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Detection of Lettuce mosaic virus (LMV) in lettuce (*Lactuca sativa*) seed by triplex seed extract RT-qPCR (SE-qPCR) pre-screen assay

Validation report, March 2026

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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 labs 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.

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SUMMARY

Lettuce mosaic virus (LMV) can infect species belonging to Asteraceae, including lettuce. The main control measure is to evaluate seed productions using immunological and PCR based techniques. Naktuinbouw and Rijk Zwaan each developed an LMV targeted primer and probe set for the detection of LMV in seed samples. This report focuses on the validation of the two primer and probe sets in a multiplex LMV seed extract (SE)-qPCR as an indirect pre-screening assay next to the current seed ELISA as screening step and seedling ELISA as confirmatory test for suspect seed lots. The analytical specificity, analytical sensitivity, selectivity, repeatability, and the reproducibility of the assay are evaluated. The diagnostic performance of the LMV seed extract qPCR is evaluated by comparison of the assay against the current LMV seed ELISA. In addition, the robustness of the assay regarding the use of different RNA extraction methods is evaluated.

In silico analysis and testing of the LMV SE-qPCR using RNA from LMV and non-target virus isolates (ArMV, TBRV, and TSWV) demonstrated the analytical specificity of the LMV SE-qPCR. The analytical sensitivity of the LMV qPCR met the requirements by detecting at least six synthetic oligonucleotides, resembling LMV sequences, per µL template with >95% confidence. Moreover, the SE-qPCR was able to detect an LMV infection equivalent to single infected seed in a subsample of 1,000 seeds. Analysis of different lettuce SEs with the same amount of LMV revealed that the seed matrix only limitedly (<3 Cq) impacted the SE-qPCR, adhering to the selectivity requirements. Preparation of the LMV spiked SEs was performed on three time points by the same operator and produced the same qualitative (positive or negative) outcome. This demonstrated the repeatability of the LMV SE-qPCR assay. The reproducibility of the assay was evaluated through a comparative test (CT) where a total of eight laboratories from the Netherlands, France, Spain, and Japan participated in. All laboratories identified subsamples of a high LMV infected seed lot as positive and a healthy lettuce seed lot as negative, leading to excellent concordance scores. The concordance for the medium LMV infected samples was lower (96.88%) but deemed sufficient.

The column-based approach for RNA extraction in the comparative test was compared with a magnetic bead-based approach for the same sample set to assess robustness. The column-based approach yielded lower Cq values compared to the magnetic bead-based approach. Nevertheless, the extraction method did not change the outcome of the LMV SE-qPCR for the high and medium samples, demonstrating the robustness of the LMV SE-qPCR. The magnetic bead-based approach yielded less false positive results for the healthy lettuce seed samples.

Results from the LMV SE-qPCR, LMV seedling ELISA, and seed ELISA for the same lettuce seed lots were compared for evaluation of the diagnostic performance. All methods identified the same lettuce seed lots as positive demonstrating the diagnostic sensitivity of the LMV SE-qPCR in relation to the seedling ELISA. The diagnostic specificity of the LMV SE-qPCR was lower (66.7%) due to false positive results in relation to the seed and seedling ELISA. Nonetheless, the specificity was deemed sufficient for the LMV SE-qPCR as a pre-screening assay for which a superior sensitivity is more important than specificity.

The data presented in this validation report show that the LMV targeted SE-qPCR assay is suitable for the detection of LMV in lettuce seeds and demonstrate the validity of the LMV SE-qPCR as an optional pre-screening assay in the ISHI LMV detection method.

1. INTRODUCTION

Lettuce mosaic virus (LMV), now called *Potyvirus lactucae*, belonging to the genus *Potyvirus* of the family *Potyviridae*, was first described in 1921 and is detected worldwide. The virus can infect lettuce (*Lactuca sativa*) and other species belonging to the family Asteraceae (German-Retana *et al.*, 2008). LMV is aphid transmitted and tends to be more problematic for field crops as compared to greenhouse productions, which have better insect control. Isolates of LMV are commonly divided into three groups: LMV-Yar, LMV-Greek and LMV-RoW. Isolates within the LMV-RoW group are further subdivided into LMV-Common, which are seedborne and non-resistance breaking, and LMV-Most, which are seedborne and resistance breaking (Krause-Sakate *et al.*, 2002). There are no seed treatments described to date, and the main control measure is to evaluate seed productions. LMV can be detected by immunological (e.g., Enzyme-Linked Immunosorbent Assay (ELISA)), as well as Polymerase Chain Reaction (PCR)-based techniques.

The current ISHI protocol describes an indirect ELISA-based assay detecting LMV on lettuce seeds and seedlings. Naktuinbouw and Rijk Zwaan collaborated in developing a seed extract (SE)-qPCR assay consisting of primer pairs LMV-NAKT and LMV-RZ2, targeting gene sequences of LMV coat and P3 protein, respectively. Implementing these qPCR assays as a pre-screening step before the existing ISHI seedling ELISA assay would facilitate a swift detection of healthy seed lots as it enables an increase in subsample size due to a higher sensitivity, requiring less subsamples to be tested for the same sample size, which would improve the efficiency of the current method.

The LMV SE-qPCR may be included to the LMV detection method as a pre-screening step. The seed and seedling ELISA are intended to remain an integral part of the LMV detection method where the seed ELISA is regarded as a screening step (potentially following the proposed LMV SE-qPCR), and the seedling ELISA is used as a confirmatory test for suspect seed lots. This report focuses on validation of the LMV targeted SE-qPCR. The final workflow, with incorporation of the seed ELISA and seedling ELISA, is presented in Figure 1.

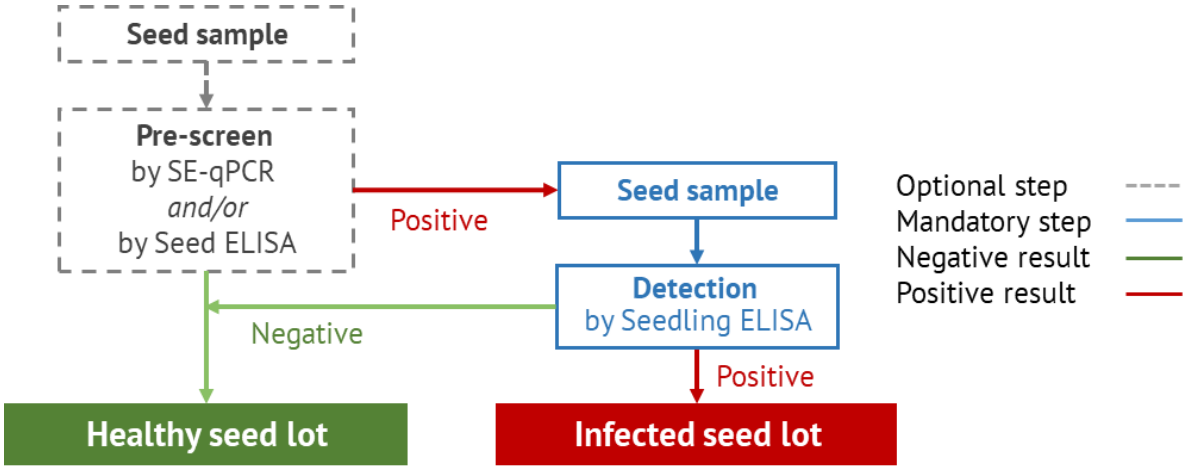


Figure 1. Method process workflow for the detection of LMV in lettuce seeds.

2. OBJECTIVES

The aim of the present validation plan is to determine whether the proposed LMV SE-qPCR (see protocol in Annex A) is fit for purpose as a pre-screening assay according to the ISHI validation guidelines. The requirements and experimental setup for validation are detailed in this validation plan for each performance characteristic, focusing on the analytical specificity, analytical sensitivity, selectivity, repeatability, reproducibility, and diagnostic performance of the LMV SE-qPCR. In addition, the robustness of the assay regarding the use of different RNA extraction methods is evaluated. During the evaluation of the diagnostic performance, the LMV SE-qPCR performance will be compared to that of the LMV seed ELISA. If the LMV SE-qPCR is deemed fit for purpose, it will be incorporated into the LMV detection protocol, not replacing the seed ELISA and/or seedling ELISA.

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 all tested LMV strains or LMV positive seed lots (n = 21) are detected with the LMV targeted primer and probe sets in the LMV triplex SE-qPCR, and the internal amplification control (IAC), when used, is in the expected range in the triplex SE-qPCR.

Furthermore, at least 95% of non-LMV selected strains or LMV negative seed lots (n = 37) must give negative qPCR result.

Experimental setup

The specificity of the LMV-NAKT and LMV-RZ2 primer and probe sets was previously determined *in silico* (Annex B). This analysis revealed that the LMV-NAKT and LMV-RZ2 primer and probe sequences matched sequences from LMV-Common and LMV-Most isolates (overcoming $mo1^1$ and $mo1^2$ resistance genes in lettuce; Krause-Sakate *et al*., 2002), albeit that the LMV-NAKT primer and probe set had single mismatches located in the middle or 5' end of the oligonucleotide, with different sequences. The LMV-RZ2 reverse primer had a single mismatch in the middle of the oligonucleotide with a single LMV sequence (Annex B). While the LMV triplex SE-qPCR showed amplification for naturally LMV infected seed lots, partial LMV genome sequences obtained for these seed lots were different. This revealed that the two LMV primer and probe sets enabled detection of various LMV in seeds (Annex B). While the LMV-RZ2 primer and probe set matches the sequence of an LMV-Yar isolate and the LMV-NAKT primer and probe set seems to be able to detect LMV-Greek isolates, it is not clear if both LMV groups would be detected by both primer and probe sets. Considering that these clusters are not known to contain seedborne LMV strains (Krause-Sakate *et al*., 2002), their detection was not found to be mandatory.

The previously obtained specificity data were complemented by performing the LMV triplex SE-qPCR using RNA (and 100× diluted RNA) from 12 LMV isolates that were collected from at least four different geographical locations (Table 1). A total of five Tomato black ring virus (TBRV, now named *Nepovirus nigranuli*), five *Arabis mosaic virus* (ArMV, now named *Nepovirus arabis*), and one *Tomato spotted wilt virus* (TSWV, now named *Orthotospovirus tomatomaculae*) were included as non-target isolates. These viruses are not closely related to LMV, but can be found in lettuce

(EPPO, 2020; 2025) and, therefore, the LMV targeted SE-qPCR should not yield positive signals that would erroneously be interpreted as confirmation for the presence of LMV.

Table 1. Origin and details for used isolates for inclusivity and exclusivity.

Virus	Isolate	Country of origin	Provided by
LMV	5386	The Netherlands	BASF
	PV-0286	Denmark	
	PV-2799	Iran	
	LMV0	Unknown	Geves
	LMV9	Unknown	
	10516 (from lettuce)	United States	Rijk Zwaan
	LS234 (from lettuce)	Unknown	
	LS252 (from endive)	Unknown	
	LSI (from lettuce)	Unknown	
	Most	Unknown	
	1	The Netherlands	Syngenta
	2	Unknown	
ArMV	PV-0046	Unknown	DSMZ
	PV-0215	Unknown	
	PV-0216	Denmark	
	PV-0217	South Bohemia, Czech Republic	
	WUR	Boskoop, The Netherlands	Prime Diagnostics
TBRV	Infected good-king-Henry leaf	Unknown	Prime Diagnostics
	Infected lettuce leaf	Unknown	
	Infected shallot leaf	Unknown	
	Infected tulip leaf	Unknown	
	Infected potato leaf (PV-0070)	Unknown	DSMZ
TSWV		Unknown	Prime Diagnostics
DLVd		The Netherlands	Rijk Zwaan

LMV isolate 5386, 10516, LS234, LS252, LSI, and Most as well as the five ArMV isolates were provided as infected plant leaf material. RNA extraction for these isolates was performed by transferring a small amount of leaf material to a 1.5 mL microcentrifuge tube together with a 1.4 mm diameter metal ball. Following a grinding step of 30 sec at 1300 rotation per minute (rpm) using a Geno/Grinder 2010, the ground leaf material was mixed with 1 mL extraction buffer (EB) that was prepared according to protocol and spiked with Dahlia latent viroid (DLVd, now named *Hostuviroid latensdahliae*). Subsequently, 100 µL of the leaf suspensions was used as input for RNA extraction using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) starting by mixing the suspension with RLT buffer. RNA extraction for LMV isolate PV-0286, PV-2799, LMV0, and LMV9 as well as the TBRV isolates was performed as above, but instead of DLVd spiked EB, 1 mL (or 750 µL for the TBRV isolates) unspiked PBS was used to suspend the ground leaf material. Isolate PV-0286 and PV-0799 were provided in general extraction buffer 3 (GEB3; Agdia, Elkhart, IN) of

which 100 µL was used as input for RNA extraction. RNA from the TSWV isolate was extracted at Rijk Zwaan using an internal protocol for which leaf material is ground and suspended in guanidine-based lysis buffer (not spiked with DLVd) followed by purification of the RNA using the Sbeadex plant maxi kit in combination with the KingFisher platform. LMV isolate 1 and 2 were kindly provided by Syngenta as RNA extracts.

In addition, the LMV triplex SE-qPCR was performed on nine confirmed naturally LMV infected seed lots produced in NL and 26 LMV negative seed lots, as determined using the LMV seedling ELISA. The seed lots were chosen to include black and white lettuce seeds obtained from different countries (Table 2). One seed sub sample was tested for all seed lots, except for SE 33, 34, and 35, for which three subsamples were tested.

The results of the LMV SE-qPCR were analysed qualitatively.

Results

Obtained Cq values are presented in Table C.1 in Annex C and are visualized in Figure 2. The results of the LMV SE-qPCR were analysed qualitatively, where Cq values <32 for either of the two LMV targeted primer and probe sets ("LMV-RZ2" or "LMV-NAKT") are considered positive and Cq values >32 are considered negative. See Annex D for the explanation of the determination of the 32 Cq cut-off.

PCR results of the PCR grade water (negative template control; NTC) did not show a relevant positive PCR result and results of SE from an LMV negative seed lot used as negative process control (NPC), as well as a negative extraction control (NEC), did not show a Cq value below 33.66 for both LMV targeted primer and probe sets (Figure 2 and Table C.1). Furthermore, none of the ArMV, TBRV and TSWV isolates showed low Cq values for both LMV targeted primer and probe sets, whereas RNA from LMV infected leaves showed low Cq signals varying from Cq 11 to 27 Cq. PCR signals were also obtained using the DLVd targeted primer and probe set (used as the IAC in the LMV SE-qPCR) for RNA from DLVd (Cq 23), for the target and non-target isolates with varying Cq values between 28 and 37, and for SE from LMV positive and negative seed lots with Cq values between 25 and 29. The Cq values with the DLVd targeted primer and probe set for SE were around the expected value of 28. The relatively high DLVd Cq values or no signal with the DLVd primer and probe set for the target and non-targeted isolates are due to using the extraction buffer that was not spiked with DLVd or was spiked with a different DLVd concentration, and subsequently used for the Qiagen or Sbeadex based RNA extraction that did not yield a Cq value with the DLVd primer and probe set. The PCR results for the TBRV and TSWV isolates did not show any amplification signal with the LMV targeted primer and probe sets as well as the DLVd primer and probe set. Although the DLVd signal is not essential for interpreting PCR results for the target isolates, its absence as the IAC for TBRV and TSWV means that successful PCR cannot be confirmed for these isolates. Specificity of the LMV-NAKT and LMV-RZ2 primers for ArMV, TBRV, and TSWV was further explored by employing the Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), which uses the Primer3 program (version 2.5.0; Ye, *et al*., 2012) on 07 August 2025 using default parameters and selecting TRBV, ArMV, and TSWV sequences in the "nt" and "RefSeq Representative Genome Database" databases. This did not show any predicted amplification product, indicating that the LMV-NAKT and LMV-RZ2 primer and probe sets will likely not yield non-specific PCR amplification for ArMV, TBRV, and TSWV.

Table 2. Origin and details for used seed batches for specificity.

Seed batch	Country of origin	Colour seed	LMV seedling ELISA result
1	Chile	White	Negative
2	Chile	Black	Negative
3	India	Black	Negative
4	United States	White	Negative
5	United States	White	Negative
6	India	White	Negative
7	United States	White	Negative
8	Zambia	White	Negative
9	Zambia	White	Negative
10	United States	Black	Negative
11	Chile	White	Negative
12	Chile	White	Negative
13	Chile	White	Negative
14	Chile	White	Negative
15	Chile	White	Negative
16	Chile	White	Negative
17	Chile	White	Negative
18	Chile	White	Negative
19	Chile	Black	Negative
20	Chile	White	Negative
21	Chile	White	Negative
22	Chile	White	Negative
23	Chile	White	Negative
24	Chile	White	Negative
25	Chile	White	Negative
26	Chile	White	Negative
27	The Netherlands	White	Positive
28	The Netherlands	White	Positive
29	The Netherlands	Black	Positive
30	The Netherlands	Black	Positive
31	The Netherlands	Black	Positive
32	The Netherlands	White	Positive
33	The Netherlands	Black	Positive
34	The Netherlands	White	Positive
35	The Netherlands	White	Positive
36	Chile	White	LMV seedling ELISA not performed. Considered healthy (See section 3.5).
37	The Netherlands	White	

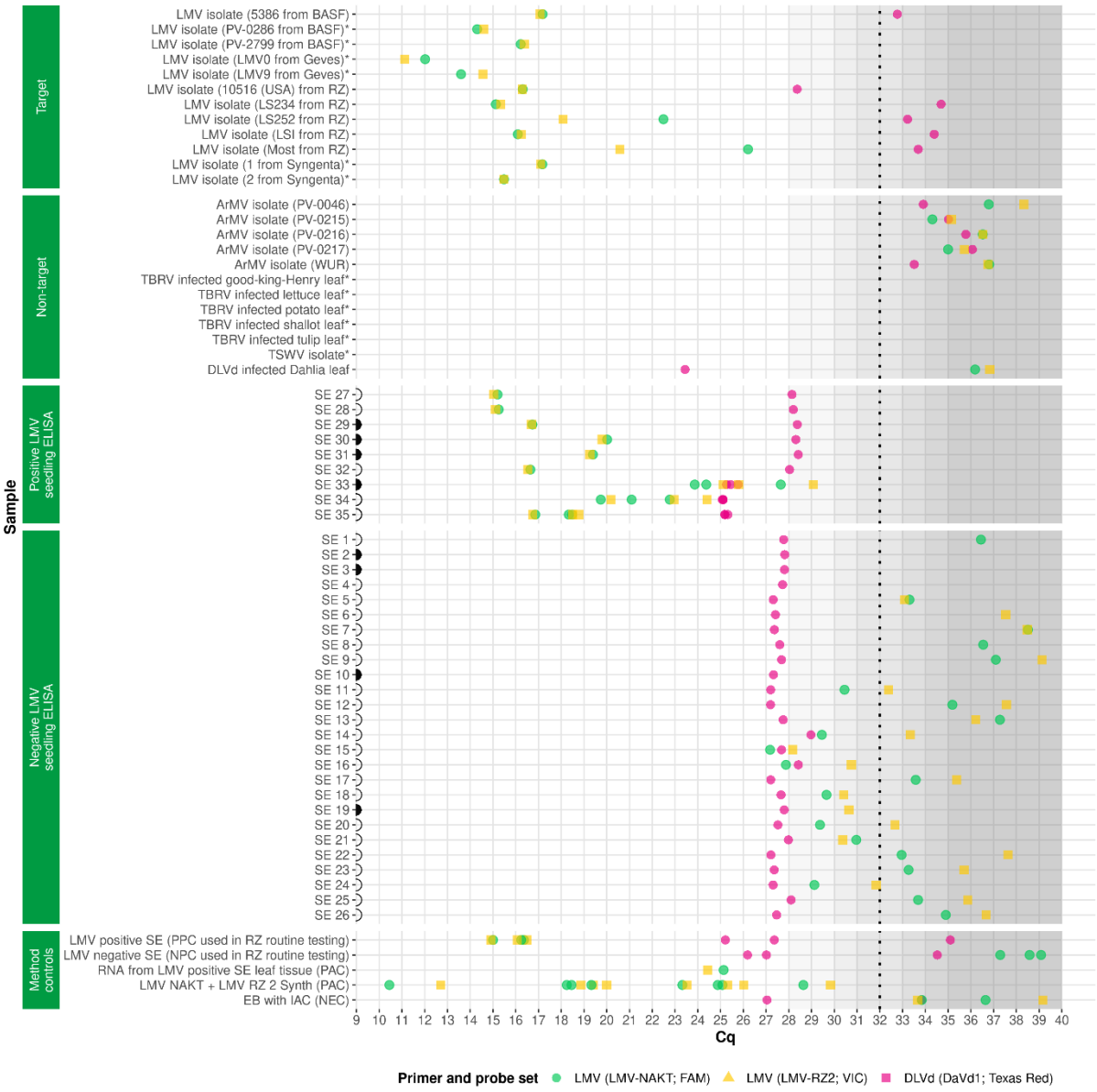


Figure 2. Obtained Cq values of the LMV triplex PCR for RNA from SE and leaf extracts. The shades in the grey background represents the range for which the SE-qPCRs give 100% sensitivity and different levels of specificity (Cq = 28-29: 93%; Cq = 30: 79%; Cq = 31-32: 69%; Cq = 33: 66%; Cq = 34: 52%; Cq ≥ 35: ≤ 49%). The dotted line indicates the chosen Cq cut-off that is applied to discriminate between LMV positive and negative lettuce seed samples. Open and solid black semi-circles on the right of the SE lot numbers indicate if the lettuce seed was white or black, respectively. *: Isolate was not spiked with DLVd during RNA extraction and no signal with the DLVd targeted primer and probe set (DaVd1) primer and probe set is expected.

Cq values for SE from an LMV positive seed lot, used as positive process control (PPC), as well as from SE 27-35 that were previously detected as positive for LMV using the LMV seedling ELISA, varied between 14 to 30 with both LMV targeted primer and probe sets (Figure 2). Cq values from SE from LMV negative seed lots, as determined by the LMV seedling ELISA, predominantly showed Cq >32 or did not yield a Cq. However, some seed lots showed Cq values <32, even with Cq values as low as 27 with one of the LMV targeted primer and probe sets (Figure 2). This suggests that when the LMV SE-qPCR is performed routinely, the LMV SE-qPCR may provide a positive outcome for seed lots that have a negative outcome with the LMV seedling ELISA.

With a Cq 32 cut-off, none of the non-target isolates tested here were found positive with the LMV targeted qPCR, while nine out of 26 (= 34.6%) of samples that were found negative using the LMV seedling ELISA were found positive using the LMV SE-qPCR (Figure 2). Note that for four out of nine only one of the qPCR primer and probe sets gave a positive result. This reveals that less than the set requirement of at least 95% of the non-target isolates and LMV negative seed lots were found negative with the LMV qPCR. However, the seed lots were chosen to include those that were negative for LMV, but also those with varying LMV infection levels determined by the LMV SE-qPCR and negative with the LMV seedling ELISA. The latter group of seed lots are overrepresented in the chosen seed lot set used here (Table 2). In practice, less than 5% of all seed lots tested with the LMV SE-qPCR are found non-negative and require confirmation using the LMV seedling ELISA (Data not shown). Furthermore, the LMV SE-qPCR also detects RNA from non-infectious LMV. This would lead to a positive result for the LMV SE-qPCR but will be found negative in the follow up seedling ELISA.

Conclusion

All tested LMV strains (n = 12) and LMV positive seed batches (n = 9) were detected as positive by the LMV qPCR with Cq <32. All of the non-target virus isolates (ArMV, TBRV, and TSWV; n = 11) resulted in negative outcomes with the LMV qPCR and based on *in silico* analysis are not predicted to yield a PCR product. These findings are in agreement with the requirement of at least 95%, for specificity. Seed lots with false positive results with the LMV SE-qPCR are expected to occur infrequently (<5% of seed lots tested under routine conditions). Therefore, the LMV SE-qPCR is suitable as pre-screening assay. Seed lots with positive results with the LMV SE-qPCR are evaluated further using, for example, the LMV seedling ELISA as a follow up, which would discriminate between truly LMV negative and LMV positive seed lots.

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 will be met when the LMV targeted qPCR assays are able to detect at least 100 copies of synthetic single stranded DNA (ssDNA) oligonucleotides, with similar LMV sequence identity and carrying the target sites of the primers and probes, with at least 95% confidence and a PCR efficiency between 90% and 110%. Furthermore, it is required that the SE-qPCR assay, including seed preparation, RNA extraction, and the LMV targeted qPCRs, enables for the detection of one infected seed in 1,000 seeds.

Experimental setup

The analytical sensitivity of the LMV targeted qPCR assays as well as the complete LMV SE-qPCR detection assay, including seed preparation, RNA extraction and the LMV qPCR, was determined. The technical approach for both assessments is detailed below.

Analytical sensitivity of the LMV targeted qPCR assays.

ssDNA oligonucleotides were used to determine the LOD as well as the efficiency of both primer and probe sets to detect LMV, circumventing the need for actual infectious LMV (Figure 3).

This approach provides data on the performance of the qPCR itself, leaving out possible bias by the seed preparation and RNA extraction that are part of the complete SE-qPCR assay. Assuming a molecular mass (provided by the supplier, Integrated DNA Technologies, Coralville, IA) of 51,307.1 g mol^{-1} for the LMV-RZ2 synthetic ssDNA oligonucleotide and 37,477.3 g mol^{-1} for the

LMV-NAKT synthetic ssDNA oligonucleotide, six independent 10-step serial 10-fold dilution series in PCR grade water were prepared from a combination of the oligonucleotides with the highest dilution containing one of each oligonucleotide per 100 µL solution.

LMV RZ 2 amplicon region from sequence from LMV NCBI Reference sequence NC_003605.1:
GAGGACTCGTGGCGCGCATTGCCATTGTGTGGAAAATTATCAGCAATGCGAGTCTCGCGGCGATGGCGGGACACCTCTACTCCCGAAGCAATCC
CA***ACAGGTGCCGCAGATTTGAAAGGCAG***ATACAGTATCTCGGTTGGGTCTGTTTCCAAAAGCGCGATCTTGC

LMV RZ 2 synthetic oligonucleotide (166 nt):
GAGGACTCGTGGCGCGCATTGCCATTGTGTGGAAAATTATCAGCAATGCGAGTCTCGCG**TCCCGTCGCTCAAGTGGCG**ACTCCCGAAGCAATCC
CA***ACAGGTGCCGCAGATTTGAAAGGCAG***ATACAGTATCTCGGTTGGGTCTGTTTCCAAAAGCGCGATCTTGC

LMV NAKT amplicon region from sequence from LMV NCBI Reference sequence NC_003605.1:
TTAATTCTGAACGGATTGATGGTTTGGTGTATAGAAAACGGG***ACATCCCCGAATATAA***ATGGAACATGGGTGATGATGGACAGTGAAGAACAAG

LMV NAKT synthetic oligonucleotide (120 nt):
TTAATTCTGAACGGATTGATGGTTTGGTGTATAGAAAACGG**CGCTCATTGAGGTTGTGGACAGGAAA**G***ACATCCCCGAATATAA***ATGGAACATG
GGTGATGATGGACAGTGAAGAACAAG

Figure 3. The predicted amplicon (with 15 nt flanking region) and proposed synthetic oligonucleotide for the LMV-NAKT and LMV-RZ2 primer and probe sets based on the LMV reference sequence (NC_003605.1). Forward and reverse primers are underlined, and probe sequences are indicated in bold italic. The added sequence for the synthetic oligonucleotides to distinguish the synthetic oligonucleotides from the natural LMV sequence is indicated in bold green and match sequences from *Fusarium* targeted probes (FusA for LMV-RZ2 and FusE for LMV-NAKT).

The LMV triplex qPCR was performed three times in 96-well plates, according to the protocol (Section 2 “RT-qPCR” in Annex A) for the oligonucleotide dilutions. The LOD for both assays was expressed as the number of oligonucleotides per µL of PCR template solution for which 18 out of 18 replicates (to meet a confidence of >95%) were detected with a Cq value standard deviation of maximally 0.3. The latter gives insights into the precision of the qPCR.

Analytical sensitivity of the LMV SE-qPCR assay.

The analytical sensitivity of the complete SE-qPCR assay, including seed preparation, RNA extraction and the qPCR with the two LMV targeted primer and probe sets, was assessed by spiking each of 10 subsamples of 985 seeds each from a healthy lettuce seed lot, used as negative process control (NPC), with 15 seeds from an LMV infected seed lot that is used as PPC. In addition, several subsamples with 1,000 seeds from a healthy lettuce seed lot were used as NPC. Seeds were ground and suspended in 10 mL extraction buffer spiked with DLVd, according to steps 1.2 to 1.4 in the LMV SE-qPCR protocol in Annex A. This was followed by 15 times dilution and a subsequent 10 times dilution (yielding a 150 times dilution of the original SE) of the suspensions in the extract from the LMV negative seed lot (NPC). The 15 times and 150 diluted SE would have an LMV load equivalent to one seed and 0.1 seed, respectively, from the LMV positive seed lot of a total of 1000 seeds (Figure 4). An aliquot of 100 µL of each seed suspension was used as input for the RNeasy Plant Mini Kit based RNA extraction, according to the manufacturer’s instructions, followed by the LMV SE-qPCR that was performed in duplicate (see steps 1.5 to 2.3 in the LMV SE-qPCR protocol in Annex A). The resulting data was used to assess the capacity of the LMV pre-screening SE-qPCR assay (seed sample preparation, RNA extraction and purification, and the qPCR itself) to detect a single naturally infected seed in a total (diluted) sample of 1,000 seeds. The Qiagen kit-based extraction approach was chosen as it can readily be performed in conventional molecular laboratories. An automated process, such as the Sbeadex plant maxi kit (LGC Genomics, Teddington, Middlesex, UK) in combination with the KingFisher platform (ThermoFisher scientific, Waltham, MA, USA), could be preferred for laboratories that test samples in high throughput. Therefore, the KingFisher approach was also evaluated during the comparative test (CT; See “Reproducibility” and “Robustness”) to demonstrate its validity.

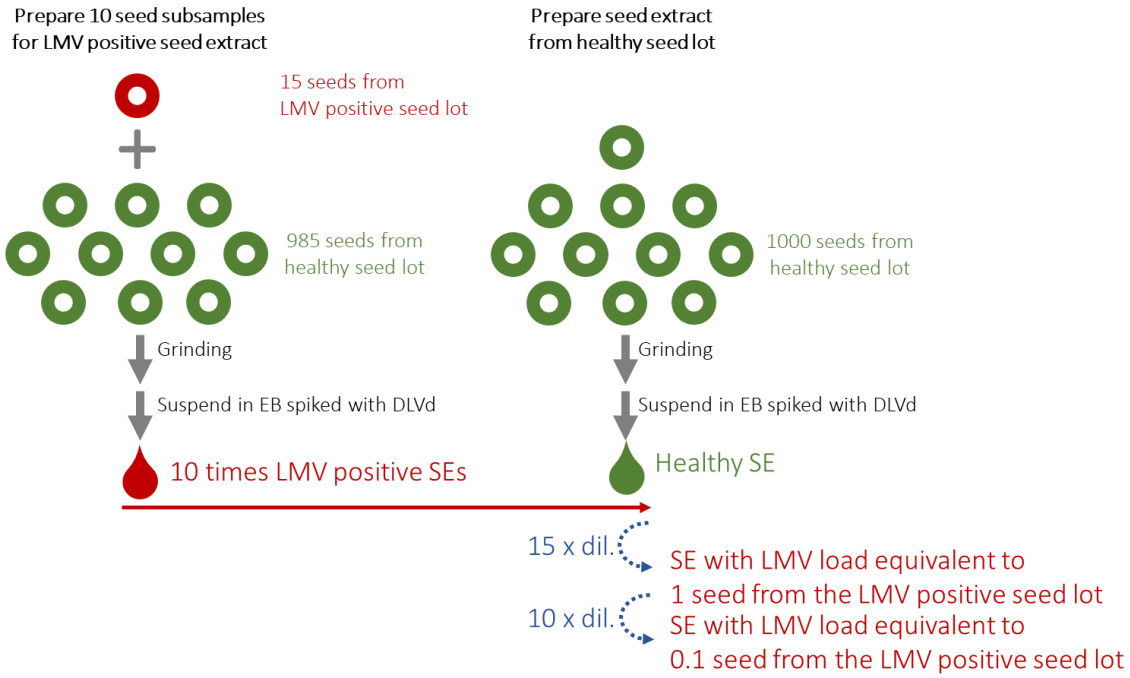


Figure 4. Schematic representation of obtaining SE from 10 subsamples consisting of 15 seeds from an LMV positive seed lot and 985 seeds from a healthy lettuce seed lot of which the SE is diluted 15 times and 150 times to obtained SE with an LMV load equivalent to one seed and 0.1 seed, respectively.

Results

Analytical sensitivity of the LMV targeted SE-qPCR assays.

Cq values are included in Table E.1 in Annex E and are visualized in Figure 5. The PCR results of the PCR grade water (NTC) did not yield any Cq value. When the two ssDNA oligonucleotides were diluted together in PCR grade water and used as PCR template mimicking the presence of the two targets in a single sample, a Cq value was obtained for all of the 18 replicates (six independent dilution series, performed in triplicate) with the number of target molecules at least six target copies per µL (Figure 5). Precision of the PCR dropped for PCR reactions with $\leqslant 6$ or $\geqslant 6 \times 10^7$ copies per µL for the LMV-NAKT primer and probe set and $\leqslant 60$ or $\geqslant 6 \times 10^6$ copies per µL for the LMV-RZ2 primer and probe set, as illustrated by standard deviations in Cq values > 0.3 obtained for replicate PCRs for these concentrations of the two oligonucleotides (Figure 5).

Analytical sensitivity of the LMV SE-qPCR assay.

The Cq values are included in Table E.2 in Annex E and are visualized in Figure 6. The PCR results of the PCR grade water (NTC) did not show any Cq values (Table E.2).

Results of SE from an LMV negative seed lot used as NPC did not show a Cq value < 38 with both LMV targeted primer and probe sets (Table E.2). As expected, the synthetic ssDNA oligonucleotides, used as positive amplification control (PAC), and RNA from SEs from the LMV positive seed lot, used as PPC, showed low Cq values (Cq <26; Table E.2).

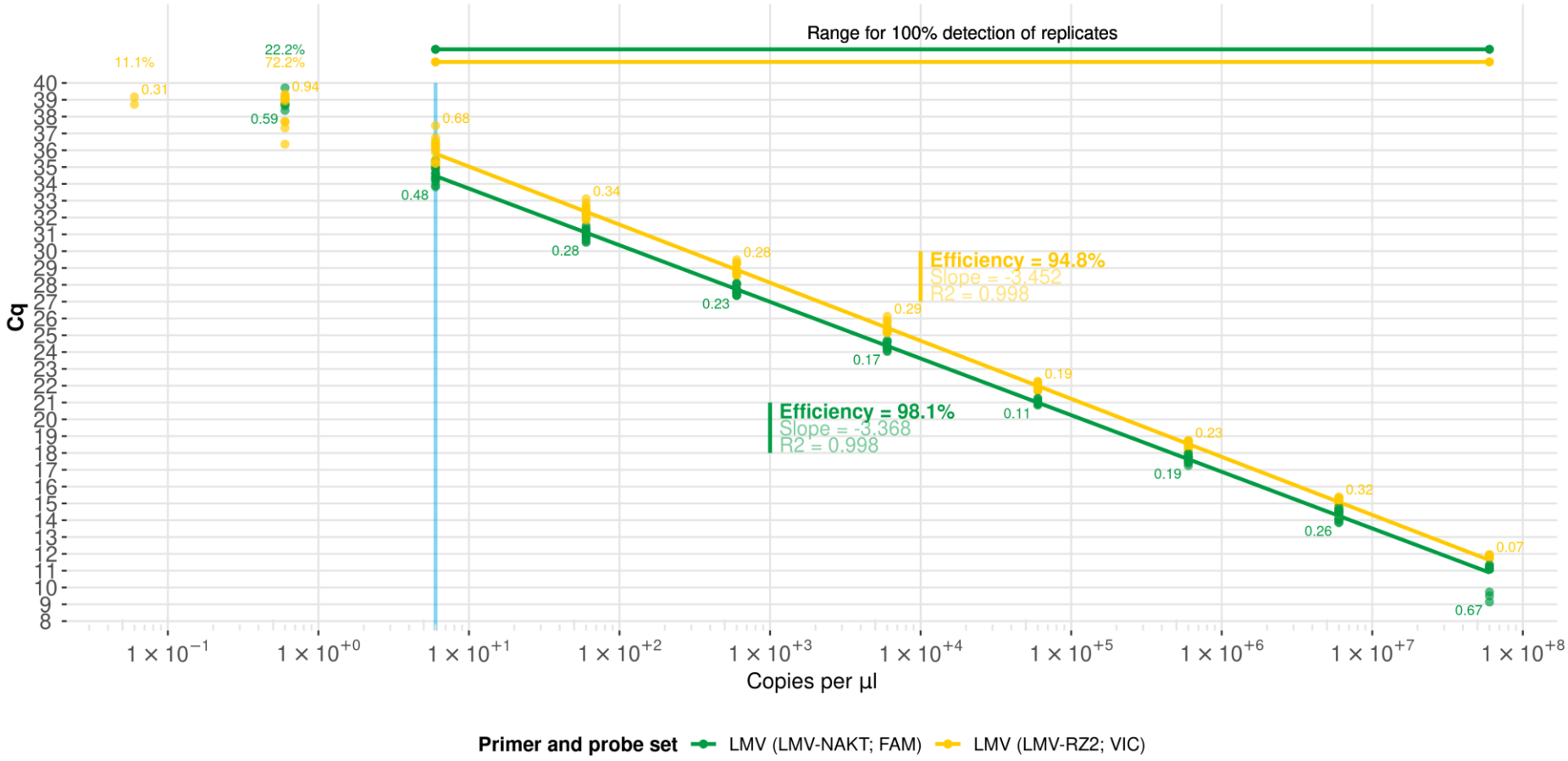


Figure 5. Visualisation of Cq values and corresponding curve of standard dilutions series using the LMV-NAKT and LMV-RZ2 synthetic oligonucleotides as template for the LMV targeted triplex qPCR (in 96-well plate). Bars above the graph indicate the range for which the two primer and probe sets yielded 100% detection of replicates. Rates above the graph indicate percentage of replicates detected. Numbers next to the data points indicate standard deviations in Cq values for replicates for each oligonucleotide concentration and primer and probe sets. The blue line indicates the lowest concentration ssDNA oligonucleotides (six target copies per µL) for which all 18 replicates (six independent dilution series, performed in triplicate) yielded a Cq value.

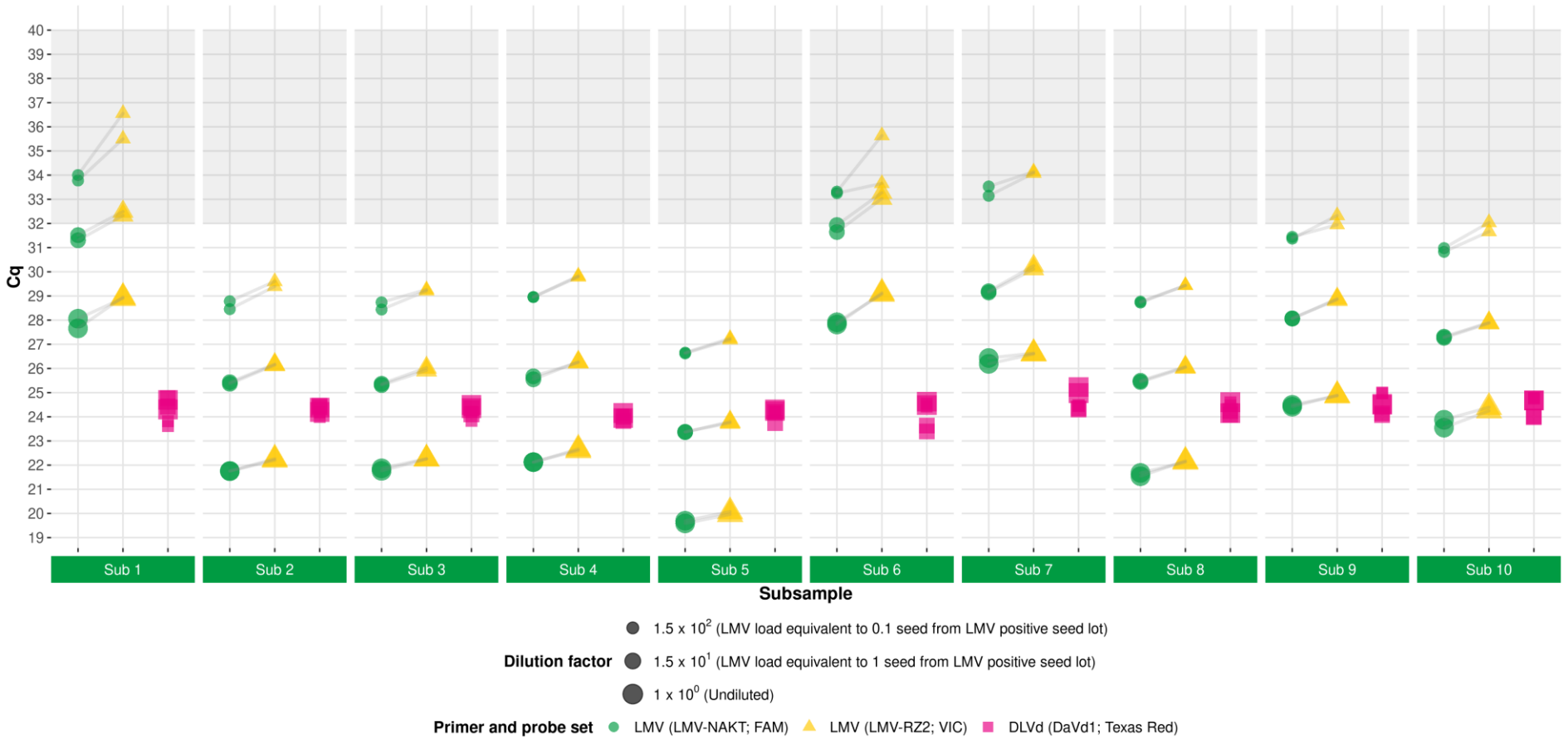


Figure 6. Obtained Cq values of the duplicate LMV triplex PCR for RNA from dilutions of seed extracts from 10 subsamples (Sub 1-10) for which 15 seeds from an LMV positive lettuce seed lot was mixed with 985 seeds from an LMV negative lettuce seed lot (upper panel). The grey lines connect SE-qPCR signals from the LMV-NAKT and LMV-RZ2 primer and probe set obtained from the same well. The grey background represents the range for which the SE-qPCRs are considered negative (Cq 32-40).

All 10 SE subsamples derived from 15 seeds from an LMV positive lot mixed with 985 seeds from an LMV negative lettuce lot, yielded a positive PCR signal with Cq values <32 for the LMV-NAKT and LMV-RZ2 primer and probe sets. More importantly, all 15-time diluted SE subsamples, having an LMV load equivalent to a single seed, also yielded a positive PCR signal, though for two subsamples (Subs 1 and 6) only the LMV-NAKT primer and probe set yielded Cq values <32 (Figure 6). Considering that at least one of the two LMV primer and probe set need to yield Cq values <32 in this validation study, these diluted SE subsamples were also deemed positive for LMV. Interestingly, the LMV SE-qPCR also yielded positive PCR signals for SE of seven out of 10 subsamples (Subs 2-5, 8-10) with an LMV load equivalent to 0.1 seed from the positive seed lot (Figure 6). This indicates that the LMV SE-qPCR is sufficiently sensitive to detect LMV even when the LMV load per seed is low.

Conclusion

Both LMV targeted primer and probe sets (LMV-NAKT and LMV-RZ2), detected the synthetic oligonucleotides with a concentration of at least six target copies per µL with >95% confidence. The PCR efficiency for an oligonucleotide concentration of 6 to 6×10^7 copies per µL was between 90 and 110%. In addition, the SE-qPCR assay detected an equivalent LMV load of a single seed (and even an order of magnitude lower) from an LMV infected seed lot in a total of 1,000 seeds tested. Therefore, the target for analytical sensitivity was deemed achieved.

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 LMV triplex SE-qPCR results for extracts from different lettuce varieties produced in different countries spiked with the same amount of LMV do not deviate more than three Cq.

Experimental setup

A total of 1,000 seeds from LMV negative seed lots (two (black seeds), four (white seeds) and six (white seeds); Table 2) from three different lettuce varieties produced in Chile, United States, and India were transferred together with a 14 mm metal ball to a tube, after which the seeds were ground and suspended in 10 mL extraction buffer according to steps 1.2 to 1.4 in the LMV SE-qPCR protocol in Annex A. During assessment of the LMV infection level of the LMV positive seed lot used for "Analytical sensitivity of the LMV SE-qPCR assay" (Section 3.2), whereby 10 subsamples of a single seed of the LMV positive seed lot were mixed with 999 seeds each from a healthy lettuce seed lot and analysed according to the LMV SE-qPCR protocol in Annex A (Data is included in Table F.1 in Annex F), SE from sub 7 showed a relatively high LMV signal (Figure F.1 in Annex F) and 100 µL was therefore used here as spike and added to 900 µL of each of the LMV negative seed extracts (SEs). The spiked SEs (a 10-time dilution of the original spike) were diluted in a four-step serial 10-fold dilution series with the highest dilution being 10^5 (Figure 7). Based on the Cq values obtained for sub 7 during assessment of the "Analytical sensitivity", the 10^2-time dilution of this extract in LMV negative SE is expected to give a Cq value just below the determined cut-off of 32. Considering that the LMV positive seed extract is a homogenous suspension, the amount of LMV added to each of the LMV negative SEs is assumed to be the same and Cq from the spiked LMV negative seed lots can be used to determine a potential matrix effect of the different lettuce seed varieties.

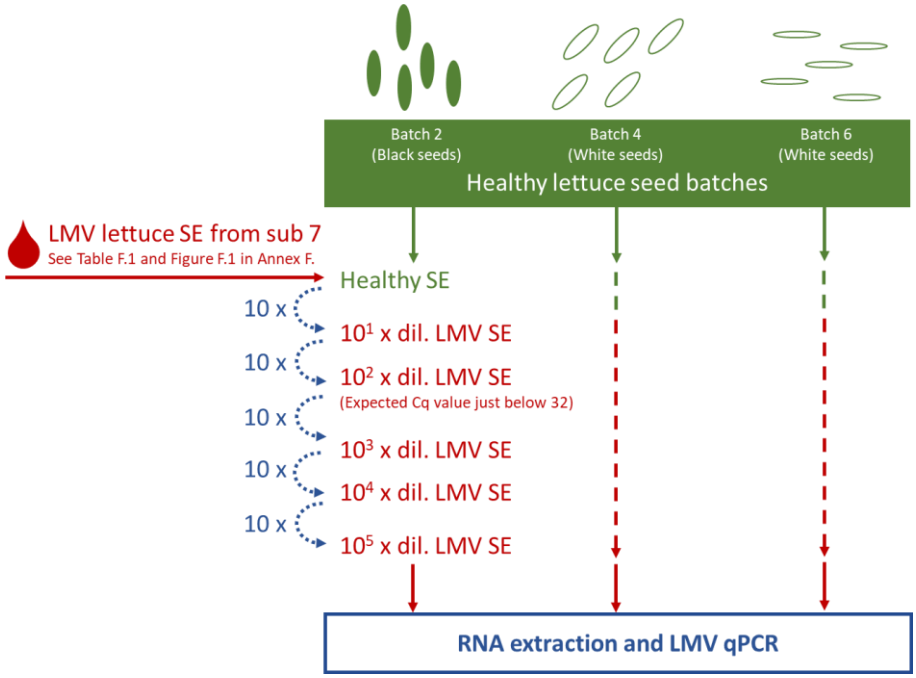


Figure 7. Schematic representation of spiking different LMV negative (healthy) SE from seed batch 2, 4, 6 (Table 2) with LMV positive SE (Sub 7 acquired during assessment of the LMV infection level of the LMV positive seed lot used for "Analytical sensitivity of the LMV SE-qPCR assay" (Section 3.2; Figure F.1) followed by preparation of a dilution series in healthy SE, RNA extraction and the LMV qPCR.

Three aliquots of 100 µL from the same LMV spiked and unspiked SEs was used as input for RNA extraction by the same operator where extraction took place on one of three different time points using the RNeasy Plant Mini Kit (Qiagen) and followed by the LMV triplex SE-qPCR (see steps 1.5 to 2.3 in the LMV SE-qPCR protocol in Annex A). The deviation in obtained Cq values between the different lettuce seed varieties was used to assess the impact of the seed matrix on the detection of LMV. The deviation in Cq values between the three time points was used to assess the repeatability of the LMV SE-qPCR based detection method of which results are included in Section 3.4.

Results

The Cq values from the LMV targeted triplex SE-qPCR are included in Table F.2 in Annex F. Considering the small differences in Cq values obtained at the three time points (see "Repeatability"; Section 3.4), Cq values were averaged and are visualized in Figure 8.

The SE-qPCR results of the PCR grade water (NTC) did not show a Cq value with the two LMV targeted primer and probe sets and the DLVd targeted primer and probe set. Results of SE from a LMV negative seed lot used as NPC did not show a Cq value below 35.43 for both LMV targeted primer and probe sets (Table F.2). As expected, the synthetic ssDNA oligonucleotides, used as PAC, showed Cq values between 25.7 and 28.2 and RNA from SEs from the LMV positive seed lot, used as PPC, showed Cq values between 19.6 and 20.5 (Table F.2).

The difference in average Cq of the LMV-NAKT, LMV-RZ2, and DLVd primer and probe sets between LMV spiked SE from lettuce batch 2, 4, and 6 was all below three, even when the spike was diluted 10,000 times and the Cq values were higher than the cut-off of 32 (Figure 8). Furthermore, the same trend was also observed when the analysis was performed for each time point separately (analysis not shown).

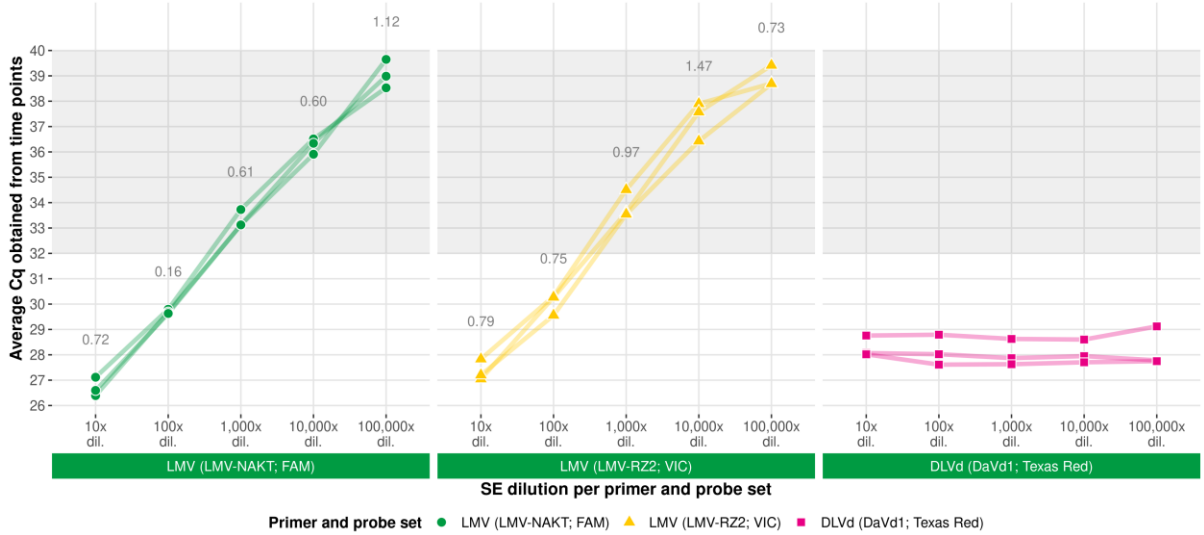


Figure 8. Averaged Cq values of the LMV triplex qPCR performed at three time points for RNA from dilutions of lettuce SE spiked with LMV positive SE (Sub 7 acquired during assessment of the LMV infection level of the LMV positive seed lot used for "Analytical sensitivity of the LMV SE-qPCR assay" (Section 3.2; Figure F.1)). Deviations in Cq of the two LMV targeted primer and probe sets between SE 2, 4, and 6 are added above points per SE dilution factor. The grey background represents the range for which the SE-qPCRs are considered negative (Cq 32-40).

Conclusion

Using SE prepared as indicated in the protocol and RNA extracted using the Qiagen kit, the impact of the lettuce SE matrix on the detection of LMV was deemed unbiased as deviations in Cq values for LMV-NAKT, LMV-RZ2, and DLVd primer and probe sets between SEs from different lettuce batches, spiked with LMV, was <3 Cq. Therefore, the requirement for selectivity was achieved.

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 laboratory.*

The repeatability requirements will be met when the accordance in the SE-qPCR outcome between triplicate reactions performed in the same laboratory by the same operator for the two LMV targeted SE-qPCR assays performed for the same RNA is higher than 90%.

Experimental setup

The approach described under "Selectivity" (Section 3.3) was performed at three separate time points, all on the same day by the same operator. The obtained qualitative results from the LMV SE-qPCR reactions are analysed according to Langton *et al.* (2002) to determine the accordance.

Results

Considering the small differences in Cq values obtained for the three seed lots (2, 4 and 6) spiked with LMV positive SE (see "Selectivity"; Section 3.3), Cq values were averaged and are visualized in Figure 9 for comparison of the results obtained at the three time points.

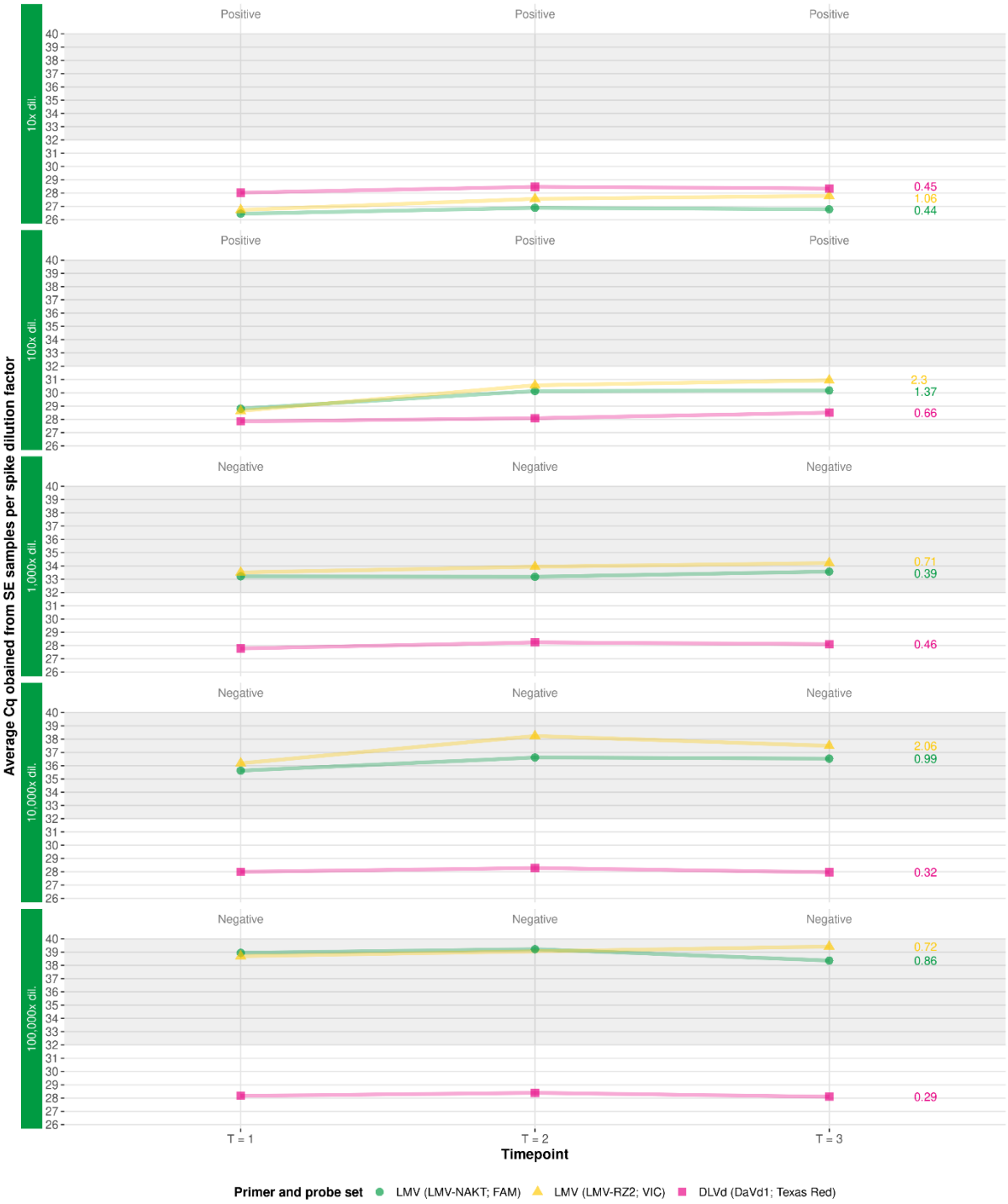


Figure 9. Comparison of averaged Cq values obtained from lettuce SE spiked with LMV positive SE (Sub 7 acquired during assessment of the LMV infection level of the LMV positive seed lot used for "Analytical sensitivity of the LMV SE-qPCR assay" (Section 3.2; Figure F.1)) at three time points (T = 1, T = 2, and T = 3). The outcome (Positive or Negative) of the LMV SE-qPCR at the three time points is added to the top of the figure. Deviations in Cq of the two LMV targeted primer and probe sets between the time points are added to the rights of points for T = 3. The grey background represents the range for which the SE-qPCRs are considered negative (Cq 32-40).

The outcome of the LMV SE-qPCR at the three different time points was calculated when a reaction was deemed positive if one or both of the LMV targeted primer and probe sets yielded a Cq value of < 32. This revealed that the outcome for all LMV SEs with 10 and 100 times diluted LMV spike SE (Figure 9) as well as the undiluted LMV positive seed extract ("Sub 7") and PPC (Table F.2 in Annex F) was positive at the three time points of analysis. In addition, LMV SEs with 1,000, 10,000, and 100,000 times diluted LMV spike SE, unspiked SEs, and NPC did not yield Cq values or yielded Cq values that were >32 and were concluded as negative for the three time points. Based on these results and following the formula below, the accordance was 100% (Langton *et al*., 2002). A 100% accordance was also calculated when the analysis was performed for each seed lot separately instead of using the outcome based on averaged Cq values.

$$Accordance = k * (k-1) + (n-k) * (n-k-1)/n(n-1)$$

n: number results

k: number of positive results

While not the focus of assessing "Repeatability", the deviation in Cq values between the three time points for each of the primer and probe set in the LMV triplex SE-qPCR was calculated (Figure F.2 in Annex F). This revealed that the deviation was generally <2 Cq and indicates that SE preparation for the RNA extraction, the RNA extraction itself, and the LMV SE-qPCR at different times did not have a profound impact on the outcome of Cq values. This notion was also true for low LMV infected samples considering that the Cq values for SE with 100 times diluted LMV positive SE spike were just below the Cq cut-off of 32.

Conclusion

RNA extractions and the LMV triplex SE-qPCR from LMV spiked lettuce SE in different concentrations performed by the same operator at three time points yielded the same outcome and an accordance of 100%. Therefore, the requirement for repeatability was achieved.

3.5 Reproducibility

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

The reproducibility requirements will be met when the concordance among participating laboratories is >90%.

Experimental setup

To find suitable seed lots for the CT of the LMV SE-qPCR, the CT organizer evaluated LMV infection homogeneity using at least ten subsamples of 1,000 seeds for six lettuce seed lots (SE 28, 29, 33, 35-37 (Table 2). The subsamples were tested using the LMV SE-qPCR, according to the protocol in Annex A.

Prior to the CT, a Pre-CT was organized to give participants an opportunity to gain proficiency in the method (Annex G for Pre-CT setup and results) and to facilitate setting the Cq cut-off discriminating between LMV positive and negative seed lots. For this purpose, the Pre-CT organizer provided five subsamples of 1,000 seeds from healthy lettuce seed lots SE 36 and 37 as well as from the homogenous low LMV infected SE 33, together with the NPC, PPC, IAC and PAC (Table G.1; Annex G) to six of the eight CT participants. The two remaining participants were

based outside the EU, and the complexity of organizing documentation for material transfer and transport was deemed prohibitive for their inclusion in the Pre-CT.

For the CT, the organizer provided sets of a total of 20 subsamples of 1,000 seeds (Table 3) in 50 mL tubes with a stainless-steel grinding bullet (14 mm) to all eight CT participants. Each sample set consisted of three subsamples from homogenous high LMV infected SE 28 and 29 (six samples in total), eight subsamples that were prepared by mixing 100 seeds from LMV homogenously high infected SE 35 and 900 seeds from healthy lettuce SE 36 that mimicked a subsample from a medium homogenously LMV infected seed lot (Figure H.1 in Annex H), and six subsamples from healthy SE 37. The order of the seed samples from the different seed lots with different infection levels was randomized and blind coded (1-20) to CT participants for the sample LMV infection level. The order of the subsample was the same for each set sent to participants.

The sample sets were supplemented with an NPC and PPC that are used routinely by the CT organizer as controls for detection of LMV in seed lots (Table 3). These controls differ from the healthy and infected seed samples to be tested by the participants in that they are part of different seed lots. The CT organizer also provided a microcentrifuge tube with 1 mL of synthetic RNA resembling the complete genome of Dahlia latent viroid (DLVd; 342 nt + 7 nt as a result of cloning and synthesis) as IAC (Table 3) and was added to 1 L seed extraction buffer in Step 1.1 of the protocol in Annex A.

Table 3. Seed samples and controls provided by the CT organizer to participants of the CT.

Item	LMV infection level	Quantity	Label	Expected Cq value		
				LMV		DLVd
				LMV-NAKT	LMV-RZ2	DaVd1
Seed samples	High, Medium, and Healthy[a]	20 × 1,000 seeds[b]	1-20			
	Low (SE 33 in Table 2)	3 × 1,000 seeds[b]	Training Low LMV[d]			
	NPC	1 × 1,000 seeds from LMV negative seed lot[b]	NPC	No Cq Or >Cq cut-off	No Cq Or >Cq cut-off	26-30
	PPC	1 × 1,000 seeds from LMV positive seed lot[b]	PPC	<20	<20	26-30
Empty tubes[c]	-	5	-			
DLVd synthetic RNA (IAC)	-	1.25 mL in 1.5 mL microcentrifuge tube	IAC	No Cq	No Cq	26-30
LMV synthetic ssDNA (PAC)	-	50 µL in 1.5 mL microcentrifuge tube	PC	26-32	26-32	No Cq

[an] Infection level of samples will not be known to participants.

[b] Seed samples will be provided in 50 mL tubes with stainless steel grinding bullet 14 mm.

[c] Empty tubes are provided by the CT organizer to test grinding settings.

[d] Sub samples from a low LMV infection level were provided for training purposes (e.g., Grinding settings and RNA extraction) of the method of which the results are not included in the analysis of the CT results.

The RNA extraction was performed using to the RNeasy Plant Mini Kit (Qiagen; step 1.2 to 1.6 of the protocol). The LMV qPCRs were requested to be performed using Ultraplex 1-Step ToughMix (QuantaBio, Beverly, MA) in duplicate (see Step 2 of the protocol in Annex A) including for a provided PAC (Table 3) that consisted of a mixture of synthetic ssDNA resembling the amplified sequence of the LMV-NAKT and LMV-RZ2 primer and probe sets. The CT participants were requested to keep the seed samples and controls at 4 °C upon arrival until the analysis could be performed.

To identify potential stability issues, the organizer analysed a CT sample set before shipment of the sample sets to the participants, after the last European participant sent their data, and after the last non-EU participant sent their data. The CT organizer maintained the extra sample sets, including the IAC and PAC, at room temperature during transport of the samples to participants and until the last CT participants completed their analyses.

The Cq values and the participant's appraisal for each seed sample (either positive or negative) were entered in a MS Excel file (Annex H) along with the general information and relevant observations and sent to the CT organizer. The Cq values from each participant for samples from each seed lot as well as the controls were compared. Furthermore, concordance in results of participants was analysed according to the method developed by Langton *et al*. (2002) for concordance.

Results

Homogeneity assessment

The Cq values from the LMV targeted triplex SE-qPCR of the homogeneity test are included in Annex H, Table H.1 and visualized in Figure H.1. The SE 28, 29, and 35 were deemed homogenous high LMV infected seed lots, because all 10 subsamples of these seed lots yielded Cq values <20 with both LMV targeted primer and probe sets (Figure H.1). The SE 33 was considered a homogenous low LMV infected seed lot, because all 10 subsamples yielded Cq values between 24 and the Cq cut-off of 32. While two subsamples for SE 36 yielded no Cq values with the LMV targeted primer and probe sets, Cq values for the remaining subsamples were between 29 and 39. Considering that only one subsample of SE 36 produced Cq values between 29 and 31. The SE 36 was used in the Pre-CT to assist in determining a suitable Cq cut-off (Annex G) and is used in the CT, not as a healthy seed lot, but as seed lot to prepare a medium LMV infected seed lot. The medium LMV infected seed lot produced Cq values between 21, and 28.5 (Table H.1, Figure H.1). None of the subsamples of SE 37 yielded Cq values for the LMV targeted primer and probe sets (Figure H.1). Therefore, this seed lot was considered healthy.

Pre-CT

Results of the pre-CT affirmed a generally accepted notion that quantitative differences between laboratories prevent the use of the same Cq cut-off by the CT participants (Annex G). However, only high and medium LMV infected seeds were used in the CT. Therefore, relatively low Cq values were expected, and the chance of false negative results was low. Therefore, a fixed Cq cut-off of 32 was employed for all participating laboratories during CT. This circumvented also the need for each laboratory to set an individual Cq cut-off, which would be practically prohibitive.

Stability assessment

The Cq values with the LMV-NAKT and LMV-RZ2 primer and probe set obtained by the organizing laboratory before shipment, after the last participating European laboratory sent in their data, and after the last non-EU laboratory sent in their data for samples from high and medium LMV infected seed lots as well as from healthy lettuce seed lot samples were sufficiently similar (Figure 10). However, a larger range in Cq values (Cq 16 - 27) was observed for the high LMV infected seed lots analysed before samples were shipped compared to that observed after the last participating laboratory sent in their data (Cq 14 - 19; Figure 10). More importantly, all samples from the high and medium LMV infected seed lots yielded Cq <32 for the LMV targeted primer and probe sets and scored positive and none of the samples from the healthy seed lot yielded Cq values <32 and score negative, regardless of when the organizing laboratory analysed the sample sets (Table H.2; Figure 10).

The PPCs scored positive regardless of the timing of testing by the organizing laboratory but yielded slightly different Cq values of 14 to 14.8 before shipment, from 15.1 to 15.4 after the last European participating laboratory sent their results, and from 15.8 to 16.3 after the last non-EU participating laboratory sent their results (Table H.2). This variation also encompasses the different Cq values obtained from the two LMV targeted primer and probe sets and could be at least partially due to using different thermocyclers although they were in the same laboratory. Nonetheless, the observed variation was deemed acceptable also considering that these were different subsamples from the same seed lot and were not technical replicates.

The NTC and NPC analysed by the organizing laboratory yielded negative qPCR results regardless of when the analysis was performed. The provided DLVd synthetic RNA as the IAC in the LMV SE-qPCR yielded very similar Cq values in samples analysed before shipment and after the results from the European participants were received (Table H.2 and Figure H.2 in Annex H). Interestingly, this was not observed for the synthetic ssDNA resembling the amplified sequence of the LMV-NAKT and LMV-RZ2 primer and probe sets that was used as PAC for the LMV SE-qPCR and showed substantially higher Cq values for the LMV targeted primer and probe sets in the analysis after the results from the participants were received (Figure H.2 in Annex H). Similar observations were made during the Pre-CT (Figure G.3 in Annex G). While this would indicate that the relatively short ssDNA of the PAC may not be as stable for prolonged storage at room temperature compared to the longer RNA of the DLVd IAC used here, the participating laboratories stored their PAC and IAC refrigerated (~4 °C) and showed Cq values for the PAC that were more in line with those obtained by the organizing laboratory before the samples and controls were sent to the participants (Figure H.2 in Annex H).

The PAC aliquots stored at RT during the time the participants were performing their analysis and a PAC aliquot stored at -80 °C were taken along during the final analysis by the CT organizer after the non-EU participants handed in their results. Results of the -80 °C stored PAC showed Cq values that were very similar to those obtained for the PAC in the analysis performed before the sample sets were sent to the participants, while the PAC maintained at room temperature again showed high Cq values similar to those obtained for the PAC in the analysis performed after the EU participants returned their results (Figure H.2 in Annex H). Therefore, the presumed stability issues with the PAC seem to occur only after a prolonged time and could be at least partially circumvented by cold storage of the ssDNA.

Based on the above, the LMV infection levels in the seed samples were deemed stable in time and the results from the participating laboratories were accepted as valid.

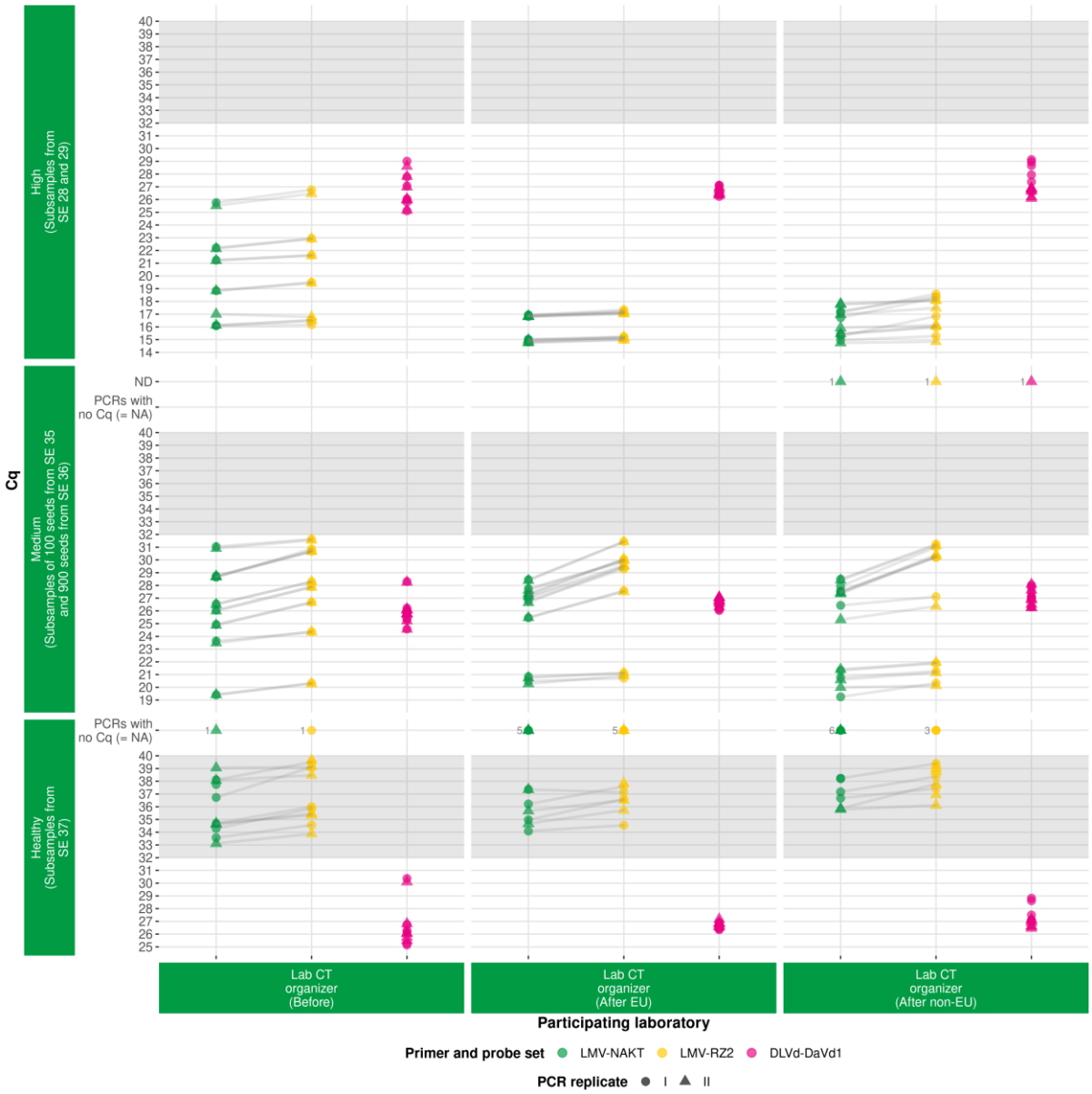


Figure 10. Obtained Cq values from the two LMV targeted primer and probe sets (LMV-NAKT and LMV-RZ2) and DLVd that is used as an IAC, for subsamples from high (SE 28 and 29) and medium (100 seeds from SE 35 and 900 seeds from SE 36) infected seed subsamples as well healthy seed subsamples (SE 37) performed as part of the CT **by the CT organizer**. The grey background represents the range for which the SE-qPCRs were considered negative (Cq >32) in the CT. The grey lines connect qPCR signals from the LMV-NAKT and LMV-RZ2 primer and probe sets from the same reaction. The number of reactions yielding no Cq value (NA: not available) and the number of reactions that was not determined (ND) are added to the top of each graph.

CT

In total, eight ISHI member laboratories (four from the Netherlands, one from France, one from Spain, and two from Japan) joined the LMV SE-qPCR CT, followed the protocol for the samples, and submitted their data of the LMV SE-qPCR. The European based laboratories received their sample sets in November 2024, and the non-European participants received their sample sets in December 2024. The Cq values from the LMV targeted triplex SE-qPCR, coded per laboratory to preserve anonymity, are included in Table H.2 (Annex H) and Figure 11 for subsamples of the high and medium infected as well as of the healthy seed.

All laboratories showed negative results (Cq <32) with LMV qPCR for the NTC and NPC (Table H.2; Figure H.2 in Annex H). As expected, the PPC yielded positive results with Cq values <19. The PAC was also positive when analysed by the participating laboratories 1-6 (Cq 25 - 26 for LMV-NAKT and 24.7 - 28.5 for LMV-RZ2; Figure H.2 in Annex H). The Cq values for laboratories 7 and 8 were considerably higher (Figure H.2 in Annex H). A lower level in variations could have been expected considering that the concentration of the synthetic ssDNA in the PAC was the same for each laboratory. Similar to observations in the pre-CT, these findings seem to indicate that the different laboratories may use different settings of the PCR baseline above the background fluorescence or have a varying efficiency to detect LMV. Alternatively, the PAC may have degraded to some extent during transport (see also findings of the CT organizer for the PAC above). However, there was no clear evidence that transport duration had a profound effect on the Cq values of the subsamples (data not shown).

Similar to the PAC, the concentration of synthetic DLVd used as the IAC was also the same for each laboratory albeit that the participants prepared their own seed extraction buffer and spiked it with the provided IAC. This may at least partially explain the varying Cq values with the DLVd targeted primer and probe set that did not follow the same trend of the PAC.

Unexpectedly, laboratory 5 showed Cq values > 34 with the DLVd targeted primer and probe set for the seed subsamples (Figure 11 and Figure H.2 in Annex H). Upon investigation, participant 5 indicated that these values may be due to using older primers and probes that could have been degraded. Cq values by laboratory 5 for high LMV infected samples were relatively low (Cq <22 for at least one of the LMV assays (Figure 11) and, therefore, should be regarded as positive. Healthy samples yielded Cq values >32, but without the expected signal for the IAC should be regarded as “Inconclusive”. Several medium LMV infected samples yielded Cq values >32 with both LMV targeted primer and probe sets and combined with the high Cq values for DLVd were deemed “Inconclusive”. Considering the aberrant DLVd Cq values from laboratory 5, the results of this laboratory were excluded from analysis of the accordance and concordance (see below). In contrast to laboratory 5, laboratory 3 showed Cq values for DLVd that were substantially lower than that obtained by the other participants (Figure 11 and Figure H.2 in Annex H). This was particularly apparent for seed subsamples with a signal from the LMV-NAKT and LMV-RZ2 primer and probe sets. These relatively low Cq values with the DLVd targeted primer and probe set can likely be attributed to using the ABY label for this primer and probe set next to the HEX label that was used by participant 3 for the LMV-RZ2 primer and probe set. These labels have an overlapping emission spectrum leading to crosstalk.

Importantly, all participating laboratories correctly identified the high infected seed subsamples as positive with Cq values <32 and scored all samples of the healthy lettuce seed lot as negative with Cq values >32 (Figure 11). All laboratories, except laboratory 4, scored samples from the medium LMV infected seed lot as positive. Laboratory 4 scored one subsample of the medium LMV infected seed lot as (false) negative with >32 or no Cq value with the LMV targeted primer and probe sets (Figure 11).

The accordance per participating laboratory, including that of the CT organizer using data obtained from the sample set analysed before shipment, the concordance, and the concordance odds ratio (COR) were calculated according to Langton *et al.* (2002) and visualized in Figure 12.

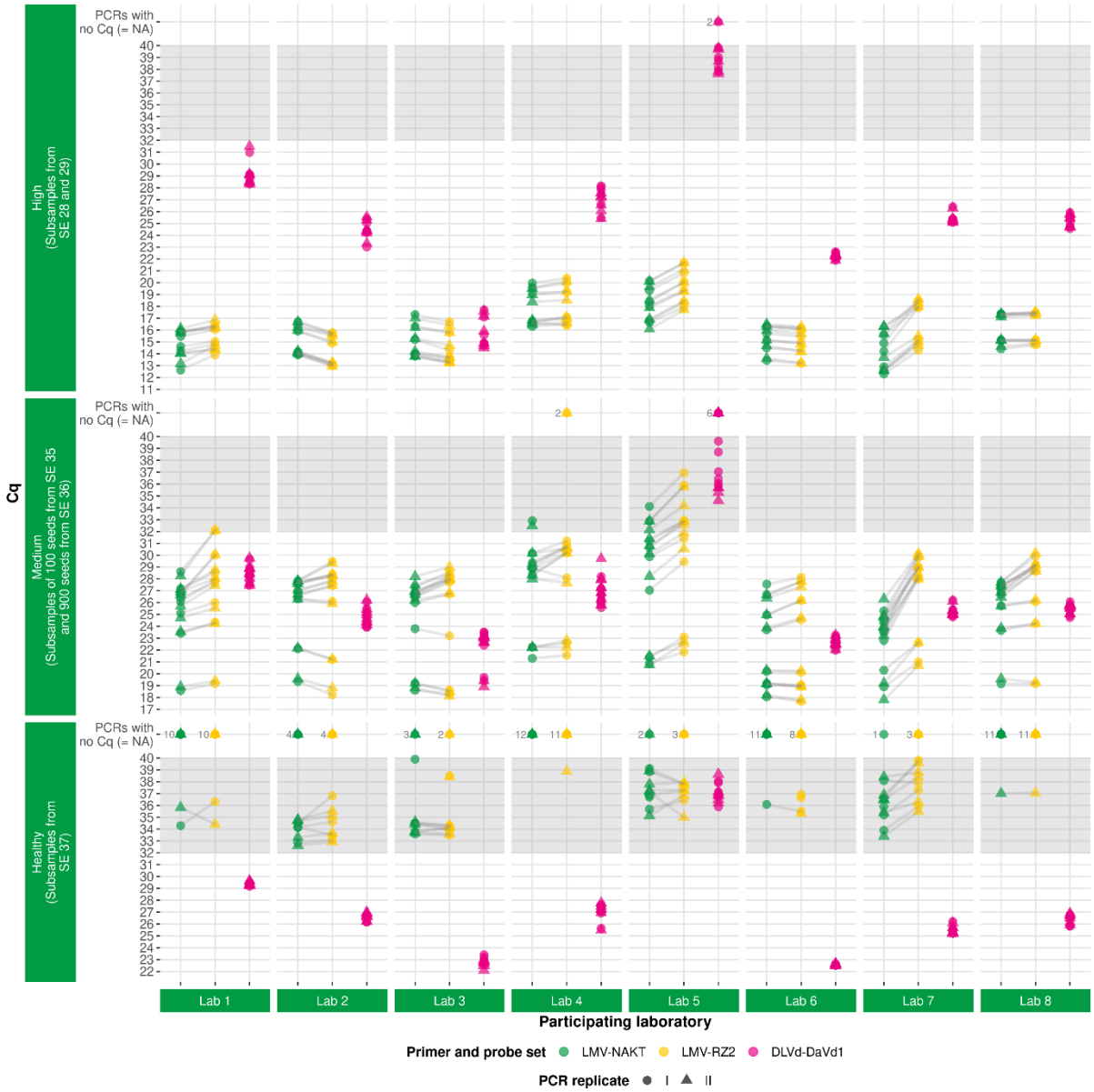


Figure 11. Obtained Cq values from the two LMV targeted primer and probe sets (LMV-NAKT and LMV-RZ2) and DLVd that is used as an IAC, for subsamples from high (SE 28 and 29) and medium (100 seeds from SE 35 and 900 seeds from SE 36) infected seed subsamples as well healthy seed subsamples (SE 37) performed as part of the CT **by the participating laboratories**. The grey background represents the range for which the SE-qPCRs were considered negative (Cq >32) in the CT. The grey lines connect qPCR signals from the LMV-NAKT and LMV-RZ2 primer and probe sets from the same reaction. The number of reactions yielding no Cq value (NA: not available) and the number of reactions that was not determined (ND) are added to the top of each laboratory.

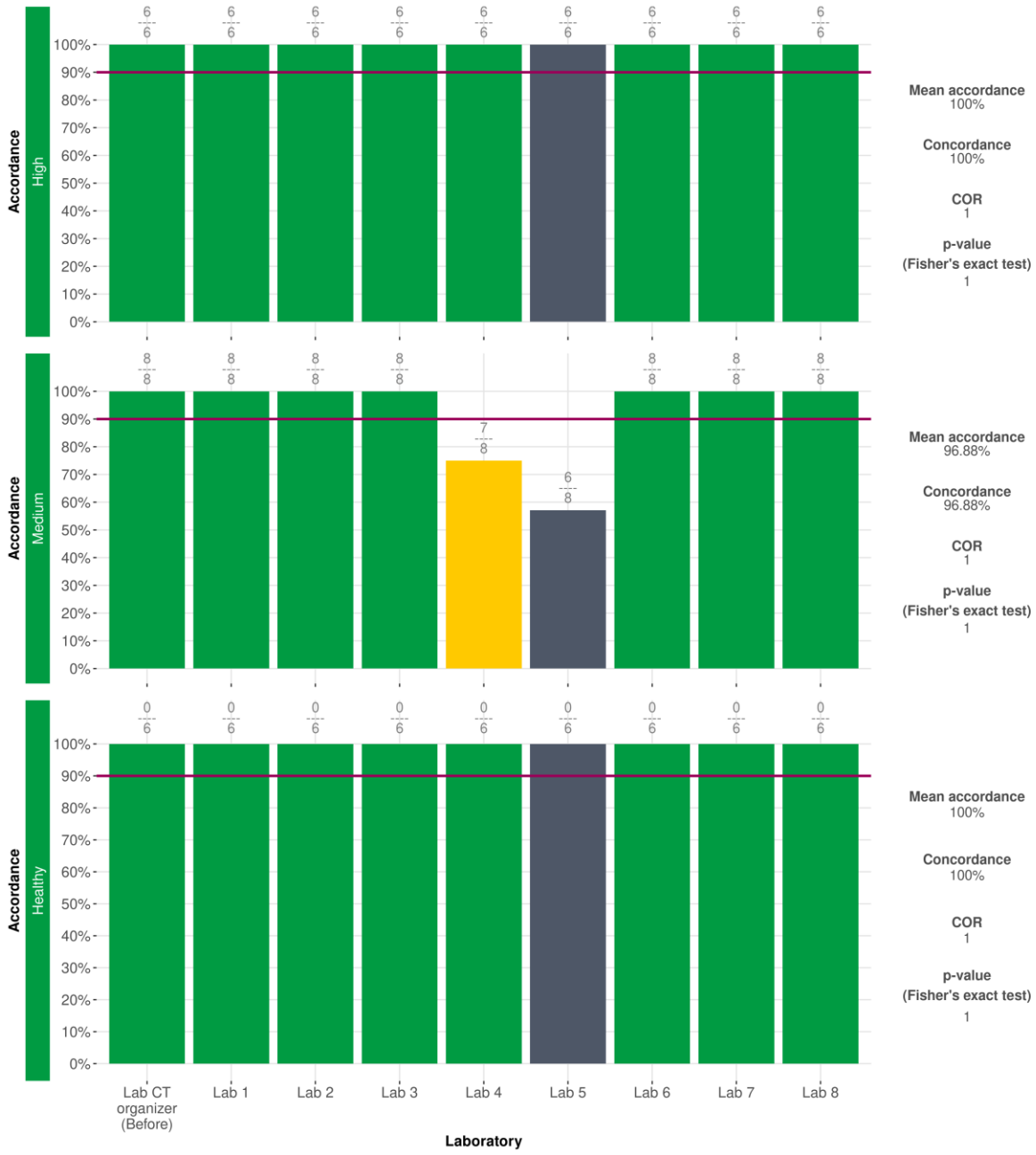


Figure 12. Visualization of the accordance and concordance analysis according to Langton *et al.* (2002) when a Cq cut-off of 32 is used to discriminate between positive and negative samples. The purple line indicates the required minimum accordance value per participating laboratory. The values above each bar represent the number of samples giving a positive outcome (above dashes) and the total number of samples tested (below dashes). Samples analysed by CT participant "Laboratory 5" are considered 'Inconclusive' due to aberrant Cq values of DLVd as the IAC and are indicated with the grey bar. These samples are excluded from accordance and concordance analysis.

The COR (see formula below) represents the relative chance of obtaining the same outcome when samples are analysed in the same laboratory compared to analysis in different laboratories and indicate the degree to which results vary between laboratories.

$$COR = \frac{\text{Accordance } \times (100 - \text{Concordance})}{Concordance \times (100 - Accordance)}$$

Considering that all participating laboratories correctly identified subsamples of the high LMV infected seed lots as positive and the samples of the healthy seed lot as negative, it was no surprise that these LMV infection levels produced excellent scores of 100% for the accordance for each laboratory and concordance were obtained as well as a COR of 1 (Figure 12).

The accordance score for laboratory 4 was lower than 80% due to the false negative results for one of the medium LMV infected samples. This brought the average accordance value down to 96.88%. The concordance for the medium LMV infected samples was sufficient with a score of 96.88% and a COR of 1.

Conclusion

The CT assessed the reproducibility in the outcome of the LMV SE-qPCR across laboratories to detect LMV in subsamples of the same LMV infected or healthy lettuce seed lots. Despite that the laboratories used samples from the same seed lots and were provided with the same controls, differences in Cq values were observed. This means that results between laboratories should only be compared qualitatively, and that results can be compared quantitatively within a single laboratory provided that the assay is performed in the same way. In the current CT, a negative outcome for the PAC, consisting of a mixture of synthetic ssDNA resembling the amplified sequence of the LMV targeted primer and probe sets, was obtained when it was stored at room temperature in contrast to the positive outcome when the PAC was analysed shortly after preparation or after cold storage. This indicates that these types of controls are suitable for in-house use, but less suitable when prolonged temperature fluctuations are expected, such as with shipment at ambient temperatures. Synthetic RNA, here used as IAC, appeared to be less hampered by prolonged storage at room temperature and, therefore, could be used in future CTs as controls that can be shipped with minor risk of degradation during shipment. The high LMV infected seed lots and healthy lettuce seed lots yielded 100% for concordance and a COR of 1. The concordance for the medium LMV infected seed lot was lower, but sufficient with a score >90% when a Cq cut-off 32 was chosen. Together, the results from the CT showed that reproducibility requirements are met.

3.6 Robustness

Definition: *A measure of the capacity of the method to maintain reliable results when (small) deliberate variations are introduced.*

While the RNeasy Plant Mini Kit (Qiagen), used for RNA extraction in the LMV SE-qPCR protocol (Annex A), is readily available, an automated process, such as the Sbeadex plant maxi kit in combination with the KingFisher platform, may be preferred to increase sample throughput. Therefore, results from the LMV SE-qPCR as performed using extraction buffer and RNeasy Plant Mini Kit (Qiagen), described in Annex A, were compared with those obtained for RNA extracted using the Sbeadex plant maxi kit (LGC Genomics) in combination with the KingFisher platform to demonstrate the validity of both approaches for LMV detection by SE-qPCR. The LMV SE-qPCR will be deemed sufficiently robust when the qPCR is performed using RNA from both RNA extraction methods and yield the same qualitative outcome.

Experimental setup

In addition to analysing the CT sample sets of 20 subsamples of 1,000 seeds (six high LMV infected subsamples, eight medium LMV infected subsamples, six subsamples from a healthy lettuce seed lot), NPC, and PPC (Table 3), according to the LMV SE-qPCR protocol in Annex A using the RNeasy Plant Mini Kit (Qiagen; hereafter referred to as the "QG" method), two additional

sample sets from the CT were analysed using the in-house protocol of the CT organizer. This protocol uses the same grinding step as used in Step 1.3. in Annex A using the Geno/Grinder 2010, followed by adding 20 mL GH+, supplemented with DLVd as the IAC, as extraction buffer and RNA extraction with the Sbeadex plant maxi kit (LGC Genomics) in combination with the KingFisher platform (ThermoFisher Scientific; hereafter referred to as the "KF" method). The KF method was performed with a sample set before shipment of the sample sets to the CT participants and a sample set after the last European CT participant sent their data to the CT organizer. Cq values from the QG and KF methods are compared for samples per LMV infection level as well as for the NPC and PPC.

Results

The Cq values from the LMV targeted triplex SE-qPCR, performed on RNA obtained using the QG and KF method, are included in Table H.2 in Annex H and in Figure 13. The Cq values for the two LMV targeted primer and probe sets and the DLVd primer and probe set were lower for the PPC (and Cq values for DLVd of the NPC) when the RNA was extracted using the QG method (Figure 13). This could also be observed for DLVd in the high and medium LMV infected samples as well as the healthy lettuce samples.

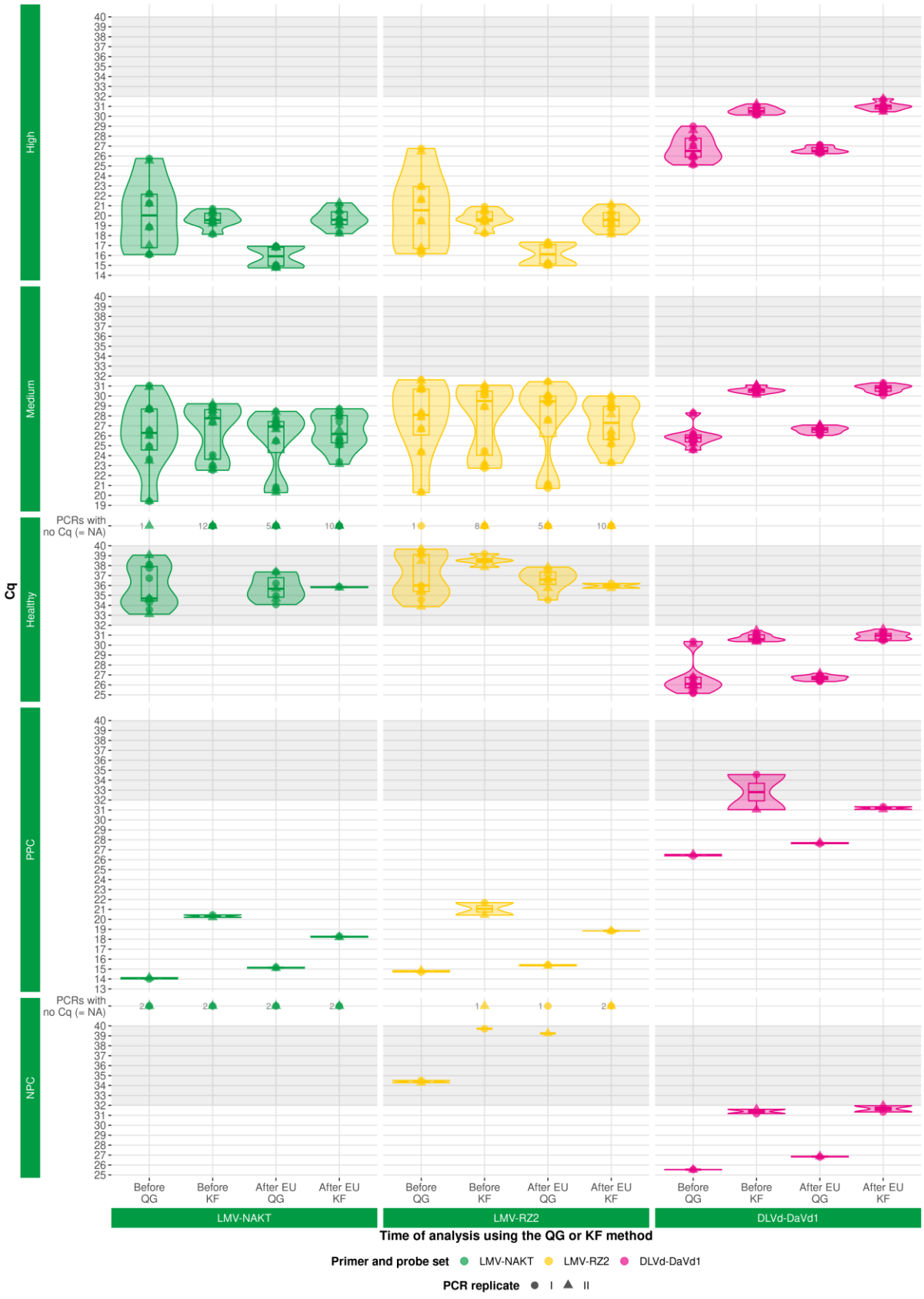
However, this trend was less prominent for the LMV targeted primer and probe set in the high LMV infected samples and was not evident in results for the medium LMV infected samples. The latter was likely due to the variation in LMV infection level in the individual samples in the sample sets or to some extent stochastics in detection, making it complicated to determine the impact of the different RNA extraction methods for these samples. Comparison of the DLVd levels was also complicated by the difference in the DLVd spike concentration in the extraction buffer for the QG and GH+ buffer in the KF method. Nonetheless, the results do indicate that the LMV SE-qPCR with RNA obtained using the QG method appears to be more sensitive compared to the KF method.

At the qualitative level, all samples from the high and medium LMV infected seed lot would be deemed positive and samples from the healthy seed lot would be deemed negative regardless of which method was used for RNA extraction.

Conclusion

Together these results suggest that the RNA extraction method does have an impact on the Cq values with the QG method appearing to yield lower Cq values compared to RNA extracted using the KF method. However, qualitatively, the RNA extraction method did not have an impact on the outcome of the LMV SE-qPCR and suggests that both RNA extraction methods are suitable and are sufficiently robust for RNA extraction as part of the LMV SE-qPCR for detection of LMV.

Figure 13 (next page). Combined boxplot and violin plot (kernel density distributions) of Cq values obtained using the LMV SE-qPCR performed in duplicate for subsamples from high and medium infected seed subsamples, healthy seed subsamples, PPC, and NPC samples performed as part of the CT by the CT organizer before the samples were shipped to the CT participants and after the European CT participants sent back their data (see also Figure 11). The grey background represents the range for which the SE-qPCRs were considered negative (Cq >32) in the CT. The number of reactions yielding no Cq value (if any) are added at the top of each graph. The results from the CT organizer using RNeasy Plant Mini Kit (Qiagen) for RNA extraction are indicated in this figure with "QG" and results from the Sbeadex plant maxi kit in combination with the KingFisher platform are indicated with "KF".


High
Medium
Healthy
PPC
NPC
Cq
PCRs with no Cq (= NA)
Before QG
Before KF
After EU QG
After EU KF
LMV-NAKT
LMV-RZ2
DLVd-DaVd1
Time of analysis using the QG or KF method
Primer and probe set
LMV-NAKT
LMV-RZ2
DLVd-DaVd1
PCR replicate
I
II

3.7 Diagnostic performance

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

Considering that the LMV SE-qPCR is a pre-screening method, the diagnostic sensitivity (no false negatives) is more important than the diagnostic specificity (no false positives), as false positives will be identified by the confirmation LMV seedling ELISA assay.

The LMV SE-qPCR is expected to have a relatively high sensitivity due to detection of RNA from non-infectious LMV or when no diseased seedlings are obtained and are identified using the LMV seedling ELISA. Consequently, the LMV SE-qPCR will possibly yield false positive signals (in relation to results from the LMV seedling ELISA). The performance requirement for diagnostic performance of the SE-qPCR will be met when the diagnostic sensitivity is >90% and a lower threshold of >40% for diagnostic specificity to cope with the expected false positive results compared to the LMV seedling ELISA.

The diagnostic performance of the seed ELISA will be evaluated alongside the SE-qPCR.

Experimental setup

Five lettuce seed batches (SE 2, 9, 18, 31, and 32, Table 2) were tested using the LMV seed ELISA performed by a proficient ISHI member. The SEs were transported from the laboratory performing the seed ELISA to the CT organiser after completion of the LMV seed ELISA followed by an RNA extraction and the LMV triplex qPCR. The seedling ELISA (Annex I) and LMV SE-qPCR (Annex A) were performed by the CT organiser. Twenty subsamples of 500 seeds each were used for the seed ELISA, 20 subsamples of 100 seeds each were used for the seedling ELISA, and three subsamples of 1,000 seeds each were used for the LMV SE-qPCR. Five subsamples for seed batches that were positive for the seedling ELISA were selected followed by an RNA extraction and the LMV triplex qPCR.

The diagnostic sensitivity and specificity were calculated according to the formula in Table 4 using the LMV seedling ELISA as reference assay.

Table 4. Calculation of the diagnostic sensitivity and specificity based on true and false positive and negative results.

		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

Cq values from the **LMV targeted triplex SE-qPCR** are included in Table J.1 in Annex J and are visualized in Figure 14. The SE-qPCR results of the PCR grade water (NTC) did not show any relevant positive qPCR result and results of SE from an LMV negative seed lot used as NPC did not obtain any Cq value below 38.2 for both LMV targeted primer and probe sets (Figure 14 and Table J.1). As expected, the synthetic ssDNA oligonucleotides, used as positive amplification control (PAC), and RNA from SEs from the LMV positive seed lot, used as PPC, showed positive

results with relatively low Cq values (Figure 14). A negative SE-qPCR results was obtained for subsamples of seed lot 2 and 9 (Cq 33.6 - 38.7 or no Cq). Subsamples for seed lot 18 yielded a positive result with Cq values <32 for at least one of the two LMV targeted primer and probe sets. The lowest Cq values were obtained with SE from subsamples of seed lots 31 and 32 (Cq 16 – 20), yielding a clear positive result (Figure 14).

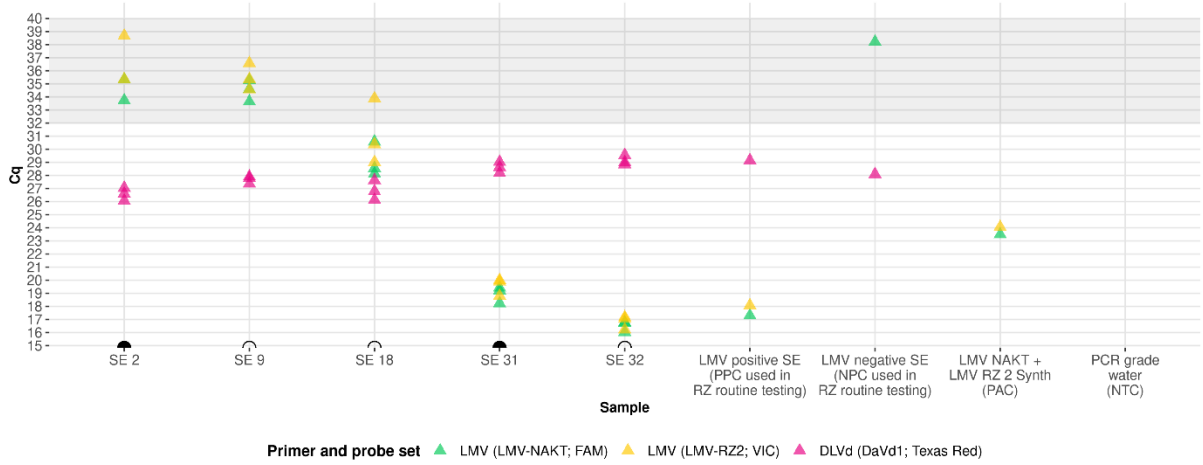


Figure 14. Obtained Cq values of the LMV triplex qPCR for RNA from SE from five seed batches. The grey background represents the range for which the SE-qPCRs are considered negative (Cq 32-40). Open and solid black semi-circles on the right of the SE lots indicate if the lettuce seeds were white or black, respectively.

Results from the **seedling ELISA** are included in Table J.2 in Annex J and are visualized in Figure 15. The positive LMV ELISA control (PC in Figure 15) and seedling extract from an LMV positive seed lot (PPC in Figure 15) showed high absorbance measurements and an expected positive ELISA outcome in relation to results for the LMV negative seed lot (NPC) that were used to set the ELISA absorbance cut-off (two-times the values of the mean NPC absorbance; Figure 15).

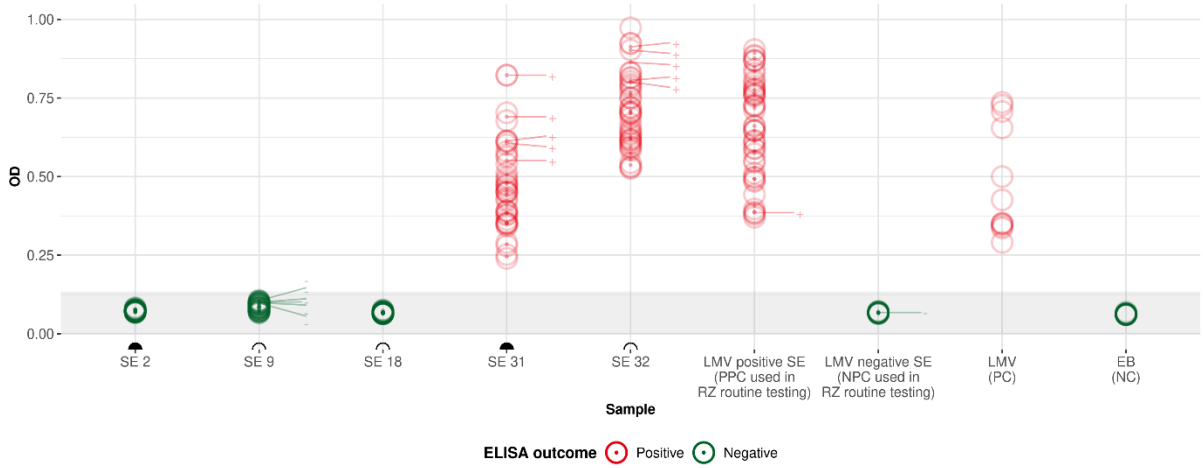


Figure 15. Absorbance (optical density) measurements from LMV seedling ELISA for SE from five seed batches. The grey background represents two times the absorbance measurements of the NPC above which the ELISA outcome is considered positive. Open and solid black semi-circles above the SE lots indicate if the lettuce seeds were white or black, respectively. Average absorbances are indicated with dots and are linked to "+" and "-" that indicate the outcome of the LMV confirmation qPCR as "positive" ("LMV-RZ2" and/or "LMV-NAKT" primer set yields Cq <32) and "negative" ("LMV-RZ2" and "LMV-NAKT" primer set yields Cq ⩾32), respectively. OD: Optical density.

Absorbance measurements obtained for seed lots 2, 9 and 18 were lower than the cut-off. Seed lots 31 and 32 showed values that passed that cut-off. RNA extraction was performed for seedling extracts used for the ELISA followed by the LMV targeted qPCR as a confirmation of the ELISA results (see Annex I). This confirmed the negative result for lot 9 and the positive result (Cq <30) for lots 31 and 32 and were therefore concluded positive for the LMV seedling ELISA.

Results from the **seed ELISA** are included in Table J.3 in Annex J and are visualized in Figure 16. The positive LMV ELISA control (PC in Figure 16) showed high absorbance measurements between 1.205 and 1.415 (4.6 – 5.4 times higher than the cut-off used to discern positive and negative samples (two-times the values of the mean NPC absorbance); Figure 16) and, therefore, yielded an expected positive ELISA outcome. Similarly to the results for the seedling ELISA, absorbances were lower than the cut-off for all subsamples from seed lots 2, 9 and 18, whereas absorbances were higher than the cut-off, and therefore deemed positive, for all subsamples from seed lots 31 and 32. The LMV targeted qPCR for RNA from SE used for the LMV seed ELISA confirmed the outcome for seed lots 2, 9, 31, and 32. Interestingly, most subsamples for seed lot 18 showed a positive LMV qPCR result although the seed ELISA was negative. This suggests that the LMV SE-qPCR is more sensitive compared to the seed ELISA. Regardless, LMV in this seed lot is highly likely not to be infectious as the seedling ELISA for this lot was negative (Figure 15).

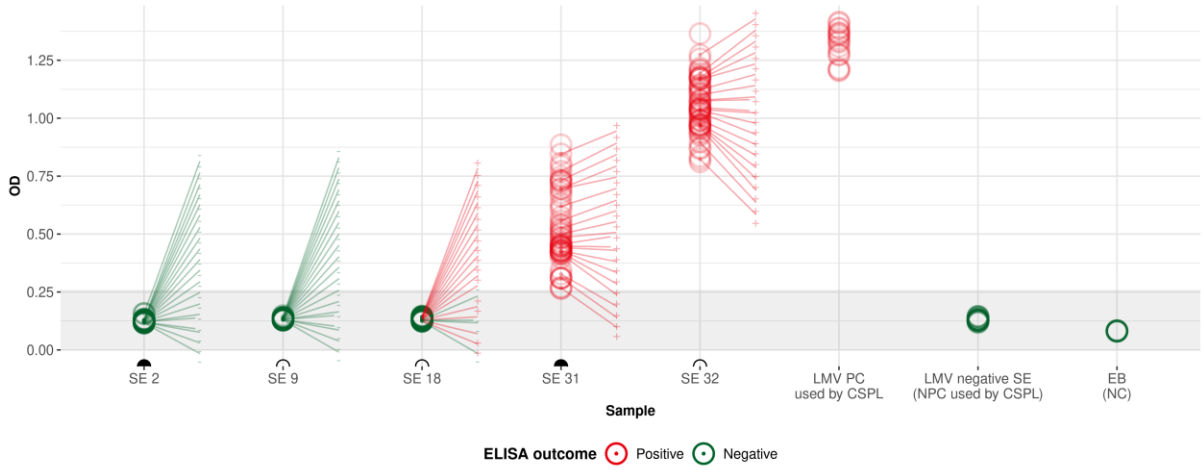


Figure 16. Absorbance (optical density) measurements from LMV seed ELISA for SE from five seed batches. The grey background represents two-times above the absorbance measurements of the NPC above which the ELISA outcome is considered positive. Open and solid black semi-circles to the right of the SE lots indicate if the lettuce seeds were white or black, respectively. Average absorbances are indicated with dots and are linked to "+" and "-" that indicate the outcome of the LMV confirmation qPCR as "positive" ("LMV-RZ2" or "LMV-NAKT" primer set yields Cq <32) and "negative" ("LMV-RZ2" and "LMV-NAKT" primer set yields Cq ⩾32), respectively. OD: Optical density.

Regardless of whether the seedling or seed ELISA was used as reference assay, seed lots 2 and 9 were detected as true negative, seed lot 18 was a false positive, and seed lots 31 and 32 were true positive with the SE-qPCR in relation to the ELISA assays. Based on these results, the diagnostic sensitivity was 100% and the diagnostic specificity was 66.7% (Table 5).

For completeness, the diagnostic sensitivity and diagnostic specificity of the seed ELISA in relation to the seedling ELISA was calculated showing excellent scores of 100% for both metrics (Table 6).

Table 5. Calculation of the diagnostic sensitivity and specificity based on true and false positive and negative results for the LMV SE-qPCR in relation to the LMV seedling ELISA.

		Validated method result / independent assessment	
		Positive	**Negative**
Test outcome	**Positive**	TP = 2	FP = 1
	Negative	FN = 0	TN = 2
		DIAGNOSTIC SENSITIVITY = 2 / (2 + 0) × 100% = 100%	**DIAGNOSTIC SPECIFICITY = 2 / (1 + 2) × 100% = 66.7%**

Table 6. Calculation of the diagnostic sensitivity and specificity based on true and false positive and negative results for the LMV seed ELISA in relation to the LMV seedling ELISA.

		Validated method result / independent assessment	
		Positive	**Negative**
Test outcome	**Positive**	TP = 2	FP = 0
	Negative	FN = 0	TN = 3
		DIAGNOSTIC SENSITIVITY = 2 / (2 + 0) × 100% = 100%	**DIAGNOSTIC SPECIFICITY = 2 / (1 + 2) × 100% = 100%**

Conclusion

Considering that diagnostic sensitivity was >90% and the diagnostic specificity was >40%, the requirement for diagnostic performance was achieved.

4. CONCLUSION

The data presented in this validation report for analytical specificity, analytical sensitivity, selectivity, repeatability, reproducibility, robustness in terms of RNA extraction, and diagnostic performance show that the LMV targeted SE-qPCR assay is suitable for detection of LMV in lettuce seed and demonstrate validity of the LMV SE-qPCR as an optional pre-screening assay in the ISHI LMV detection method.

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6. ANNEXES

Annex A. Protocol for detection of Lettuce mosaic virus (LMV) in Lettuce (Lactuca sativa) Seed by triplex Seed Extract RT-qPCR (SE-qPCR) pre-screen assay

PRE-SCREEN BY SEED EXTRACT RT-qPCR

For PCR methods, *in-house* method optimization is often necessary, see https://worldseed.org/document/ishi-veg-best-practices-pcr/ for information.

Subsample size

The subsample size for the LMV SE-qPCR pre-screening step is 1,000 seeds.

Materials

- PBS Tween buffer (see Table A.1)
- Seed extraction buffer (see Table A.2)
- *Dahlia Latent Viroid* (DLVd) spike (Table A.3)
  Provided by the CT organizer
- RNA purification kit and equipment
  Qiagen RNeasy Plant Mini Kit should be used for the CT (Qiagen, Hilden, Germany)
- 50 mL centrifuge tube
- Stainless steel grinding bullet ø14 mm
- Geno/Grinder 2010 (SPEX® SamplePrep LLC, Metuchen, NJ, USA; or equivalent apparatus)
- Controls (Table A.3) Provided by the CT organizer
- 1.5 mL RNase Free tubes
- RNAse free water
- 96 well PCR plate
- 96 well (heat) seal
- qPCR mix, primers and probes (Table A.4), and equipment
  QuantaBio Ultraplex 1-Step ToughMix was used for validation

Table A.1. 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
Deionised water	950 mL*

*Adjust the volume to 1 L.

Table A.2. Seed extraction buffer (pH 7.4).

Compound	Amount/L
Polyvinyl pyrrolidone (PVP 10.000-15.000)	20.0 g
PBS Tween buffer (See Table A.1)	Up to 1 L

Spike solution

The spike solution is prepared by taking a leaf from a plant infected by DLVd and making an extract of it in PBS. 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 solution.

Table A.3. Types of controls used.

Control type	Description
Internal amplification control (IAC)	Spike solution added to the samples aiming for a Cq value between 26 and 30
	Synthetic RNA resembling the genome of DLVd using the DaVd1 primer and probe set aiming for a Cq value between 26 and 30
Negative process control (NPC)	Healthy or LMV free lettuce seeds
Positive process control (PPC)	Positive (LMV infected) lettuce seed
Positive amplification control (PAC)	LMV synthetic ssDNA oligonucleotide aiming for a Cq value between 26 and 32
	LMV RNA aiming for a Cq value between 26 and 32
	LMV cDNA aiming for a Cq value between 26 and 32
Negative template control (NTC)	PCR grade water

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

Name	Sequence (5' – 3')[a]	Source
DaVd1 Fw	GCT CCG CTC CTT GTA GCT TT	Naktuinbouw, Botermans *et al.*, 2020
DaVd1 Rv	AGG AGG TGG AGA CCT CTT GG	
DaVd1 Probe	TxR-X– CTG ACT CGA GGA CGC GAC CG - IBRQ	
LMV RZ 2 Fw	GCA YTR CCA TTG TGT GGA AAA TT	Rijk Zwaan
LMV RZ 2 Rv	TGG AAA CAG AYC CAA CCG AGA TAC	
LMV RZ 2 Probe	YakYel – ACA GGY GCC GCA GAT TTG AAA GGC AG - IBFQ	
LMV NAKT Fw	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[b]	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) to the seed extraction buffer (Table A.2).

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 A.3) 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. For example, seeds may also be ground at 1,100 rpm for 3 min using a Seed Shaker.

1.4. Add 10 mL seed extraction buffer (Table A.2), spiked with DLVd as IAC, 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 isolation kit is used, it is necessary to verify its performance.

2. RT-qPCR

2.1. Prepare the RT-qPCR mix with the primer, probe and the Ultraplex 1-Step ToughMix RT-qPCR (4×; Quantabio) TaqMan mix components, as described in Table A.5.

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

Table A.5. Preparation of the RT-qPCR mix.

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
Quantabio 1-Step ToughMix RT-qPCR (4×)	6.25	1×
PCR grade water	9.50	
Sample RNA	5.00	
Total volume	**25.00**	

2.2. Distribute 20 µL of the RT-qPCR master mix in PCR tubes or a 96-well plate and add 5 µL RNA sample as template. Perform the PCR for each RNA sample in duplicate.

Note: If different PCR mix and amplification programs are used, it is necessary to verify their performance.

2.3. Seal the plate and perform the PCR reaction in a real-time PCR instrument, according to the conditions described in Table A.6.

Notes: All samples and controls should be tested in duplicate as described in Best Practices for PCR Assays in Seed Health Tests. The assay has been validated using the Ultraplex 1-Step ToughMix (4×; Quantabio). If a different qPCR mix and amplification program is used, it is necessary to verify their performance.

Table A.6. PCR conditions.

Repeats	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/or PC.

4. Interpretation and decisions

Cq cut-off values must be established by each laboratory for their positive, internal amplification, and/or inhibition 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.

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 A.7).

Test results are only valid when all included controls presented in Table A.3 give expected results.

Table A.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
Positive or negative	Positive	Positive or negative	LMV RNA detected	LMV seedling ELISA
Negative	Negative	Positive	No LMV RNA detected	No follow-up needed
Negative	Negative	Negative	IAC failure	Repeat RNA extraction and/or LMV SE-qPCR. The SE-qPCR cannot be interpreted as negative when the same results are obtained.

Annex B. Specificity assessment of the "LMV-RZ2" and the "LMV-NAKT" LMV targeted primer and probe sets

Two TaqMan based PCR primer and probe sets have been developed separately. One set was designed by Naktuinbouw, designated "LMV NAKT", that was presented on ISHI-NL workgroup Gouda 16052014 (Powerpoint presentation from Carine van Soest, *et al*. 2014). The second set, designated LMV RZ 2, was designed by Rijk Zwaan based on 24 LMV genome sequences that were available. The Naktuinbouw assay targets a 63 nucleotides (nt) region at approximately 9.4 kb of the LMV genome and falls in the gene coding for coat protein. The Rijk Zwaan assay targets a 135 nt region in the gene coding for the P3 protein at 3.5 kb of the entire LMV genome. The larger amplicon size of the LMV RZ 2 assay enables sequencing of the PCR-product to gain more insights in the sequence diversity among LMV strains.

The LMV RZ 2 primer and probe sequences were aligned with 24 sequences of LMV strains, belonging to the LMV-Yar, LMV-Common, and LMV-Most clusters (Krause-Sakate *et al*., 2002), that were present in the NCBI DNA databases revealed that they matched all sequences except for a single nucleotide mismatch towards the 5´ end of the reverse primer with a single sequence (Figure B.1). The primer and probe sequences of the LMV NAKT set were aligned with 16 sequences derived from LMV genome sequences found in published data and 22 LMV sequences that were obtained by sequencing LMV isolates from leaf material (10) and seeds (12; Figure B.2). This showed that the forward and reverse primer had three mismatches with LMV sequences, albeit that the mismatches were located in the middle and towards the 5' end. The probe sequence had one mismatch with the LMV sequences (Figure B.2). An alignment of the LMV NAKT set with sequence accession KJ161194, belonging to the LMV-Yar, revealed that the forward primer had two mismatches, while the probe and the reverse primer had no mismatch (Data not shown). While there are no sequence data available for LMV strains belonging to the LMV-Greek cluster that cover the region targeted by the LMV RZ primer and probe set, the LMV NAKT set matched sequences of accession Z78229, belonging to the LMV-Greek cluster. These results indicate that the two LMV targeted primer and probe sets are expected to enable detection of LMV strains belonging to the LMV-Common and LMV-Most clusters and that detection of strains from the LMV-Yar and LMV-Greek by one of the two primer and probe sets is likely. However, LMV strains from the latter clusters are not known to be seedborne (Krause-Sakate *et al*., 2002). Therefore, the notion that LMV strains from the LMV-Yar and LMV-Greek cluster could potentially be missed by both primer and probe sets is deemed not detrimental.

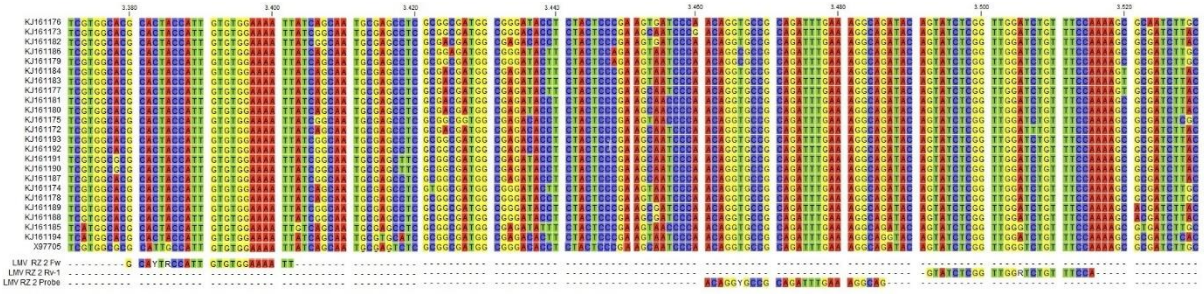

Figure B.1. Alignment of the LMV RZ 2 primer and probe sequences with 24 partial LMV genome sequences present in the NCBI DNA databases.

The two primer and probe sets were tested using RNA from two different LMV strains: a "Most" LMV strain, known for breaking mo1 resistance, and a "regular" (LMV-Common) strain that is unable to infect *mo1*[1] or *mo1*[2] plants. RNA from the regular LMV strain in combination with the LMV NAKT primer and probe set showed a sigmoidal shaped curve. This was also observed for

the LMV RZ 2 primer and probe set, albeit that the Cq values were 0.5 Cq higher. While RNA from the "Most" LMV (LMV-Most) strain with the LMV RZ 2 primer and probe set also showed a sigmoidal curve, the PCR with the LMV NAKT primer and probe set did not (Figure B.3). Nonetheless, amplification did occur.

Partial LMV genome sequences derived from naturally LMV infected seed lots that had a positive reaction with the LMV triplex SE-qPCR were analysed to gain insights in the coverage of the two LMV targeted primer and probe sets. This showed that the obtained sequences were different and demonstrated that the two LMV primer and probe sets enabled detection of various LMV strains (Figure B.4).

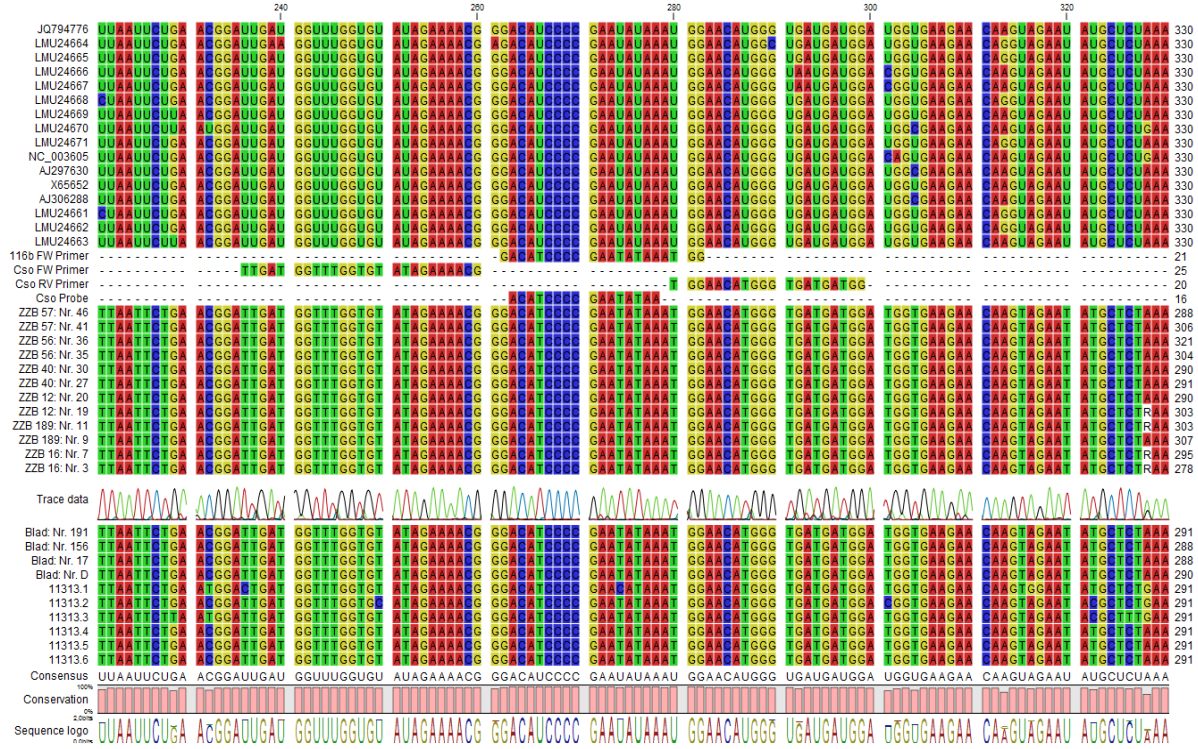

Figure B.2. Alignment of the LMV NAKT primer (Cso FW/RV Primer) and probe (Cso Probe) sequences with 16 sequences derived from LMV genome sequences found in published data and 22 LMV sequences that were obtained by sequencing LMV isolates found in leaf material (10) and seeds (12; Powerpoint presentation from Carine van Soest *et al.* (2014)).

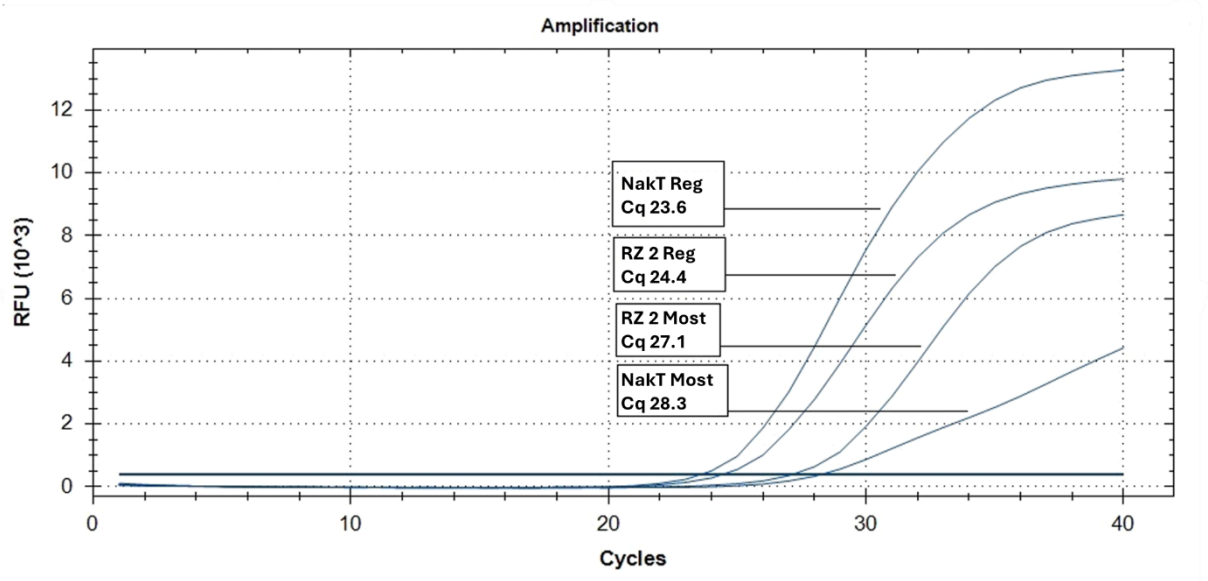

Figure B.3. Amplification curves from qPCRs with LMV NAKT and LMV RZ 2 primer and probe set using RNA from two different strains (Most: LMV strain breaking *mo1* resistance; and regular: LMV strain are unable to infect *mo1*[1] *or mo1*[2] plants).

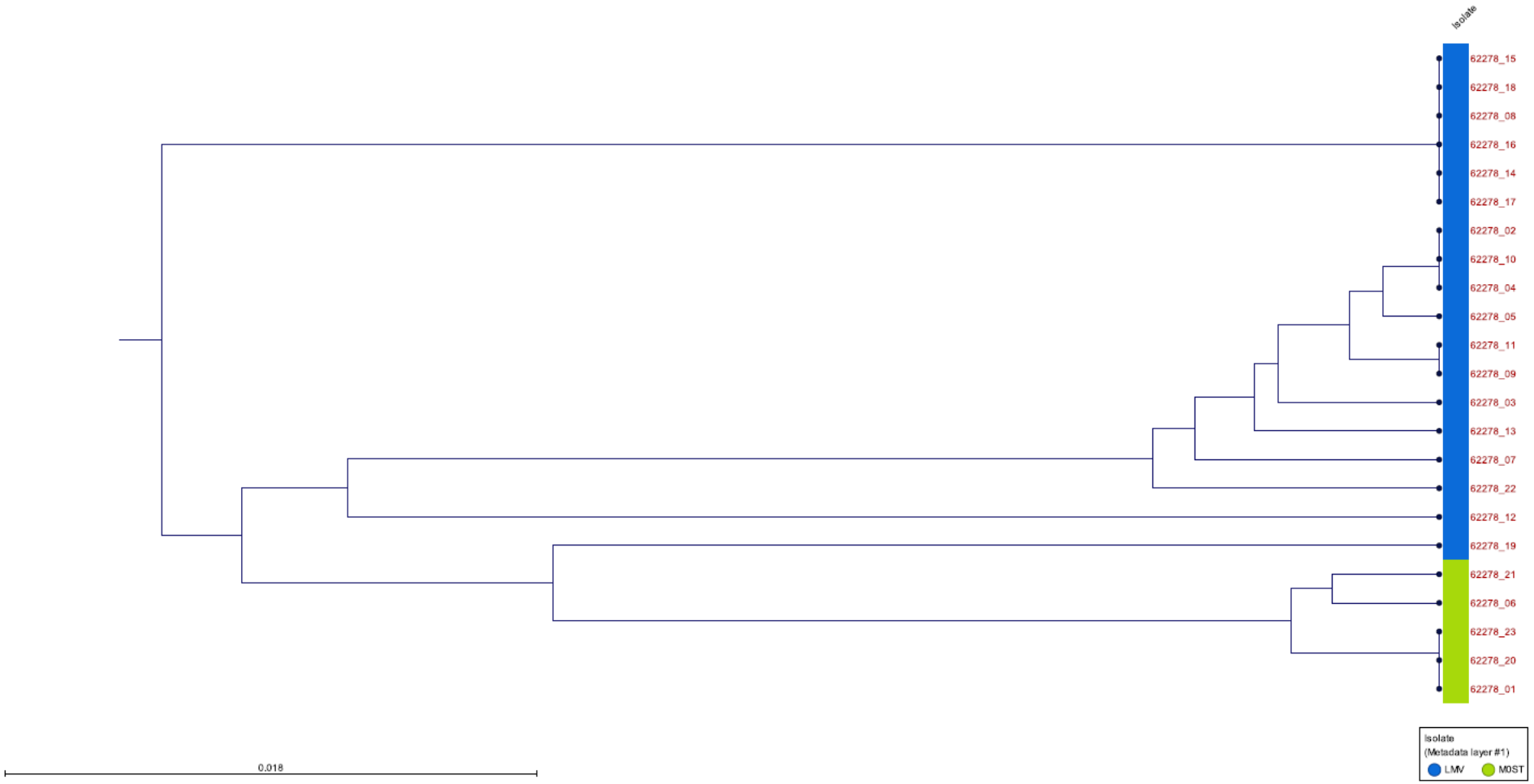

Figure B.4. Phylogenetic tree of partial regular LMV (Blue) and LMV Most (Green) genome sequences obtained from naturally LMV infected seed lots that were identified as LMV positive using the two LMV targeted SE-qPCRs.

Summary and conclusion

- The LMV NAKT and LMV RZ two primer and probe sets match LMV sequences derived from DNA databases and those obtained *in house* by sequencing.
- Both primer sets detect various regular (non-“Most”) LMV and LMV “Most” strains.

Annex C. Data from analytical specificity assessment

Table C.1. Obtained Cq values of the two LMV targeted primer and probe sets (LMV-NAKT (FAM channel) and LMV-RZ2 (VIC channel)) and DLVd (TxR channel) that is used as an IAC, for LMV isolates, non-target isolates, LMV infected roots, and extract from LMV negative as well as extract from LMV infected seed lots. All samples tested were in 96-well format.

Plate	Group	Sample	Sub	Dilution factor	PCR replicate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
I	Target	LMV isolate (5386 from BASF)		1×10^{0}	I	17.18	17.07	32.77
III	Target	LMV isolate (PV-0286 from BASF)		1×10^{0}	I	14.31	14.61	No Cq[g]
III	Target	LMV isolate (PV-2799 from BASF)		1×10^{0}	I	16.22	16.38	No Cq[g]
III	Target	LMV isolate (LMV0 from Geves)		1×10^{0}	I	12.02	11.11	No Cq[g]
III	Target	LMV isolate (LMV9 from Geves)		1×10^{0}	I	13.60	14.55	No Cq[g]
II	Target	LMV isolate (10516 (USA) from RZ)		1×10^{0}	I	16.31	16.29	28.37
I	Target	LMV isolate (LS234 from RZ)		1×10^{0}	I	15.13	15.34	34.69
I	Target	LMV isolate (LS252 from RZ)		1×10^{0}	I	22.49	18.07	33.22
I	Target	LMV isolate (LSI from RZ)		1×10^{0}	I	16.09	16.25	34.39
I	Target	LMV isolate (m0st from RZ)		1×10^{0}	I	26.21	20.58	33.69
III	Target	LMV isolate (1 from Syngenta)		1×10^{0}	I	17.17	17.10	No Cq[g]
III	Target	LMV isolate (2 from Syngenta)		1×10^{0}	I	15.48	15.48	No Cq[g]
I	Non-target	ArMV isolate (PV-0046)		1×10^{0}	I	36.79	38.33	33.91
I	Non-target	ArMV isolate (PV-0215)		1×10^{0}	I	34.31	35.15	35.02
I	Non-target	ArMV isolate (PV-0216)		1×10^{0}	I	36.52	36.53	35.78
I	Non-target	ArMV isolate (PV-0217)		1×10^{0}	I	35.00	35.72	36.07
I	Non-target	ArMV isolate (WUR)		1×10^{0}	I	36.81	36.75	33.51
I	Non-target	TBRV infected good-king-Henry leaf		1×10^{0}	I	No Cq	No Cq	No Cq[g]

Plate	Group	Sample	Sub	Dilution factor	PCR replicate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
I	Non-target	TBRV infected lettuce leaf		1×10^{0}	I	No Cq	No Cq	No Cq[g]
I	Non-target	TBRV infected potato leaf		1×10^{0}	I	No Cq	No Cq	No Cq[g]
I	Non-target	TBRV infected shallot leaf		1×10^{0}	I	No Cq	No Cq	No Cq[g]
I	Non-target	TBRV infected tulip leaf		1×10^{0}	I	No Cq	No Cq	No Cq[g]
I	Non-target	TSWV isolate		1×10^{0}	I	No Cq	No Cq	No Cq[g]
III	Non-target	DLVd infected Dahlia leaf		1×10^{0}	I	36.19	36.84	23.44
II	Positive LMV seedling ELISA	SE 27[e]		1×10^{0}	I	15.20	15.03	28.14
II	Positive LMV seedling ELISA	SE 28[e]		1×10^{0}	I	15.25	15.11	28.20
II	Positive LMV seedling ELISA	SE 29[f]		1×10^{0}	I	16.73	16.69	28.37
II	Positive LMV seedling ELISA	SE 30[f]		1×10^{0}	I	20.02	19.79	28.32
II	Positive LMV seedling ELISA	SE 31[f]		1×10^{0}	I	19.39	19.25	28.42
II	Positive LMV seedling ELISA	SE 32[e]		1×10^{0}	I	16.65	16.52	28.03
IV	Positive LMV seedling ELISA	SE 33[f]	I	1×10^{0}	I	27.65	29.08	25.45
IV	Positive LMV seedling ELISA	SE 33[f]	II	1×10^{0}	I	24.37	25.80	25.26
IV	Positive LMV seedling ELISA	SE 33[f]	III	1×10^{0}	I	23.87	25.11	25.77
IV	Positive LMV seedling ELISA	SE 34[e]	I	1×10^{0}	I	22.77	24.41	25.11
IV	Positive LMV seedling ELISA	SE 34[e]	II	1×10^{0}	I	21.09	22.96	25.08
IV	Positive LMV seedling ELISA	SE 34[e]	III	1×10^{0}	I	19.74	20.19	25.12
IV	Positive LMV seedling ELISA	SE 35[e]	I	1×10^{0}	I	18.33	18.50	25.19
IV	Positive LMV seedling ELISA	SE 35[e]	II	1×10^{0}	I	16.86	16.74	25.32
IV	Positive LMV seedling ELISA	SE 35[e]	III	1×10^{0}	I	18.48	18.77	25.19

Plate	Group	Sample	Sub	Dilution factor	PCR replicate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
II	Negative LMV seedling ELISA	SE 1[e]		1×10^{0}	I	36.44	No Cq	27.78
II	Negative LMV seedling ELISA	SE 2[f]		1×10^{0}	I	No Cq	No Cq	27.82
II	Negative LMV seedling ELISA	SE 3[f]		1×10^{0}	I	No Cq	No Cq	27.81
II	Negative LMV seedling ELISA	SE 4[e]		1×10^{0}	I	No Cq	No Cq	27.73
II	Negative LMV seedling ELISA	SE 5[e]		1×10^{0}	I	33.30	33.09	27.32
II	Negative LMV seedling ELISA	SE 6[e]		1×10^{0}	I	No Cq	37.54	27.41
II	Negative LMV seedling ELISA	SE 7[e]		1×10^{0}	I	38.51	38.48	27.37
II	Negative LMV seedling ELISA	SE 8[e]		1×10^{0}	I	36.54	No Cq	27.60
II	Negative LMV seedling ELISA	SE 9[e]		1×10^{0}	I	37.10	39.12	27.68
II	Negative LMV seedling ELISA	SE 10[f]		1×10^{0}	I	No Cq	No Cq	27.33
II	Negative LMV seedling ELISA	SE 11[e]		1×10^{0}	I	30.45	32.37	27.20
II	Negative LMV seedling ELISA	SE 12[e]		1×10^{0}	I	35.19	37.56	27.20
II	Negative LMV seedling ELISA	SE 13[e]		1×10^{0}	I	37.28	36.22	27.75
II	Negative LMV seedling ELISA	SE 14[e]		1×10^{0}	I	29.45	33.34	28.97
II	Negative LMV seedling ELISA	SE 15[e]		1×10^{0}	I	27.18	28.18	27.68
II	Negative LMV seedling ELISA	SE 16[e]		1×10^{0}	I	27.88	30.75	28.42
II	Negative LMV seedling ELISA	SE 17[e]		1×10^{0}	I	33.58	35.38	27.21
II	Negative LMV seedling ELISA	SE 18[e]		1×10^{0}	I	29.66	30.41	27.66
II	Negative LMV seedling ELISA	SE 19[f]		1×10^{0}	I	No Cq	30.64	27.80
II	Negative LMV seedling ELISA	SE 20[e]		1×10^{0}	I	29.37	32.66	27.52
II	Negative LMV seedling ELISA	SE 21[e]		1×10^{0}	I	30.97	30.37	27.98

Plate	Group	Sample	Sub	Dilution factor	PCR replicate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
II	Negative LMV seedling ELISA	SE 22[e]		1×10^{0}	I	32.96	37.63	27.21
II	Negative LMV seedling ELISA	SE 23[e]		1×10^{0}	I	33.27	35.7	27.36
II	Negative LMV seedling ELISA	SE 24[e]		1×10^{0}	I	29.12	31.84	27.31
II	Negative LMV seedling ELISA	SE 25[e]		1×10^{0}	I	33.68	35.87	28.10
II	Negative LMV seedling ELISA	SE 26[e]		1×10^{0}	I	34.90	36.68	27.47
I	Method controls	LMV positive SE (PPC used in RZ routine testing)		1×10^{0}	I	16.36	16.49	35.10
II	Method controls	LMV positive SE (PPC used in RZ routine testing)		1×10^{0}	I	16.24	16.06	27.36
IV	Method controls	LMV positive SE (PPC used in RZ routine testing)	V	1×10^{0}	I	15.00	14.91	25.21
I	Method controls	LMV negative SE (NPC used in RZ routine testing)		1×10^{0}	I	39.09	No Cq	34.52
II	Method controls	LMV negative SE (NPC used in RZ routine testing)		1×10^{0}	I	37.29	No Cq	27.02
IV	Method controls	LMV negative SE (NPC used in RZ routine testing)	V	1×10^{0}	I	38.58	No Cq	26.19
II	Method controls	RNA from LMV positive SE leaf tissue (PAC)		1×10^{0}	I	25.14	24.43	No Cq
I	Method controls	LMV NAKT + LMV RZ 2 Synth (PAC)[a]		1×10^{11}	I	28.64	29.84	No Cq
III	Method controls	LMV NAKT + LMV RZ 2 Synth (PAC)[a]		1×10^{11}	I	23.32	23.53	No Cq
IV	Method controls	LMV NAKT + LMV RZ 2 Synth (PAC)[a]		1×10^{11}	I	24.88	25.32	No Cq
I	Method controls	LMV NAKT + LMV RZ 2 Synth (PAC)[b]		1×10^{10}	I	25.08	26.02	No Cq
I	Method controls	LMV NAKT + LMV RZ 2 Synth (PAC)[c]		1×10^{8}	I	18.24	18.86	No Cq
I	Method controls	LMV NAKT + LMV RZ 2 Synth (PAC)[c]		1×10^{8}	II	18.45	19.41	No Cq

Plate	Group	Sample	Sub	Dilution factor	PCR replicate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
II	Method controls	LMV NAKT + LMV RZ 2 Synth (PAC)[c]		1×10^{8}	I	19.33	20.00	No Cq
II	Method controls	LMV NAKT + LMV RZ 2 Synth (PAC)[d]		1×10^{6}	I	10.45	12.71	No Cq
I	Method controls	EB with IAC (NEC)			I	36.65	39.17	33.83
II	Method controls	EB with IAC (NEC)			I	33.84	33.66	27.04
III	Method controls	EB with IAC (NEC)			I	36.09	36.57	27.15
I	Method controls	PCR grade water (NTC)			I	No Cq	No Cq	No Cq
II	Method controls	PCR grade water (NTC)			I	No Cq	No Cq	No Cq
III	Method controls	PCR grade water (NTC)			I	No Cq	No Cq	No Cq
IV	Method controls	PCR grade water (NTC)			I	No Cq	No Cq	No Cq
IV	Method controls	PCR grade water (NTC)			II	No Cq	No Cq	No Cq
IV	Method controls	PCR grade water (NTC)			III	No Cq	No Cq	No Cq
IV	Method controls	PCR grade water (NTC)			IV	No Cq	No Cq	No Cq
IV	Method controls	PCR grade water (NTC)			V	No Cq	No Cq	No Cq
IV	Method controls	PCR grade water (NTC)			VI	No Cq	No Cq	No Cq

[a] A mixture of synthetic ssDNA with a concentration of 6×10^{2} copies per µL.
[b] A mixture of synthetic ssDNA with a concentration of 6×10^{3} copies per µL.
[c] A mixture of synthetic ssDNA with a concentration of 6×10^{5} copies per µL.
[d] A mixture of synthetic ssDNA with a concentration of 6×10^{7} copies per µL.
[e] White seeds.
[f] Black seeds.
[g] Isolate was not spiked with DLVd during RNA extraction and no signal with the DLVd targeted primer and probe set (DaVd1) primer and probe set is expected.

Annex D. Determination of the Cq Cut-off value for the validation of the LMV SE-qPCR

The Cq values from LMV negative and positive SE (SE 1-35), negative processing controls (NPCs), and positive processing controls (PPCs) from the analysis performed under "Analytical specificity" (Section 3.1, Figure 2; Table C.1 in Annex C) were used in a Response Operator Characteristic (ROC) curve analysis to further evaluate the Cq cut-off for the LMV triplex SE-qPCR. Using this analysis, Cq cut-offs from 1 to 40 in steps of a single Cq were evaluated, where the outcome of the PCR is positive if at least one of the two primer and probe sets ("LMV-RZ2" or "LMV-NAKT") yields a Cq value under each of the tested cut-offs. This qualitative outcome (positive vs negative) was compared to the outcome of the LMV seedling ELISA to determine true positive and negative as well as false positive and negative outcomes to ultimately calculate the (diagnostic) sensitivity and (diagnostic) specificity of the LMV triplex SE-qPCR. Results of the ROC curve analysis are visualized in Figure D.1 and show that sensitivity was 100% when a Cq cut-off of at least 28 was used and a specificity of 100% was obtained when a Cq cut-off lower than 25 was used. This indicates that cut-offs chosen >28 Cq would provide an excellent sensitivity, but that this would come at the expense of specificity. Considering that the LMV SE-qPCR is a pre-screening method, and it is crucial that no false negative results are obtained, which is more important than to avoid false positive results, this compromise is acceptable. Based on these findings a Cq cut-off for the LMV SE-qPCR of Cq 32 was chosen and applied during this validation to discriminate between LMV positive and negative lettuce seed samples.

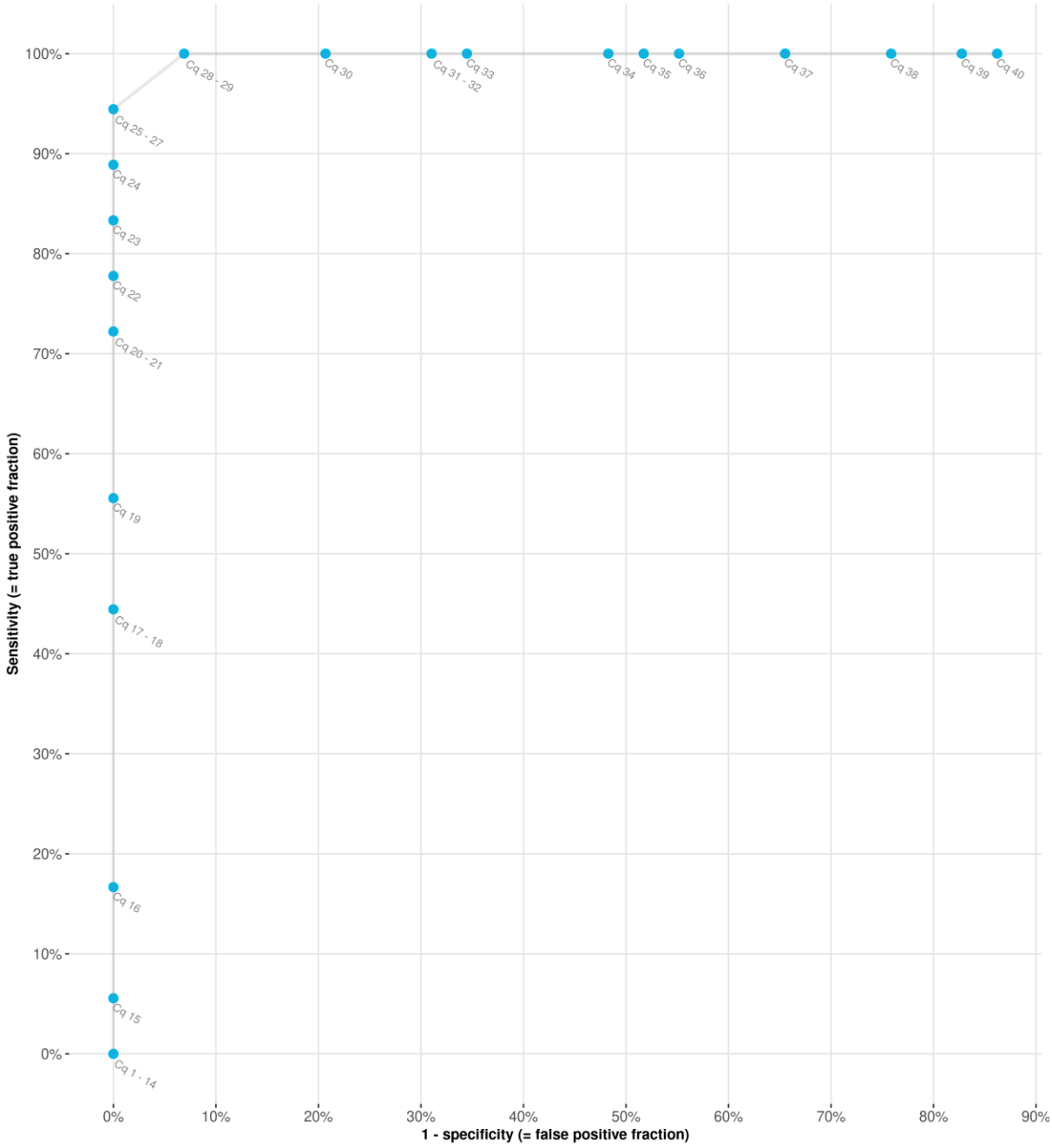

Figure D.1. Receiver operating characteristic (ROC) curve for the LMV triplex SE-qPCR.

Annex E. Data from analytical sensitivity assessment

Table E.1. Obtained Cq values with the two LMV targeted primer and probe sets (LMV-NAKT (FAM channel) and LMV-RZ2 (VIC channel)) and DLVd (TxR channel) that is used as an IAC, for LMV-NAKT and LMV-RZ2 synthetic oligonucleotide dilution series. All samples tested were in the 96-well format.

Sample	Copies per µL	PCR replicate	Plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
PCR grade water		I	I	No Cq	No Cq	No Cq
PCR grade water		I	II	No Cq	No Cq	No Cq
PCR grade water		II	I	No Cq	No Cq	No Cq
PCR grade water		II	II	No Cq	No Cq	No Cq
PCR grade water		III	I	No Cq	No Cq	No Cq
PCR grade water		III	II	No Cq	No Cq	No Cq
PCR grade water		IV	I	No Cq	No Cq	No Cq
PCR grade water		IV	II	No Cq	No Cq	No Cq
LMV positive control		I	I	23.00	23.09	No Cq
LMV positive control		I	II	23.16	23.33	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series I)	6×10^{-2}	I	I	No Cq	No Cq	No Cq
		II	I	No Cq	No Cq	No Cq
		III	I	No Cq	No Cq	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series II)	6×10^{-2}	I	I	No Cq	No Cq	No Cq
		II	I	No Cq	No Cq	No Cq
		III	I	No Cq	No Cq	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series III)	6×10^{-2}	I	I	No Cq	No Cq	No Cq
		II	I	No Cq	No Cq	No Cq
		III	I	No Cq	38.73	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series IV)	6×10^{-2}	I	II	No Cq	No Cq	No Cq
		II	II	No Cq	No Cq	No Cq
		III	II	No Cq	No Cq	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series V)	6×10^{-2}	I	II	No Cq	No Cq	No Cq
		II	II	No Cq	No Cq	No Cq
		III	II	No Cq	No Cq	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series VI)	6×10^{-2}	I	II	No Cq	No Cq	No Cq
		II	II	No Cq	39.17	No Cq
		III	II	No Cq	No Cq	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series I)	6×10^{-1}	I	I	No Cq	No Cq	No Cq
		II	I	39.71	39.02	No Cq
		III	I	No Cq	No Cq	No Cq
	6×10^{-1}	I	I	No Cq	38.79	No Cq

Sample	Copies per µL	PCR replicate	Plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
LMV NAKT + LMV RZ 2 Synth (Dilution series II)		II	I	No Cq	37.71	No Cq
		III	I	No Cq	No Cq	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series III)	6×10^{-1}	I	I	No Cq	37.68	No Cq
		II	I	No Cq	37.32	No Cq
		III	I	No Cq	36.36	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series IV)	6×10^{-1}	I	II	No Cq	38.93	No Cq
		II	II	38.36	39.01	No Cq
		III	II	No Cq	39.13	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series V)	6×10^{-1}	I	II	38.75	39.34	No Cq
		II	II	No Cq	No Cq	No Cq
		III	II	38.64	No Cq	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series VI)	6×10^{-1}	I	II	No Cq	39.09	No Cq
		II	II	No Cq	39.24	No Cq
		III	II	No Cq	39.21	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series I)	6×10^{0}	I	I	34.26	35.43	No Cq
		II	I	34.99	35.04	No Cq
		III	I	35.10	36.20	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series II)	6×10^{0}	I	I	34.28	35.03	No Cq
		II	I	35.39	35.16	No Cq
		III	I	34.30	36.09	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series III)	6×10^{0}	I	I	35.41	36.74	No Cq
		II	I	34.60	36.36	No Cq
		III	I	34.45	35.86	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series IV)	6×10^{0}	I	II	34.89	36.54	No Cq
		II	II	35.28	36.36	No Cq
		III	II	34.64	37.46	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series V)	6×10^{0}	I	II	34.63	36.28	No Cq
		II	II	34.14	35.23	No Cq
		III	II	33.83	35.23	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series VI)	6×10^{0}	I	II	34.93	36.45	No Cq
		II	II	34.38	35.39	No Cq
		III	II	33.90	36.04	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series I)	6×10^{1}	I	I	31.27	32.46	No Cq
		II	I	31.19	32.00	No Cq
		III	I	31.38	32.22	No Cq
LMV NAKT + LMV RZ 2 Synth	6×10^{1}	I	I	30.88	32.57	No Cq
		II	I	30.66	32.31	No Cq

Sample	Copies per µL	PCR replicate	Plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
(Dilution series II)		III	I	31.13	32.31	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series III)	6×10^{1}	I	I	31.21	32.06	No Cq
		II	I	30.83	31.89	No Cq
		III	I	31.07	32.29	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series IV)	6×10^{1}	I	II	31.28	33.12	No Cq
		II	II	31.50	32.86	No Cq
		III	II	31.11	32.54	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series V)	6×10^{1}	I	II	30.57	31.85	No Cq
		II	II	30.52	32.33	No Cq
		III	II	31.10	31.93	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series VI)	6×10^{1}	I	II	30.85	32.69	No Cq
		II	II	30.75	32.09	No Cq
		III	II	30.90	32.40	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series I)	6×10^{2}	I	I	27.69	28.90	No Cq
		II	I	27.70	28.78	No Cq
		III	I	27.47	28.67	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series II)	6×10^{2}	I	I	27.74	28.71	No Cq
		II	I	27.67	28.77	No Cq
		III	I	27.61	28.65	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series III)	6×10^{2}	I	I	27.53	28.63	No Cq
		II	I	27.61	28.68	No Cq
		III	I	27.51	28.52	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series IV)	6×10^{2}	I	II	28.10	29.22	No Cq
		II	II	28.04	29.28	No Cq
		III	II	28.03	29.31	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series V)	6×10^{2}	I	II	27.37	28.60	No Cq
		II	II	27.36	28.74	No Cq
		III	II	27.37	28.70	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series VI)	6×10^{2}	I	II	27.49	28.73	No Cq
		II	II	27.54	28.88	No Cq
		III	II	27.51	29.49	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series I)	6×10^{3}	I	I	24.28	25.21	No Cq
		II	I	24.26	25.26	No Cq
		III	I	24.25	25.16	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series II)	6×10^{3}	I	I	24.30	25.25	No Cq
		II	I	24.24	25.25	No Cq
		III	I	24.21	25.23	No Cq

Sample	Copies per µL	PCR replicate	Plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
LMV NAKT + LMV RZ 2 Synth (Dilution series III)	6×10^{3}	I	I	24.28	25.18	No Cq
		II	I	24.25	25.22	No Cq
		III	I	24.24	25.20	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series IV)	6×10^{3}	I	II	24.70	25.83	No Cq
		II	II	24.60	25.88	No Cq
		III	II	24.60	25.61	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series V)	6×10^{3}	I	II	24.03	25.22	No Cq
		II	II	24.22	25.24	No Cq
		III	II	24.09	25.14	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series VI)	6×10^{3}	I	II	24.34	25.37	No Cq
		II	II	24.26	25.47	No Cq
		III	II	24.27	26.13	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series I)	6×10^{4}	I	I	21.09	21.71	No Cq
		II	I	20.91	21.72	No Cq
		III	I	21.00	21.73	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series II)	6×10^{4}	I	I	21.01	21.79	No Cq
		II	I	20.92	21.72	No Cq
		III	I	20.97	21.77	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series III)	6×10^{4}	I	I	21.02	21.80	No Cq
		II	I	20.96	21.70	No Cq
		III	I	21.00	21.79	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series IV)	6×10^{4}	I	II	21.21	22.19	No Cq
		II	II	21.26	22.27	No Cq
		III	II	21.11	22.19	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series V)	6×10^{4}	I	II	20.84	21.93	No Cq
		II	II	20.91	21.98	No Cq
		III	II	20.92	21.98	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series VI)	6×10^{4}	I	II	21.06	22.10	No Cq
		II	II	21.06	22.08	No Cq
		III	II	20.99	22.05	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series I)	6×10^{5}	I	I	17.75	18.49	No Cq
		II	I	17.50	18.15	No Cq
		III	I	17.25	18.04	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series II)	6×10^{5}	I	I	17.42	18.14	No Cq
		II	I	17.51	18.28	No Cq
		III	I	17.41	18.13	No Cq
	6×10^{5}	I	I	17.63	18.33	No Cq

Sample	Copies per µL	PCR replicate	Plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
LMV NAKT + LMV RZ 2 Synth (Dilution series III)		II	I	17.58	18.28	No Cq
		III	I	17.61	18.33	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series IV)	6×10^{5}	I	II	17.95	18.65	No Cq
		II	II	17.94	18.75	No Cq
		III	II	17.78	18.69	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series V)	6×10^{5}	I	II	17.48	18.51	No Cq
		II	II	17.52	18.44	No Cq
		III	II	17.62	18.55	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series VI)	6×10^{5}	I	II	17.80	18.72	No Cq
		II	II	17.72	18.64	No Cq
		III	II	17.72	18.63	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series I)	6×10^{6}	I	I	14.35	14.98	No Cq
		II	I	14.46	15.04	No Cq
		III	I	14.26	14.95	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series II)	6×10^{6}	I	I	13.89	14.47	No Cq
		II	I	14.07	14.66	No Cq
		III	I	14.04	14.63	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series III)	6×10^{6}	I	I	13.85	14.38	No Cq
		II	I	14.06	14.67	No Cq
		III	I	14.39	15.07	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series IV)	6×10^{6}	I	II	14.77	15.38	No Cq
		II	II	14.63	15.34	No Cq
		III	II	14.48	15.24	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series V)	6×10^{6}	I	II	14.52	15.26	No Cq
		II	II	14.55	15.26	No Cq
		III	II	14.53	15.29	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series VI)	6×10^{6}	I	II	14.56	15.30	No Cq
		II	II	14.38	15.24	No Cq
		III	II	14.44	15.21	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series I)	6×10^{7}	I	I	11.30	11.80	No Cq
		II	I	11.20	11.85	No Cq
		III	I	9.730	11.80	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series II)	6×10^{7}	I	I	11.14	11.79	No Cq
		II	I	9.50	11.72	No Cq
		III	I	11.09	11.76	No Cq
LMV NAKT + LMV RZ 2 Synth	6×10^{7}	I	I	11.18	11.77	No Cq
		II	I	11.09	11.76	No Cq

Sample	Copies per µL	PCR replicate	Plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
(Dilution series III)		III	I	9.14	11.74	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series IV)	6×10^7	I	II	11.35	11.96	No Cq
		II	II	11.20	11.94	No Cq
		III	II	11.09	11.85	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series V)	6×10^7	I	II	11.23	11.91	No Cq
		II	II	11.08	11.86	No Cq
		III	II	11.09	11.85	No Cq
LMV NAKT + LMV RZ 2 Synth (Dilution series VI)	6×10^7	I	II	11.21	11.91	No Cq
		II	II	11.15	11.92	No Cq
		III	II	11.08	11.85	No Cq

Table E.2. Obtained Cq values with the 2 LMV targeted primer and probe sets (LMV-NAKT (FAM channel) and LMV-RZ2 (VIC channel)) and DLVd (TxR channel) that is used as an IAC, for dilutions of 10 seed extracts (SE 1-10; RNA extracted using the Qiagen column-based method) for which 15 seeds from an LMV positive lettuce seed lot (seed lot P) were mixed with 985 seeds from an LMV negative lettuce seed lot (seed lot N). All samples tested were in the 96-well format.

Sample	Sub	Dilution factor	PCR replicate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
985 seed lot N + 15 seed lot P	1	1×10^{0}	I	27.66	28.95	24.70
985 seed lot N + 15 seed lot P	1	1×10^{0}	II	28.06	28.91	24.30
985 seed lot N + 15 seed lot P	1	1.5×10^{1} [b]	I	31.30	32.33	24.78
985 seed lot N + 15 seed lot P	1	1.5×10^{1} [b]	II	31.52	32.49	24.42
985 seed lot N + 15 seed lot P	1	1.5×10^{2} [c]	I	33.78	35.50	23.62
985 seed lot N + 15 seed lot P	1	1.5×10^{2} [c]	II	34.00	36.55	23.81
985 seed lot N + 15 seed lot P	2	1×10^{0}	I	21.76	22.26	24.38
985 seed lot N + 15 seed lot P	2	1×10^{0}	II	21.75	22.21	24.20
985 seed lot N + 15 seed lot P	2	1.5×10^{1} [b]	I	25.43	26.18	24.46
985 seed lot N + 15 seed lot P	2	1.5×10^{1} [b]	II	25.36	26.14	24.38
985 seed lot N + 15 seed lot P	2	1.5×10^{2} [c]	I	28.78	29.60	23.99
985 seed lot N + 15 seed lot P	2	1.5×10^{2} [c]	II	28.45	29.40	24.18
985 seed lot N + 15 seed lot P	3	1×10^{0}	I	21.76	22.25	24.49
985 seed lot N + 15 seed lot P	3	1×10^{0}	II	21.87	22.26	24.36
985 seed lot N + 15 seed lot P	3	1.5×10^{1} [b]	I	25.31	25.92	24.10
985 seed lot N + 15 seed lot P	3	1.5×10^{1} [b]	II	25.37	26.03	24.36
985 seed lot N + 15 seed lot P	3	1.5×10^{2} [c]	I	28.44	29.21	24.21
985 seed lot N + 15 seed lot P	3	1.5×10^{2} [c]	II	28.74	29.27	23.83
985 seed lot N + 15 seed lot P	4	1×10^{0}	I	22.12	22.67	24.16
985 seed lot N + 15 seed lot P	4	1×10^{0}	II	22.12	22.61	23.94
985 seed lot N + 15 seed lot P	4	1.5×10^{1} [b]	I	25.55	26.28	24.00
985 seed lot N + 15 seed lot P	4	1.5×10^{1} [b]	II	25.68	26.25	23.83
985 seed lot N + 15 seed lot P	4	1.5×10^{2} [c]	I	28.96	29.83	24.08
985 seed lot N + 15 seed lot P	4	1.5×10^{2} [c]	II	28.95	29.79	24.10
985 seed lot N + 15 seed lot P	5	1×10^{0}	I	19.70	20.07	24.31
985 seed lot N + 15 seed lot P	5	1×10^{0}	II	19.59	19.97	24.24
985 seed lot N + 15 seed lot P	5	1.5×10^{1} [b]	I	23.38	23.75	23.73
985 seed lot N + 15 seed lot P	5	1.5×10^{1} [b]	II	23.36	23.80	24.19
985 seed lot N + 15 seed lot P	5	1.5×10^{2} [c]	I	26.66	27.25	24.21
985 seed lot N + 15 seed lot P	5	1.5×10^{2} [c]	II	26.62	27.19	24.20
985 seed lot N + 15 seed lot P	6	1×10^{0}	I	27.82	29.13	24.49
985 seed lot N + 15 seed lot P	6	1×10^{0}	II	27.91	29.07	24.63
985 seed lot N + 15 seed lot P	6	1.5×10^{1} [b]	I	31.65	33.03	23.64

Sample	Sub	Dilution factor	PCR replicate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
985 seed lot N + 15 seed lot P	6	1.5×10^{1} [b]	II	31.93	33.27	23.40
985 seed lot N + 15 seed lot P	6	1.5×10^{2} [c]	I	33.32	35.64	24.53
985 seed lot N + 15 seed lot P	6	1.5×10^{2} [c]	II	33.25	33.66	24.43
985 seed lot N + 15 seed lot P	7	1×10^{0}	I	26.43	26.63	24.98
985 seed lot N + 15 seed lot P	7	1×10^{0}	II	26.19	26.60	25.23
985 seed lot N + 15 seed lot P	7	1.5×10^{1} [b]	I	29.20	30.25	24.30
985 seed lot N + 15 seed lot P	7	1.5×10^{1} [b]	II	29.14	30.11	24.35
985 seed lot N + 15 seed lot P	7	1.5×10^{2} [c]	I	33.53	34.14	24.45
985 seed lot N + 15 seed lot P	7	1.5×10^{2} [c]	II	33.14	34.08	24.48
985 seed lot N + 15 seed lot P	8	1×10^{0}	I	21.54	22.17	24.61
985 seed lot N + 15 seed lot P	8	1×10^{0}	II	21.68	22.13	24.15
985 seed lot N + 15 seed lot P	8	1.5×10^{1} [b]	I	25.49	26.07	24.09
985 seed lot N + 15 seed lot P	8	1.5×10^{1} [b]	II	25.44	26.04	24.25
985 seed lot N + 15 seed lot P	8	1.5×10^{2} [c]	I	28.72	29.44	24.46
985 seed lot N + 15 seed lot P	8	1.5×10^{2} [c]	II	28.77	29.43	24.60
985 seed lot N + 15 seed lot P	9	1×10^{0}	I	24.42	24.89	24.50
985 seed lot N + 15 seed lot P	9	1×10^{0}	II	24.49	24.86	24.53
985 seed lot N + 15 seed lot P	9	1.5×10^{1} [b]	I	28.06	28.89	24.15
985 seed lot N + 15 seed lot P	9	1.5×10^{1} [b]	II	28.07	28.84	24.07
985 seed lot N + 15 seed lot P	9	1.5×10^{2} [c]	I	31.44	31.95	24.99
985 seed lot N + 15 seed lot P	9	1.5×10^{2} [c]	II	31.37	32.33	24.98
985 seed lot N + 15 seed lot P	10	1×10^{0}	I	23.55	24.25	24.67
985 seed lot N + 15 seed lot P	10	1×10^{0}	II	23.88	24.39	24.68
985 seed lot N + 15 seed lot P	10	1.5×10^{1} [b]	I	27.31	27.90	23.99
985 seed lot N + 15 seed lot P	10	1.5×10^{1} [b]	II	27.27	27.87	24.01
985 seed lot N + 15 seed lot P	10	1.5×10^{2} [c]	I	30.98	32.04	24.78
985 seed lot N + 15 seed lot P	10	1.5×10^{2} [c]	II	30.82	31.66	24.76
LMV positive SE (PPC used in RZ routine testing)		1×10^{0}	I	17.80	18.06	24.52
LMV positive SE (PPC used in RZ routine testing)		1×10^{0}	II	18.00	18.12	24.53
LMV NAKT + LMV RZ 2 Synth (PAC)[a]		1×10^{11}	I	25.75	25.43	No Cq
LMV NAKT + LMV RZ 2 Synth (PAC)[a]		1×10^{11}	II	25.71	25.45	No Cq
LMV negative SE (NPC used in RZ routine testing)		1×10^{0}	I	38.40	39.96	23.74
LMV negative SE (NPC used in RZ routine testing)		1×10^{0}	II	38.65	39.59	23.94

Sample	Sub	Dilution factor	PCR replicate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
PCR grade water (NTC)			I	No Cq	No Cq	No Cq
PCR grade water (NTC)			II	No Cq	No Cq	No Cq

[a] A mixture of synthetic ssDNA with a concentration of 6×10^2 copies per µL.

[b] 15 times dilution of SE to obtain an LMV load equivalent to 1 seed from the LMV positive seed lot.

[c] 150 times dilution of SE to obtain an LMV load equivalent to 0.1 seed from the LMV positive seed lot.

Annex F. Data from selectivity and repeatability assessment

Table F.1. Obtained Cq values with the two LMV targeted primer and probe sets (LMV-NAKT (FAM channel) and LMV-RZ2 (VIC channel)) and DLVd (TxR channel) that is used as an IAC, for dilutions of 10 seed extracts (SE 1-10; RNA extracted using the Qiagen column-based method) for which one seed from an LMV positive lettuce seed lot (seed lot P) was mixed with 999 seeds from an LMV negative lettuce seed lot (seed lot N). All samples tested were in the 96-well format.

Sample	Sub	Dilution factor	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
999 seed lot N + 1 seed lot P	Sub 1	1×10^{0}	30.39	30.59	28.31
999 seed lot N + 1 seed lot P	Sub 1	1×10^{1}	34.11	34.78	26.33
999 seed lot N + 1 seed lot P	Sub 1	1×10^{2}	36.93	39.49	26.15
999 seed lot N + 1 seed lot P	Sub 1	1×10^{3}	No Cq	No Cq	26.17
999 seed lot N + 1 seed lot P	Sub 2	1×10^{0}	32.22	32.28	28.00
999 seed lot N + 1 seed lot P	Sub 2	1×10^{1}	34.74	36.73	26.56
999 seed lot N + 1 seed lot P	Sub 2	1×10^{2}	No Cq	No Cq	26.28
999 seed lot N + 1 seed lot P	Sub 2	1×10^{3}	38.41	No Cq	26.26
999 seed lot N + 1 seed lot P	Sub 3	1×10^{0}	33.83	34.50	28.58
999 seed lot N + 1 seed lot P	Sub 3	1×10^{1}	38.44	37.74	26.62
999 seed lot N + 1 seed lot P	Sub 3	1×10^{2}	No Cq	No Cq	26.38
999 seed lot N + 1 seed lot P	Sub 3	1×10^{3}	No Cq	No Cq	26.29
999 seed lot N + 1 seed lot P	Sub 4	1×10^{0}	29.09	29.44	28.04
999 seed lot N + 1 seed lot P	Sub 4	1×10^{1}	33.78	34.49	26.58
999 seed lot N + 1 seed lot P	Sub 4	1×10^{2}	37.29	38.82	26.44
999 seed lot N + 1 seed lot P	Sub 4	1×10^{3}	No Cq	No Cq	26.06
999 seed lot N + 1 seed lot P	Sub 5	1×10^{0}	35.48	35.72	27.91
999 seed lot N + 1 seed lot P	Sub 5	1×10^{1}	38.58	No Cq	26.69
999 seed lot N + 1 seed lot P	Sub 5	1×10^{2}	38.49	No Cq	26.24
999 seed lot N + 1 seed lot P	Sub 5	1×10^{3}	No Cq	No Cq	26.18
999 seed lot N + 1 seed lot P	Sub 6	1×10^{0}	29.73	30.48	28.06
999 seed lot N + 1 seed lot P	Sub 6	1×10^{1}	33.94	34.84	26.69
999 seed lot N + 1 seed lot P	Sub 6	1×10^{2}	36.60	No Cq	26.24
999 seed lot N + 1 seed lot P	Sub 6	1×10^{3}	No Cq	No Cq	26.33
999 seed lot N + 1 seed lot P	Sub 7	1×10^{0}	21.40	22.07	28.29
999 seed lot N + 1 seed lot P	Sub 7	1×10^{1}	25.22	26.14	26.64
999 seed lot N + 1 seed lot P	Sub 7	1×10^{2}	28.34	29.22	26.24
999 seed lot N + 1 seed lot P	Sub 7	1×10^{3}	31.75	32.92	26.27
999 seed lot N + 1 seed lot P	Sub 8	1×10^{0}	35.51	38.41	28.51
999 seed lot N + 1 seed lot P	Sub 8	1×10^{1}	35.43	36.21	27.03
999 seed lot N + 1 seed lot P	Sub 8	1×10^{2}	No Cq	No Cq	26.82
999 seed lot N + 1 seed lot P	Sub 8	1×10^{3}	No Cq	No Cq	26.42

Sample	Sub	Dilution factor	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
999 seed lot N + 1 seed lot P	Sub 9	1×10^{0}	36.94	37.36	28.87
999 seed lot N + 1 seed lot P	Sub 9	1×10^{1}	38.4	No Cq	27.19
999 seed lot N + 1 seed lot P	Sub 9	1×10^{2}	No Cq	No Cq	26.59
999 seed lot N + 1 seed lot P	Sub 9	1×10^{3}	No Cq	No Cq	26.88
999 seed lot N + 1 seed lot P	Sub 10	1×10^{0}	32.52	34.77	28.94
999 seed lot N + 1 seed lot P	Sub 10	1×10^{1}	33.67	35.17	27.10
999 seed lot N + 1 seed lot P	Sub 10	1×10^{2}	No Cq	No Cq	26.63
999 seed lot N + 1 seed lot P	Sub 10	1×10^{3}	No Cq	No Cq	26.64
LMV positive SE (PPC used in RZ routine testing)	IV	1×10^{0}	17.53	17.99	26.66
LMV NAKT + LMV RZ 2 Synth (PAC)[a]		1×10^{11}	24.88	25.32	No Cq
LMV negative SE (NPC used in RZ routine testing)	IV	1×10^{0}	No Cq	No Cq	26.38
PCR grade water (NTC; PCR replicate I)			No Cq	No Cq	No Cq
PCR grade water (NTC; PCR replicate II)			No Cq	No Cq	No Cq
PCR grade water (NTC; PCR replicate III)			No Cq	No Cq	No Cq
PCR grade water (NTC; PCR replicate IV)			No Cq	No Cq	No Cq
PCR grade water (NTC; PCR replicate V)			No Cq	No Cq	No Cq
PCR grade water (NTC; PCR replicate VI)			No Cq	No Cq	No Cq

[a] A mixture of synthetic ssDNA with a concentration of 6×10^{2} copies per µL.

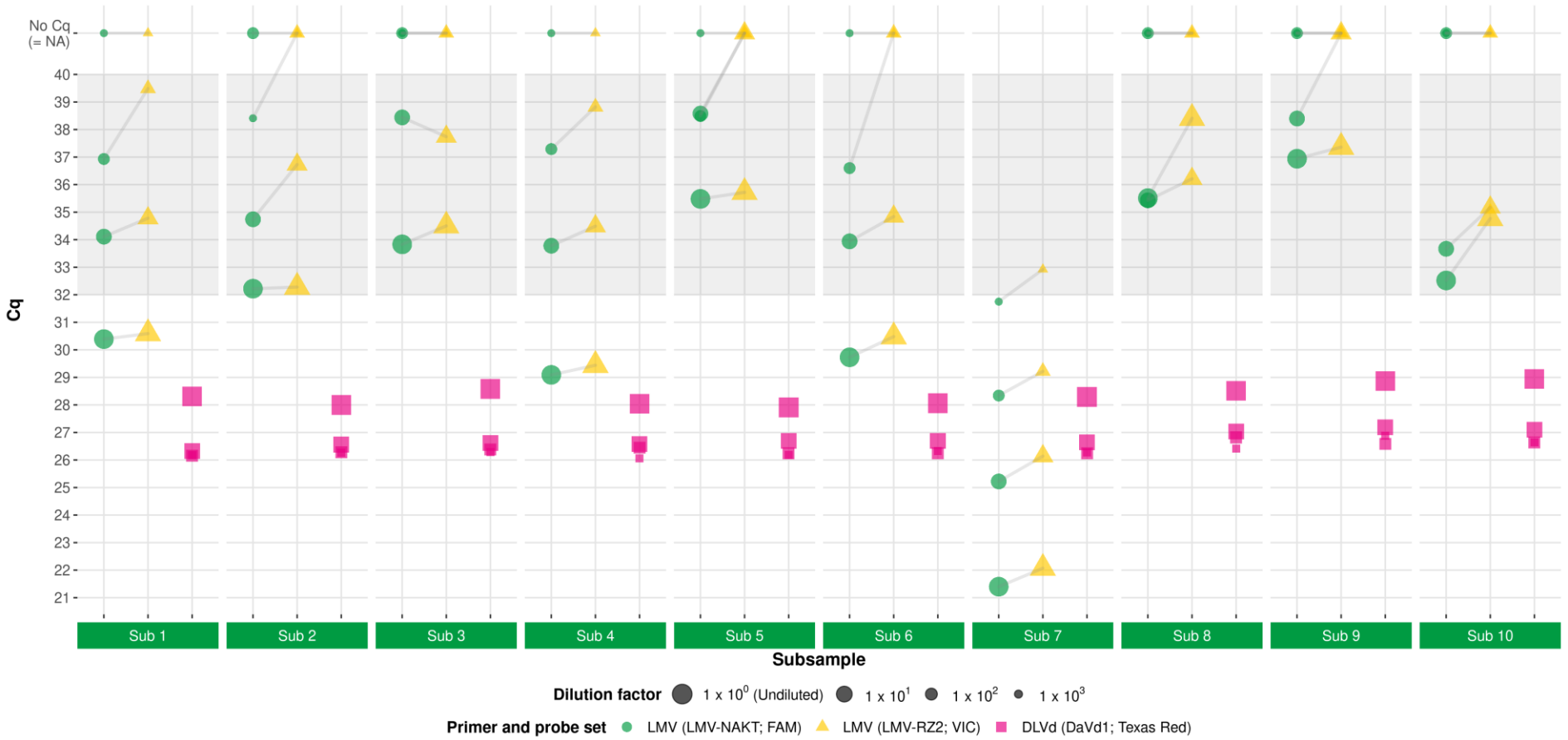

Figure F.1. Obtained Cq values of the LMV triplex PCR for RNA from dilutions of seed extracts from 10 subsamples (Sub 1-10) for which one seed from an LMV positive lettuce seed lot was mixed with 999 seeds from an LMV negative lettuce seed lot (upper panel). The grey lines connect SE-qPCR signals from the LMV-NAKT and LMV-RZ2 primer and probe set obtained from the same well. The grey background represents the range for which the SE-qPCRs are considered negative (Cq 32-40). PCRs yielding no Cq value (NA: not available) are added to the top of the graph.

Table F.2. Obtained Cq values with the two LMV targeted primer and probe sets (LMV-NAKT (FAM channel) and LMV-RZ2 (VIC channel)) and DLVd (TxR channel) that is used as an IAC, for dilutions of lettuce SE spiked with LMV positive SE (Sub 7 acquired during assessment of the LMV infection level of the LMV positive seed lot used for "Analytical sensitivity of the LMV SE-qPCR assay" (Section 3.2; Figure F.1)). All samples tested were in the 96-well format.

Plate	Sample	Dilution factor	Extraction replicate (Time point)	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
I	Unspiked SE 2	1×10^0	I (T = 1)	No Cq	No Cq	27.72
II	Unspiked SE 2	1×10^0	II (T = 2)	No Cq	No Cq	28.10
III	Unspiked SE 2	1×10^0	III (T = 3)	No Cq	No Cq	28.36
I	Unspiked SE 4	1×10^0	I (T = 1)	No Cq	No Cq	26.99
II	Unspiked SE 4	1×10^0	II (T = 2)	No Cq	No Cq	27.03
III	Unspiked SE 4	1×10^0	III (T = 3)	No Cq	No Cq	26.67
I	Unspiked SE 6	1×10^0	I (T = 1)	No Cq	No Cq	27.23
II	Unspiked SE 6	1×10^0	II (T = 2)	No Cq	No Cq	27.20
III	Unspiked SE 6	1×10^0	III (T = 3)	No Cq	39.41	27.40
IV	LMV negative SE (NPC used in RZ routine testing)	1×10^0	I (T = 1)	35.43	35.63	32.35
II	LMV negative SE (NPC used in RZ routine testing)	1×10^0	II (T = 2)	No Cq	No Cq	32.00
IV	LMV negative SE (NPC used in RZ routine testing)	1×10^0	III (T = 3)	No Cq	No Cq	32.13
I	PCR grade water (NTC)			No Cq	No Cq	No Cq
II	PCR grade water (NTC)			No Cq	No Cq	No Cq
III	PCR grade water (NTC)			No Cq	No Cq	No Cq
IV	PCR grade water (NTC)			No Cq	No Cq	No Cq
I	Sub 7 (999 seed lot N + 1 seed lot P)	1×10^0	I (T = 1)	22.46	23.18	31.92
II	Sub 7 (999 seed lot N + 1 seed lot P)	1×10^0	II (T = 2)	23.55	24.54	32.19
III	Sub 7 (999 seed lot N + 1 seed lot P)	1×10^0	III (T = 3)	24.29	25.90	33.59
IV	LMV positive SE (PPC used in RZ routine testing)	1×10^0	I (T = 1)	19.81	20.31	31.09
II	LMV positive SE (PPC used in RZ routine testing)	1×10^0	II (T = 2)	19.63	20.43	31.18

Plate	Sample	Dilution factor	Extraction replicate (Time point)	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
IV	LMV positive SE (PPC used in RZ routine testing)	1×10^{0}	III (T = 3)	19.77	20.3	32.02
I	LMV NAKT + LMV RZ 2 Synth (PAC)[a]	1×10^{10}		26.28	26.06	No Cq
II	LMV NAKT + LMV RZ 2 Synth (PAC)[a]	1×10^{10}		26.91	26.61	No Cq
III	LMV NAKT + LMV RZ 2 Synth (PAC)[a]	1×10^{10}		25.75	25.82	No Cq
IV	LMV NAKT + LMV RZ 2 Synth (PAC)[a]	1×10^{10}		27.99	28.14	No Cq
IV	Spiked SE 2	1×10^{1}	I (T = 1)	27.13	27.23	27.96
IV	Spiked SE 2	1×10^{2}	I (T = 1)	28.99	28.63	28.01
IV	Spiked SE 2	1×10^{3}	I (T = 1)	34.04	34.45	27.78
I	Spiked SE 2	1×10^{4}	I (T = 1)	35.33	36.03	28.47
I	Spiked SE 2	1×10^{5}	I (T = 1)	38.70	38.70	28.77
II	Spiked SE 2	1×10^{1}	II (T = 2)	27.22	28.17	29.28
II	Spiked SE 2	1×10^{2}	II (T = 2)	30.20	30.82	28.99
II	Spiked SE 2	1×10^{3}	II (T = 2)	33.23	34.39	29.07
II	Spiked SE 2	1×10^{4}	II (T = 2)	37.45	39.09	29.08
II	Spiked SE 2	1×10^{5}	II (T = 2)	No Cq	No Cq	29.32
III	Spiked SE 2	1×10^{1}	III (T = 3)	26.99	28.10	29.04
III	Spiked SE 2	1×10^{2}	III (T = 3)	30.18	31.47	29.37
III	Spiked SE 2	1×10^{3}	III (T = 3)	33.92	34.68	29.02
III	Spiked SE 2	1×10^{4}	III (T = 3)	36.76	38.58	28.25
III	Spiked SE 2	1×10^{5}	III (T = 3)	38.36	No Cq	29.27
I	Spiked SE 4	1×10^{1}	I (T = 1)	26.15	26.63	28.31
I	Spiked SE 4	1×10^{2}	I (T = 1)	29.37	29.78	28.13
I	Spiked SE 4	1×10^{3}	I (T = 1)	32.79	33.08	28.19
I	Spiked SE 4	1×10^{4}	I (T = 1)	35.25	36.21	28.04
I	Spiked SE 4	1×10^{5}	I (T = 1)	No Cq	No Cq	28.02
II	Spiked SE 4	1×10^{1}	II (T = 2)	26.70	27.30	28.11
II	Spiked SE 4	1×10^{2}	II (T = 2)	29.88	30.34	28.00
II	Spiked SE 4	1×10^{3}	II (T = 2)	33.31	33.47	27.72
II	Spiked SE 4	1×10^{4}	II (T = 2)	36.47	39.02	28.02
II	Spiked SE 4	1×10^{5}	II (T = 2)	39.65	No Cq	27.93
III	Spiked SE 4	1×10^{1}	III (T = 3)	26.33	27.21	27.79
III	Spiked SE 4	1×10^{2}	III (T = 3)	29.81	30.69	27.93

Plate	Sample	Dilution factor	Extraction replicate (Time point)	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
III	Spiked SE 4	1×10^{3}	III (T = 3)	33.26	34.31	27.70
III	Spiked SE 4	1×10^{4}	III (T = 3)	36.02	37.49	27.79
III	Spiked SE 4	1×10^{5}	III (T = 3)	No Cq	39.41	27.40
I	Spiked SE 6	1×10^{1}	I (T = 1)	26.05	26.33	27.81
I	Spiked SE 6	1×10^{2}	I (T = 1)	28.05	27.48	27.39
I	Spiked SE 6	1×10^{3}	I (T = 1)	32.81	33.00	27.37
I	Spiked SE 6	1×10^{4}	I (T = 1)	36.30	36.28	27.50
I	Spiked SE 6	1×10^{5}	I (T = 1)	39.19	38.69	27.70
II	Spiked SE 6	1×10^{1}	II (T = 2)	26.73	27.23	28.02
II	Spiked SE 6	1×10^{2}	II (T = 2)	30.31	30.54	27.25
II	Spiked SE 6	1×10^{3}	II (T = 2)	33.01	33.96	27.93
II	Spiked SE 6	1×10^{4}	II (T = 2)	35.92	36.60	27.76
II	Spiked SE 6	1×10^{5}	II (T = 2)	38.78	No Cq	27.92
III	Spiked SE 6	1×10^{1}	III (T = 3)	27.00	28.05	28.23
IV	Spiked SE 6	1×10^{2}	III (T = 3)	30.53	30.65	28.20
IV	Spiked SE 6	1×10^{3}	III (T = 3)	33.54	33.66	27.59
IV	Spiked SE 6	1×10^{4}	III (T = 3)	36.80	36.40	27.86
III	Spiked SE 6	1×10^{5}	III (T = 3)	No Cq	No Cq	27.63

[a] A mixture of synthetic ssDNA resembling the amplified sequence of the LMV-NAKT and LMV-RZ2 primer and probe sets each with a concentration of 6,000 copies per µL.

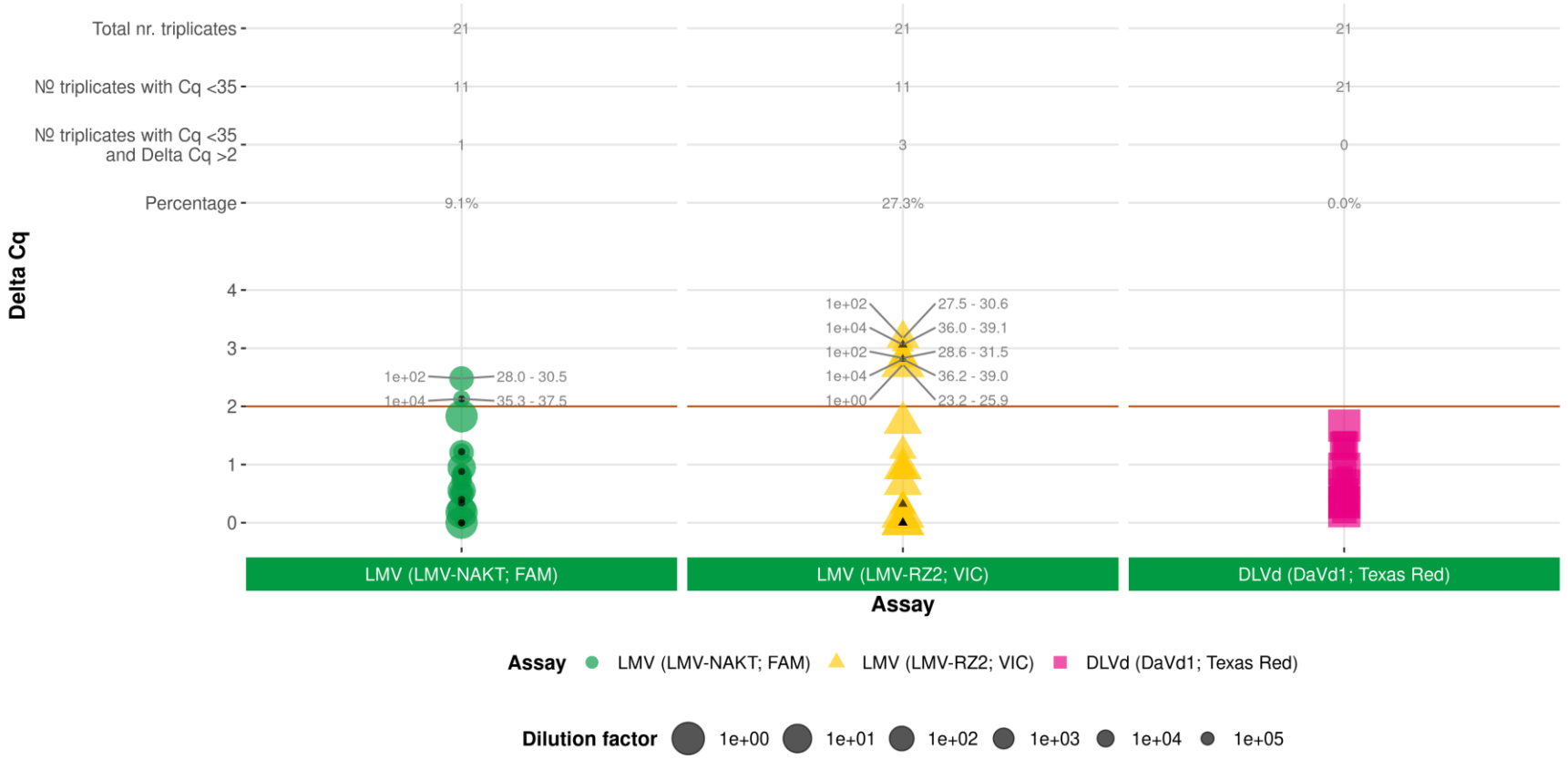

Figure F.2. Calculated difference in Cq values between triplicates of the primer and probe sets in the LMV triplex SE-qPCR for RNA derived from dilutions of lettuce SE spiked with LMV positive SE (Sub 7 acquired during assessment of the LMV infection level of the LMV positive seed lot used for "Analytical sensitivity of the LMV SE-qPCR assay" (Section 3.2; Figure F.1)). The dilution factor (left of points) and minimum and maximum Cq values (right of points) from the triplicate SE-qPCRs is added for differences that exceeded 2 Cq. Black dots indicate a difference in Cq between replicates where the highest Cq of the triplicate was above 35. The percentage of triplicate with deviations >2 Cq and Cq values < 35 are indicated per primer and probe set in the top part of the figure.

Annex G. LMV SE-qPCR Pre-CT

Objective

Considering that the LMV SE-qPCR is not implemented in most of the laboratories that participate in the final comparative test (CT), the pre-CT is organized for a selection of laboratories that will join the final CT to give an opportunity to participants to gain proficiency in the method and to facilitate setting the Cq cut-off discriminating between LMV positive and negative seed lots. The pre-CT serves as preparation for the final CT that addresses reproducibility, defined as the degree of similarity in results when the method is performed across laboratories with replicates of the same subsamples, of the LMV targeted SE-qPCR pre-screen assay to detect LMV in lettuce seed.

Experimental setup

The CT organizer provided five subsamples of 1,000 seeds from two healthy lettuce seed lots: "Healthy I" and "Healthy II" as well as from a low level LMV infected lettuce seed lot (Table G.1). Homogeneity assessment of the seed lots, whereby 10 subsamples of each lot were tested according to the LMV SE-qPCR described in Annex A, revealed that seed lot "Healthy I" differs from seed lot "Healthy II" with the former yielding high Cq values (Cq >29) and the latter yielding no Cq values. Seed lot "Low" was positive for LMV as determined using the LMV seedling ELISA as performed by the CT organizer based on the ISHI protocol (https://worldseed.org/wp-content/uploads/2017/07/Lettuce_LMV_July2017.pdf) and yielded Cq values between 24 and 32 for all 10 subsamples during homogeneity testing. Considering the relatively high Cq values this seed lot was deemed to have a low, but homogenous, LMV infection.

Table G.1. Seed samples and controls provided by the CT organizer to laboratories participating in the CT.

Item	LMV infection level	Quantity	Label	Storage conditions
Seed samples	Low	5 × 1,000 seeds	Low 1-5	4 ºC
	Healthy	5 × 1,000 seeds	Healthy I 1-5	4 ºC
	Healthy	5 × 1,000 seeds	Healthy II 1-5	4 ºC
	Healthy	1 × 1,000 seeds from LMV negative seed lot	NPC	4 ºC
	High	1 × 1,000 seeds from LMV positive seed lot	PPC	4 ºC
DLVd synthetic RNA (IAC)	-	1.25 mL in 2 mL microcentrifuge tube	IAC	4 ºC
LMV synthetic ssDNA (PAC)	-	50 µL in 1.5 mL microcentrifuge tube	PAC	4 ºC

The sample sets were supplemented with a negative process control (NPC) and positive process control (PPC) that are used routinely by the CT organizer as controls for detection of LMV in seed lots. These controls differ from the healthy and infected seed samples to be tested by the participants in that they are part of different seed lots.

The seed samples were tested by each participant according to the LMV SE-qPCR protocol described in Annex A using seed extraction buffer supplemented with synthetic RNA resembling the complete genome of Dahlia latent viroid (DLVd; 342 nt + 7 nt as a result of cloning and synthesis) as internal amplification control (IAC; Annex A - Step 1.1.), the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) for RNA extraction, and the Ultraplex 1-Step ToughMix (QuantaBio, Berverly, MA) for the qPCR. The qPCRs were requested to be performed in duplicate including for a provided positive amplification control (PAC) that consisted of a mixture of synthetic ssDNA resembling the amplified sequence of the LMV-NAKT and LMV-RZ2 primer and probe sets. Furthermore, the participant was requested to determine a suitable Cq cut-off to discriminate between LMV and negative seed samples. This cut-off is ideally placed above Cq values obtained for samples from the low infected seed lot and below Cq values from Healthy I and (if any) from II (Figure G.1).

To identify potential stability issues, the organizer analysed a sample set before shipment of the sample sets to the participants and after the last participant sent their data.

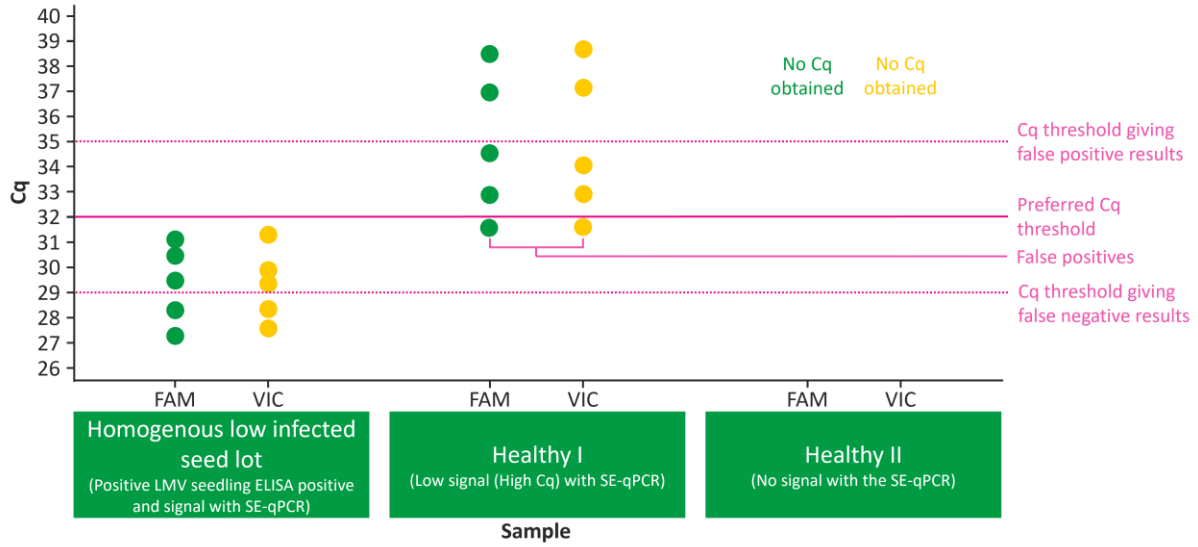

Figure G.1. Example for determining the preferred Cq cut-off for LMV SE-qPCR based on Cq values obtained for samples from homogenous low infected seed lot and samples from healthy lot I and II.

Cq values and the participant's interpretation (either positive or negative) were entered in a MS Excel file (Annex H) along with the general information and relevant observations and sent to Rijk Zwaan as the CT organizer. The Cq values from each participant for samples from each seed lot as well as the controls were compared.

Results

In total, six ISHI member laboratories (four from the Netherlands, one from France, and one from Spain) joined the LMV SE-qPCR pre-Ct, followed the protocol for the samples, within 12 days of each other, and submitted their data of the LMV SE-qPCR. Cq values from the LMV targeted triplex SE-qPCR, coded per laboratory to preserve anonymity, are included in Table G.2 below and are visualized in Figure G.2 for subsamples of the healthy and low infected seed lots and in figure G.3 for the controls.

Cq values from analysis of sample sets by the organizing laboratory before shipment and after the last participating laboratory sent in their data were sufficiently similar for the low LMV infected and healthy seed lots with the LMV targeted primer and probe sets as well with the DLVd

targeted primer and probe set (Figure G.2). In contrast to results obtained before shipment, the LMV-RZ2 primer and probe set did yield a Cq values >32 for replicate PCRs of one sample analysed after results from all participants were received. This sample would have been scored as negative if it not for the Cq <32 obtained with the LMV-NAKT primer and probe for the same RNA sample. Taken together, the results from the participating laboratories were accepted as valid.

Figure G.2. Obtained Cq values from the two LMV targeted primer and probe sets (LMV-NAKT and LMV-RZ2) and DLVd that is used as an IAC, for subsamples from healthy and low LMV infected lettuce seed lots performed as part of the Pre-CT by different laboratories. The grey background represents the range for which the SE-qPCRs are considered negative and are based on the suggested Cq cut-off by each of the participants. The grey lines connect qPCR signals from the LMV-NAKT and LMV-RZ2 primer and probe sets from the same reaction. The number of reactions yielding no Cq value and the number of reactions that was not determined (ND) are added to the top of each graph.

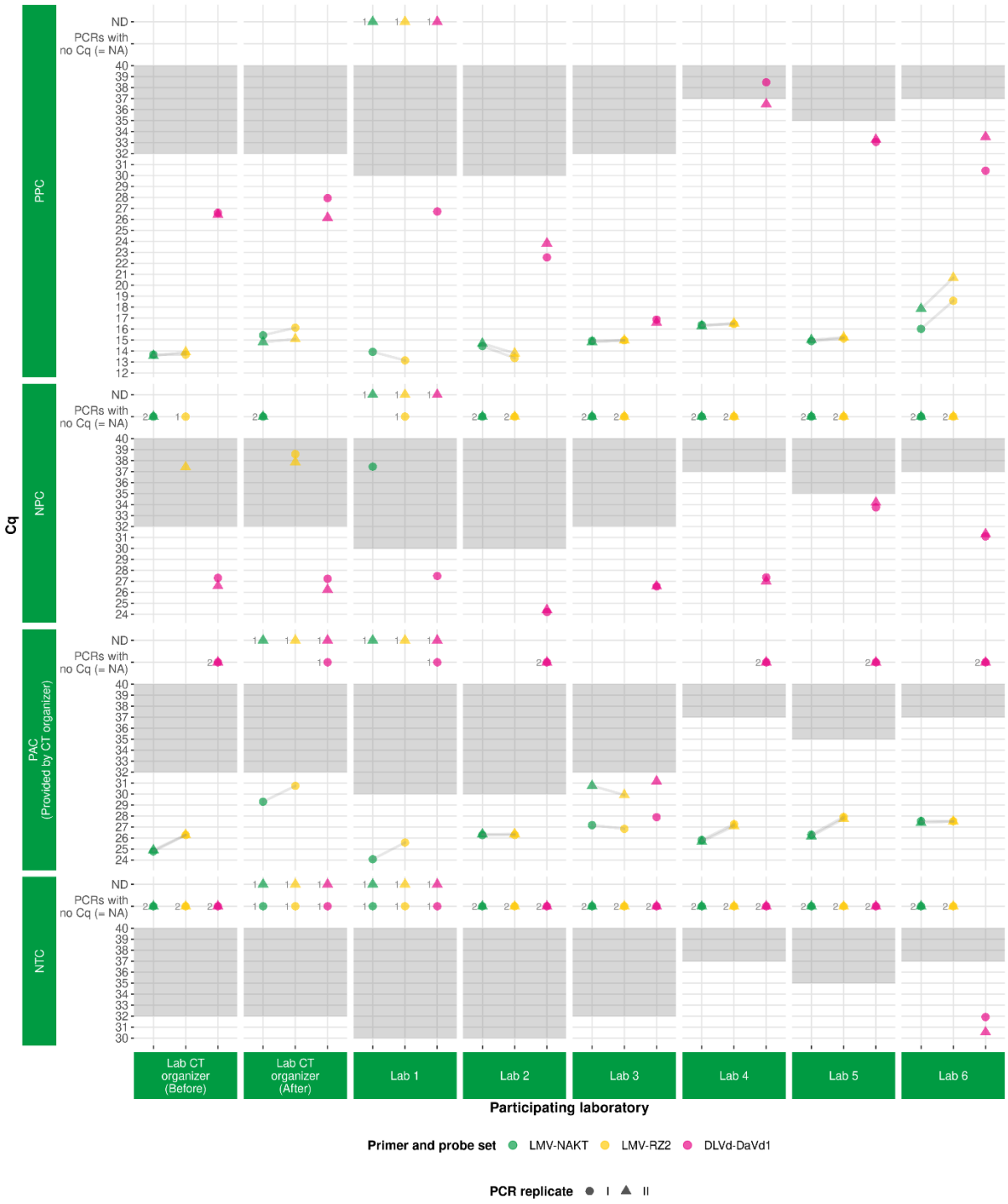

Figure G.3. Obtained Cq values from the two LMV targeted primer and probe sets (LMV-NAKT and LMV-RZ2) and DLVd that is used as an IAC, for controls performed as part of the Pre-CT by different laboratories. The grey background represents the range for which the SE-qPCRs are considered negative and are based on the suggested Cq cut-off by each of the participants. The grey lines connect qPCR signals from the LMV-NAKT and LMV-RZ2 primer and probe sets from the same reaction. The number of reactions yielding no Cq value and the number of reactions that was not determined (ND) are added to the top of each graph.

None of the laboratories showed a Cq value lower than their suggested Cq cut-off value with the two LMV targeted primer and probe sets for their negative template controls (NTC) as well as the NPC (Figure G.3). However, laboratory 6 did show unexpected Cq values with the primer and probe set targeted to DLVd for the NTC. As expected, all laboratories showed relatively low Cq

values for the PPC and Cq values lower than their suggested Cq cut-offs for the PAC (Figure G.3). Interestingly, Cq values for the PAC did vary among the different laboratories. Considering that the concentration of the synthetic ssDNA in the PAC was the same for each laboratory, similar Cq values were expected. These findings may seem to indicate that the different laboratories have a varying efficiency to detect LMV, but it cannot be ruled out that the PAC may have degraded to some extent during transport.

Similar to the PAC, the concentration of synthetic DLVd used as the IAC was also the same for each laboratory albeit that the participants prepared their own seed extraction buffer and spiked it with the provided IAC. This may at least partially explain the varying Cq values with the DLVd targeted primer and probe set of which the trend did not match that of the PAC.

In contrast to what was expected based on homogeneity testing, no Cq values were obtained for samples from seed lot "Healthy I" except for few Cq values >38 obtained by laboratory 6. Samples from seed lot "Healthy II" also did not yield Cq values, except for a single Cq >37 by laboratory 4. All laboratories reported Cq values for all samples from the low LMV infected seed lot, except for laboratory 3 that received four instead of five samples from this lot and laboratory 6 who did not obtain a signal from two replicate PCRs from one of five samples (Figure G.2). Cq values for the low LMV infected seed lot varied considerably between the different laboratories with laboratory 1 showing the lowest with the LMV targeted primer and probe sets. This also resulted in a variation in suggested cut-offs from Cq 30 to 37 (grey background in Figures G.2 and G.3) that could only be based on Cq values for the low infected seed lot as no Cq values being obtained for samples from seed lot "Healthy I".

Conclusion

The Pre-CT was successful in giving laboratories an opportunity to gain proficiency in the LMV SE-qPCR and to determine a suitable Cq cut-off to discriminate between LMV positive and negative seed lots in the future such as for the LMV SE-qPCR CT. Despite that the laboratories used samples from the same seed lots and were provided the same controls, differences in Cq values were profound and also led to differences in placement of the Cq cut-off. This reaffirms the notion that quantitatively, differences between laboratories may be evident. However, the final CT will predominantly focus on comparison of qualitative scorings (positive *vs.* negative) LMV infected seed lots to determine the degree of similarity in results when the method is performed across laboratories for the same samples. High and medium level LMV infected seed lots will be used for this purpose and will yield lower Cq values compared to those obtained here for the low infected seed lot. Consequently, the risk of obtaining false negative results is negligible. This also provides an opportunity to use a fixed Cq cut-off for all participating laboratories during the final CT, circumventing the need to have every laboratory set a Cq cut-off, which would be practically prohibitive. Considering that a Cq cut-off of 32, as used by the CT organizer, would have led to false negative results by some laboratories, a higher Cq cut-off of 35 for the final CT is recommended.

Table G.2. Obtained Cq values from the two LMV targeted primer and probe sets (LMV-NAKT and LMV-RZ2) and DLVd that is used as an IAC, for subsamples from healthy and low LMV infected lettuce seed lots performed as part of the Pre-CT by different laboratories. ND: Not Determined.

Lab number	Date test	Seed lot designation	Sub sample	PCR replicate	Suggested Cq cut-off	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
Lab CT organizer (Before)	8 May 2024	Low	1	I	32	26.48	28.14	27.00
				II	32	26.48	28.16	26.69
			2	I	32	26.40	27.90	26.37
				II	32	26.30	27.84	26.40
			3	I	32	25.28	26.52	26.23
				II	32	25.41	26.47	26.29
			4	I	32	26.38	27.88	26.39
				II	32	26.50	28.16	26.43
			5	I	32	27.20	28.61	26.67
				II	32	27.12	28.54	26.35
Lab CT organizer (Before)	8 May 2024	Healthy I	1	I	32	36.69	No Cq	26.94
				II	32	36.15	No Cq	26.37
			2	I	32	35.64	No Cq	26.68
				II	32	35.51	No Cq	26.71
			3	I	32	No Cq	No Cq	26.33
				II	32	No Cq	No Cq	25.79
			4	I	32	No Cq	No Cq	26.49
				II	32	No Cq	No Cq	27.01
			5	I	32	No Cq	No Cq	26.52
				II	32	No Cq	No Cq	26.52
Lab CT organizer (Before)	8 May 2024	Healthy II	1	I	32	39.26	No Cq	26.35
				II	32	No Cq	No Cq	26.37
			2	I	32	No Cq	No Cq	26.49
				II	32	No Cq	No Cq	26.26
			3	I	32	No Cq	No Cq	26.21
				II	32	No Cq	37.96	26.81
			4	I	32	No Cq	No Cq	27.37
				II	32	No Cq	No Cq	26.57
			5	I	32	No Cq	No Cq	27.23
				II	32	No Cq	No Cq	25.97
Lab CT organizer (Before)	8 May 2024	PPC	1	I	32	13.68	13.68	26.60
				II	32	13.56	13.91	26.43
		NPC	1	I	32	No Cq	No Cq	27.31

Lab number	Date test	Seed lot designation	Sub sample	PCR replicate	Suggested Cq cut-off	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
Lab CT organizer (Before)	8 May 2024			II	32	No Cq	37.40	26.58
Lab CT organizer (Before)	8 May 2024	PAC (Provided by CT organizer)		I	32	24.77	26.27	No Cq
				II	32	24.90	26.28	No Cq
Lab CT organizer (Before)	8 May 2024	PAC (Optional: provided by CT participant)		I	32	ND	ND	ND
				II	32	ND	ND	ND
Lab CT organizer (Before)	8 May 2024	NTC		I	32	No Cq	No Cq	No Cq
				II	32	No Cq	No Cq	No Cq
Lab CT organizer (After)	16 Jul 2024	Low	1	I	32	25.75	26.14	25.80
				II	32	25.99	26.50	27.05
			2	I	32	25.15	25.12	25.94
				II	32	25.26	25.70	27.66
			3	I	32	24.50	24.64	25.78
				II	32	25.00	25.57	27.76
			4	I	32	28.76	30.36	26.02
				II	32	29.20	31.12	28.11
			5	I	32	30.58	32.44	25.87
				II	32	31.06	32.44	26.50
Lab CT organizer (After)	16 Jul 2024	Healthy I	1	I	32	No Cq	No Cq	25.71
				II	32	No Cq	No Cq	28.49
			2	I	32	36.13	36.14	25.50
				II	32	36.14	36.42	27.22
			3	I	32	No Cq	No Cq	25.71
				II	32	38.20	No Cq	27.44
			4	I	32	No Cq	No Cq	25.64
				II	32	38.07	No Cq	27.01
			5	I	32	No Cq	38.44	25.68
				II	32	No Cq	No Cq	27.18
Lab CT organizer (After)	16 Jul 2024	Healthy II	1	I	32	No Cq	No Cq	25.32
				II	32	No Cq	No Cq	26.46
			2	I	32	No Cq	No Cq	25.38
				II	32	38.32	No Cq	26.26
			3	I	32	No Cq	No Cq	25.60
				II	32	No Cq	No Cq	26.66
			4	I	32	33.76	34.26	25.30

Lab number	Date test	Seed lot designation	Sub sample	PCR replicate	Suggested Cq cut-off	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
				II	32	33.99	36.13	26.43
			5	I	32	No Cq	No Cq	25.91
				II	32	No Cq	No Cq	27.05
Lab CT organizer (After)	16 Jul 2024	PPC	1	I	32	15.44	16.13	27.93
				II	32	14.82	15.09	26.15
Lab CT organizer (After)	16 Jul 2024	NPC	1	I	32	No Cq	38.59	27.23
				II	32	No Cq	37.85	26.24
Lab CT organizer (After)	16 Jul 2024	PAC (Provided by CT organizer)		I	32	29.30	30.74	No Cq
				II	32	ND	ND	ND
Lab CT organizer (After)	16 Jul 2024	PAC (Optional: provided by CT participant)		I	32	ND	ND	ND
				II	32	ND	ND	ND
Lab CT organizer (After)	16 Jul 2024	NTC		I	32	No Cq	No Cq	No Cq
				II	32	ND	ND	ND
Lab 1	5 Jun 2024	Low	1	I	30	25.95	26.91	26.02
				II	30	ND	ND	ND
			2	I	30	25.29	27.21	26.08
				II	30	ND	ND	ND
			3	I	30	ND	ND	ND
				II	30	ND	ND	ND
			4	I	30	25.51	27.02	26.07
				II	30	ND	ND	ND
			5	I	30	25.60	27.09	24.96
				II	30	ND	ND	ND
Lab 1	5 Jun 2024	Healthy I	1	I	30	No Cq	No Cq	26.09
				II	30	ND	ND	ND
			2	I	30	No Cq	No Cq	25.94
				II	30	ND	ND	ND
			3	I	30	No Cq	No Cq	25.42
				II	30	ND	ND	ND
			4	I	30	No Cq	No Cq	26.09
				II	30	ND	ND	ND
			5	I	30	No Cq	No Cq	25.73
				II	30	ND	ND	ND
Lab 1	5 Jun 2024	Healthy II	1	I	30	No Cq	No Cq	26.22
				II	30	ND	ND	ND

Lab number	Date test	Seed lot designation	Sub sample	PCR replicate	Suggested Cq cut-off	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
			2	I	30	No Cq	No Cq	25.49
				II	30	ND	ND	ND
			3	I	30	No Cq	No Cq	26.17
				II	30	ND	ND	ND
			4	I	30	No Cq	No Cq	26.08
				II	30	ND	ND	ND
			5	I	30	No Cq	No Cq	25.95
				II	30	ND	ND	ND
Lab 1	5 Jun 2024	PPC	1	I	30	13.93	13.15	26.71
				II	30	ND	ND	ND
Lab 1	5 Jun 2024	NPC	1	I	30	37.44	No Cq	27.49
				II	30	ND	ND	ND
Lab 1	5 Jun 2024	PAC (Provided by CT organizer)		I	30	24.06	25.58	No Cq
				II	30	ND	ND	ND
Lab 1	5 Jun 2024	PAC (Optional: provided by CT participant)		I	30	ND	ND	ND
				II	30	ND	ND	ND
Lab 1	5 Jun 2024	NTC		I	30	No Cq	No Cq	No Cq
				II	30	ND	ND	ND
Lab 2	13 Jun 2024	Low	1	I	30	28.46	29.32	23.42
				II	30	28.38	29.08	23.61
			2	I	30	27.06	27.54	24.84
				II	30	27.22	27.49	24.95
			3	I	30	29.26	30.04	23.49
				II	30	29.19	29.84	23.76
			4	I	30	28.19	28.54	23.70
				II	30	28.30	29.02	23.93
			5	I	30	26.75	27.48	23.95
				II	30	26.74	27.38	24.08
Lab 2	13 Jun 2024	Healthy I	1	I	30	No Cq	No Cq	23.62
				II	30	No Cq	No Cq	23.59
			2	I	30	No Cq	No Cq	23.51
				II	30	No Cq	No Cq	23.62
			3	I	30	No Cq	No Cq	23.40
				II	30	No Cq	No Cq	23.87
			4	I	30	No Cq	No Cq	23.59
				II	30	No Cq	No Cq	23.20
			5	I	30	No Cq	No Cq	23.73

Lab number	Date test	Seed lot designation	Sub sample	PCR replicate	Suggested Cq cut-off	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
				II	30	No Cq	No Cq	23.09
Lab 2	13 Jun 2024	Healthy II	1	I	30	No Cq	No Cq	24.60
				II	30	No Cq	No Cq	24.24
			2	I	30	No Cq	No Cq	23.06
				II	30	No Cq	No Cq	22.71
			3	I	30	No Cq	No Cq	22.25
				II	30	No Cq	No Cq	22.85
			4	I	30	No Cq	No Cq	22.69
				II	30	No Cq	No Cq	22.87
			5	I	30	No Cq	No Cq	22.50
				II	30	No Cq	No Cq	23.02
Lab 2	13 Jun 2024	PPC	1	I	30	14.45	13.37	22.53
				II	30	14.69	13.80	23.82
Lab 2	13 Jun 2024	NPC	1	I	30	No Cq	No Cq	24.20
				II	30	No Cq	No Cq	24.40
Lab 2	13 Jun 2024	PAC (Provided by CT organizer)		I	30	26.26	26.27	No Cq
				II	30	26.35	26.37	No Cq
Lab 2	13 Jun 2024	PAC (Optional: provided by CT participant)		I	30	30.05	28.65	No Cq
				II	30	29.74	28.67	No Cq
Lab 2	13 Jun 2024	NTC		I	30	No Cq	No Cq	No Cq
				II	30	No Cq	No Cq	No Cq
Lab 2	13 Jun 2024	PAC DLVd (Provided by CT organizer)		I	30	No Cq	No Cq	29.43
				II	30	No Cq	No Cq	28.86
Lab 2	13 Jun 2024	PAC DLVd (Provided by CT participant)		I	30	No Cq	No Cq	25.49
				II	30	No Cq	No Cq	25.93
Lab 3	13 Jun 2024	Low	1	I	32	30.26	32.17	26.37
				II	32	30.07	32.06	26.14
			2	I	32	29.53	31.75	26.33
				II	32	29.69	32.08	26.19
			3	I	32	28.40	30.38	26.62
				II	32	27.42	29.39	25.86
			4	I	32	28.38	28.38	25.86
				II	32	28.74	28.87	25.87
			5	I	32	28.76	30.73	25.97
				II	32	28.60	30.6	25.89
Lab 3		Healthy I	1	I	32	No Cq	No Cq	25.87

Lab number	Date test	Seed lot designation	Sub sample	PCR replicate	Suggested Cq cut-off	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
	13 Jun 2024			II	32	No Cq	No Cq	25.73
			2	I	32	No Cq	No Cq	25.85
				II	32	No Cq	No Cq	25.84
			3	I	32	No Cq	No Cq	25.68
				II	32	No Cq	No Cq	25.84
			4	I	32	No Cq	No Cq	26.27
				II	32	No Cq	No Cq	25.80
			5	I	32	No Cq	No Cq	25.69
				II	32	No Cq	No Cq	25.52
Lab 3	13 Jun 2024	Healthy II	1	I	32	No Cq	No Cq	26.16
				II	32	No Cq	No Cq	26.32
			2	I	32	No Cq	No Cq	26.09
				II	32	No Cq	No Cq	26.15
			3	I	32	No Cq	No Cq	25.84
				II	32	No Cq	No Cq	25.90
			4	I	32	No Cq	No Cq	25.60
				II	32	No Cq	No Cq	25.58
			5	I	32	No Cq	No Cq	25.45
				II	32	No Cq	No Cq	25.42
Lab 3	13 Jun 2024	PPC	1	I	32	14.93	14.97	16.87
				II	32	14.81	14.99	16.61
Lab 3	13 Jun 2024	NPC	1	I	32	No Cq	No Cq	26.54
				II	32	No Cq	No Cq	26.56
Lab 3	13 Jun 2024	PAC (Provided by CT organizer)		I	32	27.16	26.83	27.89
				II	32	30.75	29.91	31.16
Lab 3	13 Jun 2024	PAC (Optional: provided by CT participant)		I	32	30.96	29.89	31.14
				II	32	30.95	30.29	31.56
Lab 3	13 Jun 2024	NTC		I	32	No Cq	No Cq	No Cq
				II	32	No Cq	No Cq	No Cq
Lab 4	18 Jun 2024	Low	1	I	37	29.87	32.21	29.81
				II	37	29.06	31.05	28.96
			2	I	37	35.69	37.07	28.71
				II	37	35.94	36.25	28.59
			3	I	37	29.37	31.20	28.56
				II	37	28.72	30.34	28.34
			4	I	37	27.97	28.34	28.67
				II	37	27.84	28.10	28.24

Lab number	Date test	Seed lot designation	Sub sample	PCR replicate	Suggested Cq cut-off	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
			5	I	37	30.98	32.91	28.59
				II	37	31.26	32.57	28.31
Lab 4	18 Jun 2024	Healthy I	1	I	37	No Cq	No Cq	27.47
				II	37	No Cq	No Cq	27.44
			2	I	37	No Cq	No Cq	27.80
				II	37	No Cq	No Cq	27.26
			3	I	37	No Cq	No Cq	27.58
				II	37	No Cq	No Cq	27.66
			4	I	37	No Cq	No Cq	27.86
				II	37	No Cq	No Cq	27.58
			5	I	37	No Cq	No Cq	27.78
				II	37	No Cq	No Cq	27.31
Lab 4	18 Jun 2024	Healthy II	1	I	37	No Cq	No Cq	29.76
				II	37	No Cq	37.35	29.41
			2	I	37	No Cq	No Cq	28.62
				II	37	No Cq	No Cq	27.98
			3	I	37	No Cq	No Cq	28.85
				II	37	No Cq	No Cq	29.00
			4	I	37	No Cq	No Cq	29.10
				II	37	No Cq	No Cq	29.07
			5	I	37	No Cq	No Cq	29.32
				II	37	No Cq	No Cq	28.70
Lab 4	18 Jun 2024	PPC	1	I	37	16.38	16.46	38.48
				II	37	16.29	16.54	36.50
Lab 4	18 Jun 2024	NPC	1	I	37	No Cq	No Cq	27.36
				II	37	No Cq	No Cq	27.00
Lab 4	18 Jun 2024	PAC (Provided by CT organizer)		I	37	25.81	27.25	No Cq
				II	37	25.66	27.09	No Cq
Lab 4	18 Jun 2024	PAC (Optional: provided by CT participant)		I	37	12.74	12.72	No Cq
				II	37	12.89	12.74	No Cq
Lab 4	18 Jun 2024	NTC		I	37	No Cq	No Cq	No Cq
				II	37	No Cq	No Cq	No Cq
Lab 5	17 Jun 2024	Low	1	I	35	28.58	29.94	31.59
				II	35	28.71	30.15	31.80
			2	I	35	30.43	32.53	31.57
				II	35	30.52	32.26	31.55
			3	I	35	31.38	32.58	31.79

Lab number	Date test	Seed lot designation	Sub sample	PCR replicate	Suggested Cq cut-off	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
				II	35	30.99	33.41	32.05
			4	I	35	28.10	29.46	31.39
				II	35	27.98	29.40	31.14
			5	I	35	29.76	31.23	31.18
				II	35	29.53	31.08	30.87
Lab 5	17 Jun 2024	Healthy I	1	I	35	No Cq	No Cq	31.57
				II	35	No Cq	No Cq	31.22
			2	I	35	No Cq	No Cq	31.16
				II	35	No Cq	No Cq	30.79
			3	I	35	No Cq	No Cq	30.71
				II	35	39.59	No Cq	30.76
			4	I	35	No Cq	No Cq	31.24
				II	35	No Cq	No Cq	31.31
			5	I	35	No Cq	No Cq	31.22
				II	35	No Cq	No Cq	30.77
Lab 5	17 Jun 2024	Healthy II	1	I	35	No Cq	No Cq	31.98
				II	35	No Cq	No Cq	32.16
			2	I	35	No Cq	No Cq	31.89
				II	35	No Cq	No Cq	30.77
			3	I	35	No Cq	No Cq	31.44
				II	35	No Cq	No Cq	30.64
			4	I	35	No Cq	No Cq	32.68
				II	35	No Cq	No Cq	31.61
			5	I	35	No Cq	No Cq	32.39
				II	35	No Cq	No Cq	32.51
Lab 5	17 Jun 2024	PPC	1	I	35	14.90	15.15	33.05
				II	35	15.02	15.25	33.27
Lab 5	17 Jun 2024	NPC	1	I	35	No Cq	No Cq	33.73
				II	35	No Cq	No Cq	34.17
Lab 5	17 Jun 2024	PAC (Provided by CT organizer)		I	35	26.30	27.91	No Cq
				II	35	26.15	27.76	No Cq
Lab 5	17 Jun 2024	PAC (Optional: provided by CT participant)		I	35	ND	ND	ND
				II	35	ND	ND	ND
Lab 5	17 Jun 2024	NTC		I	35	No Cq	No Cq	No Cq
				II	35	No Cq	No Cq	No Cq
Lab 6	11 Jun 2024	Low	1	I	37	30.11	31.37	29.15
				II	37	30.04	31.54	29.23

Lab number	Date test	Seed lot designation	Sub sample	PCR replicate	Suggested Cq cut-off	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
			2	I	37	33.50	36.13	31.24
				II	37	31.97	34.90	30.14
			3	I	37	29.59	30.59	28.78
				II	37	29.45	30.46	28.84
			4	I	37	No Cq	No Cq	33.30
				II	37	No Cq	No Cq	No Cq
			5	I	37	29.39	31.99	31.09
				II	37	30.91	33.26	31.92
Lab 6	11 Jun 2024	Healthy I	1	I	37	No Cq	No Cq	28.89
				II	37	No Cq	No Cq	29.60
			2	I	37	38.78	37.70	27.75
				II	37	38.77	No Cq	27.70
			3	I	37	No Cq	No Cq	29.58
				II	37	No Cq	No Cq	28.16
			4	I	37	No Cq	No Cq	29.49
				II	37	No Cq	No Cq	28.83
			5	I	37	No Cq	No Cq	28.39
				II	37	No Cq	No Cq	28.16
Lab 6	11 Jun 2024	Healthy II	1	I	37	No Cq	No Cq	33.61
				II	37	No Cq	No Cq	No Cq
			2	I	37	No Cq	No Cq	30.16
				II	37	No Cq	No Cq	29.04
			3	I	37	No Cq	No Cq	30.30
				II	37	No Cq	No Cq	29.83
			4	I	37	No Cq	No Cq	31.65
				II	37	No Cq	No Cq	30.19
			5	I	37	No Cq	No Cq	No Cq
				II	37	No Cq	No Cq	No Cq
Lab 6	11 Jun 2024	PPC	1	I	37	16.02	18.58	30.43
				II	37	17.86	20.68	33.51
Lab 6	11 Jun 2024	NPC	1	I	37	No Cq	No Cq	31.09
				II	37	No Cq	No Cq	31.31
Lab 6	11 Jun 2024	PAC (Provided by CT organizer)		I	37	27.54	27.52	No Cq
				II	37	27.39	27.50	No Cq
Lab 6	11 Jun 2024	PAC (Optional: provided by CT participant)		I	37	ND	ND	ND
				II	37	ND	ND	ND
Lab 6		NTC		I	37	No Cq	No Cq	31.91

Lab number	Date test	Seed lot designation	Sub sample	PCR replicate	Suggested Cq cut-off	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
	11 Jun 2024			II	37	No Cq	No Cq	30.53

Annex H. Data from reproducibility assessment

The data submission file is provided below as MS Excel file.

LMV SE-qPCR (Pre-)CT data_sheet_

Table H.1. Obtained Cq values from the two LMV targeted primer and probe sets (LMV-NAKT and LMV-RZ2) and DLVd that is used as an IAC, for 10 subsamples of 1,000 seeds for seven seed lots for evaluation of the LMV infection homogeneity as part of the preparation for the LMV SE-qPCR (Pre-)CT. All samples tested were in the 96-well format.

Plate	Sample	Sub	PCR replicate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
I	SE 28	1	I	14.88	14.54	27.32
		2	I	14.27	13.90	26.93
		3	I	14.90	14.51	27.56
		4	I	14.82	14.49	27.51
		5	I	15.03	14.71	27.12
		6	I	14.61	14.27	26.78
		7	I	14.50	14.22	27.21
		8	I	15.16	14.70	27.21
		9	I	14.79	14.49	27.38
		10	I	14.62	14.16	26.93
I	SE 29	1	I	16.77	16.42	26.58
		2	I	16.49	16.15	26.89
		3	I	16.68	16.35	26.71
		4	I	16.74	16.48	27.07
		5	I	16.60	16.27	27.14
		6	I	16.55	16.29	26.80
		7	I	17.24	16.97	26.19
		8	I	17.11	16.76	26.48
		9	I	16.89	16.64	27.16
		10	I	16.66	16.48	27.00
I	SE 33	1	I	27.57	28.55	27.53
		2	I	27.34	28.47	27.86
		3	I	25.91	27.00	27.74
		4	I	28.33	29.60	28.11
		5	I	30.29	31.20	27.92
		6	I	28.10	29.14	28.00
		7	I	24.69	25.53	27.26
		8	I	25.06	25.97	27.46

Plate	Sample	Sub	PCR replicate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
		9	I	26.26	27.55	27.60
		10	I	26.14	26.86	27.34
I	SE 35	1	I	19.85	19.80	26.84
		2	I	18.79	18.50	26.36
		3	I	18.31	18.23	27.11
		4	I	19.09	19.19	26.74
		5	I	17.17	16.81	27.21
		6	I	18.08	17.97	27.06
		7	I	18.33	18.18	27.00
		8	I	18.87	18.92	27.39
		9	I	18.12	17.81	27.10
		10	I	17.68	17.47	27.24
II	SE 35	11	I	20.40	20.98	29.89
II	SE 35 (100 seeds) + 36 (900 seeds)	1	I	21.65	22.11	29.54
		2	I	21.70	22.24	29.20
		3	I	25.10	25.58	30.00
		4	I	25.75	26.63	29.79
		5	I	21.70	22.10	29.13
		6	I	25.07	26.58	29.12
		7	I	26.08	26.51	29.34
		8	I	26.88	28.48	29.60
		9	I	21.06	21.40	28.96
		10	I	26.59	28.18	30.09
I	SE 36	1	I	No Cq	37.73	26.90
		2	I	No Cq	No Cq	26.56
		3	I	38.20	No Cq	26.64
		4	I	38.30	No Cq	26.64
		5	I	36.07	38.42	26.97
		6	I	38.47	38.83	26.47
		7	I	29.41	30.44	27.13
		8	I	38.42	37.54	26.81
		9	I	33.91	34.87	27.11
		10	I	No Cq	No Cq	26.72
II	SE 36	11	I	38.25	No Cq	30.53
IV	SE 37	1	I	No Cq	No Cq	26.58
		2	I	No Cq	No Cq	26.41
		3	I	No Cq	No Cq	26.51

Plate	Sample	Sub	PCR replicate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
		4	I	No Cq	No Cq	26.77
		5	I	No Cq	No Cq	26.43
		6	I	No Cq	No Cq	26.47
		7	I	No Cq	No Cq	26.48
		8	I	No Cq	No Cq	26.82
		9	I	No Cq	No Cq	26.96
		10	I	No Cq	No Cq	26.90
I	LMV NAKT + LMV RZ 2 Synth (PAC)[a]		I	26.46	27.55	No Cq
II	LMV NAKT + LMV RZ 2 Synth (PAC)[a]		I	26.85	28.04	No Cq
IV	LMV NAKT + LMV RZ 2 Synth (PAC)[a]		I	29.85	30.62	No Cq
I	LMV NAKT + LMV RZ 2 Synth (PAC)[a]		II	26.53	27.57	No Cq
IV	LMV NAKT + LMV RZ 2 Synth (PAC)[a]		II	29.74	30.56	No Cq
I	LMV positive SE (PPC used in RZ routine testing)	V	I	15.75	15.57	26.36
II	LMV positive SE (PPC used in RZ routine testing)	VI	I	14.94	15.14	29.87
IV	LMV positive SE (PPC used in RZ routine testing)	VIII	I	16.27	15.57	25.61
I	LMV negative SE (NPC used in RZ routine testing)	V	I	37.04	36.92	26.33
II	LMV negative SE (NPC used in RZ routine testing)	VI	I	No Cq	37.33	29.88
IV	LMV negative SE (NPC used in RZ routine testing)	VIII	I	39.15	No Cq	26.51
I	PCR grade water (NTC)		I	No Cq	No Cq	No Cq
II	PCR grade water (NTC)		I	No Cq	No Cq	No Cq
IV	PCR grade water (NTC)		I	No Cq	No Cq	No Cq
I	PCR grade water (NTC)		II	No Cq	No Cq	No Cq
II	PCR grade water (NTC)		II	No Cq	No Cq	No Cq
IV	PCR grade water (NTC)		II	No Cq	No Cq	No Cq

Plate	Sample	Sub	PCR replicate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
I	PCR grade water (NTC)		III	No Cq	No Cq	No Cq
I	PCR grade water (NTC)		IV	No Cq	No Cq	No Cq
I	PCR grade water (NTC)		V	No Cq	No Cq	No Cq
I	PCR grade water (NTC)		VI	No Cq	No Cq	No Cq

[a] A mixture of synthetic ssDNA with a concentration of 6×10^3 copies per µL.

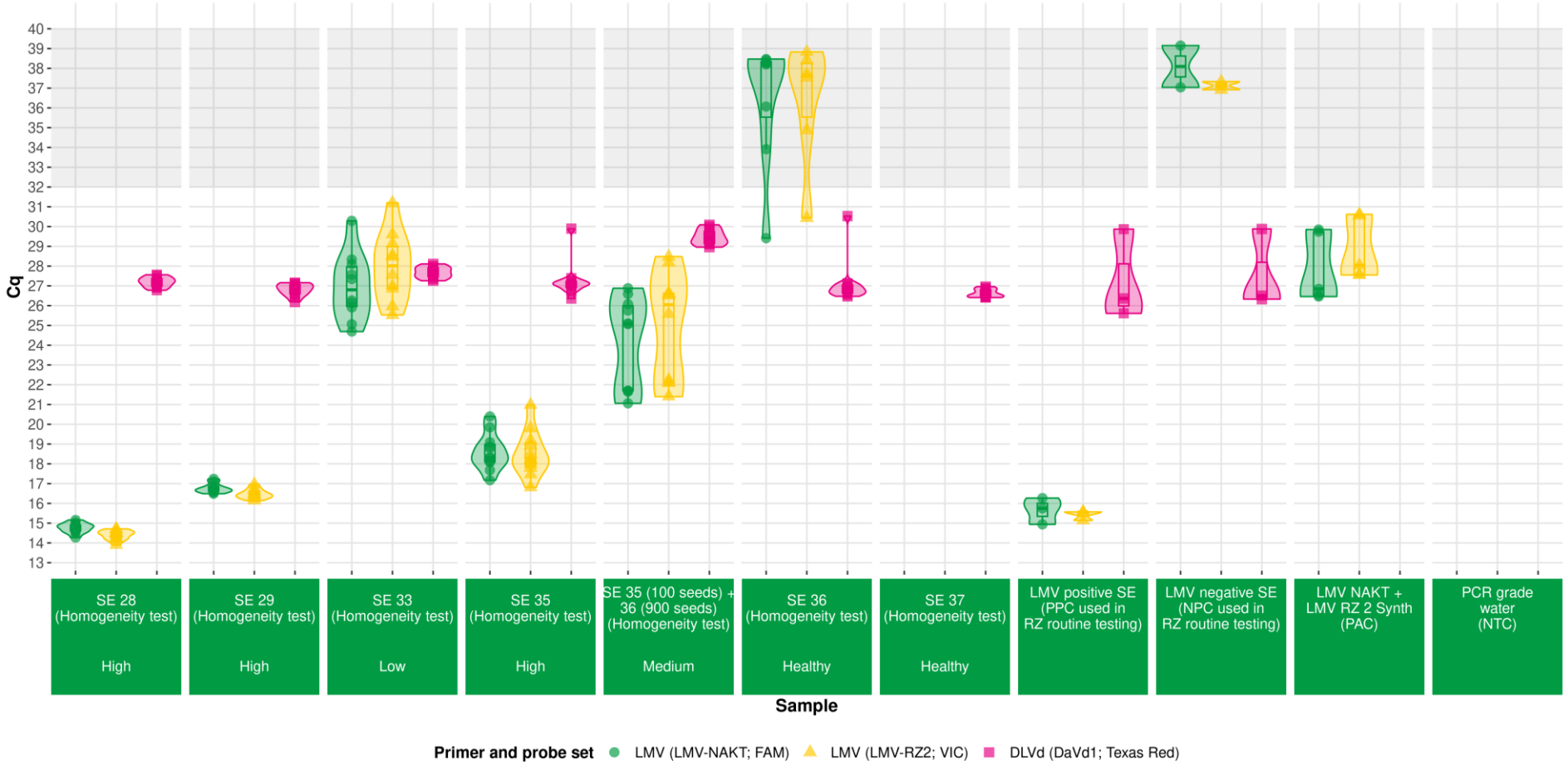

Figure H.1. Obtained Cq values in combined boxplot and violin plot (kernel density distributions) from the 2 LMV targeted primer and probe sets (LMV-NAKT and LMV-RZ2) and DLVd that is used as an IC, for 10 subsamples of 1,000 seeds from seven seed lots (as well as controls) for evaluation of the LMV infection homogeneity as part of preparation for the LMV SE-qPCR (Pre-)CT. The appraisal of the seed lot as homogenous "High", "Medium", and "Low" LMV infection as well as "healthy" is indicated below the seed lot name. The grey background represents the range for which the SE-qPCRs are considered negative (Cq 32-40).

Table H.2. Obtained Cq values from the two LMV targeted primer and probe sets (LMV-NAKT and LMV-RZ2) and DLVd that is used as an IAC, for subsamples from high and medium LMV infected seed lots as well healthy lettuce seed lots performed by different laboratories as part of the CT. ND: Not determined; Cq values < 32 for the LMV-NAKT and LMV-RZ2 primer and probe sets are highlighted in pink and unexpected high Cq values for the DLVd targeted primer and probe set are highlighted in orange. The RNA extraction as performed by the CT organizer for separate sample set using the Qiagen RNeasy Plant Mini Kit is indicated with "QG" and the in-house method of the CT organizer using GH+ as extraction buffer followed by RNA extraction with the Sbeadex plant maxi kit (LGC Genomics) in combination with the KingFisher platform (ThermoFisher scientific) is indicated with "KF".

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
Lab CT organizer (Before) QG	17 Oct 2024	High	4	I	16.09	16.17	25.11
				II	17.00	16.78	25.17
			8	I	16.11	16.49	25.79
				II	16.13	16.52	25.94
			9	I	18.84	19.50	26.05
				II	18.84	19.44	26.05
			14	I	21.24	21.63	27.07
				II	21.22	21.60	26.98
			15	I	22.21	22.94	27.78
				II	22.15	22.92	27.81
			16	I	25.76	26.77	29.01
				II	25.52	26.46	28.60
Lab CT organizer (Before) QG	17 Oct 2024	Medium	1	I	23.64	24.35	25.41
				II	23.48	24.36	25.73
			2	I	26.54	28.28	25.86
				II	26.51	28.30	26.15
			3	I	28.64	30.87	25.30
				II	28.72	30.71	25.21
			7	I	28.74	30.67	25.82
				II	28.67	30.66	25.79
			11	I	31.05	31.63	28.30
				II	30.90	31.55	28.26
Lab CT organizer (Before) QG	18 Oct 2024	Medium	18	I	26.06	27.90	26.22
				II	25.99	27.85	26.06
			19	I	24.93	26.68	24.58
				II	24.87	26.66	24.56
			20	I	19.40	20.33	26.03
				II	19.43	20.29	25.79
Lab CT organizer (Before) QG	17 Oct 2024	Healthy	5	I	34.69	36.00	25.25
				II	34.68	35.33	25.77

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
			6	I	33.57	34.56	25.15
				II	33.12	33.87	25.51
			10	I	38.11	39.51	26.29
				II	38.07	38.47	26.07
			12	I	36.73	39.11	26.13
				II	39.05	39.11	26.06
			13	I	34.29	35.85	26.75
				II	34.61	35.47	26.81
			17	I	37.75	No Cq	30.37
				II	No Cq	39.66	30.10
Lab CT organizer (Before) QG	18 Oct 2024		PPC	I	14.00	14.70	26.39
				II	14.14	14.85	26.53
Lab CT organizer (Before) QG	17 Oct 2024		NPC	I	No Cq	34.51	25.55
				II	No Cq	34.28	25.53
Lab CT organizer (Before) QG	17 Oct 2024		PAC (Provided by CT organizer)	I	25.61	27.48	No Cq
				II	25.68	27.45	No Cq
Lab CT organizer (Before) QG	17 Oct 2024		PAC (Optional: provided by CT participant)	I	ND	ND	ND
				II	ND	ND	ND
Lab CT organizer (Before) QG	17 Oct 2024		NTC	I	No Cq	33.93	No Cq
				II	No Cq	34.49	No Cq
Lab CT organizer (Before) KF	16 Oct 2024	High	4	I	19.67	19.64	30.81
				II	19.81	19.85	31.25
			8	I	18.14	18.21	30.13
				II	18.16	18.26	30.57
			9	I	19.44	19.60	30.97
				II	19.41	19.54	30.99
			14	I	20.25	20.39	30.21
				II	20.24	20.42	30.48
			15	I	19.28	19.46	30.48
				II	19.20	19.36	30.35
			16	I	20.70	20.92	30.67
				II	20.51	20.65	30.14
Lab CT organizer (Before) KF	16 Oct 2024	Medium	1	I	22.53	22.73	30.6
				II	22.60	22.85	30.35
			2	I	27.34	28.91	30.39
				II	27.34	28.91	30.11
			3	I	28.79	30.99	30.57

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
				II	28.90	30.68	30.63
			7	I	23.01	23.08	30.56
				II	23.02	23.19	31.10
			11	I	24.08	24.47	30.47
				II	23.84	24.33	30.30
			18	I	28.28	30.09	30.34
				II	28.18	30.09	30.60
			19	I	28.54	30.22	30.69
				II	28.57	30.42	30.98
			20	I	29.07	30.69	30.76
				II	29.22	31.08	31.09
Lab CT organizer (Before) KF	16 Oct 2024	Healthy	5	I	No Cq	38.49	30.78
				II	No Cq	37.83	31.49
			6	I	No Cq	No Cq	30.59
				II	No Cq	No Cq	30.35
			10	I	No Cq	No Cq	31.08
				II	No Cq	No Cq	30.51
			12	I	No Cq	39.19	31.04
				II	No Cq	No Cq	31.04
			13	I	No Cq	No Cq	30.50
				II	No Cq	No Cq	30.47
			17	I	No Cq	No Cq	30.59
				II	No Cq	38.53	30.49
Lab CT organizer (Before) KF	16 Oct 2024		PPC	I	20.45	21.68	34.57
				II	20.19	20.43	31.03
Lab CT organizer (Before) KF	16 Oct 2024		NPC	I	No Cq	39.71	31.17
				II	No Cq	No Cq	31.61
Lab CT organizer (Before) KF	16 Oct 2024		PAC (Provided by CT organizer)	I	25.34	27.39	No Cq
				II	25.22	27.59	No Cq
Lab CT organizer (Before) KF	16 Oct 2024		PAC (Optional: provided by CT participant)	I	25.65	27.20	No Cq
				II	25.62	27.20	No Cq
Lab CT organizer (Before) KF	16 Oct 2024		NTC	I	No Cq	No Cq	No Cq
				II	No Cq	No Cq	No Cq
Lab CT organizer (After EU) QG	20 Dec 2024	High	4	I	14.98	15.24	27.13
				II	14.98	15.11	26.42
			8	I	15.03	15.17	26.24
				II	14.82	15.18	26.44

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
			9	I	16.93	17.12	26.39
				II	16.81	17.12	26.82
			14	I	16.93	17.34	27.09
				II	16.86	17.23	26.97
			15	I	14.82	15.02	26.47
				II	14.75	14.97	26.57
			16	I	16.81	17.09	26.66
				II	16.82	17.01	26.33
Lab CT organizer (After EU) QG	20 Dec 2024	Medium	1	I	20.49	20.71	26.03
				II	20.28	20.92	26.59
			2	I	27.76	29.86	26.63
				II	27.64	29.96	27.02
			3	I	27.04	29.42	26.20
				II	27.15	29.58	26.74
			7	I	27.25	30.05	26.62
				II	27.35	30.07	26.81
			11	I	20.84	21.13	26.14
				II	20.75	21.11	26.31
			18	I	25.46	27.59	26.82
				II	25.47	27.52	27.04
			19	I	26.81	29.28	26.4
				II	26.65	29.50	27.08
			20	I	28.45	31.44	26.36
				II	28.39	31.43	26.83
Lab CT organizer (After EU) QG	20 Dec 2024	Healthy	5	I	34.09	34.54	26.47
				II	34.68	35.73	26.80
			6	I	No Cq	No Cq	26.79
				II	No Cq	No Cq	27.17
			10	I	37.36	37.12	26.58
				II	No Cq	No Cq	26.89
			12	I	No Cq	No Cq	26.43
				II	No Cq	37.85	26.56
			13	I	34.97	36.60	26.34
				II	35.67	36.51	26.83
			17	I	36.21	37.59	26.90
				II	37.36	No Cq	26.63
			PPC	I	15.18	15.44	27.61

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
Lab CT organizer (After EU) QG	20 Dec 2024			II	15.09	15.32	27.72
Lab CT organizer (After EU) QG	20 Dec 2024		NPC	I	No Cq	No Cq	26.81
				II	No Cq	39.24	26.88
Lab CT organizer (After EU) QG	20 Dec 2024		PAC (Provided by CT organizer)	I	33.08	34.14	No Cq
				II	32.91	34.63	No Cq
Lab CT organizer (After EU) QG	20 Dec 2024		PAC (Optional: provided by CT participant)	I	25.73	25.16	No Cq
				II	25.60	25.16	No Cq
Lab CT organizer (After EU) QG	20 Dec 2024		NTC	I	No Cq	No Cq	No Cq
				II	No Cq	No Cq	No Cq
Lab CT organizer (After non-EU) QG	27 Feb 2025	High	4	I	15.44	15.97	26.61
				II	15.46	16.04	26.89
			8	I	14.96	15.28	27.38
				II	14.75	14.84	26.21
			9	I	17.23	18.36	27.94
				II	17.81	18.09	26.09
			14	I	17.15	18.59	28.95
				II	17.77	18.07	26.77
			15	I	15.28	16.84	29.14
				II	15.93	16.14	26.85
			16	I	16.72	18.29	28.66
				II	16.98	17.48	26.64
Lab CT organizer (After non-EU) QG	27 Feb 2025	Medium	1	I	28.49	31.24	27.11
				II	28.41	31.13	26.54
			2	I	20.78	21.26	26.96
				II	20.60	21.15	26.86
			3	I	21.32	21.88	26.31
				II	21.41	21.95	26.23
			7	I	27.93	31.01	26.94
				II	ND	ND	ND
			11	I	19.25	20.33	27.50
				II	19.99	20.14	26.28
			18	I	27.48	30.19	28.03
				II	27.34	30.28	28.08
			19	I	27.57	30.23	27.15
				II	27.44	30.41	26.96
			20	I	26.43	27.12	27.85

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
				II	25.31	26.37	27.62
Lab CT organizer (After non-EU) QG	27 Feb 2025	Healthy	5	I	36.67	37.43	27.10
				II	35.79	36.11	26.63
			6	I	38.20	No Cq	27.03
				II	No Cq	39.25	26.89
			10	I	No Cq	No Cq	28.82
				II	No Cq	38.98	26.59
			12	I	38.24	39.39	27.50
				II	No Cq	36.94	26.46
			13	I	No Cq	No Cq	28.62
				II	No Cq	38.77	27.16
			17	I	37.17	38.36	27.04
				II	35.86	37.79	27.03
Lab CT organizer (After non-EU) QG	27 Feb 2025		PPC	I	16.01	16.31	26.75
				II	15.85	16.17	26.25
Lab CT organizer (After non-EU) QG	27 Feb 2025		NPC	I	No Cq	No Cq	27.14
				II	No Cq	No Cq	26.97
Lab CT organizer (After non-EU) QG	27 Feb 2025		PAC (Provided by CT organizer; Storage RT I)	I	32.01	33.53	No Cq
				II	31.61	33.87	No Cq
Lab CT organizer (After non-EU) QG	27 Feb 2025		PAC (Provided by CT organizer; Storage RT II)	I	30.80	33.36	No Cq
				II	31.07	33.57	No Cq
Lab CT organizer (After non-EU) QG	27 Feb 2025		PAC (Provided by CT organizer; Storage -80C)	I	25.46	27.39	No Cq
				II	25.15	26.74	No Cq
Lab CT organizer (After non-EU) QG	27 Feb 2025		PAC (Optional: provided by CT participant)	I	27.16	26.64	No Cq
				II	26.65	26.05	No Cq
Lab CT organizer (After non-EU) QG	27 Feb 2025		NTC	I	No Cq	No Cq	No Cq
				II	No Cq	No Cq	No Cq
Lab CT organizer (After EU) KF	20 Dec 2024	High	4	I	19.52	19.47	30.75
				II	19.43	19.36	30.92
			8	I	18.30	18.24	30.56
				II	18.19	18.12	30.45
			9	I	19.66	19.67	30.96
				II	19.81	19.91	31.03
			14	I	21.03	20.99	31.68
				II	21.29	21.16	31.77

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
			15	I	19.16	18.98	31.08
				II	18.99	18.77	31.3
			16	I	20.36	20.30	31.07
				II	20.51	20.37	30.73
Lab CT organizer (After EU) KF	20 Dec 2024	Medium	1	I	23.37	23.32	30.05
				II	23.13	23.24	30.39
			2	I	28.06	28.76	30.42
				II	28.24	28.84	30.78
			3	I	27.39	28.83	30.79
				II	26.59	28.15	30.95
			7	I	28.02	29.41	30.50
				II	27.93	29.27	30.34
			11	I	25.46	25.78	31.32
				II	25.37	25.81	31.06
			18	I	25.05	25.14	31.06
				II	25.09	25.19	30.62
			19	I	25.77	26.19	31.04
				II	25.75	26.45	31.13
			20	I	28.71	29.93	30.89
				II	28.60	30.00	31.03
Lab CT organizer (After EU) KF	20 Dec 2024	Healthy	5	I	No Cq	No Cq	30.45
				II	No Cq	No Cq	30.62
			6	I	No Cq	No Cq	31.30
				II	No Cq	No Cq	30.56
			10	I	35.88	36.21	31.13
				II	35.79	35.73	31.62
			12	I	No Cq	No Cq	31.08
				II	No Cq	No Cq	31.17
			13	I	No Cq	No Cq	31.21
				II	No Cq	No Cq	30.82
			17	I	No Cq	No Cq	30.57
				II	No Cq	No Cq	30.62
Lab CT organizer (After EU) KF	20 Dec 2024		PPC	I	18.30	18.83	31.33
				II	18.20	18.84	31.06
Lab CT organizer (After EU) KF	20 Dec 2024		NPC	I	No Cq	No Cq	31.34
				II	No Cq	No Cq	31.97
				I	33.44	34.42	No Cq

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
Lab CT organizer (After EU) KF	20 Dec 2024		PAC (Provided by CT organizer)	II	33.58	34.56	No Cq
Lab CT organizer (After EU) KF	20 Dec 2024		PAC (Optional: provided by CT participant)	I	25.98	25.30	No Cq
				II	25.95	25.21	No Cq
Lab CT organizer (After EU) KF	20 Dec 2024		NTC	I	No Cq	No Cq	No Cq
				II	No Cq	No Cq	No Cq
Lab 1	21 Nov 2024	High	4	I	12.63	13.91	29.07
				II	14.06	14.37	29.14
			8	I	14.62	15.04	29.15
				II	13.18	14.72	29.06
			9	I	15.81	16.38	28.32
				II	16.12	16.86	28.32
			14	I	15.88	16.32	28.47
				II	15.77	16.33	28.46
			15	I	14.25	14.79	30.98
				II	14.05	14.36	31.49
			16	I	15.47	16.04	28.76
				II	15.90	16.17	28.55
Lab 1	27 Nov 2024	Medium	1	I	23.42	24.34	27.46
				II	23.55	24.24	27.45
			2	I	26.75	29.99	28.27
				II	26.66	30.04	28.09
			3	I	26.09	27.93	27.74
				II	25.71	27.44	27.62
			7	I	25.11	26.01	28.51
				II	24.71	25.54	28.36
			11	I	18.58	19.17	29.64
				II	18.90	19.41	29.76
			18	I	28.61	32.06	28.96
				II	28.25	32.11	28.91
			19	I	27.18	28.52	28.38
				II	27.14	28.76	28.51
			20	I	26.43	27.68	28.74
				II	26.93	28.03	28.86
Lab 1	21 Nov 2024	Healthy	5	I	No Cq	No Cq	29.34
				II	No Cq	No Cq	29.62
			6	I	34.29	36.31	29.18

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
				II	35.83	34.42	29.29
			10	I	No Cq	No Cq	29.29
				II	No Cq	No Cq	29.26
			12	I	No Cq	No Cq	29.44
				II	No Cq	No Cq	29.38
			13	I	No Cq	No Cq	29.45
				II	No Cq	No Cq	29.63
			17	I	No Cq	No Cq	29.49
				II	No Cq	No Cq	29.26
Lab 1	21 Nov 2024		PPC	I	14.66	15.15	28.52
				II	14.33	15.03	28.85
Lab 1	21 Nov 2024		NPC	I	No Cq	No Cq	28.92
				II	No Cq	No Cq	28.86
Lab 1	21 Nov 2024		PAC (Provided by CT organizer)	I	25.10	25.28	No Cq
				II	25.10	25.46	No Cq
Lab 1	21 Nov 2024		PAC (Optional: provided by CT participant)	I	ND	ND	ND
				II	ND	ND	ND
Lab 1	21 Nov 2024		NTC	I	No Cq	No Cq	No Cq
				II	No Cq	No Cq	No Cq
Lab 2	20 Nov 2024	High	4	I	13.91	12.99	25.02
				II	14.00	12.98	25.26
			8	I	14.17	13.17	24.36
				II	14.21	13.24	24.40
			9	I	15.92	14.89	24.35
				II	15.96	15.03	24.34
			14	I	16.37	15.65	24.22
				II	16.25	15.52	24.18
			15	I	13.99	13.13	25.36
				II	14.09	13.21	25.55
			16	I	16.69	15.78	23.01
				II	16.71	15.67	23.31
Lab 2	20 Nov 2024	Medium	1	I	27.65	28.20	23.98
				II	27.78	28.31	24.13
			2	I	27.63	28.39	24.12
				II	27.59	28.36	24.41
			3	I	27.84	29.48	24.86
				II	27.83	29.32	24.97

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
			7	I	26.59	27.43	25.10
				II	26.69	27.44	25.19
			11	I	27.00	27.91	26.07
				II	27.05	27.78	26.21
			18	I	26.31	26.10	25.51
				II	26.28	25.91	25.46
			19	I	22.11	21.19	24.49
				II	22.18	21.22	24.37
			20	I	19.33	18.23	23.91
				II	19.58	18.80	24.65
Lab 2	20 Nov 2024	Healthy	5	I	34.15	33.51	26.65
				II	33.31	33.66	26.63
			6	I	No Cq	No Cq	26.69
				II	No Cq	No Cq	26.66
			10	I	34.48	36.82	26.17
				II	34.70	35.12	26.25
			12	I	No Cq	No Cq	26.7
				II	No Cq	No Cq	26.89
			13	I	32.86	33.12	26.74
				II	32.62	32.94	27.00
			17	I	34.16	34.62	26.17
				II	34.80	35.55	26.76
Lab 2	20 Nov 2024		PPC	I	15.00	14.11	23.07
				II	15.20	14.29	23.74
Lab 2	20 Nov 2024		NPC	I	No Cq	37.79	23.87
				II	No Cq	No Cq	23.64
Lab 2	20 Nov 2024		PAC (Provided by CT organizer)	I	25.00	24.72	No Cq
				II	25.23	24.95	No Cq
Lab 2	20 Nov 2024		PAC (Optional: provided by CT participant)	I	29.60	28.77	No Cq
				II	29.75	28.94	No Cq
Lab 2	20 Nov 2024		PAC DLVd (Provided by CT organizer)	I	No Cq	No Cq	28.19
				II	No Cq	No Cq	28.46
Lab 2	20 Nov 2024		PAC DLVd (Provided by CT participant)	I	No Cq	No Cq	25.65
				II	No Cq	No Cq	25.82
Lab 2	20 Nov 2024		NTC	I	No Cq	No Cq	No Cq
				II	No Cq	No Cq	No Cq
Lab 3		High	4	I	14.00	13.70	14.90

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
	18 Nov 2024			II	14.20	13.70	14.90
			8	I	13.80	13.30	14.70
				II	13.80	13.30	14.50
			9	I	17.30	16.70	17.70
				II	17.00	16.40	17.60
			14	I	16.30	15.80	17.10
				II	16.20	15.80	17.20
			15	I	14.00	13.50	14.90
				II	13.80	13.20	14.70
			16	I	15.20	14.30	15.60
				II	15.30	14.70	15.90
Lab 3	18 Nov 2024	Medium	1	I	19.20	18.60	19.70
				II	19.20	18.50	19.40
			2	I	26.00	26.70	23.50
				II	26.20	26.80	23.20
			3	I	18.60	18.30	19.40
				II	18.80	18.10	18.90
			7	I	26.80	28.30	22.80
				II	26.70	28.10	22.60
			11	I	23.80	23.20	22.40
				II	28.20	29.00	22.70
			18	I	27.30	28.60	23.50
				II	27.50	28.40	23.10
			19	I	26.50	27.70	23.30
				II	26.90	27.90	23.00
			20	I	26.50	27.90	23.10
				II	27.00	28.00	23.10
Lab 3	18 Nov 2024	Healthy	5	I	39.90	No Cq	22.60
				II	No Cq	No Cq	22.50
			6	I	33.80	34.10	23.20
				II	33.80	34.10	23.00
			10	I	No Cq	38.50	22.70
				II	No Cq	38.40	22.10
			12	I	34.50	33.90	22.90
				II	34.60	34.30	22.80
			13	I	33.60	33.50	22.80
				II	33.70	33.60	22.70

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
			17	I	34.50	34.30	23.40
				II	34.40	34.20	22.90
Lab 3	18 Nov 2024		PPC	I	14.70	14.30	15.80
					14.80	14.20	15.70
Lab 3	18 Nov 2024		NPC	I	No Cq	No Cq	23.30
					No Cq	No Cq	22.80
Lab 3	18 Nov 2024		PAC (Provided by CT organizer)	I	25.70	26.10	27.70
					25.50	26.00	27.60
Lab 3	18 Nov 2024		PAC (Optional: provided by CT participant)	I	30.20	29.50	30.90
					30.00	29.40	30.60
Lab 3	18 Nov 2024		NTC	I	No Cq	No Cq	No Cq
					No Cq	No Cq	No Cq
Lab 4	28 Nov 2024	High	4	I	16.74	17.12	26.63
				II	16.63	17.08	26.54
			8	I	16.66	16.37	25.47
				II	16.83	16.96	25.40
			9	I	19.13	19.22	28.06
				II	18.98	19.25	27.28
			14	I	19.57	19.93	27.85
				II	18.39	18.53	26.06
			15	I	16.31	16.52	28.16
				II	16.48	16.65	27.56
			16	I	19.96	20.37	27.47
				II	19.53	20.15	27.20
Lab 4	28 Nov 2024	Medium	1	I	22.25	22.58	26.67
				II	22.20	22.82	26.84
			2	I	21.30	21.55	26.06
				II	22.23	22.23	29.70
			3	I	29.37	30.78	28.19
				II	29.29	30.69	27.98
			7	I	32.91	No Cq	25.59
				II	32.48	No Cq	27.93
			11	I	28.84	30.26	26.64
				II	29.08	30.65	27.26
			18	I	28.33	30.19	27.18
				II	28.34	30.16	27.23
			19	I	30.15	31.20	25.85

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
				II	30.16	30.57	25.81
			20	I	28.90	28.09	26.25
				II	28.02	27.68	26.30
Lab 4	28 Nov 2024	Healthy	5	I	No Cq	No Cq	27.62
				II	No Cq	No Cq	27.77
			6	I	No Cq	No Cq	27.50
				II	No Cq	No Cq	27.50
			10	I	No Cq	No Cq	27.13
				II	No Cq	No Cq	27.21
			12	I	No Cq	No Cq	26.92
				II	No Cq	No Cq	26.96
			13	I	No Cq	No Cq	25.64
				II	No Cq	No Cq	25.47
			17	I	No Cq	No Cq	27.45
				II	No Cq	38.86	27.27
Lab 4	28 Nov 2024		PPC	I	16.84	17.02	27.45
				II	16.98	17.09	27.74
Lab 4	28 Nov 2024		NPC	I	No Cq	38.61	26.06
				II	No Cq	No Cq	26.04
Lab 4	28 Nov 2024		PAC (Provided by CT organizer)	I	25.99	28.24	36.74
				II	26.04	28.52	No Cq
Lab 4	28 Nov 2024		PAC (Optional: provided by CT participant)	I	ND	ND	ND
				II	ND	ND	ND
Lab 4	28 Nov 2024		NTC	I	No Cq	No Cq	No Cq
				II	No Cq	No Cq	No Cq
Lab 5	3 Dec 2024	High	4	I	16.65	17.94	39.82
				II	16.07	17.71	39.79
			8	I	18.05	19.36	No Cq
				II	17.87	19.28	No Cq
			9	I	19.34	20.80	39.00
				II	20.11	21.67	39.67
			14	I	18.38	20.09	37.82
				II	18.55	20.01	37.64
			15	I	16.81	18.35	38.79
				II	16.93	18.45	38.65
			16	I	20.15	21.66	38.13

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
				II	19.62	21.12	37.85
Lab 5	3 Dec 2024	Medium	1	I	27.04	29.44	No Cq
				II	28.21	30.56	No Cq
			2	I	21.40	23.10	No Cq
				II	20.76	22.53	No Cq
			3	I	30.65	32.87	36.43
				II	30.79	32.61	35.31
			7	I	34.11	36.93	37.04
				II	32.90	35.76	35.69
			11	I	29.87	31.43	38.70
				II	30.13	31.88	No Cq
			18	I	31.21	32.88	36.03
				II	31.41	32.88	35.67
			19	I	20.87	21.84	39.62
				II	21.50	22.74	No Cq
			20	I	32.88	35.88	35.86
				II	32.13	34.18	34.60
Lab 5	3 Dec 2024	Healthy	5	I	36.70	No Cq	37.92
				II	37.79	37.92	37.14
			6	I	38.85	37.79	37.13
				II	37.14	34.98	36.94
			10	I	39.11	37.81	36.91
				II	38.93	No Cq	36.51
			12	I	35.68	36.44	38.04
				II	35.12	36.88	38.64
			13	I	No Cq	No Cq	36.77
				II	No Cq	37.67	36.81
			17	I	36.86	37.26	35.88
				II	37.28	37.29	36.19
Lab 5	3 Dec 2024		PPC	I	16.75	18.12	37.95
				II	16.53	18.24	37.71
Lab 5	3 Dec 2024		NPC	I	No Cq	No Cq	35.26
				II	No Cq	No Cq	35.10
Lab 5	3 Dec 2024		PAC (Provided by CT organizer)	I	25.85	26.68	No Cq
				II	ND	ND	ND
Lab 5	3 Dec 2024		PAC (Optional: provided by CT participant)	I	ND	ND	ND
				II	ND	ND	ND

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
Lab 5	3 Dec 2024		NTC	I	No Cq	No Cq	No Cq
				II	ND	ND	ND
Lab 6	15 Nov 2024	High	4	I	13.44	13.20	21.89
				II	13.62	13.21	21.89
			8	I	14.50	14.29	22.52
				II	14.66	14.19	22.29
			9	I	16.33	16.18	22.29
				II	16.50	16.28	22.38
			14	I	15.70	15.48	22.10
				II	15.95	15.71	22.23
			15	I	15.11	14.94	22.16
				II	15.15	14.86	22.29
			16	I	16.27	16.11	22.59
				II	16.29	16.10	22.41
Lab 6	15 Nov 2024	Medium	1	I	19.17	19.05	22.53
				II	19.13	19.00	22.53
			2	I	24.96	26.19	22.87
				II	24.97	26.17	22.94
			3	I	19.06	18.93	22.35
				II	19.20	18.90	22.40
			7	I	20.19	20.12	22.49
				II	20.29	20.24	22.55
			11	I	18.05	17.69	21.99
				II	18.19	17.77	22.01
			18	I	27.56	28.12	23.24
				II	26.39	27.33	23.25
			19	I	23.71	24.55	22.57
				II	23.87	24.72	22.51
			20	I	26.67	27.77	23.03
				II	26.65	27.78	22.83
Lab 6	15 Nov 2024	Healthy	5	I	36.08	35.49	22.60
				II	No Cq	35.33	22.63
			6	I	No Cq	36.94	22.61
				II	No Cq	No Cq	22.64
			10	I	No Cq	No Cq	22.53
				II	No Cq	No Cq	22.66
			12	I	No Cq	36.65	22.51

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
				II	No Cq	No Cq	22.49
			13	I	No Cq	No Cq	22.52
				II	No Cq	No Cq	22.47
			17	I	No Cq	No Cq	22.56
				II	No Cq	No Cq	22.61
Lab 6	15 Nov 2024		PPC	I	14.55	14.17	22.14
				II	14.58	14.37	22.16
Lab 6	15 Nov 2024		NPC	I	No Cq	No Cq	23.02
				II	No Cq	No Cq	22.91
Lab 6	15 Nov 2024		PAC (Provided by CT organizer)	I	25.42	27.05	No Cq
				II	25.40	27.06	No Cq
Lab 6	15 Nov 2024		PAC (Optional: provided by CT participant)	I	ND	ND	ND
				II	ND	ND	ND
Lab 6	15 Nov 2024		NTC	I	No Cq	No Cq	No Cq
				II	No Cq	No Cq	No Cq
Lab 7	3 Dec 2024	High	4	I	12.90	15.20	25.40
				II	13.70	15.50	25.40
			8	I	12.60	15.00	25.20
				II	12.60	15.00	25.10
			9	I	14.90	18.50	26.40
				II	15.70	18.60	26.30
			14	I	15.60	17.90	25.30
				II	16.30	18.00	25.10
			15	I	12.30	14.30	25.30
				II	12.60	14.70	25.30
			16	I	14.20	17.90	25.10
				II	16.30	18.30	25.10
Lab 7	3 Dec 2024	Medium	1	I	18.90	21.00	25.20
				II	17.80	20.70	25.30
			2	I	24.50	28.90	25.40
				II	24.80	29.00	25.40
			3	I	22.80	28.00	25.00
				II	23.20	28.20	25.00
			7	I	25.30	30.00	24.90
				II	26.30	30.10	25.00
			11	I	24.30	29.00	26.20
				II	24.00	29.00	26.10

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
			18	I	23.50	29.80	24.80
				II	24.90	29.90	25.00
			19	I	23.60	28.30	25.20
				II	23.90	28.00	25.10
			20	I	20.30	22.60	25.00
				II	19.20	22.60	25.00
Lab 7	3 Dec 2024	Healthy	5	I	33.90	35.90	25.40
				II	33.40	35.50	25.30
			6	I	35.90	No Cq	25.60
				II	38.40	38.80	25.70
			10	I	36.60	39.80	26.20
				II	36.50	38.40	26.10
			12	I	35.20	37.30	25.40
				II	35.40	36.30	25.30
			13	I	No Cq	No Cq	25.20
				II	36.50	No Cq	25.20
			17	I	38.10	37.90	25.40
				II	36.90	39.60	25.30
Lab 7	3 Dec 2024		PPC	I	12.60	15.40	25.50
				II	14.30	15.50	25.60
Lab 7	3 Dec 2024		NPC	I	No Cq	No Cq	25.20
				II	No Cq	No Cq	25.20
Lab 7	3 Dec 2024		PAC (Provided by CT organizer)	I	27.40	30.90	No Cq
				II	27.30	31.10	No Cq
Lab 7	3 Dec 2024		PAC (Optional: provided by CT participant)	I	13.80	16.20	No Cq
				II	13.60	16.20	No Cq
Lab 7	3 Dec 2024		NTC	I	No Cq	No Cq	No Cq
				II	No Cq	No Cq	No Cq
Lab 8	19 Feb 2025	High	4	I	14.41	14.76	25.91
				II	14.62	14.85	25.74
			8	I	15.12	15.11	25.42
				II	15.16	15.15	25.81
			9	I	17.35	17.34	24.57
				II	17.29	17.44	25.39
			14	I	17.37	17.47	24.72
				II	17.36	17.53	24.77
			15	I	15.16	15.18	25.56

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
				II	15.12	15.11	25.46
			16	I	17.25	17.40	25.00
				II	17.09	17.22	24.70
Lab 8	19 Feb 2025	Medium	1	I	26.68	28.63	25.61
				II	26.45	28.68	25.75
			2	I	27.41	29.06	25.63
				II	27.49	28.85	25.58
			3	I	23.66	24.23	25.80
				II	23.84	24.23	25.81
			7	I	27.27	29.19	25.65
				II	27.37	29.20	25.73
			11	I	19.15	19.16	26.07
				II	19.58	19.28	25.86
			18	I	25.73	26.07	25.32
				II	25.72	26.22	25.49
			19	I	27.71	29.91	24.74
				II	27.72	30.18	25.02
			20	I	26.90	29.04	24.90
				II	26.88	29.19	25.09
Lab 8	19 Feb 2025	Healthy	5	I	No Cq	No Cq	26.65
				II	No Cq	No Cq	26.75
			6	I	No Cq	No Cq	26.73
				II	No Cq	No Cq	26.68
			10	I	No Cq	No Cq	25.82
				II	No Cq	No Cq	26.14
			12	I	No Cq	No Cq	26.44
				II	No Cq	No Cq	26.50
			13	I	No Cq	No Cq	26.75
				II	No Cq	No Cq	26.90
			17	I	No Cq	No Cq	25.83
				II	37.01	37.05	25.93
Lab 8	19 Feb 2025		PPC	I	15.67	15.99	25.15
				II	15.81	15.94	25.50
Lab 8	19 Feb 2025		NPC	I	No Cq	No Cq	25.49
				II	No Cq	No Cq	25.41
Lab 8	19 Feb 2025		PAC (Provided by CT organizer)	I	32.74	37.63	No Cq
				II	32.66	36.52	No Cq

Lab number	Date test	Infection level	Sample or control	PCR replicate	LMV-NAKT	LMV-RZ2	DLVd-DaVd1
Lab 8	19 Feb 2025		PAC (Optional: provided by CT participant)	I	17.22	18.15	26.88
				II	17.11	18.08	27.00
Lab 8	19 Feb 2025		NTC	I	No Cq	No Cq	No Cq
				II	No Cq	No Cq	No Cq

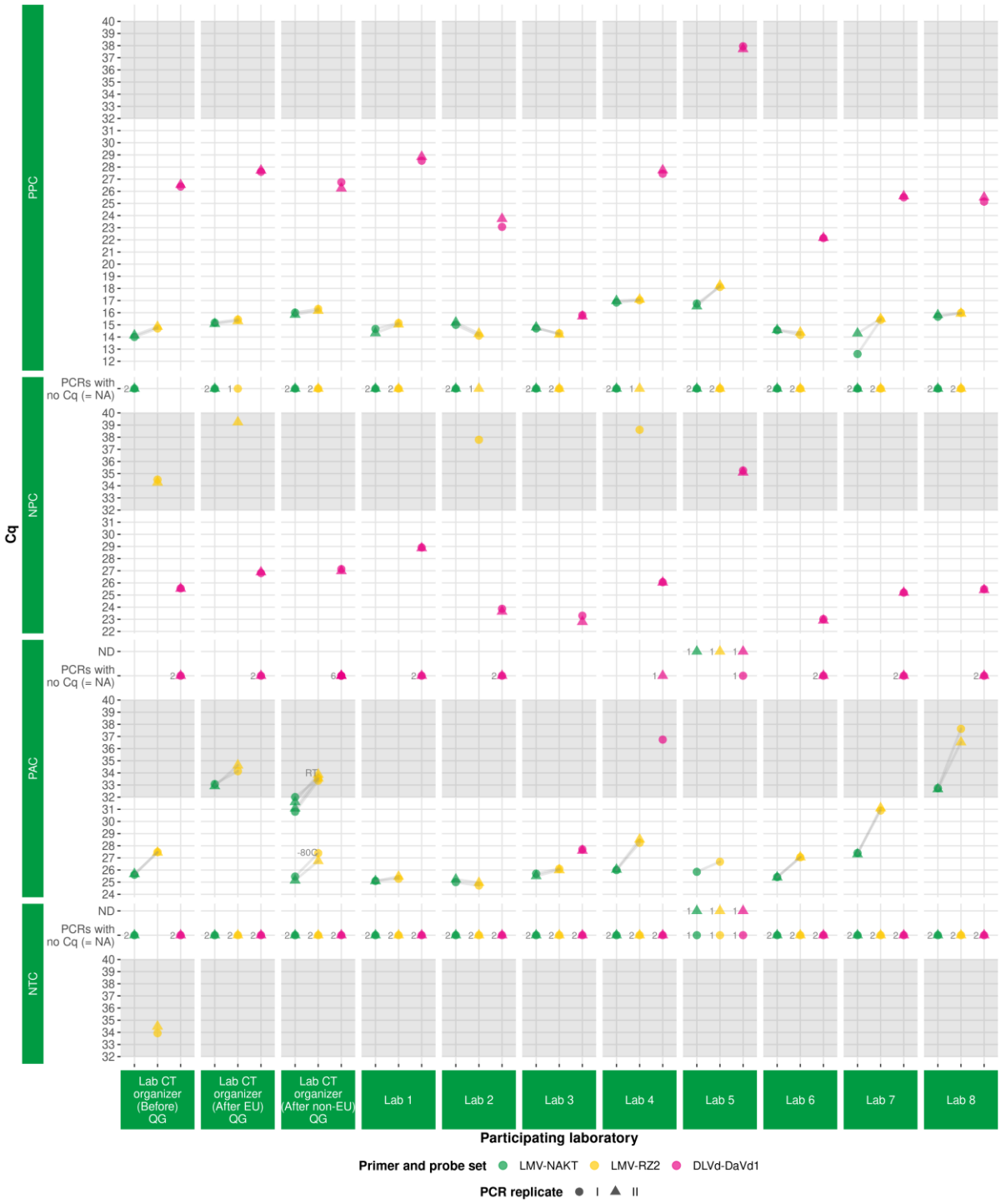

Figure H.2. Obtained Cq values from the two LMV targeted primer and probe sets (LMV-NAKT and LMV-RZ2) and DLVd that is used as an IAC, for controls performed as part of the CT by the CT organizer and participating laboratories. The grey background represents the range for which the SE-qPCRs were considered negative (Cq >32) in the CT. The grey lines connect qPCR signals from the LMV-NAKT and LMV-RZ2 primer and probe sets from the same reaction. The number of reactions yielding no Cq value and the number of reactions that was not determined (ND) are added to the top of each graph. The deviation in storage of PAC aliquots by the CT organizer and tested after all participants returned their results are indicated in the graph.

Annex I. Protocol RZ LMV Seedling ELISA

Materials

- Filter paper
- Flat container to hold filter
- Leaf juice extractor
- 96-well microtiter plate
- Coating LMV antiserum (Prime Diagnostics)
- Conjugaat LMV antiserum (Prime Diagnostics)
- Positive control ELISA (Prime Diagnostics)
- Buffers (Table I.1, I.2, I.3)
- Controls (Table I.4)
- Phosphatase substrate
- Sbeadex plant maxi kit (LGC Genomics)
- KingFisher 96/flex (ThermoFisher scientific)
- GH+ extraction buffer
- DLVd-stock (IAC, added to GH+ buffer)
- RT-qPCR mix, primers (Table I.6) and equipment
- 96-well PCR plate
- Thermoshaker
- Heatsealer
- 96-well plate (heat) seal
- 96-well PCR plate seal

Sample and subsample size

The minimum sample size of the LMV seedling ELISA is 2,000 seedlings and the maximum subsample size is 100 seedlings.

Spike solution

The spike solution is prepared by taking a leaf from a plant infected by DLVd and making an extract of it in PBS. The extracted is diluted to obtain a suitable concentration giving Cq values of 27-30 and aliquots are stored at -80 °C.

Table I.1. Seed extraction buffer and 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
Polyvinylpyrrolidone*	20.0 g
Tween 20	0.50 mL
Add with deionised water to 1 L, adjust pH, and autoclave buffer at 121 °C for 15 min.**	

*Only add when preparing extraction buffer; **Only autoclave when preparing conjugate buffer

Table I.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 with deionised water to 1 L, adjust pH, and autoclave at 121 °C for 15 min.	

Table I.3. Substrate buffer - pH 9.8.

Compound	Amount/L
Diethanolamine	97 g
Add with deionised water to 1 L, adjust pH, and autoclave at 121 °C for 15 min.	

Table I.4. Types of controls used.

Control type	Description
Positive processing control (PPC)	Positive (LMV infected) seed sample
Negative processing control (NPC)	Sample from healthy seeds (free of LMV)
Positive control (PC; ELISA)	LMV positive ELISA control from Prime Diagnostics
Buffer control (BC; ELISA)	The buffers and reagents used in the ELISA with no seed or tissue matrix or target pathogen
Positive amplification control (PAC; PCR)	LMV single-stranded DNA oligonucleotide aiming at a Cq value between 28 and 32
Internal amplification control (IAC)	DLVd spike
Negative template control (NTC)	Using nuclease free water as template for the LMV triplex confirmation qPCR

1. Sowing seed (sub)samples (Day 0)

1.1. Place a pleated filter paper with 25 pleats in a plastic container and saturate the paper with tap water.

1.2. Sow subsamples of 100 seeds, 25 subsamples of 100 seeds for the PPC, or 10 subsamples of the NPC in pleats of the filter paper. Sow seeds approximately 4 cm wide in the middle per pleat.

1.3. Incubate the seed samples at 15 °C (100% relative humidity) with 12 hours light and 12 hours in dark conditions per day).

1.4. After 2 days, add 20 mL tap water to each of the containers with the samples or PPC and add 10 mL tap water to the container with the NPC.

1.5. Incubate the seed samples at 18 °C (or 20 °C; 100% relative humidity) with 12 hours light and 12 hours in dark conditions).

2. Coating of the ELISA plates (Day 3 or 5)

2.1. Coat the wells of ELISA 96-wells plates with 200 µL (diluted) coating antibodies (e.g., LMV Prime diagnostics) in coating buffer (Table I.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 at ~7 °C overnight (a maximum of 14 days) before use.

3. Seedling extraction and incubation of extract (Day 6)

3.1. Collect seedlings from the pleated paper. Use seedlings from 23 pleats from the PPC and seedlings from eight pleats from the NPC.

Note: The test is stopped if seeds are insufficiently germinated.

3.2. Use an extractor with extraction buffer to collect juice from the seedling subsamples.

Note: Rinse the juice extractor with tap water after every sample. The seedling extract can be stored at ~7 °C.

3.3. Remove coating from the ELISA 96-well plates.

3.4. Add 100 µL seedling extract in duplicate in the plate. Store seedling extract at ~7 °C until further use.

3.5. Add 100 µL (diluted) conjugate antibodies (e.g., LMV Prime diagnostics) in conjugate buffer (Table I.1.).

3.6. Incubate the ELISA plate with samples overnight at ~7 °C.

4. Incubation with substrate (Day 7)

4.1. Dissolve 10 mg of phosphatase substrate per 20 mL substrate buffer (Table I.3).

4.2. Remove the seedling extract sample and conjugate buffer from the ELISA plate and rinse them well (using a washing device).

4.3. Add 200 µL substrate buffer with phosphatase substrate to the ELISA plate.

4.4. Measure after 4 hours the ELISA plate extinction using a spectrophotometer at 405 nm and 620 nm.

5. Evaluation of the LMV seedling ELISA

Seedling extract with corrected absorbance measurements that are at least 1.25 times higher than averaged corrected absorbance measurements of the NPC, are considered positive. Note: Test results are only valid when all included controls presented in Table I.5 give the expected results.

Table I.5. Expected outcome of the LMV ELISA for used controls.

Control type	Expected seedling ELISA outcome
Positive processing control (PPC)	Positive (for at least one subsample)
Negative processing control (NPC)	Negative
Positive control (PC; ELISA)	Positive
Buffer control (BC; ELISA)	Negative

6. RNA extraction from seedling extract

6.1. Retrieve seedling extracts with positive ELISA outcome from ~7 °C storage. Include extract from a subsample from the PPC and NPC.

6.2. Transfer 100 µL seedling extract to a 96-well deep well plate suitable for KingFisher based extraction. Note: Seedling extract in extraction buffer can be stored for a maximum of 3 days at ~7 °C.

6.3. Spike GH+ buffer with DLVd.

6.4. Add 500 µL spiked GH+ buffer to wells with seedling extract.

6.5. Seal the 96-well deep well plate with a heat seal using the heat sealer and incubate at 65 °C for 15 min and 850 rpm in a thermomixer.

6.6. Centrifuge 96-well deep-well plate briefly.

6.7. Transfer 100 µL of the seedling extract in GH+ to new 96-well deep-well plate with and use this as input for RNA extraction using the Sbeadex plant maxi kit in combination with the KingFisher platform.

7. LMV confirmation RT-qPCR

7.1. Prepare the RT-qPCR mix with the primer, probe and TaqMan mix components as described in Table I.6.

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

7.2. Distribute 20 µL of the RT-qPCR master mix in PCR tubes or a 96 well plate and add 5 µL RNA sample as template.

7.3. Seal the plate and perform the PCR reaction in a real-time PCR instrument, according to the conditions described in Table I.7.

Table I.6. Preparation of the SE-qPCR Master mix.

Component	For 1 reaction (in µL)	Final concentration
DaVd1 Fw (10 uM)	0.25	0.10 uM
DaVd1 Rv (10 uM)	0.25	0.10 uM
DaVd1 Probe (10 uM)	0.25	0.10 uM
LMV RZ 2 Fw (10 uM)	0.5	0.20 uM
LMV RZ 2 Rv (10 uM)	0.5	0.20 uM
LMV RZ 2 Probe (10 uM)	0.375	0.15 uM
LMV NAKT Fw (10 uM)	0.5	0.20 uM
LMV NAKT Rv (10 uM)	0.5	0.20 uM
LMV NAKT Probe (10 uM)	0.375	0.15 uM
Quantabio 1-Step ToughMix RT-qPCR (4×)	6.25	1×
PCR grade water	10.25	
Sample RNA	5	
Total volume	25	

Table I.7. PCR conditions of the LMV confirmation qPCR.

Repeats	Step	Temperature	Duration
1	cDNA synthesis	50 °C	5 min
1	Initial denaturation	95 °C	20 sec
40	Denaturation	95 °C	10 sec
	Annealing and primer extension	60 °C	30 sec

8. Evaluation of the LMV confirmation RT-qPCR

Samples with Cq values <32 are considered positive. Note: Test results are only valid when all included controls presented in Table I.8 give the expected results.

Table I.8. Expected outcome of the LMV confirmation qPCR for used controls.

Control type	Description	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
Positive processing control (PPC)	Positive (LMV infected) seed sample	Positive	Positive	Positive
Negative processing control (NPC)	Sample from healthy seeds (free of LMV)	Negative	Negative	Positive
Positive amplification control (PAC; PCR)	LMV single-stranded DNA oligonucleotide aiming at a Cq value between 28 and 32	Positive	Positive	Negative
Negative template control (NTC)	Using nuclease free water as template for the LMV triplex confirmation qPCR	Negative	Negative	Negative

9. Interpretation and decisions

For interpretation and decision making, the results from the LMV seedling ELISA and LMV confirmation qPCR need to be taken into account, see Table I.9.

Table I.9. Decision table for seed samples using the LMV seedling ELISA and confirmation qPCR outcome.

Seedling ELISA	Confirmation qPCR	Conclusion
Positive	Positive	Positive
Positive	Negative	Negative
Negative		Negative

Annex J. Data from diagnostic performance assessment

Table J.1. Obtained Cq values with the two LMV targeted primer and probe sets (LMV-NAKT (FAM channel) and LMV-RZ2 (VIC channel)) and DLVd (TxR channel) that is used as an IAC, for extract from lettuce LMV positive and negative seed lots. All qPCRs were performed on a single 96 well plate. All samples tested were in the 96-well format. NA: Not analysed.

Plate	Sample	Sub	PCR replicate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
I	LMV negative SE (NPC used in RZ routine testing)	III	I	38.2	No Cq	28.09
I	PCR grade water (NTC)		I	No Cq	No Cq	No Cq
I	LMV positive SE (PPC used in RZ routine testing)	III	I	17.29	18.07	29.15
I	LMV NAKT + LMV RZ 2 Synth (PAC)[a]		I	23.49	24.06	No Cq
I	SE 2	I	I	35.33	38.67	27.05
I	SE 2	II	I	No Cq	No Cq	26.07
I	SE 2	III	I	33.73	35.35	26.61
I	SE 9	I	I	35.27	35.35	27.36
I	SE 9	II	I	33.64	34.56	27.78
I	SE 9	III	I	34.59	36.57	27.9
I	SE 18	I	I	28.55	30.38	26.79
I	SE 18	II	I	28.13	29.02	26.13
I	SE 18	III	I	30.58	33.89	27.63
I	SE 31	I	I	18.23	18.79	28.63
I	SE 31	II	I	19.2	19.88	28.18
I	SE 31	III	I	19.42	20.00	29.03
I	SE 32	I	I	16.77	17.17	29.01
I	SE 32	II	I	16.03	16.23	28.84
I	SE 32	III	I	16.75	17.07	29.55

[a] A mixture of synthetic ssDNA with a concentration of 6×10^2 copies per µL.

Table J.2. Corrected absorbance measurements from LMV seedling ELISA and Cq values from the LMV confirmation triplex identification qPCR for lettuce seed lots. ND: Not determined; OD: Optical Density.

Sample	Sub	ELISA Replicate	Plate	Well	OD	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 2	1	I	III	A04	0.081	ND	ND	ND
SE 2	1	II	IV	A04	0.073			
SE 2	10	I	III	B05	0.070	ND	ND	ND
SE 2	10	II	IV	B05	0.069			
SE 2	11	I	III	C05	0.069	ND	ND	ND
SE 2	11	II	IV	C05	0.067			
SE 2	12	I	III	D05	0.075	ND	ND	ND
SE 2	12	II	IV	D05	0.072			
SE 2	13	I	III	E05	0.072	ND	ND	ND
SE 2	13	II	IV	E05	0.070			
SE 2	14	I	III	F05	0.071	ND	ND	ND
SE 2	14	II	IV	F05	0.072			
SE 2	15	I	III	G05	0.071	ND	ND	ND
SE 2	15	II	IV	G05	0.072			
SE 2	16	I	III	H05	0.077	ND	ND	ND
SE 2	16	II	IV	H05	0.079			
SE 2	17	I	III	A06	0.079	ND	ND	ND
SE 2	17	II	IV	A06	0.075			
SE 2	18	I	III	B06	0.074	ND	ND	ND
SE 2	18	II	IV	B06	0.071			
SE 2	19	I	III	C06	0.072	ND	ND	ND
SE 2	19	II	IV	C06	0.076			
SE 2	2	I	III	B04	0.074	ND	ND	ND
SE 2	2	II	IV	B04	0.070			
SE 2	20	I	III	D06	0.073	ND	ND	ND
SE 2	20	II	IV	D06	0.074			
SE 2	3	I	III	C04	0.071	ND	ND	ND
SE 2	3	II	IV	C04	0.070			
SE 2	4	I	III	D04	0.073	ND	ND	ND
SE 2	4	II	IV	D04	0.070			
SE 2	5	I	III	E04	0.075	ND	ND	ND
SE 2	5	II	IV	E04	0.072			
SE 2	6	I	III	F04	0.072	ND	ND	ND
SE 2	6	II	IV	F04	0.074			
SE 2	7	I	III	G04	0.068	ND	ND	ND

Sample	Sub	ELISA Replicate	Plate	Well	OD	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 2	7	II	IV	G04	0.071			
SE 2	8	I	III	H04	0.072	ND	ND	ND
SE 2	8	II	IV	H04	0.077			
SE 2	9	I	III	A05	0.083	ND	ND	ND
SE 2	9	II	IV	A05	0.074			
SE 9	1	I	III	E06	0.072	ND	ND	ND
SE 9	1	II	IV	E06	0.075			
SE 9	10	I	III	F07	0.095	ND	ND	ND
SE 9	10	II	IV	F07	0.089			
SE 9	11	I	III	G07	0.104	No Cq	No Cq	32.09
SE 9	11	II	IV	G07	0.098			
SE 9	12	I	III	H07	0.085	ND	ND	ND
SE 9	12	II	IV	H07	0.086			
SE 9	13	I	III	A08	0.091	ND	ND	ND
SE 9	13	II	IV	A08	0.082			
SE 9	14	I	III	B08	0.088	ND	ND	ND
SE 9	14	II	IV	B08	0.085			
SE 9	15	I	III	C08	0.082	ND	ND	ND
SE 9	15	II	IV	C08	0.081			
SE 9	16	I	III	D08	0.102	No Cq	No Cq	33.36
SE 9	16	II	IV	D08	0.100			
SE 9	17	I	III	E08	0.102	No Cq	No Cq	31.06
SE 9	17	II	IV	E08	0.096			
SE 9	18	I	III	F08	0.074	ND	ND	ND
SE 9	18	II	IV	F08	0.068			
SE 9	19	I	III	G08	0.068	ND	ND	ND
SE 9	19	II	IV	G08	0.067			
SE 9	2	I	III	F06	0.092	ND	ND	ND
SE 9	2	II	IV	F06	0.097			
SE 9	20	I	III	H08	0.095	ND	ND	ND
SE 9	20	II	IV	H08	0.094			
SE 9	3	I	III	G06	0.096	No Cq	No Cq	30.42
SE 9	3	II	IV	G06	0.100			
SE 9	4	I	III	H06	0.104	No Cq	No Cq	31.32
SE 9	4	II	IV	H06	0.107			
SE 9	5	I	III	A07	0.082	ND	ND	ND

Sample	Sub	ELISA Replicate	Plate	Well	OD	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 9	5	II	IV	A07	0.075			
SE 9	6	I	III	B07	0.073	ND	ND	ND
SE 9	6	II	IV	B07	0.072			
SE 9	7	I	III	C07	0.081	ND	ND	ND
SE 9	7	II	IV	C07	0.081			
SE 9	8	I	III	D07	0.091	ND	ND	ND
SE 9	8	II	IV	D07	0.081			
SE 9	9	I	III	E07	0.075	ND	ND	ND
SE 9	9	II	IV	E07	0.071			
SE 18	1	I	III	A09	0.066	ND	ND	ND
SE 18	1	II	IV	A09	0.068			
SE 18	10	I	III	B10	0.065	ND	ND	ND
SE 18	10	II	IV	B10	0.064			
SE 18	11	I	III	C10	0.066	ND	ND	ND
SE 18	11	II	IV	C10	0.065			
SE 18	12	I	III	D10	0.067	ND	ND	ND
SE 18	12	II	IV	D10	0.066			
SE 18	13	I	III	E10	0.065	ND	ND	ND
SE 18	13	II	IV	E10	0.066			
SE 18	14	I	III	F10	0.067	ND	ND	ND
SE 18	14	II	IV	F10	0.071			
SE 18	15	I	III	G10	0.064	ND	ND	ND
SE 18	15	II	IV	G10	0.066			
SE 18	16	I	III	H10	0.073	ND	ND	ND
SE 18	16	II	IV	H10	0.075			
SE 18	17	I	III	A11	0.075	ND	ND	ND
SE 18	17	II	IV	A11	0.066			
SE 18	18	I	III	B11	0.068	ND	ND	ND
SE 18	18	II	IV	B11	0.063			
SE 18	19	I	III	C11	0.065	ND	ND	ND
SE 18	19	II	IV	C11	0.065			
SE 18	2	I	III	B09	0.070	ND	ND	ND
SE 18	2	II	IV	B09	0.067			
SE 18	20	I	III	D11	0.067	ND	ND	ND
SE 18	20	II	IV	D11	0.067			
SE 18	3	I	III	C09	0.065	ND	ND	ND

Sample	Sub	ELISA Replicate	Plate	Well	OD	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 18	3	II	IV	C09	0.065			
SE 18	4	I	III	D09	0.066	ND	ND	ND
SE 18	4	II	IV	D09	0.067			
SE 18	5	I	III	E09	0.070	ND	ND	ND
SE 18	5	II	IV	E09	0.069			
SE 18	6	I	III	F09	0.067	ND	ND	ND
SE 18	6	II	IV	F09	0.064			
SE 18	7	I	III	G09	0.068	ND	ND	ND
SE 18	7	II	IV	G09	0.066			
SE 18	8	I	III	H09	0.071	ND	ND	ND
SE 18	8	II	IV	H09	0.065			
SE 18	9	I	III	A10	0.071	ND	ND	ND
SE 18	9	II	IV	A10	0.067			
SE 31	1	I	III	E11	0.424	ND	ND	ND
SE 31	1	II	IV	E11	0.392			
SE 31	10	I	V	B01	0.389	ND	ND	ND
SE 31	10	II	VI	B01	0.378			
SE 31	11	I	V	C01	0.450	ND	ND	ND
SE 31	11	II	VI	C01	0.450			
SE 31	12	I	V	D01	0.380	ND	ND	ND
SE 31	12	II	VI	D01	0.390			
SE 31	13	I	V	E01	0.348	ND	ND	ND
SE 31	13	II	VI	E01	0.353			
SE 31	14	I	V	F01	0.602	24.35	23.84	30.80
SE 31	14	II	VI	F01	0.611			
SE 31	15	I	V	G01	0.473	ND	ND	ND
SE 31	15	II	VI	G01	0.489			
SE 31	16	I	V	H01	0.280	ND	ND	ND
SE 31	16	II	VI	H01	0.290			
SE 31	17	I	V	A02	0.351	ND	ND	ND
SE 31	17	II	VI	A02	0.352			
SE 31	18	I	V	B02	0.678	22.27	21.8	31.09
SE 31	18	II	VI	B02	0.703			
SE 31	19	I	V	C02	0.569	ND	ND	ND
SE 31	19	II	VI	C02	0.580			
SE 31	2	I	III	F11	0.562	21.91	21.55	30.37

Sample	Sub	ELISA Replicate	Plate	Well	OD	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 31	2	II	IV	F11	0.541			
SE 31	20	I	V	D02	0.240	ND	ND	ND
SE 31	20	II	VI	D02	0.252			
SE 31	3	I	III	G11	0.363	ND	ND	ND
SE 31	3	II	IV	G11	0.343			
SE 31	4	I	III	H11	0.456	ND	ND	ND
SE 31	4	II	IV	H11	0.463			
SE 31	5	I	III	A12	0.451	ND	ND	ND
SE 31	5	II	IV	A12	0.432			
SE 31	6	I	III	B12	0.822	21.14	20.79	30.55
SE 31	6	II	IV	B12	0.823			
SE 31	7	I	III	C12	0.613	22.25	21.73	30.85
SE 31	7	II	IV	C12	0.614			
SE 31	8	I	III	D12	0.472	ND	ND	ND
SE 31	8	II	IV	D12	0.481			
SE 31	9	I	V	A01	0.498	ND	ND	ND
SE 31	9	II	VI	A01	0.512			
SE 32	1	I	V	E02	0.781	21.45	21.02	29.65
SE 32	1	II	VI	E02	0.831			
SE 32	10	I	V	F03	0.719	ND	ND	ND
SE 32	10	II	VI	F03	0.815			
SE 32	11	I	V	G03	0.831	21.05	20.43	30.71
SE 32	11	II	VI	G03	0.973			
SE 32	12	I	V	H03	0.803	21.58	21.07	31.39
SE 32	12	II	VI	H03	0.924			
SE 32	13	I	V	A04	0.701	ND	ND	ND
SE 32	13	II	VI	A04	0.701			
SE 32	14	I	V	B04	0.903	22.52	22.14	31.12
SE 32	14	II	VI	B04	0.923			
SE 32	15	I	V	C04	0.594	ND	ND	ND
SE 32	15	II	VI	C04	0.594			
SE 32	16	I	V	D04	0.544	ND	ND	ND
SE 32	16	II	VI	D04	0.530			
SE 32	17	I	V	E04	0.624	ND	ND	ND
SE 32	17	II	VI	E04	0.617			
SE 32	18	I	V	F04	0.618	ND	ND	ND

Sample	Sub	ELISA Replicate	Plate	Well	OD	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 32	18	II	VI	F04	0.638			
SE 32	19	I	V	G04	0.633	ND	ND	ND
SE 32	19	II	VI	G04	0.608			
SE 32	2	I	V	F02	0.652	ND	ND	ND
SE 32	2	II	VI	F02	0.645			
SE 32	20	I	V	H04	0.708	ND	ND	ND
SE 32	20	II	VI	H04	0.704			
SE 32	3	I	V	G02	0.788	21.26	20.73	31.08
SE 32	3	II	VI	G02	0.814			
SE 32	4	I	V	H02	0.769	ND	ND	ND
SE 32	4	II	VI	H02	0.750			
SE 32	5	I	V	A03	0.668	ND	ND	ND
SE 32	5	II	VI	A03	0.750			
SE 32	6	I	V	B03	0.533	ND	ND	ND
SE 32	6	II	VI	B03	0.614			
SE 32	7	I	V	C03	0.688	ND	ND	ND
SE 32	7	II	VI	C03	0.794			
SE 32	8	I	V	D03	0.603	ND	ND	ND
SE 32	8	II	VI	D03	0.720			
SE 32	9	I	V	E03	0.526	ND	ND	ND
SE 32	9	II	VI	E03	0.584			
LMV positive SE (PPC used in RZ routine testing)	1	I	I	A02	0.384	23.14	22.94	28.65
	1	II	II	A02	0.387			
	10	I	I	B03	0.829	ND	ND	ND
	10	II	II	B03	0.869			
	11	I	I	C03	0.497	ND	ND	ND
	11	II	II	C03	0.489			
	12	I	I	D03	0.397	ND	ND	ND
	12	II	II	D03	0.380			
	13	I	I	E03	0.511	ND	ND	ND
	13	II	II	E03	0.546			
	14	I	I	F03	0.660	ND	ND	ND
	14	II	II	F03	0.662			
	15	I	I	G03	0.772	ND	ND	ND
	15	II	II	G03	0.761			
	16	I	I	H03	0.778	ND	ND	ND

Sample	Sub	ELISA Replicate	Plate	Well	OD	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
	16	II	II	H03	0.841			
	17	I	I	A04	0.550	ND	ND	ND
	17	II	II	A04	0.605			
	18	I	I	B04	0.729	ND	ND	ND
	18	II	II	B04	0.725			
	19	I	I	C04	0.883	ND	ND	ND
	19	II	II	C04	0.904			
	2	I	I	B02	0.499	ND	ND	ND
	2	II	II	B02	0.485			
	20	I	I	D04	0.761	ND	ND	ND
	20	II	II	D04	0.756			
	21	I	I	E04	0.779	ND	ND	ND
	21	II	II	E04	0.807			
	22	I	I	F04	0.371	ND	ND	ND
	22	II	II	F04	0.443			
	23	I	I	G04	0.613	ND	ND	ND
	23	II	II	G04	0.615			
	3	I	I	C02	0.645	ND	ND	ND
	3	II	II	C02	0.648			
	4	I	I	D02	0.724	ND	ND	ND
	4	II	II	D02	0.711			
	5	I	I	E02	0.573	ND	ND	ND
	5	II	II	E02	0.589			
	6	I	I	F02	0.590	ND	ND	ND
	6	II	II	F02	0.646			
	7	I	I	G02	0.799	ND	ND	ND
	7	II	II	G02	0.871			
	8	I	I	H02	0.868	ND	ND	ND
	8	II	II	H02	0.886			
	9	I	I	A03	0.788	ND	ND	ND
	9	II	II	A03	0.766			
LMV negative SE (NPC used in RZ routine testing)	1	I	I	A05	0.068	38.98	No Cq	31.81
	1	II	II	A05	0.068			
	2	I	I	B05	0.067	ND	ND	ND
	2	II	II	B05	0.067			
	3	I	I	C05	0.068	ND	ND	ND

Sample	Sub	ELISA Replicate	Plate	Well	OD	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
	3	II	II	C05	0.066			
	4	I	I	D05	0.065	ND	ND	ND
	4	II	II	D05	0.068			
	5	I	I	E05	0.064	ND	ND	ND
	5	II	II	E05	0.066			
	6	I	I	F05	0.067	ND	ND	ND
	6	II	II	F05	0.068			
	7	I	I	G05	0.065	ND	ND	ND
	7	II	II	G05	0.069			
	8	I	I	H05	0.070	ND	ND	ND
	8	II	II	H05	0.072			
LMV (PC; Undiluted)		I	I	A01	0.707	ND	ND	ND
		II	I	B01	0.736			
		III	II	A01	0.727	ND	ND	ND
		IV	III	G12	0.426			
		V	III	G12	0.655	ND	ND	ND
		VI	V	G12	0.5			
LMV (PC; 10x diluted)		I	I	C01	0.352	ND	ND	ND
		II	I	D01	0.349			
		III	II	C01	0.342	ND	ND	ND
		IV	II	D01	0.336			
		V	III	H12	0.35	ND	ND	ND
		VI	V	H12	0.291			
EB (NC)		I	I	E01	0.061	ND	ND	ND
		II	I	F01	0.069			
		III	I	G01	0.062	ND	ND	ND
		IV	I	H01	0.061			
		V	I	H04	0.064	ND	ND	ND
		VI	II	E01	0.062			
		VII	II	F01	0.062	ND	ND	ND
		VIII	II	G01	0.062			
		IX	II	H01	0.062	ND	ND	ND
		X	II	H04	0.062			
		XI	III	E12	0.064	ND	ND	ND
		XII	III	F12	0.065			
		XIII	V	E12	0.062	ND	ND	ND

Sample	Sub	ELISA Replicate	Plate	Well	OD	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
		XIV	V	F12	0.063			
		XV	IV	E12	0.065	ND	ND	ND
		XVI	IV	F12	0.063			
		XVII	VI	E12	0.061	ND	ND	ND
		XVIII	VI	F12	0.060			
PCR grade water (NTC)						No Cq	No Cq	No Cq
LMV NAKT + LMV RZ 2 Synth (PAC)[a]						24.26	24.43	No Cq

[a] A mixture of synthetic ssDNA with a concentration of 6×10^2 copies per µL.

Table J.3. Corrected absorbance measurements from LMV seed ELISA and Cq values from the LMV confirmation triplex identification qPCR for lettuce seed lots. ND: Not determined.

Sample	Sub	ELISA Replicate	ELISA plate	Well	OD	RNA extraction replicate	PCR plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 2	1	I	I	A01	0.1230	I	I	No Cq	No Cq	No Cq
SE 2	1	II	II	A01	0.1270					
SE 2	1	III	III	A01	0.1250					
SE 2	2	I	I	B01	0.1280	I	I	38.87	No Cq	No Cq
SE 2	2	II	II	B01	0.1300					
SE 2	2	III	III	B01	0.1290					
SE 2	3	I	I	C01	0.1210	I	I	No Cq	No Cq	No Cq
SE 2	3	II	II	C01	0.1230					
SE 2	3	III	III	C01	0.1220					
SE 2	4	I	I	D01	0.1580	I	I	38.97	No Cq	No Cq
SE 2	4	II	II	D01	0.1560					
SE 2	4	III	III	D01	0.1570					
SE 2	5	I	I	E01	0.1200	I	I	No Cq	No Cq	No Cq
SE 2	5	II	II	E01	0.1260					
SE 2	5	III	III	E01	0.1230					
SE 2	6	I	I	F01	0.1230	I	I	38.85	No Cq	No Cq
SE 2	6	II	II	F01	0.1250					
SE 2	6	III	III	F01	0.1240					
SE 2	7	I	I	G01	0.1320	I	I	No Cq	No Cq	No Cq
SE 2	7	II	II	G01	0.1270					
SE 2	7	III	III	G01	0.1295					
SE 2	8	I	I	H01	0.1180	I	I	No Cq	No Cq	No Cq
SE 2	8	II	II	H01	0.1250					
SE 2	8	III	III	H01	0.1215					
SE 2	9	I	I	A02	0.1220	I	I	No Cq	No Cq	No Cq
SE 2	9	II	II	A02	0.1290					
SE 2	9	III	III	A02	0.1255					
SE 2	10	I	I	B02	0.1150	I	I	No Cq	No Cq	No Cq
SE 2	10	II	II	B02	0.1180					
SE 2	10	III	III	B02	0.1165					
SE 2	11	I	I	C02	0.1220	I	I	No Cq	No Cq	No Cq
SE 2	11	II	II	C02	0.1330					
SE 2	11	III	III	C02	0.1275					
SE 2	12	I	I	D02	0.1200	I	I	No Cq	38.81	No Cq

Sample	Sub	ELISA Replicate	ELISA plate	Well	OD	RNA extraction replicate	PCR plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 2	12	II	II	D02	0.1270					
SE 2	12	III	III	D02	0.1235					
SE 2	13	I	I	E02	0.1160					
SE 2	13	II	II	E02	0.1220	I	I	No Cq	No Cq	No Cq
SE 2	13	III	III	E02	0.1190					
SE 2	14	I	I	F02	0.1270					
SE 2	14	II	II	F02	0.1340	I	I	No Cq	No Cq	No Cq
SE 2	14	III	III	F02	0.1305					
SE 2	15	I	I	G02	0.1250					
SE 2	15	II	II	G02	0.1180	I	I	No Cq	No Cq	No Cq
SE 2	15	III	III	G02	0.1215					
SE 2	16	I	I	H02	0.1270					
SE 2	16	II	II	H02	0.1320	I	I	No Cq	No Cq	No Cq
SE 2	16	III	III	H02	0.1295					
SE 2	17	I	I	A03	0.1230					
SE 2	17	II	II	A03	0.1270	I	I	38.72	No Cq	No Cq
SE 2	17	III	III	A03	0.1250					
SE 2	18	I	I	B03	0.1160					
SE 2	18	II	II	B03	0.1190	I	I	No Cq	No Cq	No Cq
SE 2	18	III	III	B03	0.1175					
SE 2	19	I	I	C03	0.1190					
SE 2	19	II	II	C03	0.1240	I	I	No Cq	No Cq	No Cq
SE 2	19	III	III	C03	0.1215					
SE 2	20	I	I	D03	0.1210					
SE 2	20	II	II	D03	0.1190	I	I	No Cq	No Cq	No Cq
SE 2	20	III	III	D03	0.1200					
SE 9	1	I	I	A04	0.1430					
SE 9	1	II	II	A04	0.1490	I	I	No Cq	No Cq	No Cq
SE 9	1	III	III	A04	0.1460					
SE 9	2	I	I	B04	0.1300					
SE 9	2	II	II	B04	0.1390	I	I	No Cq	No Cq	No Cq
SE 9	2	III	III	B04	0.1345					
SE 9	3	I	I	C04	0.1280					
SE 9	3	II	II	C04	0.1320	I	I	38.74	No Cq	No Cq
SE 9	3	III	III	C04	0.1300					
SE 9	4	I	I	D04	0.1380	I	I	No Cq	No Cq	No Cq

Sample	Sub	ELISA Replicate	ELISA plate	Well	OD	RNA extraction replicate	PCR plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 9	4	II	II	D04	0.1380					
SE 9	4	III	III	D04	0.1380					
SE 9	5	I	I	E04	0.1320					
SE 9	5	II	II	E04	0.1350	I	I	No Cq	No Cq	No Cq
SE 9	5	III	III	E04	0.1335					
SE 9	6	I	I	F04	0.1310					
SE 9	6	II	II	F04	0.1330	I	I	39.40	No Cq	No Cq
SE 9	6	III	III	F04	0.1320					
SE 9	7	I	I	G04	0.1310					
SE 9	7	II	II	G04	0.1270	I	I	37.73	No Cq	No Cq
SE 9	7	III	III	G04	0.1290					
SE 9	8	I	I	H04	0.1360					
SE 9	8	II	II	H04	0.1350	I	I	34.33	No Cq	No Cq
SE 9	8	III	III	H04	0.1355					
SE 9	9	I	I	A05	0.1400					
SE 9	9	II	II	A05	0.1390	I	I	No Cq	No Cq	No Cq
SE 9	9	III	III	A05	0.1395					
SE 9	10	I	I	B05	0.1320					
SE 9	10	II	II	B05	0.1320	I	I	No Cq	No Cq	No Cq
SE 9	10	III	III	B05	0.1320					
SE 9	11	I	I	C05	0.1280					
SE 9	11	II	II	C05	0.1330	I	I	No Cq	No Cq	No Cq
SE 9	11	III	III	C05	0.1305					
SE 9	12	I	I	D05	0.1300					
SE 9	12	II	II	D05	0.1330	I	I	No Cq	No Cq	No Cq
SE 9	12	III	III	D05	0.1315					
SE 9	13	I	I	E05	0.1330					
SE 9	13	II	II	E05	0.1360	I	I	38.65	No Cq	No Cq
SE 9	13	III	III	E05	0.1345					
SE 9	14	I	I	F05	0.1310					
SE 9	14	II	II	F05	0.1340	I	I	38.68	No Cq	No Cq
SE 9	14	III	III	F05	0.1325					
SE 9	15	I	I	G05	0.1380					
SE 9	15	II	II	G05	0.1310	I	I	No Cq	No Cq	No Cq
SE 9	15	III	III	G05	0.1345					
SE 9	16	I	I	H05	0.1400	I	I	No Cq	No Cq	No Cq

Sample	Sub	ELISA Replicate	ELISA plate	Well	OD	RNA extraction replicate	PCR plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 9	16	II	II	H05	0.1380					
SE 9	16	III	III	H05	0.1390					
SE 9	17	I	I	E03	0.1340	I	I	No Cq	No Cq	No Cq
SE 9	17	II	II	E03	0.1380					
SE 9	17	III	III	E03	0.1360					
SE 9	18	I	I	F03	0.1350	I	I	No Cq	No Cq	No Cq
SE 9	18	II	II	F03	0.1340					
SE 9	18	III	III	F03	0.1345					
SE 9	19	I	I	G03	0.1330	I	I	No Cq	No Cq	No Cq
SE 9	19	II	II	G03	0.1310					
SE 9	19	III	III	G03	0.1320					
SE 9	20	I	I	H03	0.1310	I	I	No Cq	No Cq	No Cq
SE 9	20	II	II	H03	0.1290					
SE 9	20	III	III	H03	0.1300					
SE 18	1	I	I	A06	0.1470	I	II	30.44	29.73	No Cq
SE 18	1	II	II	A06	0.1410					
SE 18	1	III	III	A06	0.1440					
SE 18	2	I	I	B06	0.1420	I	II	28.15	27.79	No Cq
SE 18	2	II	II	B06	0.1450					
SE 18	2	III	III	B06	0.1435					
SE 18	3	I	I	C06	0.1340	I	II	26.46	28.21	No Cq
SE 18	3	II	II	C06	0.1340					
SE 18	3	III	III	C06	0.1340					
SE 18	4	I	I	D06	0.1280	I	II	No Cq	32.04	No Cq
SE 18	4	II	II	D06	0.1280					
SE 18	4	III	III	D06	0.1280					
SE 18	5	I	I	E06	0.1230	I	II	38.54	32.05	No Cq
SE 18	5	II	II	E06	0.1250					
SE 18	5	III	III	E06	0.1240					
SE 18	6	I	I	F06	0.1310	I	II	26.42	30.07	No Cq
SE 18	6	II	II	F06	0.1310					
SE 18	6	III	III	F06	0.1310					
SE 18	7	I	I	G06	0.1340	I	II	27.64	29.15	No Cq
SE 18	7	II	II	G06	0.1280					
SE 18	7	III	III	G06	0.1310					
SE 18	8	I	I	H06	0.1330	I	II	27.13	27.22	No Cq

Sample	Sub	ELISA Replicate	ELISA plate	Well	OD	RNA extraction replicate	PCR plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 18	8	II	II	H06	0.1300					
SE 18	8	III	III	H06	0.1315					
SE 18	9	I	I	A07	0.1390					
SE 18	9	II	II	A07	0.1410	I	II	No Cq	29.42	No Cq
SE 18	9	III	III	A07	0.1400					
SE 18	10	I	I	B07	0.1330					
SE 18	10	II	II	B07	0.1330	I	II	31.22	34.52	No Cq
SE 18	10	III	III	B07	0.1330					
SE 18	11	I	I	C07	0.1240					
SE 18	11	II	II	C07	0.1300	I	II	34.76	35.54	No Cq
SE 18	11	III	III	C07	0.1270					
SE 18	12	I	I	D07	0.1250					
SE 18	12	II	II	D07	0.1290	I	II	28.67	31.88	No Cq
SE 18	12	III	III	D07	0.1270					
SE 18	13	I	I	E07	0.1260					
SE 18	13	II	II	E07	0.1310	I	II	28.31	30.29	No Cq
SE 18	13	III	III	E07	0.1285					
SE 18	14	I	I	F07	0.1230					
SE 18	14	II	II	F07	0.1260	I	II	30.18	31.30	No Cq
SE 18	14	III	III	F07	0.1245					
SE 18	15	I	I	G07	0.1270					
SE 18	15	II	II	G07	0.1300	I	II	33.94	32.26	No Cq
SE 18	15	III	III	G07	0.1285					
SE 18	16	I	I	H07	0.1400					
SE 18	16	II	II	H07	0.1440	I	II	28.73	31.33	No Cq
SE 18	16	III	III	H07	0.1420					
SE 18	17	I	I	A08	0.1430					
SE 18	17	II	II	A08	0.1370	I	II	29.33	29.83	No Cq
SE 18	17	III	III	A08	0.1400					
SE 18	18	I	I	B08	0.1280					
SE 18	18	II	II	B08	0.1290	I	II	No Cq	30.47	No Cq
SE 18	18	III	III	B08	0.1285					
SE 18	19	I	I	C08	0.1350					
SE 18	19	II	II	C08	0.1380	I	II	29.57	30.80	No Cq
SE 18	19	III	III	C08	0.1365					
SE 18	20	I	I	D08	0.1440	I	II	31.13	31.94	No Cq

Sample	Sub	ELISA Replicate	ELISA plate	Well	OD	RNA extraction replicate	PCR plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 18	20	II	II	D08	0.1380					
SE 18	20	III	III	D08	0.1410					
SE 31	1	I	I	A09	0.8850					
SE 31	1	II	II	A09	0.8060	I	II	18.13	19.05	No Cq
SE 31	1	III	III	A09	0.8455					
SE 31	2	I	I	B09	0.5420					
SE 31	2	II	II	B09	0.5740	I	II	18.53	19.34	No Cq
SE 31	2	III	III	B09	0.5580					
SE 31	3	I	I	C09	0.7930					
SE 31	3	II	II	C09	0.7490	I	II	18.37	19.19	No Cq
SE 31	3	III	III	C09	0.7710					
SE 31	4	I	I	D09	0.3460					
SE 31	4	II	II	D09	0.3090	I	II	20.56	21.26	No Cq
SE 31	4	III	III	D09	0.3275					
SE 31	5	I	I	E09	0.6780					
SE 31	5	II	II	E09	0.7140	I	II	18.22	19.13	No Cq
SE 31	5	III	III	E09	0.6960					
SE 31	6	I	I	F09	0.4310					
SE 31	6	II	II	F09	0.3980	I	II	19.72	20.51	No Cq
SE 31	6	III	III	F09	0.4145					
SE 31	7	I	I	G09	0.4610					
SE 31	7	II	II	G09	0.4520	I	II	19.47	20.26	No Cq
SE 31	7	III	III	G09	0.4565					
SE 31	8	I	I	H09	0.4510					
SE 31	8	II	II	H09	0.4450	I	II	19.61	20.42	No Cq
SE 31	8	III	III	H09	0.4480					
SE 31	9	I	I	A10	0.6640					
SE 31	9	II	II	A10	0.7260	I	II	18.49	19.32	No Cq
SE 31	9	III	III	A10	0.6950					
SE 31	10	I	I	B10	0.5100					
SE 31	10	II	II	B10	0.5380	I	II	19.25	20.02	No Cq
SE 31	10	III	III	B10	0.5240					
SE 31	11	I	I	C10	0.6060					
SE 31	11	II	II	C10	0.6330	I	II	18.15	18.84	No Cq
SE 31	11	III	III	C10	0.6195					
SE 31	12	I	I	D10	0.4120	I	II	19.20	19.68	No Cq

Sample	Sub	ELISA Replicate	ELISA plate	Well	OD	RNA extraction replicate	PCR plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 31	12	II	II	D10	0.4440					
SE 31	12	III	III	D10	0.4280					
SE 31	13	I	I	E10	0.4190					
SE 31	13	II	II	E10	0.4230	I	II	19.23	19.98	No Cq
SE 31	13	III	III	E10	0.4210					
SE 31	14	I	I	F10	0.4680					
SE 31	14	II	II	F10	0.5030	I	II	18.85	19.29	No Cq
SE 31	14	III	III	F10	0.4855					
SE 31	15	I	I	G10	0.2640					
SE 31	15	II	II	G10	0.2710	I	II	20.76	21.25	No Cq
SE 31	15	III	III	G10	0.2675					
SE 31	16	I	I	H10	0.7280					
SE 31	16	II	II	H10	0.7360	I	II	17.83	18.5	No Cq
SE 31	16	III	III	H10	0.7320					
SE 31	17	I	I	E08	0.3090					
SE 31	17	II	II	E08	0.3110	I	II	20.17	20.53	No Cq
SE 31	17	III	III	E08	0.3100					
SE 31	18	I	I	F08	0.4340					
SE 31	18	II	II	F08	0.4500	I	II	19.15	19.72	No Cq
SE 31	18	III	III	F08	0.4420					
SE 31	19	I	I	G08	0.4260					
SE 31	19	II	II	G08	0.4290	I	II	19.59	20.17	No Cq
SE 31	19	III	III	G08	0.4275					
SE 31	20	I	I	H08	0.5180					
SE 31	20	II	II	H08	0.4820	I	II	19.38	20.12	No Cq
SE 31	20	III	III	H08	0.5000					
SE 32	1	I	IV	A01	1.3650					
SE 32	1	II	V	A01	1.1820	I	II	16.05	17.04	No Cq
SE 32	1	III	VI	A01	1.2735					
SE 32	2	I	IV	B01	1.1760					
SE 32	2	II	V	B01	0.8730	I	II	18.43	19.31	No Cq
SE 32	2	III	VI	B01	1.0245					
SE 32	3	I	IV	C01	1.0170					
SE 32	3	II	V	C01	0.9690	I	II	17.51	18.28	No Cq
SE 32	3	III	VI	C01	0.9930					
SE 32	4	I	IV	D01	1.0090	I	II	17.25	18.07	No Cq

Sample	Sub	ELISA Replicate	ELISA plate	Well	OD	RNA extraction replicate	PCR plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 32	4	II	V	D01	0.9400					
SE 32	4	III	VI	D01	0.9745					
SE 32	5	I	IV	E01	1.2170					
SE 32	5	II	V	E01	1.1700	I	II	16.48	17.22	No Cq
SE 32	5	III	VI	E01	1.1935					
SE 32	6	I	IV	F01	1.1770					
SE 32	6	II	V	F01	1.0680	I	II	16.84	17.56	No Cq
SE 32	6	III	VI	F01	1.1225					
SE 32	7	I	IV	G01	0.9900					
SE 32	7	II	V	G01	0.9320	I	II	17.80	18.46	No Cq
SE 32	7	III	VI	G01	0.9610					
SE 32	8	I	IV	H01	1.0390					
SE 32	8	II	V	H01	1.0360	I	II	17.01	17.62	No Cq
SE 32	8	III	VI	H01	1.0375					
SE 32	9	I	IV	A02	0.9660					
SE 32	9	II	V	A02	0.9710	I	II	17.86	18.54	No Cq
SE 32	9	III	VI	A02	0.9685					
SE 32	10	I	IV	B02	1.0250					
SE 32	10	II	V	B02	1.1300	I	II	17.23	18.08	No Cq
SE 32	10	III	VI	B02	1.0775					
SE 32	11	I	IV	C02	1.0400					
SE 32	11	II	V	C02	1.0520	I	II	16.22	16.68	No Cq
SE 32	11	III	VI	C02	1.0460					
SE 32	12	I	IV	D02	0.8120					
SE 32	12	II	V	D02	0.8350	I	II	16.75	17.28	No Cq
SE 32	12	III	VI	D02	0.8235					
SE 32	13	I	IV	E02	0.8680					
SE 32	13	II	V	E02	0.9260	I	II	17.02	17.41	No Cq
SE 32	13	III	VI	E02	0.8970					
SE 32	14	I	IV	F02	1.0620					
SE 32	14	II	V	F02	1.1410	I	II	16.14	16.57	No Cq
SE 32	14	III	VI	F02	1.1015					
SE 32	15	I	IV	G02	1.1440					
SE 32	15	II	V	G02	1.2080	I	II	16.35	17.17	No Cq
SE 32	15	III	VI	G02	1.1760					
SE 32	16	I	IV	H02	1.1320	I	II	16.13	16.94	No Cq

Sample	Sub	ELISA Replicate	ELISA plate	Well	OD	RNA extraction replicate	PCR plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
SE 32	16	II	V	H02	1.2070					
SE 32	16	III	VI	H02	1.1695					
SE 32	17	I	IV	A03	0.9530					
SE 32	17	II	V	A03	0.9900	I	II	17.18	17.92	No Cq
SE 32	17	III	VI	A03	0.9715					
SE 32	18	I	IV	B03	1.0430					
SE 32	18	II	V	B03	1.1070	I	II	16.73	17.31	No Cq
SE 32	18	III	VI	B03	1.0750					
SE 32	19	I	IV	C03	1.0800					
SE 32	19	II	V	C03	1.2560	I	II	16.84	17.54	No Cq
SE 32	19	III	VI	C03	1.1680					
SE 32	20	I	IV	D03	0.9560					
SE 32	20	II	V	D03	1.1110	I	II	16.62	17.43	No Cq
SE 32	20	III	VI	D03	1.0335					
LMV negative SE (NPC used by CSP Labs)		I	I	A11	0.1280			ND	ND	ND
		II	I	B11	0.1310					
		III	I	C11	0.1200					
		IV	II	A11	0.1300			ND	ND	ND
		V	II	B11	0.1290					
		VI	II	C11	0.1270					
		VII	III	A11	0.1290			ND	ND	ND
		VIII	III	B11	0.1300					
		IX	III	C11	0.1235					
		X	IV	F03	0.1310			ND	ND	ND
		XI	IV	G03	0.1380					
		XII	IV	H03	0.1430					
		XIII	IV	A04	0.1300					
		XIV	IV	B04	0.1220					
		XV	IV	C04	0.1180					
		XVI	V	F03	0.1370			ND	ND	ND
		XVII	V	G03	0.1450					
		XVIII	V	H03	0.1450					
		XIX	V	A04	0.1300					
		XX	V	B04	0.1270					
		XXI	V	C04	0.1270					
		XXII	VI	F03	0.1340			ND	ND	ND

Sample	Sub	ELISA Replicate	ELISA plate	Well	OD	RNA extraction replicate	PCR plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
		XXIII	VI	G03	0.1415					
		XXIV	VI	H03	0.1440					
		XXV	VI	A04	0.1300					
		XXVI	VI	B04	0.1245					
		XXVII	VI	C04	0.1225					
LMV PC used by CSP Labs		I	I	D11	1.2090			ND	ND	ND
		II	I	E11	1.2050					
		III	I	F11	1.2150					
		IV	II	D11	1.4100			ND	ND	ND
		V	II	E11	1.3420					
		VI	II	F11	1.3330					
		VII	III	D11	1.3095			ND	ND	ND
		VIII	III	E11	1.2735					
		IX	III	F11	1.2740					
		X	IV	D04	1.2910			ND	ND	ND
		XI	IV	E04	1.3550					
		XII	IV	F04	1.3630					
		XIII	V	D04	1.3650			ND	ND	ND
		XIV	V	E04	1.3860					
		XV	V	F04	1.4150					
		XVI	VI	D04	1.3280			ND	ND	ND
		XVII	VI	E04	1.3705					
		XVIII	VI	F04	1.3890					
EB (NC)		I	I	G11	0.0840			ND	ND	ND
		II	I	H11	0.0820					
		III	II	G11	0.0810			ND	ND	ND
		IV	II	H11	0.0810					
		V	III	G11	0.0825			ND	ND	ND
		VI	III	H11	0.0815					
		VII	IV	G04	0.0820			ND	ND	ND
		VIII	IV	H04	0.0800					
		IX	V	G04	0.0830			ND	ND	ND
		X	V	H04	0.0810					
		XI	VI	G04	0.0825			ND	ND	ND
		XII	VI	H04	0.0805					

Sample	Sub	ELISA Replicate	ELISA plate	Well	OD	RNA extraction replicate	PCR plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
LMV NAKT + LMV RZ 2 Synth (PAC; PCR rep. I)[a]							I	27.12	27.72	No Cq
LMV NAKT + LMV RZ 2 Synth (PAC; PCR rep. II) [a]							I	27.23	27.91	No Cq
LMV NAKT + LMV RZ 2 Synth (PAC; PCR rep. III) [a]							I	26.62	27.36	No Cq
LMV NAKT + LMV RZ 2 Synth (PAC; PCR rep. I) [a]							II	27.41	28.19	No Cq
LMV NAKT + LMV RZ 2 Synth (PAC; PCR rep. II) [a]							II	27.28	28.13	No Cq
LMV NAKT + LMV RZ 2 Synth (PAC; PCR rep. III) [a]							II	27.39	28.10	No Cq
NEC						II	II	37.30	37.31	No Cq
NEC						I	I	No Cq	No Cq	No Cq
NEC						I	II	39.35	No Cq	No Cq
NEC						II	I	No Cq	No Cq	No Cq
PCR grade water (NTC; PCR rep. V)							II	No Cq	No Cq	No Cq
PCR grade water (NTC; PCR rep. VII)							II	No Cq	No Cq	No Cq
PCR grade water (NTC; PCR rep. VIII)							II	No Cq	No Cq	No Cq
PCR grade water (NTC; PCR rep. I)							I	No Cq	No Cq	No Cq
PCR grade water (NTC; PCR rep. II)							I	No Cq	No Cq	No Cq

Sample	Sub	ELISA Replicate	ELISA plate	Well	OD	RNA extraction replicate	PCR plate	LMV (LMV-NAKT; FAM)	LMV (LMV-RZ2; VIC)	DLVd (DaVd1; Texas Red)
PCR grade water (NTC; PCR rep. III)							I	No Cq	No Cq	No Cq
PCR grade water (NTC; PCR rep. IV)							I	No Cq	No Cq	No Cq
PCR grade water (NTC; PCR rep. V)							I	No Cq	No Cq	No Cq
PCR grade water (NTC; PCR rep. VI)							I	No Cq	No Cq	No Cq
PCR grade water (NTC; PCR rep. I)							II	No Cq	No Cq	No Cq
PCR grade water (NTC; PCR rep. II)							II	No Cq	No Cq	No Cq
PCR grade water (NTC; PCR rep. III)							II	No Cq	No Cq	No Cq
PCR grade water (NTC; PCR rep. IV)							II	No Cq	No Cq	No Cq

[a] A mixture of synthetic ssDNA with a concentration of 6×10^3 copies per µL.