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Detection of *Candidatus* Liberibacter solanacearum on Carrot Seed by Seed Extract PCR

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Background

Candidatus Liberibacter solanacearum (Lso) is a bacterial pathogen with multiple hosts including potato, tomato, celery and carrot. Five different haplotypes of this bacterium are described of which haplotypes C, D and E are pathogenic on carrot, celery (Haapalainen, 2014) and other *Apiaceae* (Teresani et al., 2014; Hajri et al., 2017), and recently haplotype F is described on potato (Swisher Grimm and Garczynski, 2019).

The disease observed in carrots is known as 'Yellowing decline' and vegetative disorders (Munyaneza et al., 2010). Symptoms in carrot have been hypothesized to be the result of a combination of psyllid sucking damage and the plant's response to a toxin produced by Lso or a direct result of Lso itself. Although the exact interaction remains unclear (Haapalainen, 2014) part of symptom expression is still attributed to the psyllid.

Lso lives within the phloem and is transmitted by host-specific psyllids. Bertolini et al. (2014) reported that Lso could also be transmitted through carrot seeds. However, several other studies have challenged the carrot seed transmission of Lso (Haapalainen, 2014; Loiseau et al., 2017; Oishi et al., 2017; Mawassi et al., 2018). Therefore, transmission of Lso in carrot seed is debatable.

Lso is fastidious and cannot be cultured on artificial media, and therefore, the detection of Lso is solely dependent on culture independent methods. Li et al. (2009) developed a qPCR assay that directly detects Lso in DNA extracts from infected potato and tomato plant tissues. This qPCR assay has also been applied to the detection of Lso in DNA extracts from carrot seeds (Loiseau et al., 2017). However, this qPCR assay is not in full conformity with ISHI-Veg best practices and has not yet been fully validated.

In 2015 ISHI-Veg developed a qPCR method for the detection of Lso in carrot seeds using an Lso-specific primer-probe from Li et al. (2009). Although the performance of the two main components of the ISHI-Veg method, namely the DNA extraction and the Li et al. (2009) qPCR were validated independently of ISHI-Veg for analytical specificity, sensitivity and repeatability, the ISHI-Veg method was not validated for reproducibility. Proof of viable Lso bacterial cells in a seed assay is still an area that needs to be explored and a "direct" test that confirms the viability and pathogenicity of Lso is desirable (see ISF's position on indirect seed health tests, http://www.worldseed.org).

ISHI-Veg initiated a research project to validate the detection method based on the qPCR assay developed by Li et al. (2009), that not only detects Lso directly from carrot seeds, but also fulfills the criteria for being an industry standard. This report presents validation data for the Seed Extract PCR (SE-PCR) method for the detection of Lso on carrot seeds. The data presented in the report were collected from peer reviewed literature (Li et al., 2009; Loiseau et al., 2017) and from independent experiments conducted at several laboratories. These data were organized according to the *ISHI-Veg Guidelines for the Validation of Seed Health Tests. Version 1, May 2018*, see Appendix A. Within each section, the data and results are arranged by lab/study.

1. Analytical specificity

1.1 Definition: Ability of an assay to detect the target pathogen(s) (inclusivity) while excluding non-target organism(s) (exclusivity) (Appendix A).

1.2 Requirements: The analytical specificity of the assay is confirmed when the assay shows positive only with targets and negative only with non-targets included in the study.

1.3 Method of data collection: No experiment was conducted at ISHI-Veg member labs for analytical specificity. The data presented for specificity were collected from peer reviewed literature and from external laboratories.

1.3.1 Supporting data - 1

Method: The primers and probe combination LsoF+HLBp+HLBr (shortened to "Lsopr") used in the proposed SE-PCR method was adopted from the study by Li et al. (2006, 2009; Table 1). The methods and results from Li et al. (2009) study are briefly explained below (for complete details refer to Li et al. (2009).

Table 1. Lso specific primers and probe sequences.

Primer/probe name	Sequences 5'- 3'	Source
LsoF	gTC gAg CgC TTA TTT TTA ATA ggA	Li et al., 2006, 2009
HLBr	gCg TTA TCC CgT AgA AAA Agg TAg	
HLBp	FAM-AgA Cgg gTg AgT AAC gCg-BHQ1	

The authors first confirmed the sequence specificity of "Lsopr" to Lso *in silico* and then validated it against known target and non-target pathogens. In total 18 Lso isolates from different hosts and locations were included as targets (Table 2). Three isolates each from three closely related species pathogenic on citrus, namely *Ca.* L. asiaticus, *Ca.* L. africanus and *Ca.* L. americanus, were included as non-targets. Additionally, phytoplasmas and viruses that are commonly associated with potato and *Xylella fastidiosa* from grapevine & citrus were also included as non-targets (Table 2). Amplification of the plant mitochondrial cytochrome oxidase (*CoXfpr*) gene was included as an internal positive control (processing control). Positive and negative amplification controls were included in every qPCR reaction, and each reaction was performed in triplicate.

Table 2. Lso and non-Lso organisms tested by Li et al. (2009) with the "Lsopr" qPCR.

Pathogen	Host	isolates tested	Location	qPCR amplification [a]
Specific isolates				
Ca. Liberibacter solanacearum	Potato	16	Texas, Nebraska, Colorado	+
Ca. Liberibacter solanacearum	Tomato	2	Green house	+
Closely related isolates				
Ca. Liberibacter asiaticus	Citrus	3	Florida, Japan, Brazil	-
Ca. Liberibacter africanus	Citrus	3	South Africa	-
Ca. Liberibacter americanus	Citrus	3	Brazil	-
Other pathogens				
Potato leaf roll virus	Potato	1	Unknown	-
Clover proliferation phytoplasma	Potato	1	Oregon	-
Ca Phytoplasma americanum	Potato	1	Nebraska	-
Xylella fastidiosa	Grapevine	1	Brazil	-
Xylella fastidiosa	Citrus	1	California	-

[a] + = Lso positive, - = Lso negative.
Reaction considered positive (+) only if the Cq value is <37. Each qPCR reaction was performed in triplicate.

Results: Positive amplification was obtained with all 18 Lso isolates tested in qPCR assays with “Lsopr” (Samples with a Cq value below 37 were considered as positive for Lso). However, no amplification was observed with any of the non-target pathogens tested (Table 2). Positive amplification was obtained with every target and non-target organisms tested for the internal positive control gene (*CoXfpr*), suggesting that the negative amplification observed with non-target organisms with “Lsopr” is only due to the specificity of the “Lsopr” to Lso and not due to the defect of DNA and/or PCR inhibitors. Taken together these results clearly highlighted the specificity of “Lsopr” to Lso.

1.3.2 Supporting data - 2

Method: While the data presented above revealed the potential of the “Lsopr” assay to detect Lso from potato and tomato plant tissue, there were no Lso isolates from carrot included in the evaluation. Loiseau (2017) conducted an inter-laboratory comparative test (CT) to evaluate five different publicly available PCR methods for detecting Lso, including the “Lsopr” assay, using DNA extracted from positive and negative seeds of carrot and other hosts, such as celery, tomato and potato. DNA from different reference Lso isolates from several sources including the Haplotypes known in 2017 were also part of the study in which 26 labs from 14 countries participated.

Results: While data from the CT awaits further analysis, preliminary results show that positive amplification was observed with DNA from each Lso haplotype tested by qPCR, highlighting the selectivity of “Lsopr” assay in detecting the different haplotypes from different hosts, including carrot. No amplification was observed with any of non-target organisms tested, once again confirming its specificity to Lso.

1.3.3 Supporting data - 3

The recent publication from Ilardi et al., (2018) further documented the efficacy of the “Lsopr” assay. The “Lsopr” assay successfully detected all 37 Lso isolates collected from different host species (e.g. carrot, parsley, and celery) belonging to 15 different cultivars. No false positives were observed when it was tested with many known non-target prokaryotes. Loiseau et al. (2017) also documented the proficiency of the “Lsopr” (Li et al., 2009) assay for detecting Lso from carrot seeds. In this study the “Lsopr” assay was successfully used to both identify Lso infected seed lots and determine the transmission of Lso through carrot seeds.

Conclusion

No method currently exists for culturing Lso on media, which is the major limitation for developing a detection method that requires rigorous screening against a wide range of targets. However, despite this limitation the “Lsopr” assay (Li et al., 2009) has been validated against a collection of target and non-target organisms; 60 Lso isolates from plant tissue of three different crops including carrot, and more than 19 relevant non-target organisms.

Furthermore, the “Lsopr” assay has been successfully used to characterize several Lso positive carrot seed lots (>10 seed lots; equivalent to 10 isolates) for sensitivity, selectivity and repeatability experiments (refer to Section 3, 4 & 5 for further details), adding more isolates from

carrot seed to the list of specificity. However, recently an experiment conducted by ISHI-Japan in association with NARO revealed that the "Lsopr" assay also cross reacts with *Ca*. Liberibacter asiaticus and *Ca*. Liberibacter crescens but, only at higher concentrations (> 10^6 copies /µl; data not shown). Because carrot is not a host for these two species, the "Lsopr" assay should still be considered as valid for detecting Lso from carrot seed. Taken together the data presented above clearly demonstrates that the "Lsopr" assay (Li et al., 2009) can detect the target pathogen (Lso) while excluding non-target organisms, fulfilling the ISHI-Veg validation guidelines that recommend testing of 20-30 isolates each from target and non-target organisms.

2. Analytical sensitivity

2.1 Definition: Smallest amount of the target pathogen that can be detected by the assay, i.e. the limit of detection (LOD) (Appendix A).

2.2 Requirements: The analytical sensitivity of the assay is fulfilled when the assay consistently detects the lowest amount of target included in the study through series of dilution experiments.

2.3 Method of data collection: The sensitivity data for the Lso specific primers and probes (the "Lsopr" assay) were collected from the peer reviewed literature (Li et al., 2009, Loiseau et al., 2017). However, the sensitivity data for the proposed SE-PCR detection method were collected from experiments conducted in ISHI-Veg member labs. Data for the sensitivity of "Lsopr" assay and the SE-PCR are presented separately.

<u>Sensitivity of "Lsopr" assay</u>

2.3.1 Supporting data - 1

Method: Li et al. (2009) used a plasmid cloned with the Lso specific PCR fragment to determine the sensitivity of the "Lsopr" assay. They first amplified 1163 bp fragment of the 16S rDNA from Lso infected tissues using an Lso specific primer and then cloned this fragment into a plasmid, named pTXZC18. The number of copies of plasmid pTXZC18 (Lso) was determined based on the number of base pairs and molecular mass of base pair in double stranded DNA. Using this formula, the molecular mass of a single copy of pTXZC18 (Lso) was determined to be 5.658 X 10^{-6} pg. Two absolute standard curves were separately established through 10-fold serial dilutions of plasmid pTXZC18 DNA (from 9.92 X10^8 copies/µl to 99.2 copies/µl) with sterile water (Absolute standard curve-1) and with total DNA extracted from healthy potato plant tissue (Absolute standard curve-2). In addition, a 3rd standard curve was established with DNA extracted from potato samples naturally infested with Lso. For the 3rd standard curve, the number of copies of Lso in DNA extracted from naturally infested potato plant samples was determined using absolute standard curves (3.62 X 10^6), and then the standard curve was established with 10-fold serial dilutions under the background of DNA extract from healthy potato plants. All reactions were performed in triplicate.

Results: A correlation was observed between the DNA concentration and fluorescence with all three standard curves established (data not shown). The limit of detection (LOD) for the "Lsopr" assay was 10 and 100 copies of pTXZC18 (= Lso) /reaction under pure water and host DNA background, respectively. However, with naturally infested environmental samples, the LOD of "Lsopr" assay was 20 copies/reaction. Overall qPCR amplification efficiency of the "Lsopr" assay to detect Lso from the Lso infected plant material was 90.99% (Li et al., 2009). A recent study

(Ilardi et al., 2018) also used the same plasmid construct (pTXZC18 (= Lso) to determine the sensitivity of the "Lsopr" assay. The LOD of the "Lsopr" assay was determined to be 5 copies of Lso under four different seed extract backgrounds (i.e. carrot, parsley, celery and fennel).

2.3.2 Supporting data - 2

Method: Loiseau et al. (2017) studied the seed transmission of Lso in carrot using the "Lsopr" assay (Li et al., 2009). In this report data is presented from this study regarding the sensitivity of the "Lsopr" assay. To determine the correlation between the fluorescence and cell concentration the authors first generated a standard curve using Lso clones. The Lso specific PCR fragment amplified from DNA extracted from Lso infected carrot tissues (Li et al., 2009) was first cloned in plasmid and subsequently transformed into *E. coli*. The transformed *E. coli* was then used to establish a standard curve by 10-fold serial dilutions (from 2.7 X 10^7 to 2.7 X 10^1 cells). Three repetitions were performed for each dilution.

Results: The standard curve revealed a correlation between the quantification and the concentration with R^2 value of 0.993. With the established standard curve, the authors were able to successfully quantify the level of viable Lso in naturally infested seed lots, which ranged from 4.19 X 10^2 to 4.08 X 10^3 cells/seed.

<u>**Sensitivity of the whole proposed ISHI-Veg SE-PCR method**</u>

Data for the assessment of the analytical sensitivity of the SE-PCR method were collected from experiments conducted at ISHI-Veg member labs in Japan and in the Netherlands.

2.3.3 Supporting data - 3

Method: The ISHI-Japan team conducted a comparative test with four laboratories to determine the sensitivity of the ISHI-Veg method and to validate the sub-sample size (10,000 seeds/sub-sample) recommended in ISHI-Veg's method. The findings from this study have been submitted to ISHI-Veg (Enya et al., 2017). A portion of the results from the submitted report is presented here for the sensitivity of the method.

In total three sub-sample sizes were tested in this experiment: 1) 10,000 seeds (as recommended in ISHI-Veg method) 2) 1,000 seeds and 3) 500 seeds. Three carrot seed lots that were known to be infected with Lso were first tested to determine the level of Lso infection. 50 seeds were randomly selected from each of these lots and tested individually for Lso infection using the "Lsopr" assay (Li et al., 2009). Based on the results, seed lot 2 with infection level of 38% was chosen for sub-sample size and sensitivity experiments.

For each sub-sample size tested, five different levels of Lso infection (0.0%, 0.5%, 5.0%, 20.0%, and approx. 40.0%) were created manually by mixing the Lso infected seeds from seed lot 2 with healthy seeds (Table 3). Thousand Seed Weight (TSW) was used to prepare different levels of infection rate. Each infection level (treatment) was replicated three times. Each sample was processed according to the ISHI-Veg method, and the DNA was extracted using a Qiagen DNeasy Plant Mini kit by following the manufacturer's protocol. Two qPCR reactions, one specific for Lso according to the ISHI-Veg method and another one specific for plant COX gene as internal processing control were performed with DNA from each sample. In addition, two master mixes, ABI-TaqMan universal master mix II and ABI-TaqMan gene expression master mix I were compared (Table 4). In this table the data is presented only for the sub-sample size of 10,000 seeds, which is the recommended size by ISHI-Veg method.

Statistics: The data was analyzed only qualitatively (positive vs negative) based on Cq values. Samples with Cq values below 40 with the Lso specific assay were considered as positive for Lso.

Results: With the ABI-TaqMan gene expression master mix I, positive amplification was consistently observed at all laboratories, with all four levels of Lso infection tested (Table 4). In contrast, with ABI-TaqMan universal master mix II, the Lso detection among replicated samples was inconsistent, and at least one of the three replicated samples for infection levels 0.5%, 20.0% and appr. 40.0% was not detected (false negative) by at least one of the four participating laboratories, which suggests considering the use of master mix-I over master mix-II. The negative seed lots were properly identified with both master mixes in all laboratories and the positive processing control (COX gene) was amplified in every sample.

Table 3. Creation of different infection rates for each sub-sample size (Enya et al. 2017).

Appr. Infection rate (%)[a]	Sub-sample size (No. of seeds) [b]					
	500		1000		10000	
	Healthy seeds	Infected seeds	Healthy seeds	Infected seeds	Healthy seeds	Infected seeds
0.0	500	0	1000	0	10000	0
0.5	494	6	988	12	9875	125
5.0	438	62	875	125	8750	1250
20.0	250	250	500	500	5000	5000
40.0	0	500	0	1000	0	10000

[a] Different infection rates were created by mixing the seeds from known positive and negative seed lots and the percent infection was calculated based on the estimated infection rate of positive seed lot (appr. 40%).

[b] Three sub-sample sizes were tested.

2.3.4 Supporting data - 4

Method: Experiments were also conducted at an ISHI-Veg member lab (Lab 1) to determine the detection limit of the proposed ISHI-Veg method. A known Lso positive (P20.142) and negative (P20.118) carrot seed lot were used in the experiment.

Experiment 1: To determine the percent of Lso infection in the positive seed lot, 20 seeds randomly chosen from this seed lot were tested individually for Lso infection. Seven seeds from negative seed lot were also tested as negative control. First, each seed was pulverized individually using hammer (equivalent to stomacher method in ISHI-protocol) and mixed with 500 µl PBS containing Acidovorax cattleyae (Acat) as positive processing control. DNA was extracted from each processed seed with a Roche MagnaPure MP96 DNA extraction kit. Based on earlier experiments, DNA extraction with Roche MagnaPure MP96 kit resulted in the same or higher DNA yield than with the Qiagen DNeasy Plant Mini DNA extraction kit which is described in the ISHI-Veg method. qPCR reactions were performed as described in the ISHI-Veg method for both Lso and Acat. Negative and positive amplification controls were properly included in the experiment.

Results: The qPCR assay confirmed that the seed lot P20.142 was positive for Lso, and the infection rate was 60%. No Lso was detected from any of seven seeds tested from negative seed lot P20.118. The processing control (Acat) was successfully detected with every sample tested as expected (Table 5).

Table 4. qPCR results from comparative test that used sub-sample size of 10,000 seeds (Enya et al., 2017).

Infection rate (%)	Sub Sample [a]	Type of qPCR Master mix[b]													
		Universal Master mix II								Gene expression Master mix I					
		Lab A		Lab B		Lab C		Lab D[c]		Lab A		Lab B		Lab C	
		Cq	Results	Cq	Results	Cq	Results	Cq	Results	Cq	Results	Cq	Results	Cq	Results
0.0	S-1	ND	N	ND	N	ND	N	ND	N	ND	N	ND	N	ND	N
	S-2	ND	N	ND	N	ND	N	ND	N	ND	N	ND	N	ND	N
	S-3	ND	N	ND	N	ND	N	ND	N	ND	N	ND	N	ND	N
0.5	S-1	38.89	P	40	N	37.02	P	40.72	N	34.57	P	36.2	P	35.14	P
	S-2	35.24	P	38.11	P	37.98	P	37.85	P	36.07	P	37.63	P	36.61	P
	S-3	34.22	P	37.36	P	35.87	P	38.56	P	35.05	P	35.21	P	35.32	P
5.0	S-1	31.31	P	34.41	P	33.33	P	37.85	P	31.79	P	32.84	P	31.88	P
	S-2	31.21	P	33.62	P	33.58	P	34.29	P	32.35	P	33.25	P	32.53	P
	S-3	30.66	P	35.32	P	33.26	P	36.79	P	31.49	P	32.45	P	32.00	P
20.0	S-1	35.43	P	39.31	P	34.46	P	41.44	N	29.47	P	30.61	P	30.18	P
	S-2	29.66	P	32.13	P	31.29	P	33.67	P	29.86	P	30.78	P	30.65	P
	S-3	29.77	P	35.59	P	32.1	P	34.98	P	29.1	P	30.22	P	29.79	P
Appr. 40.0	S-1	29.65	P	34.68	P	30.77	P	34.57	P	28.19	P	29.52	P	29.06	P
	S-2	35.84	P	40	N	34.4	P	ND	N	27.68	P	28.79	P	28.17	P
	S-3	28.78	P	32.86	P	30.44	P	33.47	P	28.23	P	29.49	P	28.78	P

[a] Three sub-samples (S-1, S-2, and S-3) were tested for each infection rate

[b] Two master mixes were tested: ABI TaqMan Universal Master mix II, and ABI TaqMan gene expression master mix I. Sample with Cq value <40 is considered as positive for Lso.

[c] Lab D performed the Lso assay only with Universal Master mix II. False negatives are highlighted in yellow.

ND=No Cq value, N=Negative, P=Positive.

Experiment 2: To create seed samples of 10,000 seeds with different levels of Lso infection, seeds from the Lso positive seed lot P20.142 were mixed with seeds from the healthy lot P20.118. In total 9 levels of Lso infection were created (0, 0.06, 0.15, 0.30, 0.60, 6.00, 15.00, 30.00, and 60.00%) as shown in Table 6. The percent infection rate was calculated based on the infection rate of positive seed lot of 60% as determined in experiment 1. Three replicates from each Lso infection level were tested. Each sample was spiked with Acat and processed according to the proposed method. For spiking, a standardized Acat stock (100 µl /100 ml of extraction buffer) was added to get a Cq value around 30. DNA was extracted with a Roche MagnaPure MP96, and the qPCR reactions were performed as described in the protocol.

Statistical analysis: The data was analyzed only at qualitative level (positive vs negative) based on Cq values. Samples with Cq values below 40 with Lso specific assay were considered as positive for Lso.

Results: The results in Table 6 clearly showed that Lso was detected in at least two of three replications tested for every level of infection tested. Lso was detected in all 3 replicates (100%) from samples containing Lso infected seeds from 25 to 5,000/ 10,000 seeds. This result clearly suggested that the proposed ISHI-Veg method is sensitive enough to detect infection level from 0.15% with 100% confidence.

2.4 Conclusion

The “Lsopr” assay from Li et al. (2009) was efficient in detecting Lso from naturally infested Leaf (potato) and carrot seed samples. The limit of detection of “Lsopr” assay was 20 copies of Lso-target in environmental samples (Li et al., 2009).

It is also evident that the proposed ISHI-Veg method is sensitive enough to detect Lso at infection rate of 0.15% in sub-sample size of 10,000 carrot seeds. The study by Ilardi et al., 2018 validated the sample size specified in the protocol (20,000 seeds, 10,000 seeds/subsample) to successfully detect Lso from infected carrot seed lot.

Table 5. Infection rate of positive seed lot used in the sensitivity experiment.

Sample Id[a]	No. of seeds tested	Cq values [b]	
		Lso	Acat
Lso positive seed lot: P20.142	1	34.72	28.07
	2	-	28.06
	3	-	28.43
	4	33.92	28.23
	5	36.12	27.62
	6	34.53	27.76
	7	-	27.62
	8	-	28.08
	9	-	27.75
	10	34.79	28.29
	11	29.86	28.11
	12	34.54	28.40
	13	34.59	27.84
	14	-	27.71

Sample Id[a]	No. of seeds tested	Cq values [b]	
		Lso	Acat
	15	33.39	28.07
	16	29.19	27.85
	17	-	27.54
	18	32.56	27.81
	19	31.10	28.11
Lso infection rate: 12/20 (60%)	20	-	28.23
Lso negative seed lot: P20.118	1	-	28.49
	2	-	28.50
	3	-	28.41
	4	-	28.20
	5	-	28.17
	6	-	28.17
Lso infection rate: 0/7 (0%)	7	-	28.36
NEC (Negative extraction control)		-	27.64
PAC-Lso (Positive amplification control for Lso)		31.8	-
PAC-Acat (Positive amplification control for Acat)		-	28.75
NAC (Negative amplification control)		-	-

[a] P20.142; Lso positive seed lot, 20 seeds were tested individually for Lso. P20.118; Lso negative seed lot, 7 seeds were tested individually.

[b] For each sample one duplex qPCR was performed with Lso and Acat specific primers and probe. Cq values <40 is considered positive for Lso. - = No amplification.

Table 6. The limit of Lso detection in sub-sample size of 10,000 seeds with ISHI-Veg protocol.

Proportion of Lso+ and Lso- seeds in 10,000 seeds [a]		Infection rate (%) [b]	Replications	Cq values [c]	
No. of Lso+ seeds	No. of Lso- seeds			Lso	Acat
0	10,000	0.00 (0.00)	R1	ND	30.73
			R2	ND	30.70
			R3	ND	30.90
10	9,990	0.10 (0.06)	R1	34.33	30.80
			R2	33.18	30.98
			R3	ND	30.84
25	9,975	0.25 (0.15)	R1	31.61	30.69
			R2	32.03	31.06
			R3	34.44	30.56
50	9,950	0.50 (0.30)	R1	31.56	30.61
			R2	31.80	32.66
			R3	31.92	30.75
100	9,900	1.00 (0.60)	R1	32.51	30.96
			R2	31.95	30.47
			R3	32.54	31.35

Proportion of Lso+ and Lso- seeds in 10,000 seeds [a]		Infection rate (%) [b]	Replications	Cq values [c]	
No. of Lso+ seeds	No. of Lso- seeds			Lso	Acat
1,000	9,000	10.00 (6.00)	R1	28.15	30.51
			R2	28.21	30.39
			R3	28.21	30.69
2,500	7,500	25.00 (15.00)	R1	26.97	30.28
			R2	26.97	29.90
			R3	26.46	30.19
5,000	5,000	50.0 0(30.00)	R1	25.96	30.51
			R2	25.58	30.05
			R3	25.74	29.89
10,000	0	100.00 (60.00)	R1	24.40	30.26
			R2	25.27	29.91
			R3	NT	NT
PEC Positive extraction control				NT	NT
NEC Negative extraction control				ND	31.05
PAC-Lso Positive amplification control for Lso				30.75	ND
PAC-Acat Positive amplification control for Acat				ND	28.12
NTC Negative amplification control				ND	ND

[a] The seeds from Lso+ and Lso- seed lots were mixed to create different levels of infection.

[b] The values in the parenthesis are actual percent infection rate calculated based on the infection rate of the Lso+ seed lot (60%).

[c] Cq values <40 with Lso specific assay is considered as positive for Lso. Cq values <35 with Acat specific assay is considered as positive for Acat.

ND=No Cq value, NT=Not tested

3. Selectivity

3.1 Definition: The effect of different seed matrices on the ability of the method to detect target pathogen(s) (Appendix A).

3.2 Requirements: The selectivity criterion is fulfilled when the assay consistently detects the varying level of targets under different seed matrices included in the study.

3.3. Method of data collection: The selectivity data for the proposed SE-PCR detection method were collected from experiments conducted in ISHI-Veg member labs.

3.4 Supporting data

Method: An experiment was conducted at Lab 2 in the Netherlands to determine the impact of different seed matrices on the proposed method. One known Lso-positive carrot seed lot and four known Lso-negative carrot seed lots (Lot 1, 2, 3 and 4) belonging to 4 different carrot cultivars (= matrices) were used in the experiment. The level of Lso in the positive seed lot was unknown, and therefore, the infection level was theoretically assumed to be 100 % in seed extract from positive seed lot.

Ten thousand seeds each from the positive seed lot, and the four negative seed lots were processed separately according to the proposed method to obtain seed extracts. Seed extract from the positive seed lot was diluted with seed extract from the negative seed lot to obtain the theoretical percent infection level of 100%, 25%, 10%, 1%, 0.1%, 0.01% and 0% as shown in the Table 7. In the experiment a total of 4 dilutions were performed separately with seed extract from each cultivar in association with seed extract from the positive seed lot. *Acidovorax cattleyae* was used as the Internal Amplification Control. Acat suspension prepared from 3-7 days old colonies on YDC was added to each dilution series at the concentration of $OD_{600} = 0.5 \pm 0.1$.

DNA extraction and subsequent qPCRs were performed as described in the proposed protocol. Two replications were performed for each dilution. This experiment was repeated (Experiment I and II).

Table 7. Dilution scheme used in creating different infection levels of Lso

Theoretical Lso infection percentage [a]	Max. number of theoretical positive seeds	Composition of seed wash	
		Negative seed wash (µl)	Positive seed wash (µl)
100% (undiluted)	10,000	0	2222
25%	2500	1500	500
10%	1000	2000	220 from 100%
1%	100	2000	220 from 10%
0.1%	10	2000	220 from 1%
0.01%	1	2000	220 from 0,1% [b]
0%	0	2000	0

[a] Different level of Lso infection was created by diluting the seed extract from positive seed lot with seed extract from negative seed lot.

[b] 220 µl is discarded to get a final volume of 2ml.

Statistical analysis: The data were analyzed only at qualitative level (positive vs negative) based on Cq values. Samples with Cq values below 40 with Lso specific assay were considered as positive for Lso.

Results: The results are shown in Table 8. There was no difference among seed matrices tested, and the Lso was successfully detected under all four matrices tested at 'theoretical' contamination level from 100 % to 0.1%. The Cq values did not differ among seed matrices tested at contamination level from 100% to 0.1% (Table 8). However, at infection level 0.01% the Lso detection became inconsistent within each seed matrix. No amplification was observed with seed extract from all four matrices tested with 0% Lso. This experiment clearly suggested that seed matrices did not influence the outcome of the proposed method in detecting Lso from carrot seeds.

The internal amplification control (IAC; Acat) was amplified with seed extracts from all four Lso-negative cultivars/seed lots tested (Appendix B, Table B1 to B5). However, Acat was not amplified with seed extract from the 100% positive seed lot or when this lot was mixed with seed extracts from negative seed lots of Lot 2, Lot 3 and Lot 4 at 25% (Tables B3, B4 & B5). The absence of amplification of IAC in the positive seed lot could possibly be either due to the presence of PCR inhibitor interfering with the Acat assay or due to competition between Lso and Acat assays. As Lso was successfully amplified from these extracts it is likely that competition is due to the

inhibition of the IAC. The IAC was amplified from every other contamination level, including the 25% sample prepared under seed lot 1 extract background. These results clearly showed that IAC control worked well with the four negative seed matrices tested.

Table 8. Detection of Lso under different carrot seed matrices

Percent of Lso contamination [a]	Cq Values [b]							
	Lot1 [c]		Lot2 [c]		Lot3 [c]		Lot4 [c]	
	Exp-I	Exp-II	Exp-I	Exp-II	Exp-I	Exp-II	Exp-I	Exp-II
100%	24.49	28.96	24.49	28.96	24.49	28.96	24.49	28.96
25%	25.04	26.04	23.83	26.15	24.02	26.12	25.32	25.18
10%	26.51	27.33	26.78	27.08	26.98	27.75	26.90	26.06
1%	30.25	31.23	31.06	31.63	28.87	31.66	29.83	29.11
0.1%	34.76	34.64	33.66	34.92	32.66	36.13	33.07	31.92
0.01%	ND	38.62	37.67	ND	ND	ND	ND	37.71
0%	ND	ND	ND	ND	ND	ND	ND	ND

[a] Seed extract from the same Lso positive seed lot (100%) is diluted with seed extract from Lso negative seed lots to create different theoretical level of Lso infection, and therefore, Cq value for 100% infection (positive seed lot) is common for every seed lot

[b] Each value is the average of two replications. Samples are considered positive for Lso if Cq value is < 40. Dilution experiment was performed twice (Exp-1 and Exp-II) for each cultivar. ND=No Cq value

[c] Four known negative seed lots belonging to 4 different varieties.

To determine if there is any PCR inhibitor in undiluted direct seed extract from the positive batch, a 1:10 dilution was additionally prepared for each treatment described in Table 8, and subsequently tested in qPCR for amplification (Tables B1 to B5). Undoubtedly, the 1:10 dilution enhanced the amplification of IPC, and a Cq value was observed with every sample that was negative for Lso when tested as undiluted, except the contamination level 100% where a Cq value was not obtained for every sample even after dilution (Tables B1 to B5) for the Acat. It is clearly suggested that there is a potential inhibitor in this seed lot 1 (100% infected batch) which specifically interferes with the IAC assay, and the 1:10 dilution greatly enhanced the amplification in most of the cases tested. Although, ISHI-veg method expects positive amplification for Acat with every sample spiked with Acat (positive processing control), there is no reason to reject the result as the Lso specific assay revealed a clear amplification for Lso.

3.5 Other supporting data

Data from the sensitivity experiments conducted at a lab in the Netherlands and ISHI-Japan helped to validate the selectivity of the proposed method. The naturally infested seed lots used at Lab 1 (1 seed lot) and ISHI-Japan (3 seed lots) for their sensitivity experiments are completely different from the one tested at Lab 2 for selectivity, and for the level of infestation, variety, location etc. The proposed ISHI-Veg method effectively detected Lso from all the carrot seed lots tested at Labs A, B and ISHI-Japan. Additionally, another 12 different seed lots were tested at Lab 4 (see Table 9). Furthermore, Ilardi et al. (2018) showed that the sensitivity of the “Lsopr” assay was consistent (5 copies) under all four different seed matrices tested (i.e. carrot, parsley, celery and fennel).

Table 9. Data from repeatability experiment.

Seed lot[a]	Infection level	Cq values									
		Sub sample [a]									
1	High	28.10	29.25	28.24	28.71	28.44	28.90	29.00	29.54	28.94	28.97
2	High	28.80	28.59	28.74	28.93	28.89	28.73	30.29	30.00	30.05	28.77
3	High	29.94	28.79	28.13	28.41	30.18	30.00	29.77	29.8	28.72	29.15
4	Medium	30.06	30.08	30.85	30.24	30.57	30.53	32.12	31.64	32.37	31.30
5	Medium	30.76	31.04	30.43	30.82	31.01	31.52	31.67	31.53	30.80	31.71
6	Medium	30.58	30.64	30.71	31.17	30.91	30.21	33.41	31.01	32.05	30.87
7	Medium	30.67	30.59	30.61	31.09	31.30	30.78	30.55	31.95	32.41	33.43
8	Medium	30.72	31.57	31.10	31.18	31.56	31.08	31.27	31.98	31.89	31.39
9	Medium	30.48	30.86	31.56	30.67	31.66	31.93	31.06	31.74	31.85	32.08
10	Medium	30.60	30.45	30.96	32.63	31.52	31.00	32.24	30.81	32.36	31.52
11	Medium	31.84	32.01	31.88	32.23	32.33	32.52	31.12	33.47	33.37	32.15
12	Medium	33.26	32.85	32.01	32.71	32.73	33.03	32.45	32.45	32.38	32.60
13	Negative	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
14	Negative	ND	ND	ND	ND	ND	ND	ND	ND	41.66	ND

[a] Ten subsamples (10,000 seeds/subsample) were tested from each seed lot.
ND=No Cq value, Cq value <40 is considered as positive for Lso.

3.6 Conclusion

The proposed ISHI-Veg method has been tested against 5 different carrot matrices at Lab 2, 3 matrices at ISHI-Japan, 12 at Lab 4 and 1 matrix at Lab 1, and it successfully detected Lso from all seed matrices tested. The testing clearly validates the ISHI-Veg method as robust enough to detect Lso from infested carrot seeds regardless of carrot variety, laboratory and the level of Lso infestation in the seed.

4. Repeatability

4.1 Definition: Degree of similarity in results of replicates of the same seed lots when the method is performed with minimal variations in a single lab**.**

4.2 Requirements: This criterion is fulfilled when the method yields consistent results among multiple replicates (10 replicates) of the same sample.

4.3 Method of data collection: The repeatability data for the proposed SE-PCR detection method were collected from experiments conducted in an ISHI-Veg member lab.

4.4 Supporting data

Method: An experiment was conducted at Lab 4 in France to determine the repeatability of the proposed method. In total 14 carrot seed lots were used in this experiment with 3 highly positive seed lots, 9 medium positive seed lots and 2 negative seed lots for Lso. Ten subsamples of 10,000 seeds (= replicates) were tested from each seed lot. All ten subsamples of one seed lot were tested at the same time to reduce variations within one seed lot. Two lots were tested simultaneously. Subsample processing, DNA extraction and qPCR were performed as described

in the proposed method. A positive process control (Lso infected seeds) and a positive amplification control (DNA extracted from infected seeds) were included in each run.

Statistical analysis: The data was analyzed only at qualitative level (positive vs negative) based on Cq values. Samples with Cq values below 40 with Lso specific assay were considered as positive for Lso.

Results: The results are shown also in Table 9. The proposed method correctly identified all Lso positive and negative seed lots tested in this experiment. Lso was detected consistently in every subsample/replicate tested from both high and medium Lso positive seed lots. The range of Cq values observed for high and medium Lso positive seed lots were 28.10 - 30.18 and 30.21 - 33.43, respectively. No Lso was detected from any subsamples of negative seed lots tested.

4.5 Conclusions

The data from the repeatability experiment clearly shows that the proposed ISHI-Veg method is repeatable, and it consistently detects Lso from positive carrot seed lots without any variation among replicates.

5. Reproducibility

5.1 Definition: Degree of similarity in results when the method is performed across labs with replicates of the same seed lots.

5.2. Requirements: The repeatability of the method is fulfilled when the method is tested in several independent laboratories with same set of samples, and the results are consistent among the participating laboratories.

5.3 Methods and Results

Reproducibility was tested in a Comparative Test (CT) organized by Lab 1 in the Netherlands in which fourteen laboratories from 9 different countries participated (see Table 10).

Table 10. Participating laboratories in Lso carrot seed CT 2018.

Laboratory	Country
1 – 3	Netherlands
4 - 5	France
6	Spain
7 - 8	Japan
9	Italy
10	Brazil
11 - 12	USA
13	Australia
14	New Zealand

The bacterium *Acidovorax cattleya* (Acat) was used as an Internal Amplification Control (IAC) and Positive Extraction Control (PEC) to monitor the extraction of DNA and amplification in PCR. The use of an IAC is essential according to the ISF Best Practices for PCR assays in Seed Health Tests version 3.0 June 2018 (www.worldseed.org).

Laboratories were required to strictly follow the protocol (Appendix D), except where it was

explicitly stated that optimization or lab specific settings were allowed/required.

Seed samples and controls

Each laboratory received 21 sub-samples of 10,000 untreated carrot seeds each (see Table 11). The samples were composed by mixing healthy carrot seeds with naturally Lso-infected carrot seeds to obtain different levels of Lso infection (healthy, low, medium and high). The definition and determination for the levels of Lso infection are arbitrary and based on the different levels of Lso PCR signal (Cq) and the number of spiked Lso-infected carrot seeds in the samples based on earlier tests by the test organizer.

Table 11. Composition of carrot seed samples

Seed Lot	Lso Infection level	Sub samples	Sample codes	Composition of subsamples
A	healthy	3	1-9-17	10,000 seeds Lso- P20.118
B	low	3	2-10-18	9,990 seeds Lso- P20.118 + 10 seeds Lso+ P20.142
C	medium	3	3-11-16	9,500 seeds Lso P20.118 + 500 seeds Lso+ P20.142
D	high	3	4-12-19	10,000 seeds Lso+ P20.142
E	medium	3	5-13-21	9.500 seeds Lso P20.148 + 500 seeds Lso+ P20.142
F	low	3	6-14-15	9,990 seeds Lso- P20.148 + 10 seeds Lso+ P20.142
G	healthy	3	7-8-20	10,000 seeds Lso P20.118

The 21 samples of 10,000 seeds were prepared by the test organizer based on the seed count (number of seeds/gram), packed and randomly coded. Each participating lab also received an inactivated (boiled) Acat culture that was used to spike seed samples and served as an Internal Amplification Control (IAC) to validate a negative PCR and served as a Positive Extraction Control (PEC) to validate the DNA extraction process. A Positive Process Control (PPC) and a Negative Process Control (NPC) were included in the test package, as well as a Positive Amplification Control (PAC) for both Lso and Acat and a Non-Template Control (NTC). A homogeneity and stability test were performed by the Test Organizer before and after the comparative test on seed samples of each seed lot, the PPC and NPC.

An overview of the scheduled timeline in the test plan is presented in Table 12.

Table 12. Timeline for the CT Lso carrot seed 2018 (according to Test plan)

Time	Action	Person
June 2017	Draft test plan to ITG	Test organizers
July-September 2017	Statistical review test plant	Test organizers / Statistician
August-October 2017	Finalizing test plan	Test organizers
October-December 2017	Selection seed lots	Test organizers
December-February 2018	Testing samples	Test organizer
March-June 2018	Testing samples CT	Participants
September 2018	Present results	Test organizers
September-December 2018	Data analysis and writing report	Test organizers
ISHI meeting 2019	Finalization and reporting TCG	Test organizers

An overview of the schedule for sending the CT test package, the dates they were received, and the CT was performed by the participating laboratories is presented in Table 13.

Table 13. Overview of sending test package to labs participating in the CT

Lab Nr.	Date sending	#days travel	Date test started	Date test finalized	#days finalized after sending
1	14-5-2018	9	24-5-2018	25-5-2018	11
2	24-4-2018	2	13-6-2018	28-6-2018	65
3	24-4-2018	1	30-5-2018	1-6-2018	38
4	24-4-2018	1	1-5-2018	2-5-2018	8
5	27-7-2018	11	21-9-2018	23-9-2018	58
6	24-4-2018	2	11-6-2018	14-6-2018	51
7	24-4-2018	6	31-5-2018	25-6-2018	62
8	24-4-2018	3	2-8-2018	8-8-2018	106
9	24-4-2018	6	11-6-2018	14-6-2018	51
10	15-5-2018	99	27-8-2018	21-9-2018	129
11	19-6-2018	24	31-7-2018	4-9-2018	77
12	26-4-2018	13	18-10-2018	18-10-2018	175
13	5-9-2018	28	5-10-2018	25-10-2018	50
14	22-5-2018	1	19-6-2018	13-7-2018	52

STATISTICAL ANALYSIS

Homogeneity test

The homogeneity test results were analyzed using tools provided by ISTA: Seedcalc8 and probpossample-V1. These tools calculate the expected number of positive subsamples in the comparative test and stability test, respectively. Using the results of the homogeneity test, the infection rate in the sample was calculated for each non-homogenous sample. Using the infection rate and a probability of 95%, the expected number of positive subsamples for each infection level was calculated.

Stability test

The observed number of positive subsamples per Lso infection level were compared to the expected number of positive subsamples as calculated based on the homogeneity test.

Analysis of qualitative data

The primary goal of the test was to show that the method is fit for purpose when used in multiple laboratories on the same set of samples, and the results are reproducible over four infection levels: negative, low, medium and highly infected. The analysis was done at a qualitative level per sample: a final result of a sample being suspect (as a positive result cannot be confirmed with a direct method) or negative for Lso detection (Lso DNA not detected).

The expected lowest level of detection at each participating laboratory was 10 Lso infected seeds in a sample of 10,000 seeds (0.1%). This was based on earlier research results (M. Asma, personal communication ISHI-Veg meeting Egmond aan Zee 2015).

Sensitivity, specificity, accuracy and reproducibility were calculated using the following formulas:

$$\text{Sensitivity} = \Sigma PA / (\Sigma PA+\Sigma ND) \times 100; \text{Specificity} = \Sigma NA / (\Sigma NA+\Sigma PD) \times 100$$

$$\text{Accuracy} = (\Sigma NA+\Sigma PA) / (\Sigma PA+\Sigma NA+\Sigma PD+\Sigma ND) \times 100,$$ where:

PA = positive agreement (true positive)

ND = negative deviation (false negative)

NA = negative agreement (true negative)

PD = positive deviation (false positive)

Although there is no fixed rule, values over 80% for sensitivity, specificity and accuracy are deemed acceptable (ISTA, 2013). This minimum value is used here to determine whether a method is acceptable or not.

For each infection level category, accordance (repeatability of qualitative data, 'within laboratory agreement') and concordance (reproducibility of qualitative data, 'between laboratory agreement') were calculated using the method and tools developed by Langton et al. (2002). Accordance is tested by calculating the probability of having the same result within the same infection level and calculates the average of the probabilities across labs. Concordance will be calculated as the number of pairs giving the same test result within the same infection level.

COMPARATIVE TEST RESULTS

Analysis of homogeneity and stability test

Homogeneity test

A homogeneity test was done after sampling and packaging. For each of the seven seed lots, representing the four contamination levels (healthy, low-, medium- and high infected) ten samples of 10,000 seeds were tested. The homogeneity test showed that all healthy subsamples were negative, one sample low infection level subsample was positive, and all medium and high infection level subsamples were positive (see Table 14 and 15).

Stability test

The aim of the stability test is to control the stability of the infection status of samples. The test was performed by the test organizer when all participating laboratories had finished their tests. The stability test showed that 1 subsample of the healthy lot A was positive and that all high infection level subsamples were positive. For the 20 medium infected subsamples 19 subsamples were positive and for the 20 low infected subsamples 5 subsamples were positive (Tables 14 and 16). The results in Appendix B.3 show that the Cq values of the positive results for the healthy and low infection level are above 38.0. Therefore, it was concluded that the infection status of samples was stable.

Table 14. Results of the homogeneity and stability test for the seed extract PCR per seed lot and Lso infection levels. Results present the number of positive subsamples over the total number of tested subsamples based on Lso Cq cut-off <40.

Seed Lot	Lso Infection level	Homogeneity test results	Stability test results
A	healthy	0/10	1/10
B	low	0/10	4/10
C	medium	10/10	9/10
D	high	10/10	10/10
E	medium	10/10	10/10
F	low	1/10	1/10
G	healthy	0/10	0/10

Using the results of the homogeneity test and the "Impurity estimation and confidence interval tool" of Seedcalc 8.1 software, the expected sample infection rate for the low infection level seed lots was calculated to be 0.00%. Even when the results of the stability test were included, the sample infection rate was calculated to be 0.0%. This means that at a probability of 5%, the expected number of positive subsamples is zero for the low infection level. This makes this set of subsamples inappropriate for drawing conclusions on the performance of laboratories in their detection of Lso with this method. It was decided not to include the low infected seed lots B and F in the analysis of the Comparative test results.

In the other infected categories (medium and high) all subsamples are expected to be positive, and in the healthy category, none of the subsamples are expected to be positive.

Table 15. Results of the homogeneity test

sample	Cq Lso (Li)	Cq Acat (IAC)
A1	40.00	32.21
A2	40.00	31.14
A3	40.00	30.34
A4	40.00	30.46
A5	40.00	31.94
A6	40.00	31.68
A7	40.00	29.99
A8	40.00	30.22
A9	40.00	29.88
A10	40.00	30.62
B1	40.00	31.53
B2	40.00	30.84
B3	40.00	31.42
B4	40.00	31.80
B5	40.00	31.17
B6	40.00	32.65
B7	40.00	32.15
B8	40.00	32.02
B9	40.00	31.80
B10	40.00	32.19
C1	31.89	30.93
C2	32.56	30.97
C3	33.68	30.94
C4	32.66	31.64
C5	31.94	30.52
C6	34.33	31.52
C7	32.78	32.26
C8	31.53	31.06
C9	32.15	30.60
C10	31.65	31.12

sample	Cq Lso (Li)	Cq Acat (IAC)
E1	33.05	29.60
E2	33.04	30.21
E3	31.18	30.07
E4	31.59	29.72
E5	30.76	30.28
E6	31.67	30.11
E7	32.25	30.36
E8	31.87	30.24
E9	32.53	30.13
E10	31.60	30.16
F1	40.00	30.46
F2	40.00	30.49
F3	40.00	31.04
F4	40.00	30.71
F5	40.00	30.93
F6	40.00	30.58
F7	40.00	30.57
F8	40.00	30.01
F9	40.00	30.24
F10	36.16	30.34
G1	40.00	30.10
G2	40.00	30.00
G3	40.00	30.38
G4	40.00	29.94
G5	40.00	29.98
G6	40.00	29.93
G7	40.00	30.03
G8	40.00	29.90
G9	40.00	40.00
G10	40.00	40.00

sample	Cq Lso (Li)	Cq Acat (IAC)
D1	29.86	32.56
D2	29.21	32.02
D3	30.86	32.46
D4	31.05	33.09
D5	29.74	32.77
D6	31.75	32.97
D7	29.20	30.33
D8	29.18	31.06
D9	30.06	31.49
D10	30.59	32.21

sample	Cq Lso (Li)	Cq Acat (IAC)
NEC	40.00	31.25
NEC	40.00	31.03
NEC	40.00	30.58
NTC	40.00	40.00
PAC-Acat	40.00	28.98
PAC-Lso	31.64	40.00
PPC	29.06	30.89
PPC	29.11	31.47
PPC	28.79	30.99

Table 16. Results of the stability test

sample	Cq Lso (Li)	Cq Acat (IAC)
A1	40.00	35.86
A2	40.00	37.52
A3	40.00	34.55
A4	40.00	35.83
A5	40.00	35.94
A6	40.00	37.60
A7	38.74	35.41
A8	40.00	36.06
A9	40.00	36.33
A10	40.00	35.20
B1	40.00	36.59
B2	40.00	35.42
B3	40.00	34.84
B4	38.29	36.37
B5	40.00	36.50
B6	39.65	34.56
B7	40.00	34.97
B8	39.92	37.58
B9	38.00	36.80
B10	40.00	36.48
C1	40.00	40.00
C2	37.23	35.86
C3	35.46	35.50
C4	35.75	34.07
C5	35.70	34.72
C6	35.64	35.70
C7	36.93	40.00
C8	35.28	35.92

sample	Cq Lso (Li)	Cq Acat (IAC)
E1	35.58	35.11
E2	36.13	37.16
E3	34.49	35.01
E4	34.81	36.23
E5	35.16	35.72
E6	36.47	35.30
E7	36.39	40.00
E8	35.98	38.84
E9	35.46	36.77
E10	34.84	34.97
F1	40.00	35.68
F2	38.45	35.70
F3	40.00	34.18
F4	40.00	39.22
F5	40.00	38.69
F6	40.00	37.88
F7	40.00	39.17
F8	40.00	35.44
F9	40.00	38.63
F10	40.00	39.50
G1	40.00	35.03
G2	40.00	35.96
G3	40.00	35.73
G4	40.00	34.53
G5	40.00	37.33
G6	40.00	39.91
G7	40.00	40.00
G8	40.00	40.00

sample	Cq Lso (Li)	Cq Acat (IAC)
C9	36.10	36.79
C10	35.79	35.45
D1	31.99	35.72
D2	30.85	35.43
D3	32.06	35.08
D4	31.16	34.89
D5	31.46	35.33
D6	30.76	35.77
D7	31.08	34.87
D8	31.53	37.34
D9	31.62	37.10
G9	40.00	38.18
G10	40.00	40.00
NEC	40.00	36.71
NEC	40.00	36.55
NEC	40.00	36.28
NTC	40.00	40.00
PAC-Acat	40.00	32.49
PAC-Lso	33.27	40.00
PPC	30.58	35.26
PPC	30.89	36.96
PPC	31.58	37.84

Analysis of PCR controls

The controls used in the SE-PCRs are listed in the table below.

Table 17. Controls

Control type	
Positive Amplification Control (PAC)	Lso infected carrot seed sample
Negative Extraction Control (NEC)	Buffer with IAC
Negative Process Control (NPC)	Healthy carrot seed sample
Positive Process Control (PPC)	Lso infected seed sample
Internal Amplification Control (IAC)	Acat
Negative Template Control	PCR buffer
Positive Amplification Control	Positive DNA extract

The raw Cq data generated by the fourteen laboratories is presented in Appendix C and the summary of the expected and obtained results is presented in Table 18. All labs were able to detect a positive Lso-PCR with the PAC-Lso and PPC. Lab 6 was the only lab that found Cq values <30 for Lso-PCR with the PAC-Lso. The labs were also able to detect a positive Acat-PCR (Cq⩽35) with the PAC-Acat and PPC (except lab 10 with a very small deviation). Several labs found relatively high Cq values with Acat-PCR in the NEC, and NPC. Lab 10 found high Cq values for Acat in NEC, NPC and PPC. This can be caused by a pipetting error, or a decrease in the quality of the Acat suspension. None of the labs detected Lso or Acat in the NTC as was expected (data not shown).

Conclusion

No PCR amplification occurred in the NTC. All labs obtained the expected Lso-PCR result with the PAC-Lso and the PPC. Based on a Cq cutoff value of 35 for Acat, some labs found higher Cq values. This can be caused by a pipetting error, or a decrease in the quality of the Acat suspension, potentially due to transport duration and conditions.

Table 18. Expected and obtained Cq values per laboratory for the controls in the Acat and Lso SE-PCRs

	Cq values								
	PAC-Lso		PAC-Acat		NEC	PPC		NPC	
Expectation	**Lso <40**	**Acat ≥40**	**Lso ≥40**	**Acat ≤35**	**Acat ≤35**	**Lso <40**	**Acat ≤35**	**Lso ≥40**	**Acat ≤35**
Lab 1	31.0	40.0	40.0	29.5	31.2	30.1	32.3	40.0	31.2
Lab 2	31.4	40.0	40.0	31.1	31.7	27.5	30.3	40.0	30.3
Lab 3	33.3	40.0	40.0	31.4	34.6	28.8	30.2	40.0	40.0
Lab 4	33.6	40.0	40.0	32.3	37.4	32.1	34.0	40.0	32.7
Lab 5	32.7	38.6	40.0	27.7	33.7	31.8	29.7	40.0	32.5
Lab 6	26.7	40.0	40.0	30.7	29.9	27.0	31.0	39.7	29.8
Lab 7	34.1	35.5	40.0	32.2	38.2	30.0	30.8	40.0	32.1
Lab 8	33.9	40.0	40.0	31.0	33.3	31.9	33.4	40.0	31.6
Lab 9	32.8	39.7	40.0	30.3	32.5	33.5	31.0	40.0	30.5
Lab 10	33.4	40.0	40.0	32.4	37.4	35.0	35.4	40.0	38.4
Lab 11	30.4	40.0	40.0	31.1	35.8	29.7	32.8	40.0	33.1
Lab 12	33.4	40.0	40.0	31.2	37.3	29.2	32.6	40.0	33.1
Lab 13	36.5	40.0	40.0	33.7	37.0	32.4	34.5	40.0	34.1
Lab 14	33.4	40.0	40.0	29.0	34.4	30.9	31.5	40.0	32.6

Cq values represent the average of duplicate PCR reactions.

NTC: data are not shown, since all labs obtained Cq Lso ≥40 and Cq Acat ≥40.

Red cells indicate outliers from the expected Cq. Green cells indicate Cq values corresponding to the expected result.

NOTE: There is no correlation between the Lab Nr. in this table and the order of the Labs in Table 1.

Analysis of seed samples

The raw Cq data generated by the fourteen laboratories is presented in the table in Appendix C.

Based on the results of the homogeneity test it was decided that the low infection seed lots B and F will not be included in the analysis of the comparative test results. A summary of the results for Lso and Acat (IAC) for the healthy, medium and high Lso infected carrot lots is shown in Table 19 (Lso) and Table 20 (Acat).

The results in Table 19 (Lso) show that all labs did not detect Lso in the three subsamples of healthy seed lot A. Labs 2 and 6 detected Lso in one and two subsamples of the healthy seed lot G, respectively. The raw data shows that lab 2 found a Cq 34.61 for one of the PCR duplicate reactions of subsample 20. Lab 6 found high Cq values in the PCR duplicates of subsample 20 (Cq 39.56 and Cq 39.80) but relatively low Cq values for subsample 7 (Cq 32.36 and Cq 33.48). All labs were able to detect Lso in the seed samples from the medium infected lot E and high infected lot D. For the medium infected lot C, all labs except lab 9, were able to detect Lso in the seed samples. Lab 9 detected Lso only from two of three samples tested.

Table 19. Expected and obtained Lso SE-PCR results per laboratory for the healthy, medium and highly Lso infected carrot seed lots. Figures represent the number of positive subsamples based on a Cq cut-off <40 for Lso.

	Seed lot A Healthy	Seed lot C medium	Seed lot D high	Seed lot E medium	Seed lot G healthy
Expected result	**0/3**	**3/3**	**3/3**	**3/3**	**0/3**
Lab 1	0/3	3/3	3/3	3/3	0/3
Lab 2	0/3	3/3	3/3	3/3	1/3
Lab 3	0/3	3/3	3/3	3/3	0/3
Lab 4	0/3	3/3	3/3	3/3	0/3
Lab 5	0/3	3/3	3/3	3/3	0/3
Lab 6	0/3	3/3	3/3	3/3	2/3
Lab 7	0/3	3/3	3/3	3/3	0/3
Lab 8	0/3	3/3	3/3	3/3	0/3
Lab 9	0/3	2/3	3/3	3/3	0/3
Lab 10	0/3	3/3	3/3	3/3	0/3
Lab 11	0/3	3/3	3/3	3/3	0/3
Lab 12	0/3	3/3	3/3	3/3	0/3
Lab 13	0/3	3/3	3/3	3/3	0/3
Lab 14	0/3	3/3	3/3	3/3	0/3

Table 20. Expected and obtained Acat SE-PCR results per laboratory for the healthy, medium and highly Lso infected carrot seed lots. Figures represent the number of positive subsamples based on a Cq cut-off <35.

	Seed lot A Healthy	Seed lot C medium	Seed lot D high	Seed lot E medium	Seed lot G healthy
Expected result	**3/3**	**3/3**	**3/3**	**3/3**	**3/3**
Lab 1	3/3	3/3	3/3	3/3	3/3
Lab 2	3/3	3/3	3/3	3/3	3/3
Lab 3	3/3	3/3	3/3	3/3	3/3
Lab 4	3/3	3/3	3/3	3/3	3/3
Lab 5	3/3	3/3	3/3	3/3	3/3
Lab 6	3/3	3/3	3/3	3/3	3/3
Lab 7	3/3	3/3	3/3	3/3	3/3
Lab 8	3/3	3/3	3/3	2/3	3/3
Lab 9	3/3	3/3	3/3	3/3	3/3
Lab 10	0/3	3/3	3/3	3/3	3/3
Lab 11	2/3	0/3	0/3	0/3	0/3
Lab 12	3/3	3/3	3/3	3/3	3/3
Lab 13	0/3	3/3	3/3	3/3	3/3
Lab 14	3/3	2/3	0/3	3/3	2/3

The results in Table 20 (Acat) show that for the 15 subsamples belonging to three Lso infection levels, all labs except lab 7, 10, 11 and 13 amplified Acat at an expected Cq≤35.

The raw data show that:

- Lab 7 did not find amplification in one subsample from the medium infection level lot E. However, Lso was detected (Cq≤40) for this subsample.
- Lab 10 found for Acat Cq levels between 35.6 and 39.6 for all subsamples. However, Lso was detected (Cq≤40) for the medium and high infection level seed lots.
- Lab 11 found for Acat a Cq slightly above 35.0 for one subsample of healthy lot A.
- Lab 13 found for Acat Cq levels between 35.04 and 36.19. However, Lso was detected (Cq≤40) for the medium and high infection level.

Conclusion

Nearly all labs were able to detect Lso in the Lso-infected seed lots at expected levels. In the healthy seed lot A, none of the labs detected Lso. For seed lot G, lab 6 and lab 2 detected an unexpected level of Lso. This is considered as a false positive result.

For the use of Acat as an IAC, not all labs were able to detect Acat according to the expected result (Cq≤35). However, Lso was detected in nearly all the subsamples with Cq≥35 for Acat making these test results valid. Lab 10 seemed to have problems with Acat as IAC, since this lab also found high Cq values for Acat in NEC, NPC and PPC. This can be caused by a pipetting error, or a decrease in the quality of the Acat suspension during shipment or storage in the lab.

In Table 21 and Table 22 the calculated performance criteria for the SE-PCR for the healthy, medium and high Lso infected seed lots are presented. They show that sensitivity, specificity and accuracy of the SE-PCR for detection of Lso in healthy, medium and high infected seed lots, including Acat as IAC, is above 80%.

Table 21. Performance criteria SE-PCR for Lso for each carrot seed lot (N.A. Not Applicable)

Seed Lot	Lso Infection level	Sensitivity	Specificity	Accuracy	Accordance	Concordance	Concordance Odds Ratio (COR)
A	healthy	N.A.	100%	100%	100%	100%	1.0
C	medium	97.6%	N.A.	97.6%	95.2%	95.2%	1.0
D	high	100%	N.A.	100%	100%	100%	1.0
E	medium	100%	N.A.	100%	100%	100%	1.0
G	healthy	N.A.	92.9%	92.9%	90.5%	86.2%	1.05

Table 22. Performance criteria SE-PCR for the Internal Amplification Control Acat for each carrot seed lot (N.A. Not Applicable)

Seed Lot	Lso Infection level	Sensitivity	Specificity	Accuracy	Accordance	Concordance	Concordance Odds Ratio (COR)
A	healthy	83.3%	N.A	83.3%	95.2%	70.3%	1.36
C	medium	90.5%	N.A	90.5%	95.2%	81.7%	1.17
D	high	85.7%	N.A	85.7%	100%	73.6%	1.36
E	medium	90.5%	N.A	90.5%	95.2%	81.7%	1.17
G	healthy	90.5%	N.A	90.5%	95.2%	81.7%	1.17

For Lso detection, accordance of the labs is sufficiently good and show that the method is fit for purpose when applied within the same lab, e.g. the same result is obtained when the same material is repeatedly tested. The COR (Concordance Odds Ratio) being close to 1, even for the

G-lot, shows that there is only little inter laboratory variation, and the same sample tested in different laboratories would give the same result.

Accordance and concordance for Acat are lower. Technical reasons for these lower values are given in the discussion of the results below Table 20. Accordance and concordance data show that there is interlaboratory variation and the same sample tested in different laboratories might not give the same result for Acat, partly caused by the choice to set a Cq-cut-off for Acat.

Discussion

In this comparative test for the detection of Lso in carrot seeds, 14 labs from 9 different countries participated. Due to import restrictions it has been impossible to send the test package at the same moment to all participants. After 4 months all packages were sent to the participants, but even then, in several countries it took a long period before the package was received at the laboratory. Consequently, in several labs there were problems with starting the test in a timely manner, so the period between sending and finalizing the CT varied from 8 to 175 days.

In contrast to earlier results, it was not possible to consistently detect Lso in the low infected seed lot B and F with the homogeneity and stability tests performed at the organizing lab. Both lots were spiked with 10 seeds from the Lso infected carrot seed lot P20.142. Based on single seed tests, the seed lot P20.142 contained 60% Lso infected seeds. This makes the final infection percentage for lot B and F 0.06% which is probably too low to detect. Since there is no scientific information available about the biological relevance of low infection levels and it is not clear which sensitivity of the SE-PCR method is required.

Despite these difficulties, the results of this CT show that the method is reproducible in many labs and fit for purpose. Labs were free in the choice of PCR machine and PCR TaqMan mix and provided additional information (not shown in the report). In total 7 different PCR machine brands and 7 different TaqMan mixes were used (data not shown).

All labs were able to consistently detect Lso in carrot seed lots with a medium and high Lso infection level, except for one lab missing one medium sample. All labs obtained the expected Lso-PCR result with the PAC-Lso and the PPC. Based on a Cq cutoff value of 35 for Acat, some labs found unexpectedly higher Cq values. This can be caused by a pipetting error, but more likely is a decrease in the quality of the Acat suspension during transport or storage.

In this CT, it was possible to consistently detect Lso in carrot seed, at an infection level of 3.0% (medium) and 60% (high). Based on these results, we conclude that the method for detection of *Candidatus* Liberibacter solanacearum is sufficiently reproducible and repeatable.

5.4 Conclusions

Based on these results, we conclude that the method for detection of *Candidatus* Liberibacter solanacearum from carrot seed is sufficiently reproducible and repeatable.

6. Overall Conclusions

The data collected in this study shows that the SE-PCR detection method for Lso in carrot seeds meets the required validation criteria for analytical specificity, selectivity, sensitivity, repeatability and reproducibility. Furthermore, the data have shown that the method is robust, based on the use of different equipment, consumables and tests performed in different laboratories. Therefore, we regard this method as sufficiently validated and fit for purpose.

Appendix A. ISHI-Veg Performance Criteria for Validating Seed Health Tests

Version 1, May 2018

ISHI-Veg Performance Criteria and their Characteristics

Performance Criteria	Characteristics
Analytical specificity of an assay	The ability of an assay to detect the target(s) pathogens (inclusivity) while excluding non-targets (exclusivity)
Analytical sensitivity	Smallest amount of the target pathogen that can be detected i.e. the limit of detection (LOD)
Selectivity	The effect of different seed matrices on the ability of the method to detect target pathogen(s)
Repeatability	Degree of similarity in results of replicates of the same seed lots when the method is performed with minimal variations in a single lab
Reproducibility	Degree of similarity in results when the method is performed across labs with replicates of the same subsamples
Diagnostic performance	The ability of the method to detect target pathogens in known infected seed samples while excluding non-target organisms in known healthy seed samples
Post implementation surveillance	After a method has been shown to be fit for purpose evaluating its performance over time to ensure it is performing as intended

Note: Version 2 dated May 2020 that is currently in force doesn't include Post implementation surveillance.

Appendix B. Supplementary Tables B1 to B5: The Ct values from the qPCR assay determining the Selectivity. (See section 3.3 for the details of the experiment).

Table B1. Result from known positive seed lot.

Experiments	Infection rate (%)[a]	Cq values Lso[b]				Cq values Acat[b]			
		Undiluted		1/10 dilution		Undiluted		1/10 dilution	
		Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
1	100%	24.31	24.85	27.18	27.55	ND	ND	33.50	32.29
		24.42	24.39	25.50	26.50	ND	ND	ND	35.33
2		38.16	28.37	26.10	26.00	ND	ND	ND	ND
		25.41	23.89	26.20	24.79	ND	ND	30.72	29.61

Table B2. Results from Seed lot 1.

Experiments	Infection rate (%)[a]	Cq values Lso[b]				Cq values Acat[b]			
		Undiluted		1/10 dilution		Undiluted		Undiluted	
		Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
1	25.00	24.88	25.19	28.75	29.06	26.86	26.62	30.47	30.88
	10.00	26.44	26.58	31.02	30.47	27.34	27.16	32.28	31.53
	1.00	29.36	31.14	33.38	34.62	28.13	28.56	32.31	32.49
	0.10	34.32	35.19	ND	ND	28.46	28.78	32.87	33.35
	0.01	ND	ND	ND	38.30	28.76	28.62	32.77	33.30
	0.0	ND	ND	ND	ND	28.40	29.11	32.30	32.76
2	25.00	26.16	25.91	30.85	30.17	27.84	23.80	31.64	30.24
	10.00	27.11	27.54	31.09	31.65	27.41	28.06	31.51	32.01
	1.00	30.81	31.65	34.41	38.28	28.15	28.08	34.53	33.24
	0.10	35.21	34.06	ND	ND	28.52	28.17	36.07	33.14
	0.01	ND	37.23	ND	ND	28.80	28.50	36.00	33.33
	0.0	ND	ND	ND	ND	28.53	29.02	34.65	33.29

Table B3. Results from Seed lot 2.

Experiments	Infection rate (%)[a]	Cq values Lso[b]				Cq values Acat[b]			
		Undiluted		1/10 dilution		Undiluted		Undiluted	
		Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
1	25.00	24.04	23.62	28.87	27.41	ND	ND	33.55	30.32
	10.00	27.17	26.39	30.61	30.40	27.18	24.67	31.60	30.98
	1.00	31.09	31.02	34.20	34.06	27.66	27.07	31.71	31.29
	0.10	33.59	33.70	36.77	ND	27.38	27.38	31.02	31.16
	0.01	35.33	ND	ND	ND	27.57	26.93	31.78	30.42
	0.0	ND	ND	ND	ND	27.78	27.88	31.65	31.15
2	25.00	26.17	26.13	29.60	29.46	ND	ND	30.37	30.47
	10.00	27.72	26.44	31.29	31.46	28.28	29.44	32.01	31.82
	1.00	32.03	31.22	35.01	34.35	28.26	28.64	32.11	31.77
	0.10	36.10	33.74	ND	ND	29.07	28.42	33.07	29.45
	0.01	ND	ND	ND	ND	29.24	28.72	33.39	33.21
	0.0	ND	ND	ND	ND	28.72	28.62	33.08	33.92

Table B4. Results from Seed lot 3.

Experiments	Infection rate (%)[a]	Cq values Lso[b]				Cq values Acat[b]			
		Undiluted		1/10 dilution		Undiluted		Undiluted	
		Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
1	25.00	24.01	24.02	28.09	27.39	39.44	ND	30.62	29.93
	10.00	26.91	27.04	30.77	30.83	26.75	26.81	30.63	30.75
	1.00	29.01	28.72	31.92	32.13	27.24	27.41	31.57	31.69
	0.10	34.19	31.13	ND	33.72	28.78	28.82	32.31	31.64
	0.01	ND	ND	ND	ND	28.68	28.85	31.77	31.19
	0.0	ND	ND	ND	ND	27.59	27.22	31.36	31.19
2	25.00	25.97	26.24	29.78	30.08	ND	ND	32.11	32.01
	10.00	27.56	27.94	31.57	ND	29.08	29.43	32.12	ND
	1.00	31.15	32.12	36.02	34.88	29.38	29.67	33.76	33.75
	0.10	36.03	36.22	36.69	ND	29.35	29.88	32.73	33.55
	0.01	ND	ND	ND	ND	29.37	29.14	33.10	34.68
	0.0	ND	ND	ND	ND	29.40	29.28	33.37	33.93

Table B5. Results from Seed lot 4.

Experiments	Infection rate (%)[a]	Cq values Lso[b]				Cq values Acat[b]			
		Undiluted		1/10 dilution		Undiluted		1/10 dilution	
		Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
1	25.00	25.21	25.42	27.68	27.62	38.27	ND	34.28	33.11
	10.00	26.75	27.05	29.65	30.11	39.97	ND	33.76	34.91
	1.00	30.33	29.33	35.33	33.02	36.04	39.99	31.59	33.95
	0.10	32.41	33.72	ND	ND	32.49	35.28	31.77	33.92
	0.01	ND	ND	ND	ND	31.62	33.72	34.73	33.99
	0.0	ND	ND	ND	ND	32.30	32.20	ND	34.52
2	25.00	25.03	25.32	27.64	28.08	ND	ND	31.66	31.33
	10.00	26.01	26.10	29.47	29.12	ND	29.61	31.08	30.68
	1.00	29.03	29.19	34.06	32.68	29.34	30.39	32.45	31.46
	0.10	31.87	31.96	33.29	35.17	ND	29.51	31.23	31.28
	0.01	39.97	35.44	ND	ND	ND	30.80	29.70	31.04
	0.0	ND	ND	ND	ND	29.21	30.07	30.86	34.21

The foot notes are common for Tables B1 to B5.

[a] Seed extract from Lso positive seed lot is used to create different infection rate by mixing with extracts from negative seed lot (Table B2–B5), and the infection/contamination rate of extract from positive seed lot is theoretically assumed to be 100%.

[b] qPCR was performed with undiluted DNA and with 1/10 diluted DNA separately; Rep1 and Rep2 are two technical replicates.

Experiments 1 and 2: The assay was repeated once.

ND=No Cq value.

APPENDIX C: Raw data obtained in the comparative test

Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)	Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)
1	1	healthy	A	40.00	31.92	2	1	healthy	A	40.00	31.07
1	1	healthy	B	40.00	32.76	2	1	healthy	B	40.00	31.28
1	2	low	A	40.00	33.01	2	2	low	A	40.00	30.30
1	2	low	B	40.00	33.21	2	2	low	B	40.00	30.72
1	3	medium	A	34.02	31.27	2	3	medium	A	31.33	30.41
1	3	medium	B	34.09	31.41	2	3	medium	B	30.75	30.83
1	4	high	A	30.24	31.93	2	4	high	A	30.39	30.36
1	4	high	B	30.36	31.66	2	4	high	B	30.27	30.43
1	5	medium	A	31.19	31.36	2	5	medium	A	31.08	30.40
1	5	medium	B	31.28	31.56	2	5	medium	B	30.40	30.17
1	6	low	A	34.52	31.94	2	6	low	A	40.00	30.40
1	6	low	B	34.87	31.84	2	6	low	B	40.00	30.53
1	7	healthy	A	40.00	31.13	2	7	healthy	A	40.00	30.27
1	7	healthy	B	40.00	31.11	2	7	healthy	B	40.00	30.17
1	8	healthy	A	40.00	30.79	2	8	healthy	A	40.00	30.56
1	8	healthy	B	40.00	30.75	2	8	healthy	B	40.00	30.06
1	9	healthy	A	40.00	31.41	2	9	healthy	A	40.00	30.69
1	9	healthy	B	40.00	30.97	2	9	healthy	B	40.00	30.66
1	10	low	A	40.00	34.58	2	10	low	A	40.00	30.87
1	10	low	B	40.00	36.49	2	10	low	B	35.78	30.72
1	11	medium	A	33.64	31.49	2	11	medium	A	31.06	30.26
1	11	medium	B	32.72	31.51	2	11	medium	B	30.18	30.07
1	12	high	A	29.41	33.01	2	12	high	A	27.17	29.79
1	12	high	B	29.44	32.15	2	12	high	B	27.11	30.18
1	13	medium	A	33.04	30.96	2	13	medium	A	31.39	30.12

Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)	Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)
1	13	medium	B	34.87	30.85	2	13	medium	B	30.91	29.93
1	14	low	A	40.00	30.60	2	14	low	A	34.73	30.42
1	14	low	B	40.00	30.85	2	14	low	B	35.01	29.97
1	15	low	A	40.00	31.25	2	15	low	A	34.54	29.89
1	15	low	B	35.37	30.18	2	15	low	B	40.00	30.07
1	16	medium	A	33.95	32.54	2	16	medium	A	31.70	30.41
1	16	medium	B	34.81	32.87	2	16	medium	B	32.00	30.38
1	17	healthy	A	40.00	31.80	2	17	healthy	A	40.00	30.98
1	17	healthy	B	40.00	31.45	2	17	healthy	B	40.00	30.96
1	18	low	A	35.86	31.67	2	18	low	A	40.00	30.67
1	18	low	B	36.39	31.12	2	18	low	B	40.00	30.67
1	19	high	A	29.76	32.23	2	19	high	A	28.74	29.97
1	19	high	B	29.91	31.57	2	19	high	B	28.03	30.20
1	20	healthy	A	40.00	31.09	2	20	healthy	A	40.00	30.50
1	20	healthy	B	40.00	31.00	2	20	healthy	B	34.61	30.00
1	21	medium	A	34.77	30.99	2	21	medium	A	31.59	30.52
1	21	medium	B	32.57	30.54	2	21	medium	B	31.54	30.49
1	NEC	control	A	36.25	31.37	2	NEC	control	A	40.00	31.65
1	NEC	control	B	40.00	31.02	2	NEC	control	B	40.00	31.82
1	NPC	control	A	40.00	31.33	2	NPC	control	A	40.00	30.40
1	NPC	control	B	40.00	31.16	2	NPC	control	B	40.00	30.15
1	NTC	control	A	40.00	40.00	2	NTC	control	A	40.00	40.00
1	NTC	control	B	40.00	40.00	2	NTC	control	B	40.00	40.00
1	PAC-Acat	control	A	40.00	29.57	2	PAC-Acat	control	A	40.00	31.31
1	PAC-Acat	control	B	40.00	29.44	2	PAC-Acat	control	B	40.00	30.97
1	PAC-Lso	control	A	31.17	40.00	2	PAC-Lso	control	A	31.85	40.00
1	PAC-Lso	control	B	30.81	40.00	2	PAC-Lso	control	B	31.00	40.00

Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)	Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)
1	PPC	control	A	30.31	32.39	2	PPC	control	A	27.71	30.26
1	PPC	control	B	29.84	32.24	2	PPC	control	B	27.35	30.29
3	1	healthy	A	40.00	33.42	4	1	healthy	A	40.00	33.63
3	1	healthy	B	40.00	32.92	4	1	healthy	B	40.00	33.37
3	2	low	A	36.96	33.68	4	2	low	A	40.00	34.02
3	2	low	B	40.00	33.67	4	2	low	B	40.00	33.54
3	3	medium	A	34.19	32.47	4	3	medium	A	36.32	33.87
3	3	medium	B	34.22	32.73	4	3	medium	B	35.59	33.84
3	4	high	A	32.05	33.14	4	4	high	A	32.53	34.13
3	4	high	B	31.31	32.07	4	4	high	B	32.43	33.05
3	5	medium	A	37.25	31.51	4	5	medium	A	34.56	33.91
3	5	medium	B	34.08	30.89	4	5	medium	B	35.16	33.49
3	6	low	A	40.00	33.19	4	6	low	A	40.00	34.33
3	6	low	B	40.00	37.59	4	6	low	B	40.00	33.35
3	7	healthy	A	40.00	31.93	4	7	healthy	A	40.00	32.61
3	7	healthy	B	40.00	32.59	4	7	healthy	B	40.00	32.17
3	8	healthy	A	40.00	32.31	4	8	healthy	A	40.00	33.95
3	8	healthy	B	40.00	37.53	4	8	healthy	B	40.00	32.67
3	9	healthy	A	40.00	33.59	4	9	healthy	A	40.00	33.67
3	9	healthy	B	40.00	33.21	4	9	healthy	B	40.00	33.27
3	10	low	A	40.00	34.47	4	10	low	A	40.00	33.06
3	10	low	B	40.00	33.27	4	10	low	B	40.00	32.72
3	11	medium	A	33.53	32.46	4	11	medium	A	34.86	33.33
3	11	medium	B	34.26	33.43	4	11	medium	B	35.05	32.73
3	12	high	A	31.75	32.60	4	12	high	A	32.82	34.05
3	12	high	B	31.37	32.38	4	12	high	B	32.27	33.22

Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)	Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)
3	13	medium	A	33.81	31.17	4	13	medium	A	35.03	33.15
3	13	medium	B	32.92	31.21	4	13	medium	B	33.81	33.22
3	14	low	A	40.00	29.37	4	14	low	A	40.00	32.89
3	14	low	B	36.62	29.64	4	14	low	B	40.00	32.26
3	15	low	A	40.00	29.81	4	15	low	A	40.00	33.11
3	15	low	B	40.00	30.29	4	15	low	B	40.00	32.71
3	16	medium	A	33.20	31.66	4	16	medium	A	34.77	33.28
3	16	medium	B	32.96	31.07	4	16	medium	B	33.42	33.21
3	17	healthy	A	40.00	32.51	4	17	healthy	A	40.00	33.41
3	17	healthy	B	40.00	32.14	4	17	healthy	B	40.00	33.37
3	18	low	A	33.66	32.03	4	18	low	A	35.01	33.19
3	18	low	B	33.27	30.99	4	18	low	B	37.37	32.87
3	19	high	A	29.59	32.78	4	19	high	A	32.11	33.84
3	19	high	B	28.50	29.73	4	19	high	B	32.17	34.96
3	20	healthy	A	40.00	30.22	4	20	healthy	A	40.00	33.32
3	20	healthy	B	40.00	30.30	4	20	healthy	B	40.00	33.21
3	21	medium	A	32.99	31.50	4	21	medium	A	34.75	33.15
3	21	medium	B	33.38	30.76	4	21	medium	B	34.42	33.31
3	NEC	control	A	40.00	34.46	4	NEC	control	A	40.00	37.19
3	NEC	control	B	40.00	34.70	4	NEC	control	B	40.00	37.52
3	NPC	control	A	40.00	40.00	4	NPC	control	A	40.00	32.52
3	NPC	control	B	40.00	40.00	4	NPC	control	B	40.00	32.85
3	NTC	control	A	40.00	40.00	4	NTC	control	A	40.00	40.00
3	NTC	control	B	40.00	40.00	4	NTC	control	B	40.00	40.00
3	PAC-Acat	control	A	40.00	31.15	4	PAC-Acat	control	A	40.00	32.09
3	PAC-Acat	control	B	40.00	31.69	4	PAC-Acat	control	B	40.00	32.51
3	PAC-Lso	control	A	33.22	40.00	4	PAC-Lso	control	A	33.22	40.00

Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)	Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)
3	PAC-Lso	control	B	33.32	40.00	4	PAC-Lso	control	B	33.95	40.00
3	PPC	control	A	27.84	29.60	4	PPC	control	A	31.84	34.03
3	PPC	control	B	29.82	30.71	4	PPC	control	B	32.32	33.93
5	1	healthy	A	40.00	28.54	6	1	healthy	A	40.00	29.60
5	1	healthy	B	40.00	30.99	6	1	healthy	B	40.00	29.64
5	2	low	A	40.00	29.51	6	2	low	A	40.00	29.58
5	2	low	B	37.32	31.12	6	2	low	B	40.00	29.77
5	3	medium	A	34.17	29.28	6	3	medium	A	32.50	30.01
5	3	medium	B	34.91	30.13	6	3	medium	B	33.06	30.02
5	4	high	A	32.87	29.53	6	4	high	A	30.35	30.16
5	4	high	B	32.91	31.24	6	4	high	B	30.17	29.76
5	5	medium	A	35.34	33.32	6	5	medium	A	31.46	30.14
5	5	medium	B	34.06	31.36	6	5	medium	B	31.60	30.03
5	6	low	A	40.00	30.84	6	6	low	A	40.00	30.08
5	6	low	B	40.00	31.99	6	6	low	B	40.00	30.33
5	7	healthy	A	40.00	32.03	6	7	healthy	A	33.48	30.04
5	7	healthy	B	40.00	31.09	6	7	healthy	B	32.36	30.11
5	8	healthy	A	40.00	28.82	6	8	healthy	A	40.00	30.13
5	8	healthy	B	40.00	26.77	6	8	healthy	B	40.00	30.36
5	9	healthy	A	40.00	28.52	6	9	healthy	A	40.00	30.03
5	9	healthy	B	40.00	29.60	6	9	healthy	B	40.00	29.61
5	10	low	A	40.00	30.79	6	10	low	A	40.00	29.82
5	10	low	B	40.00	26.94	6	10	low	B	40.00	30.00
5	11	medium	A	31.16	29.56	6	11	medium	A	33.59	30.11
5	11	medium	B	30.67	28.49	6	11	medium	B	35.29	29.90
5	12	high	A	31.49	30.59	6	12	high	A	29.88	30.40
5	12	high	B	31.45	29.82	6	12	high	B	30.35	30.02

Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)	Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)
5	13	medium	A	34.75	30.48	6	13	medium	A	33.78	29.65
5	13	medium	B	35.15	27.84	6	13	medium	B	33.02	29.66
5	14	low	A	40.00	31.57	6	14	low	A	40.00	29.58
5	14	low	B	40.00	31.00	6	14	low	B	40.00	29.50
5	15	low	A	40.00	31.43	6	15	low	A	33.91	29.84
5	15	low	B	40.00	32.78	6	15	low	B	39.06	29.78
5	16	medium	A	32.10	29.53	6	16	medium	A	31.49	30.02
5	16	medium	B	31.38	29.91	6	16	medium	B	32.06	30.07
5	17	healthy	A	40.00	29.56	6	17	healthy	A	40.00	29.79
5	17	healthy	B	40.00	26.84	6	17	healthy	B	40.00	30.10
5	18	low	A	40.00	27.54	6	18	low	A	37.24	29.75
5	18	low	B	35.38	28.90	6	18	low	B	40.00	29.66
5	19	high	A	32.60	30.72	6	19	high	A	27.79	29.71
5	19	high	B	31.76	28.57	6	19	high	B	27.68	29.53
5	20	healthy	A	40.00	30.59	6	20	healthy	A	39.56	30.03
5	20	healthy	B	40.00	31.45	6	20	healthy	B	39.80	29.78
5	21	medium	A	33.65	31.18	6	21	medium	A	32.19	29.63
5	21	medium	B	33.52	31.52	6	21	medium	B	30.62	29.73
5	NEC	control	A	40.00	33.10	6	NEC	control	A	39.32	30.01
5	NEC	control	B	40.00	34.37	6	NEC	control	B	39.95	29.72
5	NPC	control	A	40.00	32.72	6	NPC	control	A	40.00	30.01
5	NPC	control	B	40.00	32.34	6	NPC	control	B	39.30	29.63
5	NTC	control	A	40.00	40.00	6	NTC	control	A	40.00	40.00
5	NTC	control	B	40.00	40.00	6	NTC	control	B	40.00	40.00
5	PAC-Acat	control	A	40.00	27.78	6	PAC-Acat	control	A	40.00	31.05
5	PAC-Acat	control	B	40.00	27.52	6	PAC-Acat	control	B	40.00	30.39
5	PAC-Lso	control	A	32.49	38.66	6	PAC-Lso	control	A	26.87	40.00

Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)	Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)
5	PAC-Lso	control	B	32.92	38.52	6	PAC-Lso	control	B	26.56	40.00
5	PPC	control	A	31.76	30.62	6	PPC	control	A	27.15	31.20
5	PPC	control	B	31.91	28.75	6	PPC	control	B	26.91	30.82
7	1	healthy	A	40.00	33.13	8	1	healthy	A	40.00	34.38
7	1	healthy	B	40.00	33.96	8	1	healthy	B	40.00	34.39
7	2	low	A	40.00	34.64	8	2	low	A	40.00	34.26
7	2	low	B	40.00	34.73	8	2	low	B	40.00	34.53
7	3	medium	A	33.54	33.92	8	3	medium	A	36.01	34.11
7	3	medium	B	33.56	33.75	8	3	medium	B	36.35	33.97
7	4	high	A	30.67	31.52	8	4	high	A	34.48	34.72
7	4	high	B	29.98	30.96	8	4	high	B	34.30	34.57
7	5	medium	A	33.37	32.99	8	5	medium	A	39.64	34.77
7	5	medium	B	34.30	33.18	8	5	medium	B	40.00	33.94
7	6	low	A	36.83	33.44	8	6	low	A	40.00	32.39
7	6	low	B	40.00	33.01	8	6	low	B	40.00	32.74
7	7	healthy	A	40.00	33.04	8	7	healthy	A	40.00	32.48
7	7	healthy	B	40.00	33.47	8	7	healthy	B	40.00	33.05
7	8	healthy	A	40.00	32.83	8	8	healthy	A	40.00	33.51
7	8	healthy	B	40.00	32.27	8	8	healthy	B	40.00	33.02
7	9	healthy	A	40.00	34.04	8	9	healthy	A	40.00	33.98
7	9	healthy	B	40.00	34.79	8	9	healthy	B	40.00	34.28
7	10	low	A	40.00	33.32	8	10	low	A	40.00	34.24
7	10	low	B	40.00	33.62	8	10	low	B	40.00	34.76
7	11	medium	A	33.97	32.81	8	11	medium	A	36.05	32.89
7	11	medium	B	34.92	33.08	8	11	medium	B	36.46	32.57
7	12	high	A	29.77	30.37	8	12	high	A	34.34	33.12
7	12	high	B	30.23	30.99	8	12	high	B	34.39	33.50

Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)	Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)
7	13	medium	A	34.18	33.24	8	13	medium	A	34.99	32.62
7	13	medium	B	34.90	32.92	8	13	medium	B	35.59	32.67
7	14	low	A	35.77	33.16	8	14	low	A	40.00	31.67
7	14	low	B	35.61	33.18	8	14	low	B	40.00	31.79
7	15	low	A	40.00	33.06	8	15	low	A	40.00	32.37
7	15	low	B	40.00	32.95	8	15	low	B	40.00	32.20
7	16	medium	A	33.02	33.34	8	16	medium	A	35.79	33.24
7	16	medium	B	33.66	33.03	8	16	medium	B	35.91	32.44
7	17	healthy	A	40.00	34.01	8	17	healthy	A	40.00	32.68
7	17	healthy	B	40.00	33.64	8	17	healthy	B	40.00	32.37
7	18	low	A	40.00	33.41	8	18	low	A	40.00	32.86
7	18	low	B	40.00	34.12	8	18	low	B	40.00	33.00
7	19	high	A	30.82	31.47	8	19	high	A	33.13	33.66
7	19	high	B	30.76	31.23	8	19	high	B	33.40	33.11
7	20	healthy	A	40.00	33.75	8	20	healthy	A	40.00	32.33
9	1	healthy	A	40.00	30.72	10	1	healthy	A	40.00	39.2
9	1	healthy	B	40.00	30.68	10	1	healthy	B	40.00	38.54
9	2	low	A	40.00	30.65	10	2	low	A	40.00	39.22
9	2	low	B	40.00	30.72	10	2	low	B	40.00	39.31
9	3	medium	A	40.00	30.85	10	3	medium	A	37.02	36.25
9	3	medium	B	36.98	30.81	10	3	medium	B	40.00	38.04
9	4	high	A	33.66	31.50	10	4	high	A	35.53	35.76
9	4	high	B	33.37	31.29	10	4	high	B	35.18	35.96
9	5	medium	A	40.00	30.24	10	5	medium	A	37.86	36.97
9	5	medium	B	36.07	30.56	10	5	medium	B	38.29	38.66
9	6	low	A	40.00	30.09	10	6	low	A	40.00	38.1
9	6	low	B	40.00	29.97	10	6	low	B	40.00	38.16

Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)	Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)
9	7	healthy	A	40.00	30.23	10	7	healthy	A	40.00	38.64
9	7	healthy	B	40.00	30.20	10	7	healthy	B	40.00	38.53
9	8	healthy	A	40.00	30.13	10	8	healthy	A	40.00	38.02
9	8	healthy	B	40.00	30.36	10	8	healthy	B	40.00	37.63
9	9	healthy	A	40.00	30.74	10	9	healthy	A	40.00	39.17
9	9	healthy	B	40.00	30.62	10	9	healthy	B	40.00	38.22
9	10	low	A	40.00	30.59	10	10	low	A	40.00	38.72
9	10	low	B	40.00	30.69	10	10	low	B	40.00	40.00
9	11	medium	A	34.70	30.41	10	11	medium	A	36.79	36.42
9	11	medium	B	34.75	30.76	10	11	medium	B	40.00	37.1
9	12	high	A	33.14	31.00	10	12	high	A	35.88	35.88
9	12	high	B	33.48	31.19	10	12	high	B	35.54	35.91
9	13	medium	A	34.96	30.37	10	13	medium	A	36.34	38.36
9	13	medium	B	36.04	30.26	10	13	medium	B	36.96	39.6
9	14	low	A	40.00	30.42	10	14	low	A	40.00	38.12
9	14	low	B	40.00	30.00	10	14	low	B	40.00	38.12
9	15	low	A	40.00	40.00	10	15	low	A	40.00	37.86
9	15	low	B	40.00	29.94	10	15	low	B	40.00	37.61
9	16	medium	A	40.00	30.53	10	16	medium	A	36.87	36.06
9	16	medium	B	40.00	31.07	10	16	medium	B	40.00	37.35
9	17	healthy	A	40.00	30.51	10	17	healthy	A	40.00	37.28
9	17	healthy	B	40.00	30.61	10	17	healthy	B	40.00	36.86
9	18	low	A	40.00	30.65	10	18	low	A	40.00	37.77
9	18	low	B	40.00	30.82	10	18	low	B	40.00	36.93
9	19	high	A	33.82	31.02	10	19	high	A	34.67	36.94
9	19	high	B	33.14	31.43	10	19	high	B	35.16	35.6
9	20	healthy	A	40.00	30.09	10	20	healthy	A	40.00	37.75

Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)	Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)
11	1	healthy	A	40.00	34.42	12	1	healthy	A	40.00	32.94
11	1	healthy	B	40.00	34.78	12	1	healthy	B	40.00	33.15
11	2	low	A	40.00	33.30	12	2	low	A	37.66	33.74
11	2	low	B	40.00	34.73	12	2	low	B	40.00	33.29
11	3	medium	A	32.00	33.48	12	3	medium	A	32.65	33.42
11	3	medium	B	32.23	33.83	12	3	medium	B	32.97	33.55
11	4	high	A	29.54	32.89	12	4	high	A	30.66	33.48
11	4	high	B	29.24	32.94	12	4	high	B	30.30	32.65
11	5	medium	A	32.54	32.86	12	5	medium	A	32.71	33.01
11	5	medium	B	32.35	32.74	12	5	medium	B	33.15	32.86
11	6	low	A	35.67	33.14	12	6	low	A	40.00	33.04
11	6	low	B	38.31	33.45	12	6	low	B	40.00	33.31
11	7	healthy	A	40.00	33.15	12	7	healthy	A	40.00	33.63
11	7	healthy	B	40.00	33.19	12	7	healthy	B	40.00	32.99
11	8	healthy	A	40.00	33.44	12	8	healthy	A	40.00	33.61
11	8	healthy	B	40.00	33.08	12	8	healthy	B	40.00	32.87
11	9	healthy	A	40.00	35.00	12	9	healthy	A	40.00	33.04
11	9	healthy	B	40.00	35.27	12	9	healthy	B	40.00	32.80
11	10	low	A	40.00	35.04	12	10	low	A	40.00	32.71
11	10	low	B	40.00	34.95	12	10	low	B	40.00	32.81
11	11	medium	A	33.62	34.16	12	11	medium	A	33.28	33.45
11	11	medium	B	33.64	33.79	12	11	medium	B	33.14	33.03
11	12	high	A	29.48	33.48	12	12	high	A	30.50	33.01
11	12	high	B	29.50	33.66	12	12	high	B	30.15	32.64
11	13	medium	A	31.45	33.81	12	13	medium	A	33.95	34.17
11	13	medium	B	31.46	33.41	12	13	medium	B	33.44	32.92
11	14	low	A	40.00	32.80	12	14	low	A	37.50	34.24

Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)	Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)
11	14	low	B	40.00	33.46	12	14	low	B	40.00	33.24
11	15	low	A	40.00	34.96	12	15	low	A	40.00	32.29
11	15	low	B	40.00	34.57	12	15	low	B	37.56	32.92
11	16	medium	A	32.64	34.05	12	16	medium	A	32.17	32.85
11	16	medium	B	32.68	33.79	12	16	medium	B	31.67	32.49
11	17	healthy	A	40.00	34.44	12	17	healthy	A	40.00	33.68
11	17	healthy	B	40.00	34.92	12	17	healthy	B	40.00	33.20
11	18	low	A	40.00	35.01	12	18	low	A	40.00	33.31
11	18	low	B	40.00	34.98	12	18	low	B	40.00	33.00
11	19	high	A	30.21	33.24	12	19	high	A	29.84	33.47
11	19	high	B	29.87	32.46	12	19	high	B	29.76	33.02
11	20	healthy	A	40.00	33.52	12	20	healthy	A	40.00	32.67
13	1	healthy	A	40.00	35.21	14	1	healthy	A	40.00	31.75
13	1	healthy	B	40.00	35.36	14	1	healthy	B	40.00	32.21
13	2	low	A	40.00	35.44	14	2	low	A	40.00	32.10
13	2	low	B	40.00	34.72	14	2	low	B	40.00	32.78
13	3	medium	A	38.39	35.30	14	3	medium	A	35.34	30.74
13	3	medium	B	39.05	35.49	14	3	medium	B	34.26	30.70
13	4	high	A	33.05	35.61	14	4	high	A	31.34	32.31
13	4	high	B	33.24	34.81	14	4	high	B	31.61	31.52
13	5	medium	A	36.64	35.30	14	5	medium	A	36.31	34.82
13	5	medium	B	36.73	34.66	14	5	medium	B	34.48	34.74
13	6	low	A	40.00	34.74	14	6	low	A	40.00	34.05
13	6	low	B	40.00	34.68	14	6	low	B	36.71	31.36
13	7	healthy	A	40.00	34.14	14	7	healthy	A	40.00	34.86
13	7	healthy	B	40.00	35.04	14	7	healthy	B	40.00	34.85
13	8	healthy	A	40.00	35.17	14	8	healthy	A	40.00	33.86

Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)	Lab	sample	Lso level	duplicate	Cq Lso (Li)	Cq Acat (IAC)
13	8	healthy	B	40.00	35.23	14	8	healthy	B	40.00	33.89
13	9	healthy	A	40.00	34.86	14	9	healthy	A	40.00	32.35
13	9	healthy	B	40.00	35.52	14	9	healthy	B	40.00	33.14
13	10	low	A	40.00	35.11	14	10	low	A	40.00	32.23
13	10	low	B	40.00	34.97	14	10	low	B	40.00	31.23
13	11	medium	A	38.58	35.51	14	11	medium	A	36.19	30.73
13	11	medium	B	36.62	36.19	14	11	medium	B	35.63	31.16
13	12	high	A	33.31	35.83	14	12	high	A	31.30	33.22
13	12	high	B	33.10	35.23	14	12	high	B	30.46	33.57
13	13	medium	A	40.00	34.40	14	13	medium	A	37.28	34.87
13	13	medium	B	38.54	34.89	14	13	medium	B	35.52	32.71
13	14	low	A	40.00	33.73	14	14	low	A	40.00	33.95
13	14	low	B	40.00	34.80	14	14	low	B	40.00	34.37
13	15	low	A	40.00	35.32	14	15	low	A	40.00	34.31
13	15	low	B	40.00	34.61	14	15	low	B	40.00	34.16
13	16	medium	A	35.93	34.70	14	16	medium	A	37.41	33.36
13	16	medium	B	36.16	34.78	14	16	medium	B	37.75	32.49
13	17	healthy	A	40.00	35.20	14	17	healthy	A	40.00	31.57
13	17	healthy	B	40.00	34.83	14	17	healthy	B	40.00	32.42
13	18	low	A	40.00	34.79	14	18	low	A	40.00	31.38
13	18	low	B	40.00	34.44	14	18	low	B	40.00	30.65
13	19	high	A	32.91	35.92	14	19	high	A	31.60	33.06
13	19	high	B	33.11	35.47	14	19	high	B	31.24	34.87
13	20	healthy	A	40.00	34.81	14	20	healthy	A	40.00	33.35

Appendix D. Comparative test Protocol SE-PCR for detection of Lso on carrot seeds

Materials

Sterile Phosphate Buffered Saline

Ingredient	Quantity for 1 l
Sodium chloride (NaCl)	8,0 g
Potassium phosphate monobasic (KH_2PO_4)	0,24 g
Sodium phosphate dibasic (Na_2HPO_4)	1,44 g
Potassium chloride (KCl)	0,20 g
Demineralized water	Add 1.000 ml

Downflow cabinet with UV light (preferred) or Laminar airflow cabinet as alternative	Microliter pipettes (e.g. Gilson, Finn) with sterile filter pipette tips (1µl – 5ml)
Glassware (e.g. flasks, conical flask etc.)	Laboratory equipment (e.g. shaker, pH meter, magnetic stirrer etc.)
TaqMan® Master Mix (e.g. Applied TaqMan Universal Mastermix II)	Real-time PCR machine (e.g. Bio-Rad CFX96, Qiagen Rotorgene-Q)
Stomacher (e.g. Interscience Bag mixer) and stomacher bags (e.g. Interscience)	PCR grade 2 ml and 1,5 ml microtubes
Qiagen DNeasy Plant Mini DNA extraction kit	

Oligonucleotides for Lso, Acat

Note: Participants can make their own choice for the dye and quencher of the probes marked with an asterix (*)

Oligonucleotides Lso: LsoF, HLBr, HLBp (Li et al., 2006; Li et al., 2009)

Oligonucleotide	Sequence 5'-3'
LsoF	gTC gAg CgC TTA TTT TTA ATA ggA
HLBr	gCg TTA TCC CgT AgA AAA Agg Tag
HLBp*	FAM-AgA Cgg gTg AgT AAC gCg-BHQ1

Oligonucleotides Acat: 2-F, 2-R, 1-Pr (Koenraadt et al., 2014)

Oligonucleotide	Sequence 5'-3'
Acat-2F	TgT AgC gAT CCT TCA CAA g
Acat-2R	TgT CgA TAg ATg CTC ACA AT
Acat-1Pr*	HEX-CTT gCT CTg CTT CTC TAT CAC g-BHQ1

1. Preparation of seed extracts and controls

1.1. Use sterile techniques during the extraction procedure

1.2. Add seed samples into their corresponding sterile stomacher bags and add 100 ml sterile PBS buffer to the seeds

1.3. Add the PPC and the NPC seed samples in their corresponding sterile stomacher bags and add 100 ml sterile PBS buffer to the seeds

1.4. As a Negative Extraction Control (NEC) add 100 ml PBS buffer in a sterile stomacher bag (without seeds)

1.5. Add 100 µl of Acat spike (OD600=0.6, a boiled bacterial suspension provided by the organizer) to each of the 21 sub-samples, PPC, NPC and NEC

1.6. Macerate all samples and controls (including NEC without seeds) in a stomacher machine until all the seeds are crushed. (Note: With InterScience Bag mixer good results were obtained by stomaching for 4 minutes at speed 3 and gap-5)

1.7. Wear gloves and prevent cross contamination between samples and controls

1.8. Pipet 2 ml of the extract from the filtered side of the stomacher bag into a 2 ml tube

1.9. For an additional in-house DNA extraction kit (King Fisher, Roche MP96, Macherey-Nagel, LGC-genomics etc.) sample an extra 2 ml of the extract from the filtered side of the stomacher bag into a 2 ml tube

2. DNA Extraction

2.1. Centrifuge the 2 ml filtered extract to pellet large seed debris (500 RCF, 1 minute). Transfer 1 ml of the supernatant to a new 1.5 ml microcentrifuge tube

2.2. Centrifuge at 10,000 RCF for 5 minutes to pellet the bacteria. Remove the supernatant and continue the DNA extraction with the Qiagen DNeasy kit on the pellet in each tube

Note: At this point it is also possible to use **in parallel** the in-house DNA extraction kit (King Fisher, Roche MP96, Macherey-Nagel, LGC-genomics etc.) next to the Qiagen Plant Mini Kit

2.3. Use the Qiagen DNeasy Plant Mini Kit. Check that the salts are dissolved in the AP1 buffer, if necessary, place at 65°C until the salts are dissolved

2.4. Disinfect the flow cabinet with 10% household chlorine solution and at least 30 minutes UV light

2.5. Add 400 µl Buffer AP1 and 4 µl RNase-A stock solution (100 mg/ml). Re-suspend the pellet, vortex and incubate at 65°C for 10 minutes

2.6. Add 130 µl Buffer P3 to the lysate, mix, and incubate for 5 min on ice or at +4°C

2.7. Centrifuge the lysate for 5 min at 20,000 RCF

2.8. Pipet the lysate into the QIA shredder Mini spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 minutes at 20,000 RCF

2.9. Transfer the flow-through into a new 1.5 ml tube without disturbing the cell-debris pellet

2.10. Add 1.5 volumes of Buffer AW1 to the cleared lysate, and mix by pipetting

2.11. Pipet 650 µl of the mixture, including any precipitate that may have formed, into the DNeasy Mini spin column (white) placed in a 2 ml collection tube. Centrifuge for 1 minute at 6,000 RCF and discard the flow-through

2.12. Add 500 µl Buffer AW2 and centrifuge for 1 minute at 6000 RCF. Discard the flow-through

2.13. Repeat the wash step by adding 500 µl Buffer AW2 to the DNeasy Mini spin column, and centrifuge for 2 minutes at 20,000 RCF to dry the membrane

2.14. Elute the extracted and purified DNA by adding 100µl AE Buffer, incubate at room temperature for 5 minutes and centrifuge into a clean 1.5 ml tube at 6,000 RCF for 1 minute

3. Seed Extract PCR assays

3.1. Test the extracts from the 21 seed samples, NTC, PPC, NPC, NEC, PAC-Acat and PAC-Lso with duplex LsoF/HLBr/HLBp-Acat TaqMan® based real-time PCR

3.2. Work on ice where possible and minimize the exposure of the probes to light

3.3. Prepare enough of the reaction mixture according to Table D1, which provides an example for the reaction mixtures. However, reaction mixture and conditions need to be checked and/or **optimized** within each laboratory *before* starting the actual comparative test.

Table D1. Reaction mixture for duplex Lso-Acat TaqMan® based real-time PCR

	Duplex Lso-Acat TaqMan		
	[Stock]	Volume 1x (µl)	[Final]
TaqMan Master Mix	2x	7.50	1x
LsoF	25 µM	0.18	300 nM
HLBr	25 µM	0.18	300 nM
HLBp	25 µM	0.12	200 nM
Acat-2F	25 µM	0.18	300 nM
Acat-2R	25 µM	0.18	300 nM
Acat-1Pr	10 µM	0.15	100 nM
Nucleic acid-free water	1 x	4.51	-
Total PCR mix	-	13.00	-
DNA sample	-	2.00	-
Total Volume	-	15.00	-

3.4. Put 13.0 µl PCR reaction mixture in a 96-well PCR plate

3.5. Add in duplicate 2.0 µl DNA in corresponding wells

3.6. Add 2.0 µl PAC-Acat, PAC-Lso, NEC, PPC, NPC and NTC in **duplicate** in corresponding wells. Run the assay based on conditions described in Table D2 for the LsoF/HLBr/HLBp-Acat TaqMan

Table D2. PCR conditions for duplex Lso-Acat TaqMan® based real-time PCR

Denaturation	10 minutes	95°C
Denaturation/Elongation	40 x	15 sec. 95°C 60 sec 60°C

4. Interpretation of SE-PCR results

4.1. The amplification curves will be analyzed with a threshold fixed above the background fluorescence at the start of the exponential amplification phase of the amplification curves.

4.2. The quantification cycle (Cq) value will be used to identify positive reactions. True positive reactions show a typical exponential increase in fluorescence.

4.3. Record **all the Cq**-values in the provided table. See Table D3 for expected values.

Table D3. The expected PCR test results

Sample	Lso Taqman	Acat Taqman	Comments
Suspect sample	Cq<40	Cq<35	
	Cq<40	Cq≥35	No detection of Acat DNA due to competition
Negative sample	Cq≥40	Cq<35	
NTC	Cq≥40	Cq<35	
PPC	Cq<40	Cq<35	
NPC	Cq≥40	Cq<35	
NEC	Cq≥40	Cq<35	
PAC-Lso	Cq<40	Cq≥35	
PAC-Acat	Cq≥40	Cq<35	
IAC (PEC)	Not applicable	Cq<35	

5. Critical points

5.1. On receiving seed samples, NPC, PPC: store below 15°C (4 to 15°C)

5.2. NTC, PAC-Lso, PAC-Acat and IAC must be stored at -20°C immediately upon arrival

5.3. Prevent DNA contamination

5.4. Store extracted DNA at -20°C in case further analysis is required

5.5. Record all results, PCR reaction mixtures and all other relevant information

5.6. Do not deviate from the proposed method unless otherwise permitted in the protocol.

5.7. Before starting the CT, get experience with the proposed method with the practice samples provided. They have an infection rate equal or little over that of a low positive seed lot. Perform the CT with persons experienced with seed health pre-screening based on molecular methods.

Appendix E. References

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