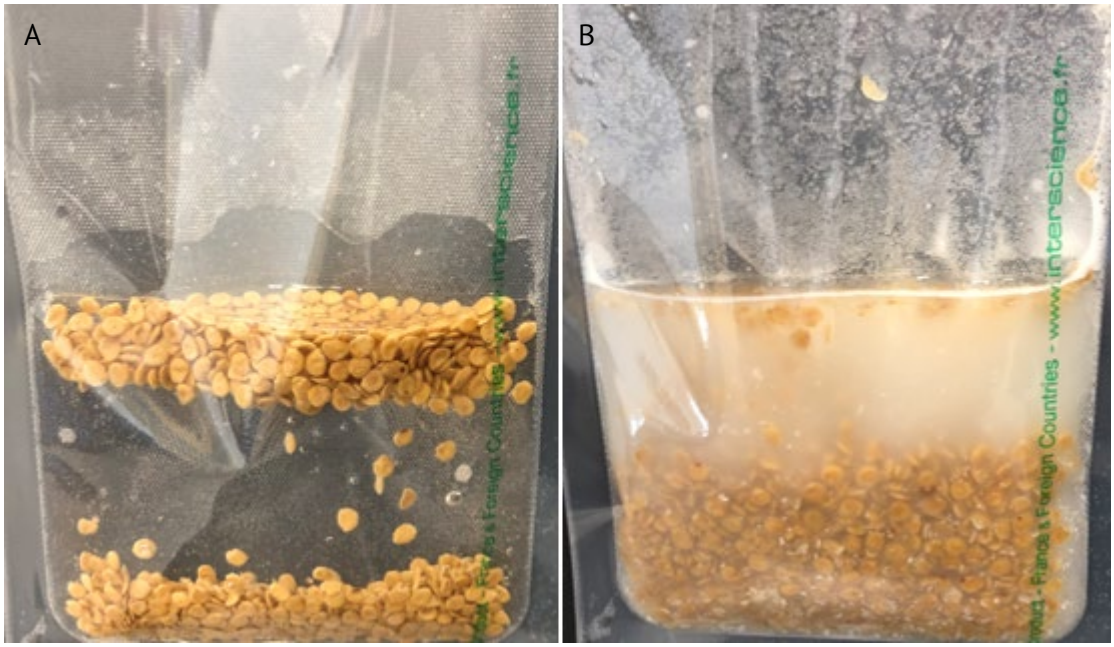

Figure A.1. A. 1,000 tomato seeds in seed extraction buffer after 30 min soaking. B. 1,000 tomato seeds after 90 sec of macerating with BagMixer.

2. RNA extraction

2.1. In case of 1,000 seed subsamples, use 100 µL from each subsample for further analysis.

In the case of 250 seed subsamples, combine 25 µL of four seed extract subsamples into a 100 µL combined sample. Use all three combined samples for further analysis.

2.2. Start RNA isolation within 1 hour after grinding.

2.3. Use the commercial RNA isolation kit RNeasy plant mini kit (Qiagen) for RNA isolation. Process the subsamples according to the supplier's instructions.

2.4. Eluate the RNA in 100 µL elution buffer.

3. Preparation of the RT-qPCR

3.1. Prepare the RT-qPCR mixture as indicated in Table A.4.

For each run, include a negative template control (NTC) and at least one positive amplification control (PAC) that give a Cq value between 28 and 32.

3.2. Perform the PCR reaction in a real-time PCR instrument according to the PCR conditions mentioned in Table A.5. All samples and controls should be tested in duplicate as described in Best Practices for PCR Assays in Seed Health Tests.

Note: Good results have been obtained by ISHI member laboratories with the UltraPlex™ 1-Step ToughMix (QuantaBio). If different qPCR mixtures and amplification programs are used, it is necessary to verify their performance.

Table A.4. RT-qPCR PepMV mix.

Component	Per reaction (in µL)	Final concentration
Bejop 133 (10 µM)	0.25	0.1 µM
Bejop 134 (10 µM)	0.25	0.1 µM
Bejop 135 (10 µM)	0.25	0.1 µM
Bejop 136 (10 µM)	0.25	0.1 µM
Bejop 137 (10 µM)	0.50	0.2 µM
Bejop 138 (10 µM)	0.50	0.2 µM
Bejop 139 (10 µM)	1.50	0.6 µM
Bejop 140 (10 µM)	1.50	0.6 µM
KL05_48 (10 µM)	0.50	0.2 µM
KL05_49 (10 µM)	0.50	0.2 µM
NAKT05_50 (10 µM)	0.50	0.2 µM
KL05_51 (10 µM)	0.50	0.2 µM
KL05_52 (10 µM)	0.50	0.2 µM
NEC BaCV F (10 µM)	0.3125	0.125 µM
NEC BaCV R (10 µM)	0.3125	0.125 µM
NEC BaCV-Probe (10 µM)	0.156	0.0625 µM
Ultraplex 1-step Toughmix (4×)	6.25	1×
RNAse free water	5.469	
Template RNA	5.0	
Total	25.00	

Table A.5. RT-qPCR conditions.

Step	Temperature	Duration
RT reaction	50 °C	10 min
Denaturation	95 °C	3 min
39 cycles	95 °C	10 sec
	60 °C	60 sec

4. Interpretation and decisions

Determine the cut-off values. Cut-off values must be established by each laboratory for their positive and internal amplification controls prior to the assay being used on routine samples. For recommendations on setting cut-off values, see Real-time PCR pre-screening in seed health methods.

In the validation study a SE-qPCR result was regarded positive with a Cq value of ⩽ 37, and negative with a Cq value > 37.

For interpretation and decision making, the results from all primer sets need to be taken into account, see Table A.6. Test results are only valid when all included controls presented in Table A.3 give the expected results.

Table A.6. Interpretation and decision table for the SE-qPCR.

Bejop	NAKT_05	BaCV (PEC)	qPCR Result	Follow-up
Positive	Positive	Positive or Negative	Target RNA for PepMV detected	Bioassay for confirmation
Negative	Negative	Positive	No target RNA for PepMV detected	Negative, no follow up
Negative	Negative	Negative	PEC/IAC failure	Repeat Extraction and/or RT-qPCR. In case of repeatable results, no conclusion can be given for this sample by PCR. Continue with ELISA and/or Bioassay
Positive	Negative	Positive or Negative	Target RNA for PepMV detected	Bioassay for confirmation
Negative	Positive	Positive or Negative	Target RNA for PepMV detected	Bioassay for confirmation

Annex B. Analytical specificity data from in-silico data analysis

Table B.1. Rate of identity of a representative selection of PepMV strains from NCBI with the ISHI PepMV SE-qPCR primers and probes.

References ID	Isolate Name	Origin	Collection date	Host	Bejop			NAKT05		
					Fw	Pr	Rv	Fw	Pr	Rv
NC004067	Sp-13	Spain	<2002	*Solanum lycopersicum*	100	100	100	100	100	100
AY509926	US1	USA	<2004	*Solanum lycopersicum*	100	100	100	100	100	100
AY509927	US2	USA	<2004	*Solanum lycopersicum*	100	100	100	100	100	100
DQ000985	Ch2	Chili	2003	*Solanum lycopersicum*	100	100	100	100	100	100
HQ663890	SAR09	Italy	2009	*Solanum lycopersicum*	95	100	100	100	100	100
MK133092	PepMV-P12-3G	Germany	2018	*Solanum lycopersicum*	100	96.4	100	100	100	100
HQ650560	P22	Poland	2005	*Solanum lycopersicum*	100	96.4	100	100	100	100
MN549397	Ca1A	Canada	2019	*Solanum lycopersicum*	100	100	95	100	100	100
HG976946	CAM1-IT	Italy	2010	*Solanum lycopersicum*	100	100	100	95	100	100
MN038407	MZ2	Spain	2014	*Solanum lycopersicum*	100	100	100	100	90	100
JQ314461	EU_EF09_60	USA	2009	*Solanum lycopersicum*	100	100	100	100	100	95
HG313805	Chi2.9	Peru	2008	*Solanum pimpinellifolium*	100	100	100	95	100	90
HG313806	Tor9	Peru	2008	*Solanum peruvianum*	100	100	100	95	100	90
HG313807	Yur1.5	Peru	2008	*Solanum peruvianum*	100	96.4	100	95	100	90
MN038405	MZ1	Spain	2014	*Solanum lycopersicum*	95	100	100	100	90	100
HQ663891	SIC1-09	Italy	2009	*Solanum lycopersicum*	95	100	100	95	100	100
AJ606361	LP-2001	Peru	2000	*Solanum peruvianum*	100	100	94.4	100	95	100
AM109896	SM74	Peru	1974	*Solanum muricatum*	100	100	94.4	100	95	100
MF422612	LP_BP0161	Switzerland	2014	*Solanum lycopersicum*	100	100	94.4	100	95	100

Annex C. Sequences of the synthetic ssDNA PepMV oligonucleotides

PC_Bejop

CTTAAAGAATCTCTAATCAATTGTCCTTACGCGCTTAATGATTATGAAGCAGACACCCTTGAGAATCTTGGTGTCACAATTAACCCCCATGCAATCCAAACACACACACATG
CCGC

PC_NAKT05

TGCCAGGTCTAACTCCTAGAGCTGACCTCACTGACACATACAAAATCATTGCCATTGCTTTCTTGTTGTCAGCTTGCATTTACTTCCAAAATAGCCACTACCAACCTGTTGC
TGGAGACAACTTGCACCGT

PC_NAKT05m

TGCCAGGGCTGACCCCTAGAGCTGACCTCACTGACACATACAAGATAATTGCAATTGCCTTCCTGTTGTCAGCCTGCATTTACTTCCAGAACAGCCACTACCAACCAGTTGC
TGGGGACAACTTGCATAGA

Annex D. Analytical specificity data from leaf and/or seed samples

Table D.1. Analytical specificity data from leaf and/or seed samples from various origins infected with PepMV. No amp.: no amplification, Rep.: replicate.

Isolate name	Genotype[b]	Origin	Year	Tissue type	Host	NAKT05		Bejop		BaCV	
						Rep. 1	Rep. 2	Rep. 1	Rep. 2	Rep. 1	Rep. 2
0148-10	EU	Netherlands	2010	Leaf	*Solanum lycopersicum*	20.09	20.17	19.47	19.56	No amp.	No amp.
0149-10	LP	Netherlands	2010	Leaf	*Solanum lycopersicum*	27.04	27.03	N/A	N/A	No amp.	No amp.
0004-11	LP	Netherlands	2011	Leaf	*Solanum lycopersicum*	15.39	15.33	14.79	14.74	No amp.	No amp.
0709-11	CH2	Netherlands	2011	Leaf	*Solanum lycopersicum*	13.17	13.16	12.45	12.26	No amp.	No amp.
0776-11	CH2	Spain	2011	Leaf	*Solanum lycopersicum*	15.07	14.90	13.98	13.96	No amp.	No amp.
0773-14	CH2	Hungary	2014	Leaf	*Solanum lycopersicum*	24.81	24.97	23.37	23.46	No amp.	No amp.
0127-15	CH2	Italy	2015	Leaf	*Solanum lycopersicum*	21.17	21.50	19.56	20.01	No amp.	No amp.
0615-16	CH2	Canada	2016	Leaf	*Solanum lycopersicum*	17.89	17.67	17.10	17.24	No amp.	No amp.
0647-16	CH2	Chili	2016	Leaf	*Solanum lycopersicum*	17.56	17.29	17.27	17.20	No amp.	No amp.
0245-17	CH2	South Africa	2017	Leaf	*Solanum lycopersicum*	15.01	14.91	14.98	14.88	No amp.	No amp.
0938-17	CH2 and LP	UK	2017	Fruit	*Solanum lycopersicum*	13.22	13.13	12.82	12.74	No amp.	No amp.
1340-18	CH2	Slovakia	2018	Leaf	*Solanum lycopersicum*	12.87	12.89	12.08	11.99	No amp.	No amp.
7739-20	LP	Brazil	2020	Leaf	*Solanum lycopersicum*	27.75	27.81	27.30	27.33	No amp.	No amp.
8319-21	CH2	France	2021	Leaf	*Solanum lycopersicum*	10.35	10.63	9.16	9.03	No amp.	No amp.
8564-21	CH2	New Zealand	2021	Leaf	*Solanum lycopersicum*	21.15	21.26	19.29	19.34	No amp.	No amp.
8934-21	US1	Canada	2021	Leaf	*Solanum lycopersicum*	15.15	15.36	13.73	13.70	No amp.	No amp.
9214-21	CH2 and LP	Poland	2021	Leaf	*Solanum lycopersicum*	11.74	11.85	11.04	11.06	No amp.	No amp.
DB1-10	EU	Netherlands	2010	Leaf	*Solanum lycopersicum*	19.51	19.57	18.05	18.12	No amp.	No amp.
SHR030	US1	Chili	2015	Seed	*Solanum lycopersicum*	26.47	26.43	24.84	24.82	No amp.	No amp.
SHR125	US1	Chili	2006	Seed	*Solanum lycopersicum*	28.15	28.34	26.44	26.63	No amp.	No amp.
SHR126	US1	Chili	2003	Seed	*Solanum lycopersicum*	23.84	23.46	19.33	19.12	No amp.	No amp.
U287-21	CH2	Unknown	2021	Seed	*Solanum lycopersicum*	27.67	27.98	27.03	27.08	No amp.	No amp.
Tor-9[a]	PES	Peru	2008	Leaf	*Solanum lycopersicum*	22.79	22.88	20.12	20.20	No amp.	No amp.
CHI 2.9[a]	PES	Peru	2008	Leaf	*Solanum lycopersicum*	21.72	21.65	18.76	18.69	No amp.	No amp.
YUR 1.5[a]	PES[c]	Peru	2008	Leaf	*Solanum lycopersicum*	24.46	24.32	No amp.	No amp.	No amp.	No amp.

[a] RNA provided by Dr. Fernando García-Arenal Rodríguez (Plant-Virus Interaction and Co-evolution Research Group at Centro de Biotecnología y Genómica de Plantas UPM-INIA, Spain).

[b] Determined based on Sanger sequence data comparison of nucleotide 3,441 to 3,761 of PepMV reference seq NC004067 (Pep5-2: 5'-TACYTAGCHAAACCAATGGC-3' and Pep6-2: 5'-GCWCCATCTTGKGATTGRTC-3').

[c] Genotype determined based on literature (Accession HG313807); no Sanger sequence data available, because sample was gel-PCR negative.

Table D.2. Specificity data from healthy leaf and/or seed samples from various origins or infected with a non-target virus/viroid. N/A: not applicable, No amp.: no amplification.

Isolate name	Supplier	Target	Origin	Year	Tissue type	Host	NAKT05	Bejop	BaCV
							Rep. 1&2	Rep. 1&2	Rep. 1&2
0710-09	Field isolate	*Potato virus X*	Australia	2009	Leaf	*Capsicum annuum*	No amp.	No amp.	No amp.
ToRSV	Prime diagnostics	*Tomato ring spot virus*	Unknown	Unknown	Leaf	*Solanum lycopersicum*	No amp.	No amp.	No amp.
PV-0215	DSMZ	*Arabic mosaic virus*	Unknown	2021	Leaf	*Chenopodium quinoa*	No amp.	No amp.	No amp.
PV-0070	DSMZ	*Tomato black ring virus*	Unknown	1987	Leaf	*Solanum tuberosum*	No amp.	No amp.	No amp.
TRSV	Prime diagnostics	*Tobacco ringspot virus*	Unknown	2021	Leaf	*Solanum lycopersicum*	No amp.	No amp.	No amp.
TMV	Field isolate	*Tobacco mosaic virus*	Unknown	2021	Leaf	*Nicotiana tabacum*	No amp.	No amp.	No amp.
0406-05	Field isolate	*Tomato brown rugose fruit virus*	Jordan	2005	Leaf	*Solanum lycopersicum*	No amp.	No amp.	No amp.
CMV	Prime diagnostics	*Cucumber mosaic virus*	Unknown	2021	Leaf	*Capsicum annuum*	No amp.	No amp.	No amp.
TSWV	Prime diagnostics	*Tomato spotted wilt virus*	Unknown	2021	Leaf	*Solanum lycopersicum*	No amp./37.83	No amp.	No amp.
MX5	USDA	*Tomato mottle mosaic virus*	Mexico	2009	Leaf	*Solanum lycopersicum*	37.78/No amp.	No amp.	No amp.
PV-0196	DSMZ	*Alfalfa mosaic virus*	Unknown	1988	Leaf	*Solanum lycopersicum*	No amp.	No amp.	No amp.
BaCV	Field isolate	*Bacopa chlorosis virus*	Netherlands	2016	Leaf	*Sutera cordata*	No amp.	No amp.	28.15/28.44
DLVd	Field isolate	*Dahlia latent viroid*	Netherlands	2020	Leaf	*Dahlia* sp.	No amp.	No amp.	No amp.
0088-17	Field isolate	*Tomato mosaic virus*	Netherlands	2017	Leaf	*Solanum lycopersicum*	No amp.	No amp.	No amp.
TCDVd	NVWA	*Tomato chlorotic dwarf viroid*	Unknown	2014	Leaf	*Solanum lycopersicum*	No amp.	No amp.	No amp.
CEVd	NVWA	*Citrus exocortis viroid*	Unknown	2014	Leaf	*Solanum lycopersicum*	No amp.	No amp.	No amp.
CLVd	NVWA	*Columnea latent viroid*	Unknown	2014	Leaf	*Solanum lycopersicum*	No amp.	No amp.	No amp.
TPMVd	NVWA	*Tomato planta macho viroid*	Unknown	2014	Leaf	*Solanum lycopersicum*	No amp./38.73	No amp.	No amp.
Tomato	Rijk Zwaan	*Solanum lycopersicum*	Netherlands	2021	Leaf	*Solanum lycopersicum*	No amp.	No amp.	No amp.
Tomato	Rijk Zwaan	*Solanum lycopersicum*	Chile	2021	Seed	*Solanum lycopersicum*	No amp.	No amp.	No amp.
Tomato	Rijk Zwaan	*Solanum lycopersicum*	Mexico	2020	Seed	*Solanum lycopersicum*	No amp.	No amp.	No amp.
Eggplant	Rijk Zwaan	*Solanum melongena*	Netherlands	2021	Leaf	*Solanum melongena*	No amp.	No amp.	No amp.
Water	IDT	no virus	N/A	2021	N/A	N/A	No amp.	No amp.	No amp.

Annex E. Supplementary data validation report

3.2 Analytical Sensitivity Limit of D

3.2 Analytical Sensitivity Single Se

3.3 Selectivity Data.xlsx

3.4 Repeatability Data.xlsx

3.5 Reproducibility Data.xlsx

3.6 Diagnostic performance Data.xl

Annex F. Protocol for detecting *Pepino mosaic virus* (PepMV) in tomato seed by ELISA

Materials

- Seed extraction buffer (Table F.1)
- Antiserum PepMV (Prime Diagnostics)
- Phosphate substrate
- ELISA buffers (Table F.2, F.3 and F.4)
- Controls (Table F.5)

Table F.1. Seed extraction buffer.

Compound	Amount/L
Sodium chloride (NaCl)	8.0 g
Sodium phosphate dibasic heptahydrate ($Na_2HPO_4.7H_2O$)	2.17 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g
Potassium chloride (KCl)	0.2 g
Sodium sulphite (Na_2SO_3)[a]	1.0 g

[a] Add after autoclaving, use within 24 hours after preparation.

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 different positive / negative outcome.

Table F.2. Coating buffer – pH 9.6.

Compound	Amount/L
Sodium carbonate (Na_2CO_3)	1.59 g
Sodium bicarbonate ($NaHCO_3$)	2.93 g
Add de-ionized water up to 1 L, adjust pH and autoclave buffer at 121 °C for 15 min.	

Table F.3. Conjugate buffer – pH 7.4.

Compound	Amount/L
Sodium chloride (NaCl)	8.0 g
Sodium phosphate dibasic heptahydrate ($Na_2HPO_4.7H_2O$)	10.85 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.0 g
Tween 20	0.5 mL
Add de-ionized water up to 1 L, adjust pH and autoclave buffer at 121 °C for 15 min.	
Bovine Serum Albumin[a]	8.0 g

[a] Add after autoclaving, use within 24 hours after preparation.

Table F.4. Substrate buffer – pH 9.6.

Compound	Amount/L
Diethanolamine	97 mL
Add de-ionized water up to 1 L, adjust pH and autoclave buffer at 121 °C for 15 min.	

Table F.5. Types of controls used.

Control type	Description
Buffer control (BC)	The buffers and reagents used in the ELISA, with no seed/tissue matrix or target pathogen
Negative process control (NPC)	Tomato seed-free of *Pepino mosaic virus*
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 to 7 °C until the assay begins. It is recommended to perform the bioassays within 20 hours following seed extraction.

2. Preparation ELISA test

2.1. Coat the ELISA plates with coating antibodies (e.g., PepMV Prime diagnostics) in coating buffer (Table F.2).

Note: If different antisera and buffers are used, or even different lot numbers, it is necessary to verify their performance.

2.2. Store the coated plate ~7 °C overnight to maximum 24 hours before use.

3. Extraction of the virus from the seed

3.1. Soak the 250 seeds of each subsample, the positive process control (PPC), and the negative process control (NPC) in 10 mL seed extraction buffer (Table F.1) for 30 to 60 min.

3.2. Macerate the samples for 90 sec using an Interscience BagMixer or equivalent equipment.

3.3. Store seed extracts at 4 – 7 °C.

4. ELISA test

4.1. Clear out the coating buffer from the ELISA plate.

4.2. Put the seed extract of each grinded sample in duplicate 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 plate with the conjugate with the conjugate antibodies (e.g., PepMV Prime diagnostics) in conjugate buffer (Table F.3.).

4.6. Store the conjugate plate for 4 hours at 28 °C.

4.7. Dissolve 10 mg of phosphatase substrate per 10 mL substrate buffer (Table F.4.).

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 hours the ELISA plate extinction using a spectrophotometer at 405 nm and 620 nm.

5. Evaluation of the test results

5.1. To calculate the signal-to-noise (S/N) ratio for an ELISA, divide the average PPC optical density (OD) value by the average NPC OD value.

5.2. Cut-off 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 three 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)

Note: Test results are only valid when all included controls presented in Table F.5 give the expected results.

Annex G. Protocol for detecting *Pepino mosaic virus* (PepMV) in tomato seed by bioassay

Materials

- Seed extraction buffer (Table G.1)
- Controls (Table G.2)
- Grinder
- *Nicotiana benthamiana* plants
- Carborundum
- Protective mask
- Gloves
- Tap water
- ELISA necessities
- Lab disposals

General notes

- *Nicotiana benthamiana* is a systemic host for all PepMV strains tested. *N. benthamiana* is preferred over tomato as an assay plant because the systemic movement of the virus in tomato can be erratic. Furthermore, leaves of *N. benthamiana* are more easily inoculated.
- Although PepMV infection of *N. benthamiana* usually results in conspicuous symptoms, this is not always the case, and symptoms can be caused by other factors than PepMV. Therefore, ELISA of assay plants is required.

Table G.1. Seed extraction buffer.

Compound	Amount/L
Sodium chloride (NaCl)	8.0 g
Sodium phosphate dibasic dodecahydrate ($Na_2HPO_4.12H_2O$)	2.9 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.2 g
Potassium chloride (KCl)	0.2 g
Sodium sulphite (Na_2SO_3)[a]	1.0 g

[a] Add after autoclaving. use within 24 hours after preparation.

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 change in the outcome.

Table G.2. Types of controls used.

Control type	Description
Buffer control (BC)	The buffers and reagents used in the ELISA, with no seed/tissue matrix or target pathogen
Negative process control (NPC)	Tomato seed-free of *Pepino mosaic virus*
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 to 7 °C until the assay begins. It is recommended to perform the bioassays within 20 hours following seed extraction.

2. Seed extraction

2.1. Soak the 250 seeds of each subsample, the positive process control (PPC), and the negative process control (NPC) in 10 mL seed extraction buffer (Table G.1.) for 30 to 60 min.

2.2. Macerate the samples for 90 sec using an Interscience BagMixer or equivalent equipment.

2.3. Store seed extracts at 4 – 7 °C.

3. Inoculation of *Nicotiana benthamiana* plants

Assay plants should have 4-7 (nearly) fully expanded leaves and should been raised under sufficient light intensity at an average temperature of 20 - 25 °C. Avoid older assay plants that start to flower. Assay plants should have good turgor at the time of inoculation (Figure G.1).

3.1. Inoculate each seed extract, including the PPC, and NPC, on the two youngest (nearly) fully expanded leaves of two plants, going across the whole surface. Do not use the primary leaf (oldest true leaf).

Inoculate by first dusting the leaves moderately with carborundum (320 mesh grit powder, Fisher scientific or equivalent), while wearing a protective mask, followed by placing a drop of inoculum (100 – 200 µL) onto the leaf. Smear the drop with fingers (or swab) without applying pressure.

Note: Work with gloves and change them between samples or clean hands thoroughly between samples by using alkaline soap.

3.2. Rinse the plants with tap water a few minutes after inoculation.

3.3. In order to allow the assay plants to become systemically infected, incubate them for at least 14 days under controlled conditions at 25 ± 5 °C and with at least 12 hours of light per day.

4. Sampling of *Nicotiana benthamiana* assay plants and leaf extraction

4.1. For each subsample, the PPC, and the NPC, sample and pool leaf material from both assay plants, making sure that the pooled leaves weigh ~10 mg. Select younger leaves that have expanded during the preceding weeks and not the inoculated leaves.

4.2. Process samples immediately, store at 4 °C for at most 48 hours or freeze until use. If the samples were frozen, process them as soon as they are thawed.

4.3. Grind each pooled leaf sample in 0.5 mL seed extraction buffer. Process extracts immediately after grinding, store at 4 °C for a maximum of 24 hours or freeze until use.

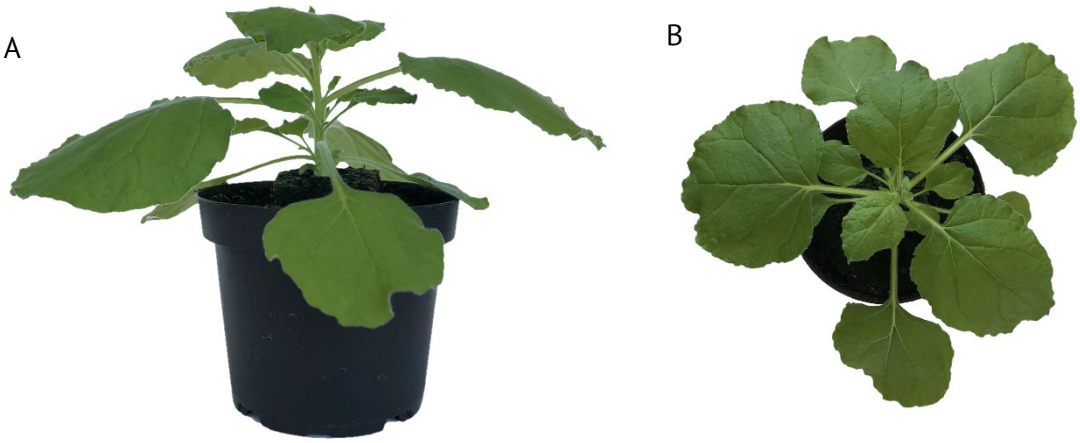

Figure G.1. *Nicotiana benthamiana* plant front view (A) and top view (B).

5. ELISA

5.1. Run a double-antibody-sandwich (DAS-)ELISA (Albrechtsen, 2006; Clark and Adams, 1977) on the extracts obtained at step 4.3. and controls mentioned in Table G.2.

5.2. A subsample is regarded as positive (infectious PepMV present) when the signal in ELISA is above the decision threshold.

Note: The source of antiserum is critical. In the comparative test study, the antiserum supplied by Wageningen University & Research Prime Diagnostics was used (https://www.wur.nl/en/show/Prime-Diagnostics2.htm). If different antisera and buffers are used, or even different lot numbers, it is necessary to verify their performance.

6. Validity of test results

Test results are only valid when all included controls presented in Table G.2 give the expected result.

REFERENCES

Albrechtsen, S.E. (2006). Testing methods for seed-transmitted viruses: principles and protocols. Wallingford, UK: CABI Publishing.

Clark, M.F. and Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology*, **34**, 475–483.