International Rules for Seed Testing 2027

Validated Seed Health Testing Methods

7-024: Detection of *pea early browning virus* and *pea seed-borne mosaic virus* in *Pisum sativum* (pea) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2026 in Calgary, Canada

Effective from 1 January 2027

Validation reports

See References. Copies are available by e-mail from the ISTA Secretariat at ista.office@ista.ch.

Please send comments, suggestions or reports of problems relating to this method to the ISTA Seed Health Committee, c/o ISTA Secretariat.

Disclaimer

Whilst ISTA has taken care to ensure the accuracy of the methods and information described in this method description, ISTA shall not be liable for any loss or damage, etc. resulting from the use of this method.

Safety precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during weighing out of ingredients. It is assumed that persons carrying out this test are in a laboratory suitable for carrying out microbiological procedures and familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic techniques. Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Note on the use of the translations

The electronic version of the International Rules for Seed Testing includes the English, French, German and Spanish versions. If there are any questions on interpretation of the ISTA Rules, the English version is the definitive version.

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7-024: Detection of *pea early browning virus* and *pea seed-borne mosaic virus* in *Pisum sativum* (pea) seed

Host: *Pisum sativum* L.
Pathogen(s): *Pea early browning virus* (PEBV); *pea seed-borne mosaic virus* (PSbMV)

Prepared by: International Seed Health Initiative for Vegetable Crops, ISF (ISHI-Veg)

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Revision history

Version 1.0, 2007-02-01
Version 1.1, 2013-01-01: Definition of sample size
Version 1.2, 2014-01-01: Common name of host added
Version 1.3, 2017-01-01: Reporting results revised
Version 1.4, 2021-01-01: Sample size revised
Version 1.5, 2024-01-01: Sample size revised
Version 1.6, 2025-01-01: Host revised
Version 1.7, 2027-01-01: Flowchart added in Methods

Background

Pea early browning virus (PEBV) and *pea seed-borne mosaic virus* (PSbMV) are seed-transmissible viruses of the pea, and therefore the detection of these viruses in pea seeds is an important tool in control strategies. The enzyme-linked immunosorbent assay (ELISA) is widely used for the detection of plant viruses (Clark & Adams, 1977), and ELISA methods have been described for the detection of PEBV and PSbMV (Hamilton & Nichols, 1978; Van Vuurde & Maat, 1985, Maury *et al.*, 1987). The test method described here was designed using this information and 20 years of laboratory experience, and evaluated in a comparative test. The method, using ground seed and a DAS-ELISA, can be used to simultaneously detect PSbMV and PEBV in a single extract. Note that the extract is tested in two microtitre plates, one for PEBV and one for PSbMV. The theoretical detection limit is one seed in 100. To ensure a 95 % probability that infestations of 0.15 % or higher are detected, it is necessary to test 20 subsamples of 100 seeds.

Treated seed

This method has not been validated for the determination of PEBV or PSbMV in treated seed. Seed treatments may affect the performance of this test.

(Definition of treatment: any process, physical, biological or chemical, to which a seed lot is subjected, including seed coatings. See 7.2.3)

Sample size

The sample size (total number of seeds to be tested) and subsample size depend on intended use, the maximum acceptable infection level and the analytical sensitivity of the method. The minimum recommended working sample size is 2000 seeds and the maximum subsample size must be 100 seeds.

Materials

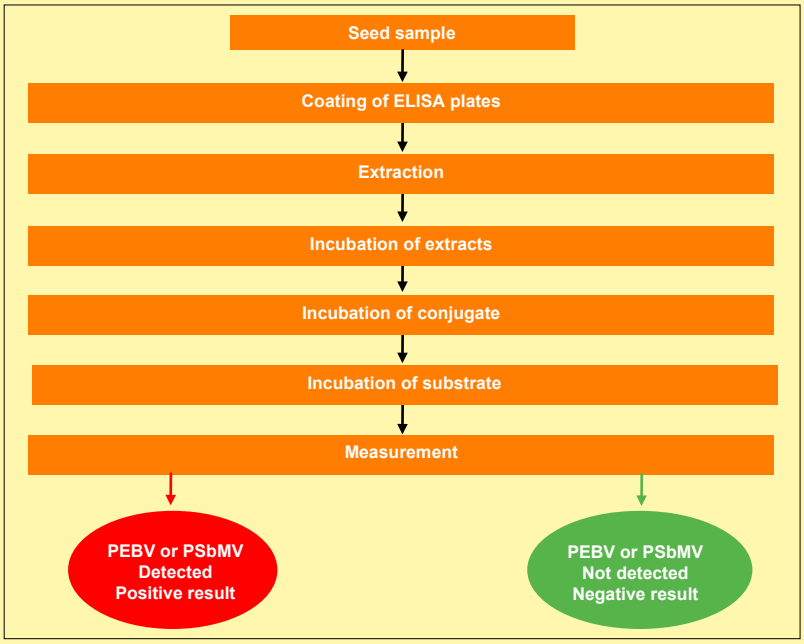
Reference material: PSbMV- and PEBV-infested seeds or standardised reference material (flour of peas containing PSbMV and/or PEBV)
Microtitre plates: 96-well plates suitable for ELISA (CCP)
Antisera: Suitable for detection of PSbMV- and PEBV-infested seeds (e.g. PRI, Wageningen, The Netherlands)
Balance: capable of weighing to the nearest 0.01 g
pH meter: capable of being read to the nearest 0.1 pH unit
Automatic pipettes: capable of pipetting to the nearest 0.001 ml
Grinder: capable of grinding peas to fine flour (e.g. Retsch grindomix GM 200)
Incubator: capable of operating at 4 ±2 °C
Incubator: capable of operating at 37 ±2 °C
ELISA plate reader.
Tubes: 10 ml

Sample preparation

This can be done in advance of the assay.

It is vital to exclude any possibility of cross-contamination between seed samples. It is therefore essential to clean all surfaces, containers, hands, etc. both before and after handling each sample.

1. Count the number of seeds in a known weight. Estimate the thousand-seed weight (TSW) as:
   TSW = (weight of seeds / number of seeds) × 1000
2. Based on the estimated TSW, weigh out subsamples of the required size into new, clean polythene bags or containers.



Methods

Critical control points are indicated by CCP.

1. Coating of ELISA plates

1.1 Add appropriate (as defined by supplier) dilution of PSbMV- and PEBV-coating serum to coating buffer. Be sure that the antisera are not only suitable for diagnostics but also for the detection of viruses in seed extracts (CCP).

1.2 Coat one plate with 100 µl (or as defined by supplier) of PSbMV-coating buffer per well. Coat another plate with 100 µl (or as defined by supplier) of PEBV-coating buffer per well.

1.3 Cover ELISA plates with lid or wrap with plastic wrap to minimise evaporation.

1.4 Incubate plates overnight at 4 ±2 °C.

2. Extraction of virus from the seed and incubation of extracts

2.1 Count or weigh 100 seed subsamples.

2.2 Grind each subsample to fine flour in a grinder (CCP).

2.3 From each subsample weigh out 0.5 g of flour and transfer to a 10 ml tube.

2.4 Add 5 ml of extraction buffer to each tube.

2.5 Vortex each tube for 15 s. Allow extract to settle for at least 5 min. on the bench to facilitate pipetting.

2.6 Remove coating buffer from ELISA plates and immediately rinse plates three times thoroughly, using PBS/Tween™ 20 to remove residues. Alternatively, use a suitable washing device (CCP).

2.7 Immediately after rinsing, pipette 100 µl (or as defined by supplier) of each seed extract into a well. Use 2 wells per subsample.

2.8 Add positive and negative controls to each ELISA plate. Use at least 2 dilutions for the positive controls: a 'low' dilution, which gives a high extinction, and a 'high' dilution, which gives an extinction just above the detection threshold (CCP). Negative controls must include a healthy seed extract.

2.9 Cover plates with lid or wrap with plastic wrap to minimise evaporation and incubate overnight at 4 ±2 °C or as defined by supplier.

3. Incubation of conjugate

3.1 Prepare appropriate dilution of PSbMV- and PEBV-conjugated antiserum using conjugate buffer as defined by the supplier.

3.2 Remove seed extracts from ELISA plates and rinse plates 3 times with washing buffer PBS/Tween™ 20

to remove residues of seed extract. Alternatively, use a suitable washing device (CCP).

3.3 Immediately after rinsing, add 100 µl (or as defined by supplier) of diluted conjugate to each well of the ELISA plate.

3.4 Cover plates with lid or wrap with plastic wrap to minimise evaporation, and incubate for 3 h at 37 ±2 °C or as defined by supplier.

4. Addition of substrate to ELISA plates

4.1 Prepare substrate solution (10 mg *para*-nitrophenol phosphate in 20 ml of substrate buffer).

4.2 Remove conjugate from ELISA plates and rinse thoroughly, either 3 times by hand using washing buffer PBS/Tween™ 20, or, alternatively, using a reliable washing device (CCP).

4.3 Add 100 µl (or as defined by supplier) of substrate solution to each well.

4.4 Incubate for 2 h at 20 ±2 °C or as defined by supplier.

4.5 Measure extinction (A_{405}) with ELISA plate reader (see General methods, Recording of ELISA extinction).

General methods

Grinding of seeds: Grind each subsample of 100 seeds to a fine flour. Be sure to use a grinder that can be cleaned thoroughly, since cross-contamination is likely during the grinding step.

Recording of ELISA extinction: Record the results for all wells in the microtitre plate. Check first whether the positive and negative controls meet the expectations, since otherwise the results of the test are invalid and the test should be repeated. It is recommended to use a negative-positive threshold of 2.5 times the background of healthy samples.

Reporting results: The result of a seed health test should indicate the scientific name of the pathogen detected and the test method used. When reported on an ISTA Certificate, results are entered under 'Other Determinations'.

The report must indicate the number of seeds tested.

In the case of a negative result (pathogen not detected in any subsample), the results must be reported as 'not detected'.

In the case of a positive result, the report must indicate the number of positive subsamples out of the total number tested.

Quality assurance

Critical control points (CCP)

- Sensitivity can be influenced by using different types of microtitre plates or materials.
- The quality of antisera from different sources is known to be variable. Therefore ensure that the antisera are suitable not only for diagnostics but also for the detection of viruses in seed extracts. Step 1.1.
- A fine flour will improve the extraction efficacy. Therefore, grind seeds for 20 s at 10 000 r.p.m. to get a fine flour. Note that some knives easily become blunt and therefore grind less efficiently with time. Step 2.2.
- Coated microtitre plates will lose activity rapidly when they are left to dry on the bench for some time. Therefore limit time that empty microtitre plates sit on bench as much as possible. Step 2.6.
- The use of appropriate positive and negative controls is very important to validate the result. Be sure that, apart from a 'high' positive control, there is always a 'low' positive control in each plate. Step 2.8.
- High backgrounds in ELISA are often caused by poor washing of the microtitre plates between the different incubation steps. Washing can be done by hand using PBS/Tween™ 20 or with a washing device. The thorough washing of microtitre plates is highly critical in several steps (2.6, 3.2 and 4.2) in the ELISA, particularly after the incubation with the conjugated antiserum. Step 4.2.

Media and solutions

Coating buffer (pH 9.6)

Na_2CO_3: 1.59 g/l
$NaHCO_3$: 2.93 g/l

Extraction buffer (0.05 M, pH 7.4)

NaCl: 8.0 g/l
KH_2PO_4: 1.0 g/l
$Na_2HPO_4 \cdot 12H_2O$: 14.5 g/l
Tween™ 20: 1.5 ml
PVP (ELISA grade, e.g. molecular weight 10 000 Da): 20.0 g

Conjugate buffer (0.05 M, pH 7.4)

NaCl: 8.0 g/l
KH_2PO_4: 1.0 g/l
$Na_2HPO_4 \cdot 12H_2O$: 14.5 g/l
Tween™ 20: 1.5 ml
BSA (ELISA grade, e.g. BSA fraction 5): 5.0 g

Substrate buffer (pH 9.6)

Diethanolamine: 97 ml
HCl (32 %): 15 ml

Washing buffer PBS/Tween™ 20 (0.05 M, pH 7.4)

NaCl: 8.0 g/l
KH_2PO_4: 1.0 g/l
$Na_2HPO_4 \cdot 12H_2O$: 14.5 g/l
Tween™ 20: 1.5 ml

All buffers

Preparation

1. Weigh or measure out all ingredients into a suitable container.
2. Adjust volume to 1000 ml with distilled/deionised water and dissolve or mix ingredients as appropriate.
3. Check the pH with a pH meter.

Storage

Store buffers as mentioned above at 4 ±2 °C for a week.

References

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Validation references

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