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Detection of *Pepino mosaic virus* (PepMV) in Tomato Seed

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Developed by ISHI

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Crop: Tomato (*Solanum lycopersicum*)

Pathogen(s): *Pepino mosaic virus* (PepMV)

Version: 6 (June 2023)

PRINCIPLE

Detection of infectious *Pepino mosaic virus* (PepMV) in seed of tomatoes is done by inoculation of *Nicotiana benthamiana* plants followed by ELISA of the new non-inoculated leaves of the plants to confirm the presence of infectious PepMV.

A seed extract qPCR (SE-qPCR) and/or ELISA may be used as a pre-screen. The test is complete if no PepMV is detected, and the seed lot is considered healthy. However, as SE-qPCR and ELISA detect both infectious and non-infectious virions, a positive pre-screen SE-qPCR and/or ELISA should be followed by a bioassay to determine the presence of infectious PepMV. The full method process workflow is presented in Figure 1.

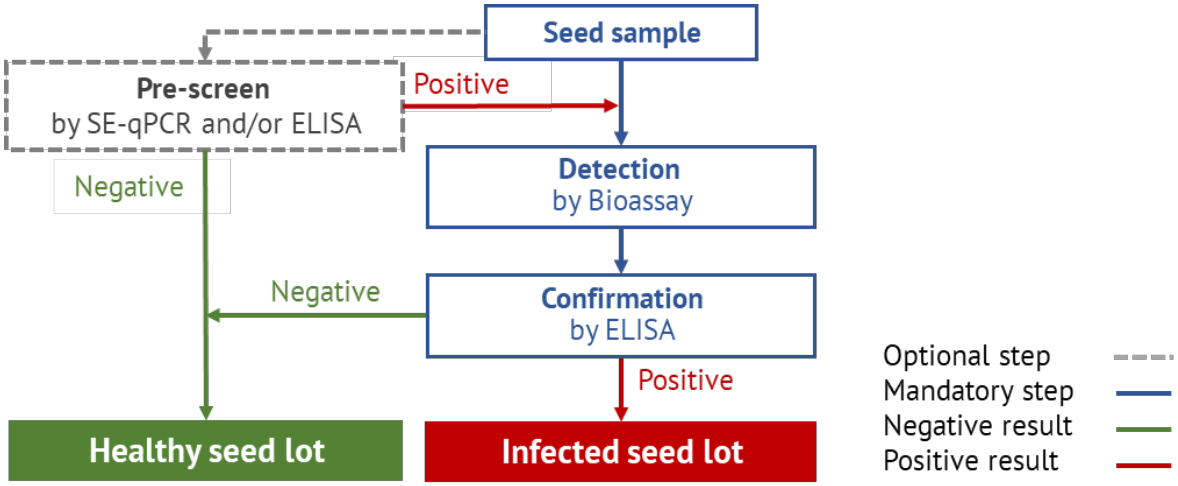

Figure 1. Method process workflow

METHOD VALIDATION

The ELISA pre-screen and bioassay were evaluated in a comparative test organised by ISHI (Krinkels, 2001).

The ELISA pre-screen and bioassay for detecting PepMV in tomato seed have been approved by the US National Seed Health System (NSHS) as a Standard B (https://seedhealth.org/seed-health-testing-methods/).

The ELISA has been shown to reproducibly detect a single PepMV infested seed in test samples in a comparative test with six participating laboratories (Krinkels, 2001). Using a dilution series of systemically infected leaves in healthy seed extracts, 10,000-fold dilutions were reproducibly

detected in both ELISA and bioassay. This sensitivity could vary depending upon laboratory conditions. It is the responsibility of the user to determine sensitivity of the assays at the time of running the assay.

The pre-screen by seed extract RT-qPCR (SE-qPCR) has been validated by ISHI (Berendsen *et al.*, 2023).

RESTRICTIONS ON USE

Before using this protocol routinely, it is necessary to verify its performance, especially when material and consumables from different suppliers are used. Technical details on the reagents/material used in the validation study (e.g., supplier's information) are provided in the protocol and the validation report.

This test method is suitable for untreated seed.

The ELISA pre-screen and bioassay are suitable for seed that has been treated using physical or chemical (acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate, etc.) processes with the aim of disinfestation/disinfection, provided that any residue, if present, does not influence the assay. It is the responsibility of the user to check for inhibition by analysis, sample spiking, or experimental comparisons.

It is recommended that the seed be checked for traces of any seed treatment that may interfere with the infectivity of the virus. This is done by comparing the infectivity of PepMV in two dilution series: one of the treated-seed extract spiked with infectious PepMV and the other with infectious PepMV in extraction buffer for the ELISA pre-screen and/or bioassay. The latter acts as a control.

Although ELISA is compatible with some seed treatment chemicals (Pataky *et al.*, 2004), seed treatments may affect the performance of this test. It is the responsibility of the user to check for such interference by analysis, sample spiking, or experimental comparisons.

METHOD EXECUTION

To ensure process standardization and valid results, it is strongly recommended to follow the Best Practices for Seed Health Tests developed by ISHI.

SAMPLE AND SUBSAMPLE SIZE

The recommended minimum sample size is 3,000 seeds with a maximum subsample size of 1,000 seeds for SE-qPCR and 250 seeds for ELISA and bioassay.

It should be noted that infested seeds (a single one being detectable in a sample) rarely lead to infected seedlings. Given this low transmission rate, less than 0.1% (Krinkels, 2001; Hanssen *et al.,* 2010), a sample size of 3,000 seeds is considered appropriate.

RATIONALE

To be able to perform the bioassay, qPCR and ELISA on the same seed-sample, PBS was chosen as the extraction buffer for all assays.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1	October 2003	First version of the protocol.
2	January 2007	Protocol updated to a new ISF template. 'Method Description' section updated, including a simplification of the ELISA method.
3	January 2009	'Restrictions on Use' section updated to include that the test is suitable for seeds treated with most chemicals with the aim of fungal disinfection with reference to Pataky *et al.,* 2004.
4	August 2011	Crop name *Solanum lycopersicum* added. 'Sensitivity and Restrictions on Use' section updated to include that it is the responsibility of the end-user to check for antagonism and to determine sensitivity of the assays at the time of running the assay.
4.1	July 2017	'Validation' section updated to include the approval from the NSHS as Standard B. 'Method Execution' section added.
5	March 2021	Protocol presented in accordance with ISHI protocol guidelines. Crop name *Lycopersicon esculentum* removed. A table with the controls added to the ELISA section.
6	June 2023	SE-qPCR pre-screen assay added. Protocol updated to the latest ISHI protocol guidelines.

Protocol for detecting *Pepino mosaic virus* (PepMV) in tomato seed

I. PRE-SCREEN BY SEED EXTRACT RT-qPCR (SE-qPCR) (OPTIONAL STEP)

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 I.1)
- Grinder (e.g., Interscience MiniMix 100)
- RT-qPCR mix, primers (Table I.2) and equipment
- Controls (Table I.3)
- Spike solution (see description below)
- RNA purification kit (e.g., Qiagen RNeasy Plant Mini Kit)
- 1.5 mL RNase Free tube
- RNAse free water
- Centrifuge

Table I.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.

Spike solution

The spike solution is prepared by using a leaf from a plant infected by *Bacopa chlorosis virus* (BaCV) to make an extract in seed extraction buffer (Table I.1). 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 I.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 I.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 samples 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 I.1).

Note: If a different seed extraction buffer is used, it must be verified by comparing against a uniform positive control material.

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 a grinder. See Figure I.1 to observe differences 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 (see section III.1).

Alternatively, grind 12 subsamples of 250 seeds directly in 10 mL seed extraction buffer containing the PEC to allow for downstream analyses.

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. If the extracts are to be used for the bioassay after SE-qPCR, the bioassay plants must be inoculated within 20 hours after extraction. Store the PPC and NPC (Table I.3) under similar conditions as the samples to validate the results.

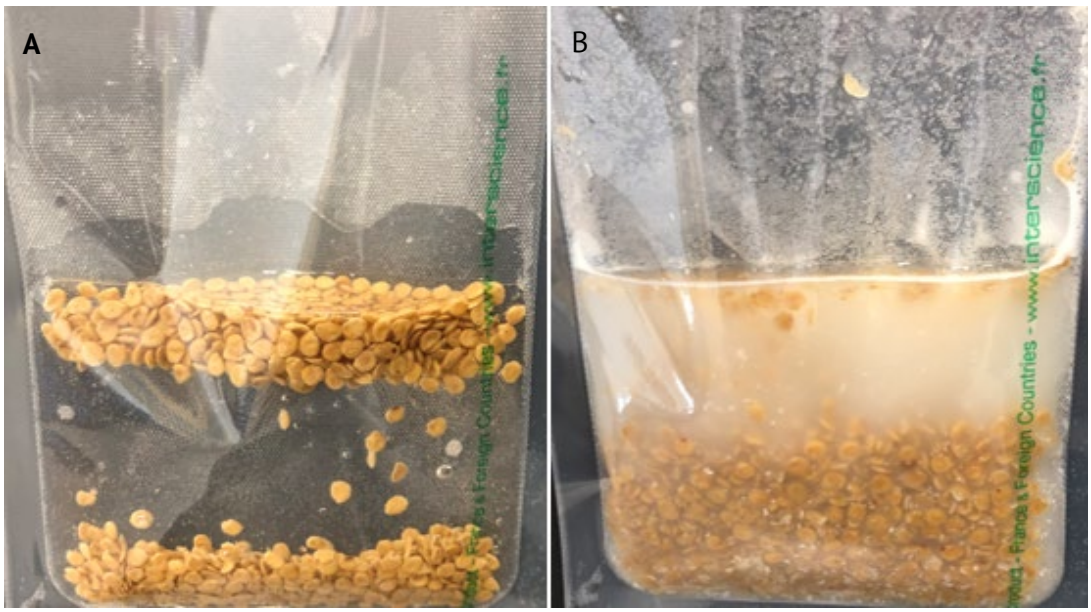

Figure I.1. A. 1,000 tomato seeds in seed extraction buffer after 30 min soaking. B. 1,000 tomato seeds after 90 sec of macerating with Interscience MiniMix 100.

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 when placed on ice or within 24 hours when stored at -20°C.

2.3. Process the subsamples for RNA extraction, as described in the manual of the RNA extraction kit.

Note: The assay has been validated with the Qiagen RNeasy Plant Mini Kit. If a different RNA isolation kit is used, it is necessary to verify its performance.

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 I.4.

For each run, include a negative template control (NTC) and at least one positive amplification control (PAC) from Table I.3. 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 I.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 I.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 I.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

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, an 'indirect' test used for pre-screening in seed health methods.

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

Table I.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 needed
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

II. PRE-SCREEN BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) (OPTIONAL STEP)

Materials

- Seed extraction buffer (Table I.1)
- Controls (Table II.1)
- Grinder
- ELISA necessities
- Lab disposables

Table II.1 Types of controls used.

Control type	Description
Positive process control (PPC)	Tomato seed with infectious PepMV or an appropriate dilution of reference material including infectious PepMV in seed extraction buffer.
Negative process control (NPC)	Tomato seed free of PepMV
Buffer control (BC)	The buffers and reagents used in the ELISA, with no seed/tissue matrix or target pathogen

1. Seed extraction

1.1. Grind the 250 seeds of each subsample, the positive process control (PPC) and the negative process control (NPC) in 10 mL seed extraction buffer (Table I.1). In case SE-qPCR was already conducted in subsamples of 250 seeds, the same extracts can be used for ELISA within 20 hours.

Note: Process extracts immediately after grinding or store at 4 °C for a maximum of 20 hours. Do not freeze. If the extracts are to be used for the bioassay after ELISA, the ELISA must be completed within 20 hours after extraction and bioassay plants inoculated within 20 hours after extraction. Store the PPC and NPC (Table II.1) under similar conditions as the samples to validate the results.

2. ELISA

2.1. Run a double-antibody-sandwich (DAS-)ELISA on the extracts (Albrechtsen, 2006; Clark and Adams, 1977).

2.2. 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 Plant Research was used (https://www.wur.nl/en/show/Prime-Diagnostics-2.htm). If different antisera and buffers are used, or even different lot numbers, it is necessary to verify their performance.

3. Validity of test results

Test results are only valid when all included controls presented in Table II.1 give the expected results. A positive test result must be followed by a bioassay (described in section III) to determine the presence of infectious PepMV.

III. DETECTION BY BIOASSAY AND CONFIRMATION BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Materials

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

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 the case at all times, and symptoms can be caused by other factors than PepMV. Therefore, ELISA of assay plants is required.

1. Seed extraction

1.1. Perform the seed extraction as described in section II.1, or use the seed extracts as obtained in section I.1 or II.1 when performing the bioassay after the pre-screen SE-qPCR or ELISA assay, respectively.

2. Inoculation of *Nicotiana benthamiana* assay plants

Assay plants should have 4–7 (nearly) fully expanded leaves and should have 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 III.1).

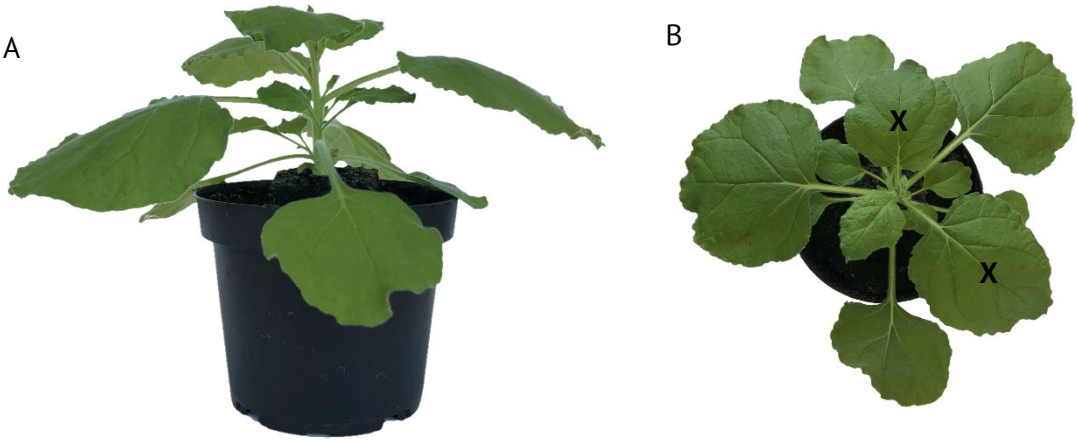

Figure III.1 Front view (A) and top view (B) of an assay plant at the latest stage suitable for inoculation; Leaves to be inoculated are marked by 'X'.

2.1. Inoculate each seed extract, including the positive process control (PPC), and negative process control (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 without applying pressure.

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

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

2.3. 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.

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

3.1. For each subsample, the PPC, and the NPC, sample and pool leaf material from both assay plants, making sure that the pooled leaves weigh 0.2–0.5 g. Select younger leaves that have expanded during the preceding weeks and not the inoculated leaves.

3.2. 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 have thawed.

3.3. Grind each pooled leaf sample in 10-12 mL ELISA extraction buffer. Process extracts immediately after grinding, store at 4 °C for a maximum of 24 hours or freeze until use.

4. ELISA

4.1. Run a double-antibody-sandwich (DAS-)ELISA (Albrechtsen, 2006; Clark and Adams, 1977) on the extracts obtained from step 3.3.

4.2. 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 Plant Research was used (https://www.wur.nl/en/show/Prime-Diagnostics-2.htm). If different antisera and buffers are used, or even different lot numbers, it is necessary to verify their performance.

5. Validity of test results

Test results are only valid when all included controls presented in Table II.1 give the expected result.

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