International Seed Federation
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Detection of Lettuce mosaic virus (LMV) in Lettuce Seed

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Developed by ISHI

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Crop: Lettuce (*Lactuca sativa*)

Pathogen(s): Lettuce mosaic virus (LMV, now *Potyvirus lactucae*)

Version: 5.0 (March 2026)

PRINCIPLE

Detection of infectious Lettuce mosaic virus (LMV), now called *Potyvirus lactucae*, in lettuce seed is done by growing out seeds on wet filter paper, followed by an enzyme-linked immunosorbent assay (ELISA) on the seedlings extract for the detection of LMV. A seed extract qPCR (SE-qPCR) and/or ELISA may be used as a pre-screen. The test is complete if no LMV 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 seedling ELISA to determine the presence of infectious LMV. The full method process workflow is presented in Figure 1.

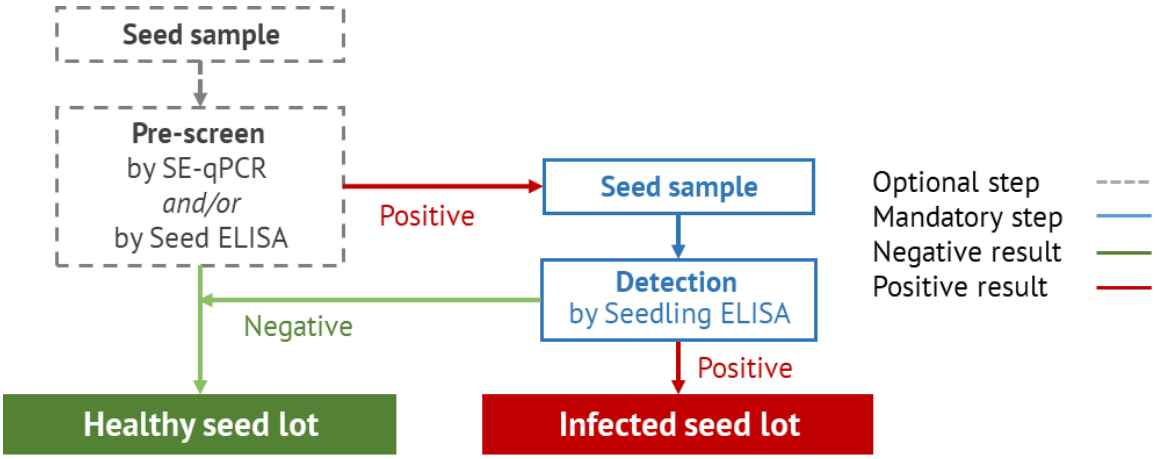

Figure 1. Method process workflow for the detection of LMV in lettuce seeds.

METHOD VALIDATION

The seed and seedling ELISA assays have been used in the seed industry since 1990 (Falk and Purcifull, 1983; Jafapour *et al.*, 1979; Maury-Chovelon, 1984; Van Vuurde and Maat, 1983; Van Vuurde and Maat, 1984). Validation was done in an ISHI comparative test (Oosterhof *et al.*, 2001).

The LMV SE-qPCR as pre-screen has been validated by ISHI (Van den Bogert *et al.*, 2026).

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 validation report. Comparison of antibodies is recommended (Borgsteede, 2015).

This method is suitable for untreated seed.

It is also suitable for seed that has been treated using physical (e.g., hot water) or chemical (e.g., acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate) 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 experimental comparisons or other means.

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

For the seed ELISA, the recommended minimum sample size is 10,000 seeds with a maximum subsample size of 500 seeds. For the SE-qPCR and seedling ELISA, the recommended minimum sample size is 2,000 seeds with a maximum subsample size of 1,000 seeds for the SE-qPCR and 100 seeds for the seedling ELISA.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1.0	July 2006	First version of the protocol.
2.0	January 2007	'Restrictions on Use' section expanded. Examples high and low extinctions positive controls included. Covering of ELISA plates during incubation added.
3.0	January 2009	'Restrictions on Use' section modified.
4.0	March 2011	'Restrictions on Use' and 'Sensitivity' sections combined.
4.1	March 2015	Reference to CT report Borgsteede *et al.* (2015) added to 'Sensitivity and Restrictions on Use'.
4.2	April 2015	Information on the Envirologix kit deleted from the 'Sensitivity and Restrictions on Use' section as the company discontinued plant pathology products for this and other ELISA kits.

Version	Date	Changes (minor editorial changes not indicated)
4.3	July 2017	'Validation' section updated to include the approval from the NSHS as Standard A. 'Method Execution' section added. 'Sensitivity and Restrictions on Use' section modified.
5.0	March 2026	SE-qPCR pre-screen assay added. Protocol updated to the latest ISHI protocol guidelines.

Protocol for detection of Lettuce mosaic virus (LMV) in Lettuce 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

- Controls (Table I.1)
- Phosphate-buffered saline (PBS)+Tween buffer (Table I.2)
- Seed extraction buffer (Table I.3)
- RNA purification kit (e.g., RNeasy Plant Mini Kit (Qiagen, Hilden, Germany)) and equipment
- Stainless steel grinding bullet ø14 mm
- Grinder (e.g., Geno/Grinder 2010 (Cole-Parmer®, Vernon Hills, IL))
- PCR grade water
- qPCR mix (e.g., Ultraplex 1-Step ToughMix (QuantaBio, Beverly, MA), primers and probes (Table I.4), and qPCR equipment
- Lab disposables

Table I.1. Types of controls used.

Control type	Description
Internal amplification control (IAC)[a]	Dahlia latent viroid[b] (DLVd, now named *Hostuviroid latensdahliae*)
Negative process control (NPC)	LMV free lettuce seeds
Negative template control (NTC)	PCR grade water (PCR mix free from any pathogen or seed)
Positive process control (PPC)	Positive (LMV infected) lettuce seed
Positive amplification control (PAC)	LMV synthetic ssDNA oligonucleotide *or*
	LMV RNA *or*
	LMV cDNA

[a] The IAC also serves as inhibition control (IC).

[b] The spike solution is prepared by a leaf from a plant infected by DLVd and making an extract of it in seed extraction buffer (Table I.3). The extract is diluted to obtain a suitable concentration, and aliquots are stored at -80 °C. Alternatively, synthetic RNA resembling the complete genome of DLVd can be used as spike. This approach was used in the validation of the LMV SE-qPCR. Other organisms such as Squash mosaic virus (SqMV; now named *Comovirus cucurbitae*) or Bacopa chlorosis virus (BaCV; genus *Ilarvirus*) may also be used, but compatibility with the LMV primers in a multiplex qPCR should be verified.

Table I.2. Phosphate-buffered saline (PBS)+Tween buffer.

Compound	Amount/L
NaCl	8.0 g
KH_2PO_4	1.0 g
$Na_2HPO_4.12H_2O$	14.5 g
Tween™ 20	0.5 mL

Table I.3. Seed extraction buffer, pH 7.4.

Compound	Amount/L
Polyvinyl pyrrolidone (PVP; mol. wt. 10,000-15,000 Da)	20.0 g
PBS+Tween buffer (See Table I.2)	Up to 1 L

Table I.4. Primer and probe sequences and references.

Name	Target	Sequence (5' – 3')	Source
DaVd1 Fw	DLVd	GCT CCG CTC CTT GTA GCT TT	Naktuinbouw; Botermans *et al.*, 2020
DaVd1 Rv		AGG AGG TGG AGA CCT CTT GG	
DaVd1 Probe[a]		TxR-X– CTG ACT CGA GGA CGC GAC CG - IBRQ	
LMV RZ 2 Fw	LMV	GCA YTR CCA TTG TGT GGA AAA TT	Van den Bogert *et al.*, 2026
LMV RZ 2 Rv		TGG AAA CAG AYC CAA CCG AGA TAC	
LMV RZ 2 Probe[a]		YakYel – ACA GGY GCC GCA GAT TTG AAA GGC AG - IBFQ	
LMV NAKT Fw	LMV	TTG ATG GTT TGG TGT ATA GAA AAC G	Naktuinbouw; Van Soest, *et al.*, 2014
LMV NAKT Rv		CCA TCA TCA CCC ATG TTC CA	
LMV NAKT Probe[ab]		FAM – ACA TCC CCG AAT ATA A – MGB - NFQ	

[a] Other fluorochrome may also be used.
[b] The use of MGB is mandatory.

1. Sample preparation and RNA extraction

1.1. Add the internal amplification control (IAC; Table I.1) to the seed extraction buffer (Table I.3).

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

1.2. Add 1,000 seeds for each sample, healthy seed lot as the NPC, and LMV infected seeds as the PPC (Table I.1) to separate 50 mL tubes each together with a grinding bullet (stainless steel grinding bullet; ø14 mm).

1.3. Grind subsamples at 1,300 rpm for 3 min using the Geno/Grinder 2010 or equivalent apparatus.

1.4. Briefly spin the tubes or tap the lid of the tubes to remove any ground seed from the lid

and add 10 mL seed extraction buffer spiked with DLVd as IAC from Step 1.1 to each subsample and mix by vortexing.

1.5. Spin the samples at 500 × *g* for 1 min and use the supernatant for RNA extraction.

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

Note: The assay has been validated with the RNeasy Plant Mini Kit (Qiagen) starting by mixing 100 µL seed slurry supernatant with 400 µL RLT buffer (provided in the RNeasy Plant Mini Kit) and proceeding according to the manufacturer's instructions. The RNA was eluted in 50 µL nuclease free water provided in the RNeasy Plant Mini Kit (Qiagen) and reloading the first eluate a second time on the same column. If a different RNA isolation kit is used, it is necessary to verify its performance.

2. RT-qPCR

2.1. Prepare the RT-qPCR mix as described in Table I.5.

In each run, include a negative template control (NTC) and at least one positive amplification control (PAC) from Table I.1 that gives a Cq value between 26 and 32.

2.2. Seal the plate and perform the RT-qPCR reaction in a real-time PCR instrument, according to the conditions described in Table I.6. All samples and controls should be tested in duplicate, as described in Best Practices for PCR Assays in Seed Health Tests.

Note: If different RT-qPCR mixtures and amplification programs are used, it is necessary to verify their performance.

Table I.5. RT-qPCR mix for the SE-qPCR.

Component	Per reaction (in µL)	Final concentration
DaVd1 Fw (10 µM)	0.50	0.20 µM
DaVd1 Rv (10 µM)	0.50	0.20 µM
DaVd1 Probe (10 µM)	0.50	0.20 µM
LMV RZ 2 Fw (10 µM)	0.50	0.20 µM
LMV RZ 2 Rv (10 µM)	0.50	0.20 µM
LMV RZ 2 Probe (10 µM)	0.375	0.15 µM
LMV NAKT Fw (10 µM)	0.50	0.20 µM
LMV NAKT Rv (10 µM)	0.50	0.20 µM
LMV NAKT Probe (10 µM)	0.375	0.15 µM
Ultraplex 1-Step ToughMix RT-qPCR (4×)	6.25	1×
PCR grade water	9.50	
Sample RNA	5.00	
Total volume	**25.00**	

Table I.6. RT-qPCR conditions.

Cycles	Step	Temperature	Duration
1	cDNA synthesis	50 °C	20 min
1	Initial denaturation	95 °C	3 min
40	Denaturation	95 °C	10 sec
	Annealing and primer extension	60 °C	1 min

3. Evaluation of test results

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

3.2. Check for exponential amplification, indicated by an S-shaped amplification curve, for LMV positive samples and compare with the PPC and PAC.

4. Interpretation and decisions

Cut-off values must be established by each laboratory for their positive control and the internal amplification control 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.

The results from the LMV targeted primer and probe sets as well as the one targeting DLVd need to be taken into account for interpretation and decision making (Table I.7).

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

Table I.7. Interpretation and decision table for the SE-qPCR.

Primer and probe set			SE-qPCR result	Follow-up
LMV NAKT	LMV RZ 2	DaVd1		
Positive	Positive or negative	Positive or negative	LMV RNA detected	LMV seedling ELISA (Section III)
Positive or negative	Positive	Positive or negative	LMV RNA detected	LMV seedling ELISA (Section III)
Negative	Negative	Positive	No LMV RNA detected	No follow-up needed
Negative	Negative	Negative	IAC failure	LMV seedling ELISA (Section III) or repeat RNA extraction and/or LMV SE-qPCR (IAC failure).

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

Materials

- Seed extraction buffer (Table I.3)
- Coating and conjugated LMV antiserum
- ELISA buffers (Tables I.2 and II.1 to II. 3)
- Crusher (e.g., Kinematica Polytron™ PT 10/35 GT Homogenizers (Fisher Scientific, Waltham, MA))
- Controls (Table II.4)
- ELISA equipment
- Para-nitrophenyl phosphate (pNPP) for substrate solution
- Lab disposables

Table II.1. Coating buffer, pH 9.6.

Compound	Amount/L
Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g

Table II.2. Conjugate buffer, pH 7.4.

Compound	Amount/L
Bovine serum albumin (BSA; or ovalbumine (OVA))	5.0 g
PBS+Tween buffer (See Table I.2)	Up to 1 L

Table II.3. Substrate buffer, pH 9.8[a].

Compound	Amount/L
$C_4H_{11}NO_2$	97 mL

[a] Adjust pH with HCl, e.g., 36.8% (v/v).

Table II.4. Types of controls used.

Control type	Description
Positive process control (PPC)	Lettuce seed with infectious LMV
Positive control (PC)	LMV positive ELISA control
Negative process control (NPC)	Lettuce seed free of LMV
Negative buffer control (NBC)	The buffers and reagents used in the ELISA, with no seed or tissue matrix or target pathogen

1. Coating of ELISA plates

1.1. Add appropriate (as defined by supplier) dilution of LMV coating serum to coating buffer (Table II.1). Ensure that the antisera are not only suitable for diagnostics but as well for the detection of viruses in extracts of seeds and seedlings.

1.2. Coat plates with 180 µL coating buffer per well.

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

1.4. Incubate ELISA plates with coating buffer overnight at 4 °C.

2. Virus extraction from seed

2.1. Weigh or count 20 × 500 seeds per sample. For each test use a positive and negative seed lot as positive process control (PPC) and negative process control (NPC), respectively, to validate the results of the test (Table II.4).

2.2. Grind the seeds of each subsample in at least 5.0 mL extraction buffer (Table I.3), using a power drive crusher. An alternative extraction device could be used if the results are comparable to those obtained using the crushed described above.

2.3. Collect at least 1.5 mL of the seed extract into a suitable container. Process extracts within 4 hours after crushing. Store extracts at 4 °C, if not processed immediately. Centrifugation can be used for clarification of seed extracts.

3. Incubation of extract on ELISA plates

3.1. Remove coating from ELISA plates and rinse them well (e.g., three times) with PBS+Tween buffer (Table I.2) to remove residues. Alternatively use a suitable washing device.

3.2. Immediately after rinsing, transfer 180 µL of each seed extract into a well. Use at least two wells per subsample.

3.3. Add to each ELISA plate the positive control (PC), and negative buffer control (NBC; Table II.4). For the PC, it is recommended to use at least two dilutions: one 'low' dilution that gives a high extinction (Optical Density (OD) above 1.00 after one-hour incubation) and a 'high' dilution that gives extinction just above the detection threshold (usually OD around 0.20 after one-hour incubation time).

3.4. Cover plates with a lid or wrap with foil to minimise evaporation and incubate ELISA plates overnight at 4 °C.

4. Incubation of conjugate on ELISA plates

4.1. Prepare the appropriate dilution of LMV-conjugated antiserum using conjugate buffer (Table II.2).

4.2. Remove seed extracts from the ELISA plates and rinse them well (e.g., three times) with PBS+Tween buffer (Table I.2) to remove residues of the seed extract. Alternatively, use a suitable washing device.

4.3. Immediately after rinsing, add 180 µL of diluted conjugate to each well of the ELISA plate, or as defined by the supplier.

4.4. Cover plates with a lid or wrap with plastic foil to minimise evaporation and incubate plates for three hours at 37 °C, or as defined by the supplier.

5. Addition of substrate to ELISA plates

5.1. Prepare the substrate solution by adding 10 mg para-nitrophenyl phosphate (pNPP) to 20 mL of substrate buffer (Table II.3). Use an alternative substrate when the antiserum is conjugated with peroxidase.

5.2. Remove the conjugate from ELISA plates and rinse them thoroughly (e.g., three times by hand) with PBS+Tween buffer (Table I.2) or alternatively use a reliable washing device.

Note: This step is especially critical for reducing the background.

5.3. Add 180 µL of substrate buffer (Table II.3) to each well and incubate in the dark for 2 hours at 20 °C, or as defined by the supplier. Cover plates with a lid or wrap with plastic foil.

5.4. Measure the extinction value (A_{405}), optical density (OD), with ELISA plate reader. First check whether the positive and negative controls (Table II.4) react according to expectations.

6. Evaluation

Test results are only valid when all included controls presented in Table II.4 give the expected results, as defined by the internal laboratory validation or by the supplier. It is recommended to use a negative-positive threshold of two-times the background of healthy negative controls.

The seed sample is considered positive when one or more subsamples gives an extinction value (A_{405}), OD, equal or above the calculated cut-off. The seed sample is considered negative when all subsamples give an extinction value (A_{405}), OD, lower than the calculated cut-off.

An LMV positive test result should be followed by a seedling ELISA (described in Section III) to confirm the presence of infectious LMV.

III. DETECTION BY SEEDLING ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Materials

- Filter paper (e.g., 3014 Pleated strips (Whatman, Maidstone, UK))
- Flat container to hold filter
- Seed extraction buffer (Table I.3)
- Coating and conjugated LMV antiserum
- ELISA buffers (Tables I.2 and II.1 to II.3)
- Crusher (e.g., Leaf juice press (Meku Erich Pollähne, Wennigsen, Germany))
- Controls (Table II.4)
- ELISA equipment
- Para-nitrophenyl phosphate (pNPP) for substrate solution
- Lab disposables

1. Coating of ELISA plates

See Section II.1

2. Virus extraction from seedlings

2.1. Weigh or count the number of subsamples needed. For each test, use a positive and negative seed lot as a positive process control (PPC) and negative process control (NPC), respectively, to validate the results of the test.

2.2. Place a filter paper (20 × 14 cm) in the seedling germination box(es) (20 × 15 × 3 cm). Alternative sizes of filter paper and box can also be used.

2.3. Transfer each subsample (100 seeds) onto the pleated filter paper. Spread the seeds throughout the pleat avoiding the edges (ca. 1-2 cm) to allow them to germinate uniformly.

2.4. Saturate the filter paper of each seedling germination box with (sterile) water.

2.5. Close the germination box(es) with a transparent lid and incubate for 2 days at 15 °C in darkness.

2.6. Add 10 mL of (sterile) water to each box. Note that the amount of water depends on the type of filter paper used.

2.7. Transfer the germination box(es) to an incubator (18-20 °C) with 8 hours of light (ca. 900 lux) per day and incubate the boxes for 4 days.

2.8. Remove seedlings from the germination box(es) and transfer them to a tube or other suitable container. Add 1.0 mL of extraction buffer and grind each subsample using a power driven crusher. An alternative extraction device (such as a roller or pneumatic press) could be used if the results are comparable to those obtained using the crusher described above.

3. Incubation of extract on ELISA plates

See Section II.3.

4. Incubation of conjugate on ELISA plates

See Section II.4.

5. Addition of substrate to ELISA plates

See Section II.5.

6. Evaluation

Test results are only valid when all included controls presented in Table II.4 give the expected results, as defined by the internal laboratory validation or by the supplier. It is recommended to use a negative-positive threshold of two-times the background of healthy negative controls.

The seed sample is considered positive when one or more subsamples gives an extinction value (A_{405}), OD, equal or above the calculated cut-off. The seed sample is considered negative when all subsamples give an extinction value (A_{405}), OD, lower than the calculated cut-off.

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