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Detection of *Pseudomonas syringae* pv. *pisi* in pea seed: Identification of suspect isolates by a PCR assay

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Detection of *Pseudomonas syringae* pv. *pisi* in pea seed: Identification of suspect isolates by a PCR assay.

1. INTRODUCTION

Pseudomonas syringae pv. *pisi* (or Psp), the causal organism of bacterial blight on pea seeds (Grondeau et al., 1993), is a significant seed-borne (Hollaway et al., 2007) and seed-transmitted bacterial pathogen (Grondeau et al., 1993; 1996; Roberts 1992; Roberts et al., 1996). Epidemiological studies have shown that Psp infected pea debris is also an important source of inoculum (Hollaway & Bretag, 1997; Hollaway et al., 2007; Grondeau et al., 1996). No effective chemical foliar or seed treatments that control this disease are currently available. Therefore, the use of healthy seed is a critical aspect of disease management strategies (Grondeau et al., 1992; Lawyer & Chun, 2001; Hollaway et al., 2007; Martín-Sanz et al., 2012).

The current method to detect Psp (ISTA Rule 7-029; see http://seedtest.org/upload/cms/user/ISTARules2018SHmethod7-029_updated20171109.pdf) is based on dilution-plating a seed wash on KBBCA and SNAC semi-selective media, optional biochemical tests on suspect colonies and a pathogenicity test (ISTA 2014). The biochemical tests allow for a reduced number of Psp suspects to be confirmed resulting in reduced time and labor in the pathogenicity test. This method, nevertheless, requires a long lead-time; nine to 19 days depending on the pathogenicity test selected and demands space making it unsuitable for high-throughput strain identification.

Strains of Psp can be grouped into two distinct genetic lineages by amplification of PCR fragments with either the AN3 (Group I) or AN7 (Group II) primers, also called the Arnold primers. Validation of the specificity of the Arnold primers was published by Arnold et al. (1996) and Martín-Sanz et al. (2012). These primer sets have been used for several years by some companies in routine seed testing.

As detection of all Psp strains requires the use of two pairs of Arnold primers to distinguish the two genetic groups within the pathovar *pisi*, two new primer pairs targeting the pathogenicity genes hopAX1 and avrRps4 were developed (Baldwin, 2015). Developmental work and validation showed that a single primer pair targeting the avrRps4 gene could be used as an alternative or in addition to the AN3-AN7 primers for the identification of *Pseudomonas syringae* pv. *pisi* strains and could replace the biochemical tests in ISTA Rule 7-029.

PCR-based assays have become the preferred tool to detect and identify plant pathogenic bacteria and offer many advantages over other assays, such as the grow-outs, bioassays and serological methods. They detect the presence of molecules (nucleic acids) specific to the target pathogen but are, however, unable to differentiate viable from non-viable pathogens and non-targets.

In line with the guidance provided in ISF's view on indirect seed health tests (see https://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-method-development/), a "negative" result when using the PCR assay indicates that the suspected isolate is not the target pathogen and the seed lot is healthy. A "positive" PCR result is indicative of a *suspect* seed lot, and the health status of the seed lot can only be determined after the pathogenicity assay.

The PCR assay for identification is optional (see Figure 1 for the workflow of the method for detecting Psp in pea seed) and the final seed health status of pea seed lots being tested for the presence of Psp can only be confirmed by a pathogenicity assay.

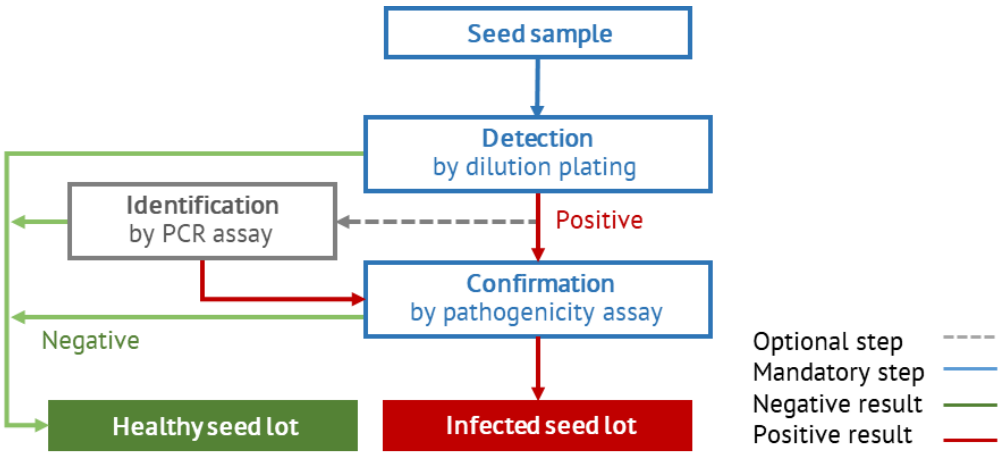


Figure 1. Workflow of the methods for detecting Psp in pea seed

2. OBJECTIVES

The objective of this study is to demonstrate that the PCR assay, a step in the method to detect *Pseudomonas syringae* pv. *pisi* in pea seed, accurately identifies suspect isolates. The assay was validated according to the ISHI-Veg guidelines for the Validation of Seed Health Tests (Version 2, May 2020).

3. METHOD VALIDATION

The protocol of the PCR assay for identifying suspect *Pseudomonas syringae* pv. *pisi* isolates is in Annex A in this document.

3.1. Analytical specificity

Definition ISHI-Veg guidelines: *The ability of an assay to detect the target(s) pathogens (inclusivity) while excluding non-targets (exclusivity).*

The requirements for analytical specificity were met when the PCR assay using the Arnold (AN3 or AN7) and avrRps4 primers gives a positive result for the Psp strains tested and a negative result for all non-Psp strains tested. The PCR results are compared with the result of the pathogenicity assay to confirm the identity of the strains; e.g. Psp or non-Psp.

Experimental approach

Data were generated from 2013 to 2015 (Baldwin, 2015) by three labs.

Inclusivity

A total of 58 *Pseudomonas syringae* pv. *pisi* strains belonging to both genetic groups (7 strains of the group I amplified by AN3 and 51 strains of the group II amplified by AN7) and 5 non-Psp strains (used as negative controls), were characterised by the three labs using a real-time PCR followed by a pathogenicity test on pea plants using the pathogenicity assay option 2 (inoculation of seedlings with a bacterial suspension made from a suspect colony) in ISTA's Rule 7-029. The PCR assay was

performed following the protocol described in Annex A but the Wu assay was not consistently run. For some samples the Wu assay was performed by one laboratory on a different suspension of the isolate than the AN3, AN7 and avrRps4 assays. Information on the diversity of the collection used (countries and year collected) was not available.

Exclusivity

A collection of 30 suspect *Pseudomonas syringae* strains isolated from pea seeds and 5 non-Psp strains, all found to be non-pathogenic on pea plants according to the pathogenicity assay in ISTA's Rule 7-029, as well as two positive controls for Psp were tested to determine if the PCR assay excluded non-Psp strains. The PCR assay was performed following the protocol described in Annex A, without inclusion of the Wu assay.

In silico analysis

In complement, an *in silico* analysis was performed for each primer pair using the Primer-blast online tool (Ye *et al.*, 2012). Default parameters were used. Primers sequences were blasted against the nucleotide collection database (nr) to check for specificity.

Results

Inclusivity

Table 1 shows that the pathogenicity test results on pea plants correlates 100% with the PCR assay results using Arnold (AN3 and AN7) and avrRps4 primers.

Table 1. Results of PCR identification, using AN3, AN7, avrRps4 and Wu primers, and the pathogenicity test for 58 Psp strains and 5 non-Psp strains.

ID	Isolate Name	Collection	PCR Reaction Cq values			Result Pathogenicity test	Psp Genetic Group
			AN3/AN7*	avrRps4	Wu**		
1	192.3.d1	Lab 1	14.27 (AN7)	13.78	NA	Pathogenic	II
2	213.3.d0	Lab 1	13.53 (AN7)	12.5	NA	Pathogenic	II
3	215.3.d0	Lab 1	13.9 (AN7)	14.39	NA	Pathogenic	II
4	L196.6.d1	Lab 1	13.66 (AN7)	12.59	NA	Pathogenic	II
5	L200.3.do	Lab 1	13.94 (AN7)	13.14	NA	Pathogenic	II
6	L201.6.d1	Lab 1	13.88 (AN7)	13.2	NA	Pathogenic	II
7	L44.3.d1	Lab 1	13.98 (AN7)	13.19	NA	Pathogenic	II
8	L45.5.d0	Lab 1	14.47 (AN7)	13.87	NA	Pathogenic	II
9	L46.4.d0	Lab 1	14.32 (AN7)	13.51	NA	Pathogenic	II
10	L50.4.d1	Lab 1	13.81 (AN3)	13.7	NA	Pathogenic	I
11	50.5.d0	Lab 1	13.77 (AN7)	12.73	NA	Pathogenic	II
12	69.3.d0	Lab 1	14.4 (AN7)	13.97	NA	Pathogenic	II
13	i32485.2	Lab 1	13.96 (AN7)	13.33	NA	Pathogenic	II
14	J67989.2.2	Lab 1	14.19 (AN3)	13.65	NA	Pathogenic	I
15	i91920.2	Lab 1	14.91 (AN3)	13.96	NA	Pathogenic	I
16	348	Lab 1	14.47 (AN7)	13.8	NA	Pathogenic	II
17	J06789.2.2	Lab 1	14.02 (AN3)	12.49	NA	Pathogenic	I
18	L66.1.d2	Lab 1	13.81 (AN7)	12.43	NA	Pathogenic	II

ID	Isolate Name	Collection	PCR Reaction Cq values			Result Pathogenicity test	Psp Genetic Group
			AN3/AN7*	avrRps4	Wu**		
19	158.2.d0	Lab 1	13.73 (AN7)	12.73	NA	Pathogenic	II
20	183.5.d0	Lab 1	14.09 (AN7)	13.1	NA	Pathogenic	II
21	187.1.d2	Lab 1	14.25 (AN7)	14.35	NA	Pathogenic	II
22	PVL1	Lab 1	13.79 (AN7)	13.23	NA	Pathogenic	II
23	I31522.3	Lab 1	13.99 (AN7)	13.17	NA	Pathogenic	II
24	I32631.2.d1.1	Lab 1	14.15 (AN3	13.46	NA	Pathogenic	I
25	i32250	Lab 1	14.43 (AN7)	14.31	NA	Pathogenic	II
26	10840.1	Lab 1	14.41 (AN7)	13.48	NA	Pathogenic	II
27	10450	Lab 1	14.74 (AN7)	13.64	NA	Pathogenic	II
28	FTK1	Lab 1	13.87 (AN7)	14.08	NA	Pathogenic	II
29	FTM1	Lab 1	14.08 (AN7)	13.8	NA	Pathogenic	II
30	Psp2	Lab 2	16.29 (AN7)	16.05	NA	Pathogenic	II
31	Psp3	Lab 2	16.82 (AN7)	16.13	NA	Pathogenic	II
32	Psp4	Lab 2	16.58 (AN7)	16.01	NA	Pathogenic	II
33	Psp5	Lab 2	17.75 (AN3)	16.61	NA	Pathogenic	I
34	Psp8	Lab 2	16.56 (AN7)	15.78	NA	Pathogenic	II
35	Psp9	Lab 2	16.78 (AN7)	16.10	NA	Pathogenic	II
36	Psp10	Lab 2	16.27 (AN7)	16.13	NA	Pathogenic	II
37	Psp11	Lab 2	15.62 (AN7)	15.58	NA	Pathogenic	II
38	Psp13	Lab 2	16.09 (AN7)	15.80	NA	Pathogenic	II
39	Psp14	Lab 2	17.45 (AN7)	17.01	NA	Pathogenic	II
40	Psp15	Lab 2	16.33 (AN7)	15.60	NA	Pathogenic	II
41	Psp16	Lab 2	16.45 (AN7)	15.91	NA	Pathogenic	II
42	Psp17	Lab 2	17.73 (AN7)	16.23	NA	Pathogenic	II
43	Psp18	Lab 2	16.33 (AN7)	15.68	NA	Pathogenic	II
44	CFBP2105	Lab 2	16.65 (AN7)	16.27	NA	Pathogenic	II
45	65-9-0	Lab 3	18.59 (AN7)	18.23	12.19	Pathogenic	II
46	65-7-0	Lab 3	19.11 (AN3)	17.76	12.01	Pathogenic	I
47	425046-2-1	Lab 3	18.3 (AN7)	17.75	12.07	Pathogenic	II
48	425046-3-2	Lab 3	18.07 (AN7)	17.51	12.86	Pathogenic	II
49	425046-2-2	Lab 3	18.78 (AN7)	17.11	12.11	Pathogenic	II
50	425052-3-2	Lab 3	18.28 (AN7)	17.77	11.91	Pathogenic	II
51	425054-2-0a	Lab 3	18.31 (AN7)	17.32	12.34	Pathogenic	II
52	425054-2-0b	Lab 3	18.57 (AN7)	17.49	12.25	Pathogenic	II
53	425054-4-1	Lab 3	18.34 (AN7)	18.32	12.25	Pathogenic	II
54	425058-2-0	Lab 3	18.07 (AN7)	17.34	12.48	Pathogenic	II
55	425058-2-2	Lab 3	18.34 (AN7)	17.34	12.14	Pathogenic	II
56	425058-5-0	Lab 3	18.41 (AN7)	17.67	12.55	Pathogenic	II
57	425058-3-0	Lab 3	17.95 (AN7)	17.04	12.38	Pathogenic	II

ID	Isolate Name	Collection	PCR Reaction Cq values			Result Pathogenicity test	Psp Genetic Group
			AN3/AN7*	avrRps4	Wu**		
58	CFBP6472	Lab 3	18.5 (AN7)	17.49	12.61	Pathogenic	II
59	*P. syringae lachrymans* 1007009	Lab 1	Negative	Negative	NA	Non-Pathogenic	NR
60	*P. syringae* (group II) strain 1213	Lab 1	Negative	Negative	NA	Non-Pathogenic	NR
61	Psp12	Lab 2	Negative	Negative	NA	Non-pathogenic	NR
62	425052-1-1	Lab 3	Negative	Negative	16	Non-pathogenic	NR
63	425052-5-1	Lab 3	Negative	Negative	14.51	Non-pathogenic	NR

Notes*:* A positive result, an amplification by real-time PCR and a same melt temperature as the positive isolate control reaction (± 1.5°C), is indicated by a green cell. A negative result, no amplification or amplification with a melt temperature different than the positive isolate control reaction (± 1.5°C), is indicated by a red cell**;** NA: missing data; NR: Not relevant; *: AN3 and AN7 primer pairs were run in duplex in the same PCR assay; **: the Wu assay (in simplex) was performed by one laboratory for some samples on a different suspension of the isolate then the AN3/AN7 and avrRps4 assays.

Exclusivity

Results presented in Table 2 show that the reaction of the three PCRs with the AN3-AN7 and avrRps4 primers led to 100% correlation with the outcome of the pathogenicity test.

Table 2. Results of PCR identification using AN3, AN7 and avrRps4 primers compared to results of pathogenicity test for 35 non-Psp strains and 2 Psp strains used as positive controls.

	Isolate Name	PCR Reaction*		Result Pathogenicity test
		AN3/AN7	avrRps4	
1	85374.D1	Negative	Negative	Non-Pathogenic
2	86880.3D03	Negative	Negative	Non-Pathogenic
3	86880.6D1	Negative	Negative	Non-Pathogenic
4	86880.4D03	Negative	Negative	Non-Pathogenic
5	86880.3D02	Negative	Negative	Non-Pathogenic
6	85983.5D0	Negative	Negative	Non-Pathogenic
7	85983.6D02	Negative	Negative	Non-Pathogenic
8	85983.6D01	Negative	Negative	Non-Pathogenic
9	85983.4D2	Negative	Negative	Non-Pathogenic
10	85983.2D0	Negative	Negative	Non-Pathogenic
11	85983.3D1	Negative	Negative	Non-Pathogenic
12	85983.2D1	Negative	Negative	Non-Pathogenic
13	85983.1D1	Negative	Negative	Non-Pathogenic
14	87274.D1	Negative	Negative	Non-Pathogenic
15	87274.D2.2	Negative	Negative	Non-Pathogenic
16	87274.D2.1	Negative	Negative	Non-Pathogenic
17	87274.D0.1	Negative	Negative	Non-Pathogenic
18	85090.D0.3	Negative	Negative	Non-Pathogenic
19	85090.D1.2	Negative	Negative	Non-Pathogenic
20	85090.D1.1	Negative	Negative	Non-Pathogenic
21	86781.D0.2	Negative	Negative	Non-Pathogenic

	Isolate Name	PCR Reaction*		Result Pathogenicity test
		AN3/AN7	avrRps4	
22	85456.6D1	Negative	Negative	Non-Pathogenic
23	86781.D0.1	Negative	Negative	Non-Pathogenic
24	85456.6D2	Negative	Negative	Non-Pathogenic
25	85456.5D0	Negative	Negative	Non-Pathogenic
26	85454.D04	Negative	Negative	Non-Pathogenic
27	85456.2D1	Negative	Negative	Non-Pathogenic
28	85456.2D0	Negative	Negative	Non-Pathogenic
29	84653.6	Negative	Negative	Non-Pathogenic
30	85277.D0	Negative	Negative	Non-Pathogenic
31	*P. syringae lachrymans* 1007009	Negative	Negative	Non-Pathogenic
32	*P. syringae* (group II) strain 1213	Negative	Negative	Non-Pathogenic
33	Psp12	Negative	Negative	Non-Pathogenic
34	425052-1-1	Negative	Negative	Non-Pathogenic
35	425052-5-1	Negative	Negative	Non-Pathogenic
1	CFBP 2105 (PC Psp group II)	Positive (AN7)	Positive	Pathogenic
2	K55817 (PC Psp group I)	Positive (AN3)	Positive	Pathogenic

Note: A positive result, an amplification by real-time PCR and a same melt temperature as the positive isolate control reaction (± 1.5°C), is indicated by a green cell. A negative result, no amplification or amplification with a melt temperature different than the positive isolate control reaction (± 1.5°C), is indicated by a red cell; PC: Positive Control; *Cq values were not available.

In silico analysis

The primer pairs were specific to Psp as no PCR product from non-Psp strains was generated (Table 3). The AN3 and AN7 primer pairs generated a product with a *P. syringae* strain from anonymous DNA but the pathovar identity was not specified. The amplicon length was 131 and 272 bp for AN3 and AN7 respectively as expected.

Table 3. Number of hits for each primer pair using Primer Blast.

Primer pair	Number of hits			Number of hits analyzed
	Psp strain	Non-Psp strain	others	
avrRps4	2	0		1446
AN3	1[1]	0	1[2]	2583
AN7	0	0	1[2]	3467

[1] With 2 mismatched at the 3'-side of the primer.

[2] *P. syringae* anonymous DNA

Conclusion

Inclusivity and exclusivity were 100% for the PCR assay independently of the primer pair used. *In silico* analysis showed that all primer pairs were specific to Psp as no PCR product from non-Psp strains was generated. The requirements for analytical specificity are met, as 100% of the target collection was detected and 100% of the non-target collection was not detected.

3.2. Analytical sensitivity

Definition ISHI-Veg guidelines: *Smallest amount of the target pathogen that can be detected i.e. the limit of detection (LOD).*

In this method the PCR is used as identification assay on bacterial suspensions after the dilution plating detection assay. The analytical sensitivity of the identification assay is, therefore, not the analytical sensitivity of the method. However, sufficient dilutions of the bacterial suspensions should be tested to ensure that the dilution used is fit for purpose.

The dilution used is fit-for-purpose when Psp strains give a positive PCR result by AN3 or AN7, avrRps4 and Wu primers (amplification and correct melting curve temperature) and non-Psp strains give a negative PCR result using the specific primers (no amplification or amplification with a different melting curve temperature) and a positive result using the Wu primers (amplification curve).

Experimental approach

Data was generated during the comparative test (CT) described in section 3.5 of this report.

A routinely used dilution of 10^7-10^8 CFU/mL for bacterial suspensions was selected for Psp. A similar concentration is used in other ISF protocols (e.g. detection of *Xanthomonas axonopodis* pv. *phaseoli* and pv. *phaseoli* var. *fuscans* in bean seeds, 2019). This operational range was tested during homogeneity and stability tests (see section 3.5) with 60 dead bacterial suspensions (25 Psp, 30 non-Psp and 5 mixed suspensions of Psp and non-Psp). The protocol in Annex A was used to prepare bacterial suspensions and run the real-time PCR.

Results

All bacterial suspensions were positive using the Wu primers (Ct ⩽ 35) and no amplification occurred in the non-template control. All Psp suspensions were positive; amplification and the melt temperature were the same as the positive isolate control reaction (± 1.5°C). All non-Psp suspensions were negative, i.e. there were no amplifications when using AN3, AN7 or avrRps4 primers (Annex B and C).

Conclusion

The results confirmed that the used dilution concentration of 10^7-10^8 CFU/mL is fit for purpose.

3.3. Selectivity

Definition ISHI-Veg guidelines: *The effect of different seed matrices on the ability of the method to detect target pathogen(s).*

To evaluate selectivity for the PCR identification assay, bacterial suspensions of Psp strains are mixed with a suspension of a look-alike saprophyte to simulate different matrices.

The requirements for selectivity will be met when the Psp isolate will be detected by the PCR assay in all matrices (mixed suspensions), by all labs.

Experimental approach

Data was generated during the CT described in this report (section 3.5).

Bacterial suspensions of five Psp strains were prepared and each was mixed with a suspension of a look-alike saprophyte in a 1:1 ratio to simulate different matrices (Table 4). The protocol presented in Annex A was used to prepare bacterial suspensions and run the real-time PCR reactions.

Table 4. Composition of the mixed bacterial suspensions and the code used in the comparative test.

Isolate codes and names		
Code	Psp isolate name	Non-Psp isolate name
23	K55817	1.2
31	18	2.10
39	L.1.1.D2	2.17
47	7	3.14
55	21	3.4

Results

The target pathogen was detected in all 5 mixed suspensions with AN3-AN7 and avrRps4 primers by all laboratories (Table 5 and Annex D).

Table 5. Results of PCR identification using AN3, AN7, avrRps4 and Wu primers for the mixed suspensions from laboratory C.

Code	Name	Expected result	AN3/AN7		avrRps4		Wu		Final result
			Cq	Melt	Cq	Melt	Cq	Melt	
23	1.2+K55817	positive	15.04	81.8 (AN3)	14.33	84.0	21.1	84.5	positive
31	2.10+18	positive	13.71	81.8 (AN3)	12.97	84.0	20.34	85.0	positive
39	2.17+L.1.1.D2	positive	16.47	85.0 (AN7)	14.64	84.0	22.15	84.5	positive
47	3.14+7	positive	14.45	85.0 (AN7)	13.48	84.0	18.44	84.5	positive
55	3.4+21	positive	16.28	85.0 (AN7)	14.34	84.0	19.51	84.7	positive

Conclusion

The different matrices used did not affect the ability of the method to target Psp strains. Therefore, the validation requirements for selectivity are met.

3.4. Robustness

Robustness indicates the variation in the results due to deviations in the procedure, circumstances or nature of the materials.

The robustness requirement for the PCR assay will be met when the same qualitative results for all four primer sets (AN3, AN7, avrRps4 and Wu) are obtained for all samples tested by laboratories using different PCR equipment and PCR mixes.

The robustness requirement for the AcrRps4 primers will be met when a PCR product will be obtained with the avrRps4 primers in all end-point PCRs with different annealing temperatures.

Experimental approach

Data was generated in 2013 (Baldwin, 2015) and during the CT described in this report (section 3.5).

During the CT laboratories were free to use different PCR equipment and PCR mixes. Twenty-five Psp suspensions, 30 non-Psp suspensions and 5 mixed suspensions of Psp and non-Psp were tested in the CT.

Additionally, the robustness of the avrRps4 primers was tested against a change in the PCR technique: an end-point PCR with different annealing temperatures was used instead of the real-time PCR. The performance of these primers in conventional PCR was tested on three Psp strains

and three non-Psp strains. Three different annealing temperatures were tested. PCR products were visualised on a 2% (w/v) agarose electrophoresis gel (Figure 2). The expected size of the amplified fragment was 114 bp.

Results

During the CT, three laboratories (coded B, C and E) obtained the same qualitative results for all primer sets (raw data presented in Annex D). These laboratories used different PCR equipment and PCR mixes (Table 6).

In the robustness test of the avrRps4 primers it was possible to distinguish the positive PCR reactions (above the 100bp marker) from the primer dimers observed in the negative reactions.

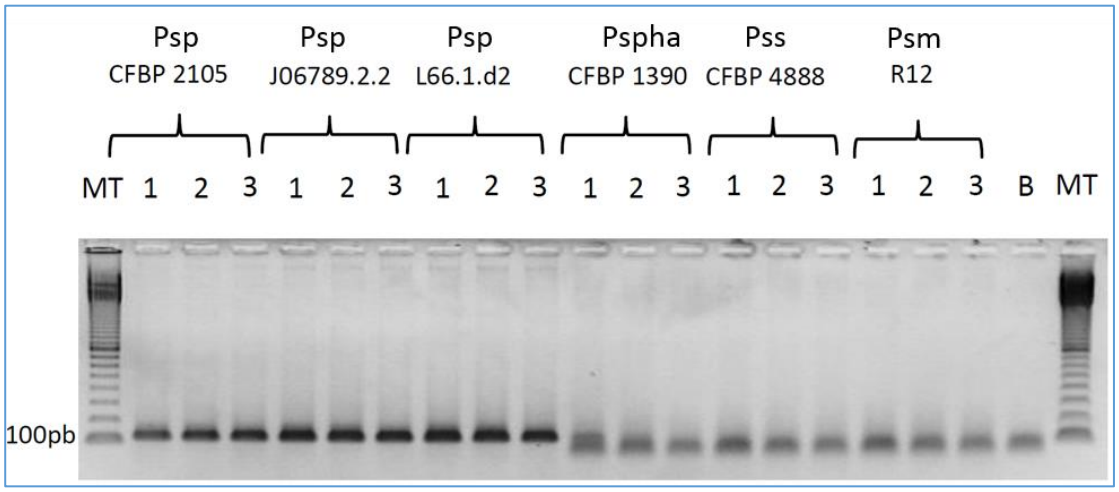


Figure 2. Gel electrophoresis of PCR products amplified with the avrRps4 primers. MT: 100bp ladder; annealing temperatures (1=58°C; 2=61°C; 3=63°C); B= Non-template control; Psp: *Pseudomnas syringae* pv. *pisi*, Pspha: *Pseudomonas savastanoi* pv. *phaseolicola*, Pss: *Pseudomonas syringae* pv *syringae*, Psm: *Pseudomonas syringae* pv. *maculicola.*

Conclusion

The PCR assay is robust for PCR equipment and PCR mixes used.

The avrRps4 PCR assay is robust in the range of PCR annealing temperatures tested and to the change of PCR technique.

3.5. Repeatability and Reproducibility

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

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

The requirements for repeatability and reproducibility will be met when the accordance and concordance of the test results obtained by the different laboratories on the tested samples are above the accepted values of 90%.

Experimental approach

Repeatability (accordance) and reproducibility (concordance) of the method were evaluated in a comparative test (CT) in which five laboratories participated using identical samples and following

the same protocol in Annex A. The five laboratories which participated in the CT were randomly coded using the letters A to E. Data generated from the CT provided quantification of variation in test performance in the same laboratory and across different laboratories.

All laboratories received a set of coded samples. The set comprised of 60 suspensions of dead bacteria in sterile water at approximately 10^7-10^8 CFU/mL. The bacteria were killed by heating them to 95°C for 10 min as specified in the proposed method (Annex A). These bacterial suspensions were divided in three categories:

- 25 *Pseudomonas syringae* pv. *pisi* isolates
- 30 non-target isolates (look-alike saprophytes from pea seeds), and
- 5 mixed suspensions of target Psp isolates and a look-alike saprophyte in a ratio of 1:1.

In addition to the samples, the following controls were included in the package:

- 'AN3 control' (positive process control 1), suspension of an isolate amplified by AN3 and avrRps4 primers but not by AN7 primers.
- 'AN7 control' (positive process control 2), suspension of an isolate amplified by AN7 and avrRps4 primers but not by AN3 primers.

Each participating lab was asked to include a negative control (non-template control or NTC). All suspensions had to be stored at -20°C upon arrival until they were processed.

Materials needed to perform the test:

- Reagents for real-time PCR
- Sterile microtubes (1.5 ml; 0.2 ml)
- Microlitre pipettes with sterile filtered tips (1 µl – 1000 µl)
- Real-time PCR equipment

Each laboratory could use their own real-time PCR equipment and PCR reagents.

PCR testing

Each sample and control were tested in three single PCR reactions using different primers:

- avrRps4F/R Psp-specific primers,
- Arnold Psp-specific primers,
- Wu universal bacterial primers (Wu et al., 2008)

A negative Psp-specific result can only be verified if there is a PCR product amplified using the Wu primers and no PCR product is amplified with any of the Psp-specific primers on the corresponding sample. A positive Psp-specific result is verified if a PCR product is amplified with at least one of the Psp-specific primers and with the Wu primers. The PCR primer pairs are run as separate reactions because the melt temperatures of the avrRps4 and Wu PCR products are too close to be easily distinguished in duplex reactions. AN3 and AN7 primer pairs are run in duplex in the same run as the melt temperature of their PCR product can be easily distinguished. The protocol for the PCRs is described in the Annex A.

Prior to the CT the set of isolates used were characterized with the avrRps4 primers and AN3-AN7 primers. The pathogenicity test was performed only by the organising laboratory.

Notation of results

Participants reported quantitative (Cq values) as well as qualitative (positive/negative) results for each subsample and each primer set, the quantification and melt curve analysis reports.

In addition, they were asked to include information on the model of PCR machine and the PCR mix for each PCR. However, statistical analysis was performed on the qualitative data only since Cq values are difficult to compare due to a multitude of factors, such as equipment, chemicals used, thresholds and primer supplier, which vary between labs and influences Cq values.

Statistical Analysis

Homogeneity

Since dead bacterial suspensions were used in this comparative test no homogeneity test was performed but the whole set of isolates was tested using AN3-AN7, avrRps4 and Wu primers following the protocol in Annex A.

Before the CT, isolates were characterised by an oxydase-test which was performed by the organising lab using the ISTA method 7-029. Psp isolates were also tested for their pathogenicity, using the pathogenicity assay option 2 in the ISTA method 7-029, before 2015 by the organising laboratory. Non-Psp isolates were tested using the pathogenicity assay by the organiser laboratory just before the CT. Mixed suspensions were not tested by the pathogenicity test.

Stability

To determine the stability of samples over time, an extra set of samples was kept at room temperature and tested by the organiser at the end of the CT, using AN3-AN7, avrRps4 and Wu primers (protocol in Annex A), once all laboratories had finished their tests.

Accordance and concordance

Accordance (repeatability of qualitative data) and concordance (reproducibility of qualitative data) were evaluated using the method developed by Langton et al. (2002). Results were analysed separately for targets (30 Psp isolates in pure culture or in mixed culture) and non-targets (30 isolates) using the ISTA online tool based on Langton's method (https://www.seedtest.org/en/tool-box-_content---1--1410.html).

For qualitative results, accordance is expressed as the probability that two samples give the same result, i.e. the number of accords divided by number of possible accords in a laboratory. The probability averaged over all laboratories gives an estimate of the repeatability of the assay.

For qualitative data, concordance is calculated by enumerating all possible pairing of results between laboratories. Concordance is calculated as the number of accords divided by the number of possible accords between laboratories giving an estimate of the reproducibility of the assay.

These measures are based on the probability of finding the same test results for identical test materials (target or non-target) within and between laboratories, respectively.

Results

All laboratories received a coded set of 60 suspensions of dead bacteria in sterile water at approximately 10^7-10^8 CFU/mL. A second identical set was sent to all the participating labs as the first was delayed at customs resulting in one of the participating labs receiving dry samples. Lab A tested both sets of samples, labs B and D the first set and lab C and E the second set. For Lab A only the test results of the first set of samples are used in the calculations.

The real-time PCR equipment and PCR reagents used by the different laboratories are summarised in Table 6.

Table 6. PCR equipment and reagents used in the CT by the different laboratories.

Laboratory	PCR equipment	PCR mix
A	ViiA 7, Thermo Fisher Scientific	QuantiTect SYBR Green, Qiagen
B	Step-One Plus Life technologies	QuantiTect SYBR Green, Qiagen
C	Rotor Gene Q, Qiagen	QuantiTect SYBR Green, Qiagen
D	CFX96, BioRad	Sso ADV Universal SYBR Green Supermix, BioRad
E	CFX-96 Real-Time System - C1000 Touch Thermal cycler, BioRad	Sso Advanced universal SYBR Green Supermix, BioRad

Homogeneity

Psp isolates were all oxydase negative (Annex B). Non-Psp strains were either oxydase negative or positive and were all non-pathogenic on pea seedlings.

Using AN3-AN7 and avrRps4 primers, a PCR product was detected before 35 cycles at the same melt temperature as the positive isolate control (± 1.5°C) for all the 25 Psp strains and the five mixed suspensions (Annex B).

For non-Psp isolates, no PCR products were detected for 13 isolates using the AN3-AN7 duplex. A late amplification product (around 30 cycles) was detected for 17 non-Psp isolates. The delta in melt temperature was above 1.5°C for 16 isolates (Annex B). One non-Psp isolate (sample 19: isolate "2.4") has a melt curve temperature of 82.7°C (AN3-control melt temperature was at 82.2°C). Using the avrRps4 primers no PCR products were detected for 25 isolates. A late amplification product (around 31 cycles) was detected for five non-Psp isolates with a delta in melt temperature above 1.5°C compare to the positive control (Annex B). Thus, final results for these five isolates are negative. Late amplifications are not problematic if the melt temperatures of the PCR product are not specific, but one must pay attention to the melting curve to correctly interpret the result.

Using the Wu primers, a PCR product was detected for all the 60 bacterial suspensions (Psp and non-Psp). An amplification was detected in water (Cq value = 27.8) but the Cq value was 3.3 points higher than that of the Cq value for the samples, with the exception of sample 24 (isolate "2.7") which had a Cq value of 26.4 (only 1.4 points lower then the water Cq value). When using universal bacterial primers, positive reactions may occur due to the presence of residual DNA in Taq enzyme reagents. The Internal Amplification Control (IAC) Cq values from reactions on suspect isolates should indicate at least 10-fold higher concentration of bacterial DNA than the IAC Cq values from the NTC reactions; the difference between Cq values should be more than 3.3 according to the best practices for PCR Assays in Seed Health Tests developed by ISHI-Veg (https://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-method-development/).

Samples 19 (non-Psp isolate "2.4") and 24 (non-Psp isolate "2.7") gave results that deviated from expectations. Particular attention was paid to these isolates during the stability analysis.

Stability

The stability test was performed once all participating laboratories had finished their tests.

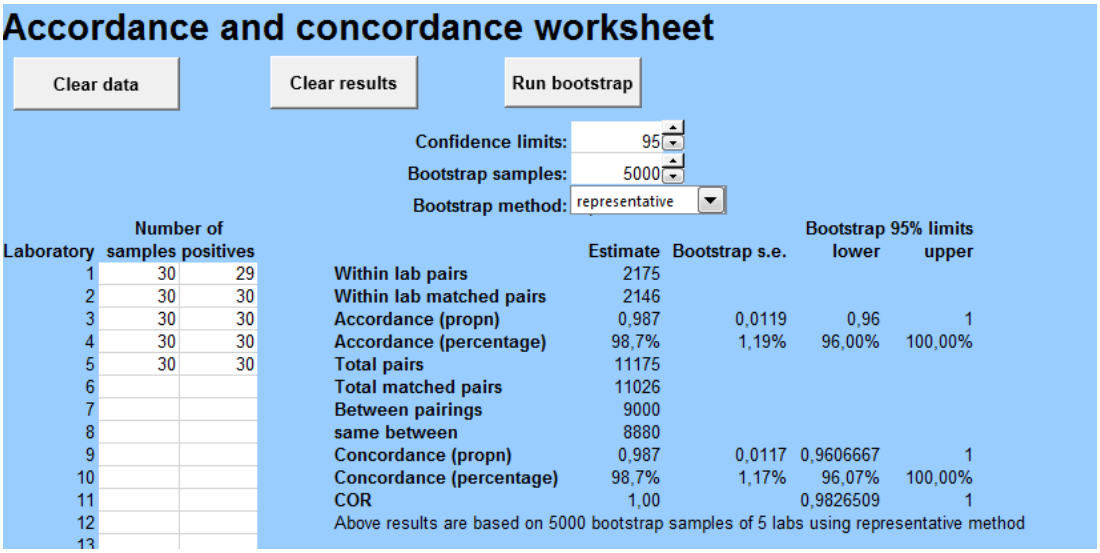
Using AN3-AN7 and avrRps4 primers, a PCR product was detected before 35 cycles at the same melt temperature as the positive isolate control (± 1.5°C) for all the 25 Psp strains and the five mixed suspensions (Annex C).

For non-Psp strains no PCR products were detected using either the AN3-AN7 duplex or the avrRps4 primers, with the exception of sample 7 (non-Psp isolate "3.14", Cq = 30.29 with a melt temperature of 84.7°C) (Annex C). The non-Psp isolate "2.4" gave no amplification curve. Considering homogeneity results, the late amplification curves observed were most probably some cross contaminations because they were not reproducible.

All amplification control reactions were positive using the Wu primers (Cq ⩽ 35) and no amplification occurred in the NTC.

Accordance and concordance

Accordance and concordance were calculated separately for target and non-target isolates. They were both at 100% for the AN3-AN7 primer set and were for the avrRps4 primer set at 98.7% (CI, confidence interval calculated by bootstrap was 96.07-100%) and 97.3% (CI = 94.77-100%) for the target isolates and the non-target isolates respectively (Figures 3 and 4). For the avrRps4 primer set, the mean accordance and concordance for target and non-target isolates was at 98%.



Accordance and concordance worksheet

Clear data | Clear results | Run bootstrap

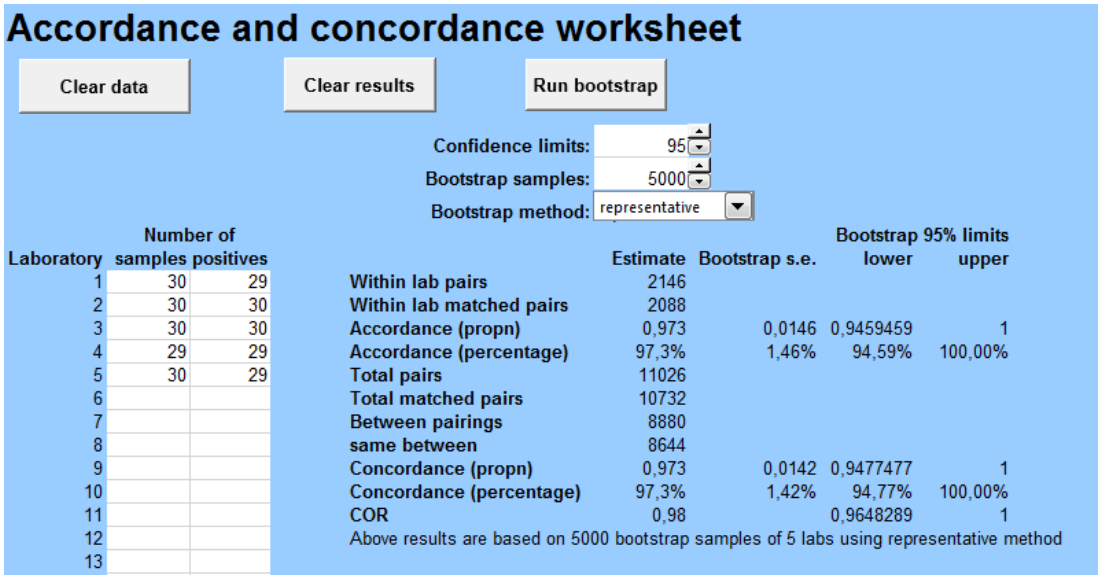
Confidence limits: 95
Bootstrap samples: 5000
Bootstrap method: representative

Laboratory	Number of samples	positives
1	30	29
2	30	30
3	30	30
4	30	30
5	30	30
6		
7		
8		
9		
10		
11		
12		
13		

	Estimate	Bootstrap s.e.	Bootstrap 95% limits lower	upper
Within lab pairs	2175			
Within lab matched pairs	2146			
Accordance (propn)	0,987	0,0119	0,96	1
Accordance (percentage)	98,7%	1,19%	96,00%	100,00%
Total pairs	11175			
Total matched pairs	11026			
Between pairings	9000			
same between	8880			
Concordance (propn)	0,987	0,0117	0,9606667	1
Concordance (percentage)	98,7%	1,17%	96,07%	100,00%
COR	1,00		0,9826509	1

Above results are based on 5000 bootstrap samples of 5 labs using representative method

Figure 3. Calculation of accordance and concordance on target isolates with avrRps4 primer set



Accordance and concordance worksheet

Clear data | Clear results | Run bootstrap

Confidence limits: 95
Bootstrap samples: 5000
Bootstrap method: representative

Laboratory	Number of samples	positives
1	30	29
2	30	30
3	30	30
4	29	29
5	30	29
6		
7		
8		
9		
10		
11		
12		
13		

	Estimate	Bootstrap s.e.	Bootstrap 95% limits lower	upper
Within lab pairs	2146			
Within lab matched pairs	2088			
Accordance (propn)	0,973	0,0146	0,9459459	1
Accordance (percentage)	97,3%	1,46%	94,59%	100,00%
Total pairs	11026			
Total matched pairs	10732			
Between pairings	8880			
same between	8644			
Concordance (propn)	0,973	0,0142	0,9477477	1
Concordance (percentage)	97,3%	1,42%	94,77%	100,00%
COR	0,98		0,9648289	1

Above results are based on 5000 bootstrap samples of 5 labs using representative method

Figure 4. Calculation of accordance and concordance on non-target isolates with avrRps4 primer set

Conclusion

The combined results for homogeneity and stability allow for the conclusion that samples were homogeneous and stable. Some cross contaminations (late amplification) were observed in two samples using the AN3-AN7 duplex but were not reproduced in the homogeneity and stability analyses.

The accordance and concordance of the AN3-AN7 and avrRps4 primer sets for both the target and non-target isolates (100% and 100%, and 98.7% and 97.3% respectively) are above the accepted values (90%) according to ISHI-Veg Guidelines for the Validation of Seed Health Tests. Therefore, the validation requirements for repeatability and reproducibility are met.

3.6. Diagnostic performance

Definition ISHI-Veg guidelines: *The ability of the method to detect target pathogens in known infected seed samples while excluding non-target organisms in known healthy seed samples*

Although there is no fixed rule, values above 95% are considered acceptable for analytical sensitivity, analytical specificity and accuracy (ISTA, 2018).

Experimental approach

Samples were analysed according to the Standard NF EN ISO 16140 (AFNOR, 2003). Diagnostic sensitivity, diagnostic specificity and accuracy of the PCR assay, the diagnostic performance of the assay in other words, were calculated according to the following mathematical formulas:

	Expected result + (target)	**Expected result - (non-target)**
Obtained result +	positive agreement (PA) +/+	positive deviation (PD) -/+
Obtained result -	negative deviation (ND) +/-	negative agreement (NA) -/-

Diagnostic sensitivity = ΣPA / (ΣPA+ΣND) x 100

Diagnostic specificity = ΣNA / (ΣNA+ΣPD) x 100

Accuracy = (ΣNA+ΣPA) / (ΣPA+ΣNA+ΣPD+ΣND) x 100

Results

Raw data from the tests run by all participants are presented in Annex D.

Laboratories set their own thresholds internally above the background noise of fluorescence. The cut-off value was fixed at 35 cycles for this CT. However, as cut-off value is dependent on equipment, material and chemical products it should be determined by each laboratory based on internal validation data. All negative samples (non-target isolates) were negative and all positive samples were positive using the AN3, AN7 primer set. The Wu primer allowed for the amplification of all suspensions with a ΔCq > 3.3 between samples and negative control (water) with the exception of samples 1 and 10 for lab D.

As sample 10 was positive using AN3-AN7 and avrRps4 primers the lack of amplification with the Wu primers is acceptable. However, sample 1 could not be verified as being negative and a re-test was required using the Wu primers, and was therefore not included in the calculation of the performance characteristics. Lab D did perform a second PCR after the CT on all samples using the Wu primers and all results were positive (Annex D). The cause was most likely a technical issue.

When using avrRps4 primers, four false positives with high Cq values (Lab A sample 56; Lab D samples 19 and 56, and Lab E sample 51) and one false negative (Lab A sample 41) results were reported (Annex D). As the Cq values of the false positives were high, cross-contamination was suspected. Laboratories were asked to run these samples a second time to determine if the false positive results were due to cross contamination or a lack of specificity of the primers, however, the original results are used in the calculation of the performance characteristics.

Lab A ran the protocol on the two sets of samples. After examining the results given by the two sets of samples it appeared that the false positive and false negative results were due to either technical problems or cross-contamination because the result was not repeated with the second, identical sample set. The false-positive results were due to cross contamination and not to lack of avrRps4 primer specificity, while the false-negative was due to a technical issue. Thus, results of the second run confirmed the suspicion of cross-contamination. Although, these results are not considered in assessing the performance of the assay, they provide valuable information on the need to minimise the risk of cross contamination when using the assay.

Lab D, after checking its results, determined both false positive results to be negative. There was an error in the analysis of the melt curve, as the melting temperature was not specific to Psp (Annex D). Thus, they did not need to run these samples a second time.

Lab E ran the protocol a second time on sample 51 that had initially given a false positive result and obtained a negative result.

Performance criteria calculated with the results that included the false positive (except samples 19 and 56 linked to an error in interpretation by Lab D) and false negative results are in Table 7.

Table 7. Positive, negative agreement and deviation calculated for each specific primer set and overall

	AN3-AN7		avrRps4		Final result (both primer sets)	
	expected result + (target)	expected result - (non- target)	expected result + (target)	expected result - (non -target)	expected result + (target)	expected result - (non-target)
Obtained result +	positive agreement PA = 150	positive deviation PD = 0	positive agreement PA = 149	positive deviation PD = 2	positive agreement PA = 150	positive deviation PD = 2
Obtained result -	negative deviation ND = 0	negative agreement NA = 149*	negative deviation ND = 1	negative agreement NA = 147*	negative deviation ND = 0	negative agreement NA = 147*

Note: Agreement and deviation are measured in numbers. The final result is determined taking the results of each primer sets into consideration, i.e. if a sample is positive with only one of the primer sets, the final result will be positive. *: One non-Psp isolate (sample 1) was not validated by one laboratory because of a negative result using the Wu primers.

- Diagnostic sensitivity (AN3-AN7) = 150 / (150+0) x 100 = **100 %**
- Diagnostic specificity (AN3-AN7) = 149 / (149+0) x 100 = **100 %**
- Accuracy (AN3-AN7) = (150+149) / (150+149+0+0) = **100%**

- Diagnostic sensitivity (avrRps4) = 149 / (149+1) x 100 = **99.3 %**
- Diagnostic specificity (avrRps4) = 147 / (147+2) x 100 = **98.6 %**
- Accuracy (avrRps4) = (149+147) / (149+147+2+1) x 100 = **98.9 %**

- Diagnostic sensitivity (PCR assay) = 150 / (150+0) x 100 = **100 %**
- Diagnostic specificity (PCR assay) = 147 / (147+2) = **98.6 %**
- Accuracy (PCR assay) = (150+147) / (150+147+2+0) x 100 = **99,3 %**

Using avrRps4 primers, the diagnostic sensitivity, diagnostic specificity and the accuracy were at 99.3%, 98.6% and 98.9% respectively. Using the AN3-AN7 primer set, diagnostic sensitivity, diagnostic specificity and accuracy were all at 100%. For the assay, using both primer pairs, diagnostic sensitivity was 100% while diagnostic specificity and accuracy were 98.6% and 99.3% respectively.

Conclusion

Due to cross contamination of samples or during the PCR process in the CT, two laboratories out of five found the PCR assay using the Arnold primers to be more specific, sensitive and accurate than the assay with avrRps4 primers. This result is, however, mitigated by the results of the test run on the second set of samples, which revealed that the assay with the avrRps4 primers was just as accurate as the assay with the AN3-AN7 primers. The false positive results were due to cross contamination and not due to lack of specificity of avrRps4 primers, while the false negative was due to a technical issue.

However, performance criteria based on the initial results, i.e. without retesting, of the avrRps4 primer set (diagnostic sensitivity, diagnostic specificity, and accuracy at 99.3%, 98.6% and 98.9% respectively) are above the accepted values (95%) according to ISTA guidelines (ISTA, 2018). Therefore, the validation requirements for diagnostic performance are met. If retesting results had been considered for performance criteria calculation, the values for all parameters would have been higher.

4. CONCLUSION

The performance criteria measured during method validation confirm that the PCR assay is fit for purpose as an optional step for identification of suspect Psp isolates before the pathogenicity assay.

The CT results stress the importance of a good analysis of the melt curve and the risk of cross contamination leading to a false positive. However, since any suspect isolate after a positive PCR result should be confirmed by a pathogenicity test (Figure 1), the risk of false positive is greatly reduced.

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6. ANNEXES

Annex A: PCR assay for identifying suspect *Pseudomonas syringae* pv. *pisi* isolates

SYBR-green PCR method

The methods defined here are guidelines. The concentration of volumes of each reagent may need to be modified according to the suppliers' guidelines or to optimise PCR sensitivity.

Primers (Table A1)

- Use the avrRps4 primers for Psp-specific DNA amplification. The amplification with these primers will give a product size of 114bp.
- Use the Arnold primers for Psp-specific DNA amplification. The amplification with these primers will give a product size of 272bp or 132bp according to the isolate being tested.
- Use the Wu primers for universal bacterial DNA amplification. The amplification with these primers will give a product size of 228bp.

Table A1. Primer sequences and source

Primer	Source	Sequences 5'-3'	fragment
avrRps4F	Baldwin 2015	GAGGCCAACCCAGCCGAAA	114 bp
avrRps4R		TGATTCTGCGGTCTTCGTTTCTG	
AN7/2	Arnold et al (1996)	AACGGCGAGGGTTGTGGAAA	272 bp
Pspi3		TCACTCCGAGCTCCTCACTA	
AN3/1		CACCCAGCGCATTACTAGGA	132 bp
AN3/2		CCAGCACCCAGATTGAGACT	
WuF	Wu et al. (2008)	CAACGCGAAGAACCTTACC	228 bp
WuR		ACGTCATCCCCACCTTCC	

Templates

- For the samples and controls prepare the PCR mix as indicated below in Table A2 – A4, add 2 µL of heat killed bacterial suspension (95°C for 10 min) in each reaction tube. During each amplification run, add a PCR negative control (replace the bacterial suspension with molecular biology grade water) and the positive controls provided (uncoded) with the sample set. Perform the PCR with the amplification program as indicated in Table A5.

Reaction Mixes

- Carry out PCR reactions in thin walled PCR tubes or plates for use in real-time PCR machines in a final volume of 15 µL
- Other PCR reagents may be used but may require additional optimisation of the PCR conditions.
- Real-time PCR Mix (Qiagen Quantitect SYBR mastermix)

Table A2. avrRps4 SYBR-green (*P. syringae* pv. *pisi* specific reaction mix)

Mix	Initial concentration	Volume (µL)
Water		3.5
Qiagen Quantitect SYBR 2x	2x	7.5
avrRps4F	10 µM	1.0
avrRps4R	10 µM	1.0
Heat killed bacterial suspension	10^{7}-10^{8} CFU/mL	2.0
Total		15.0

Table A3. Arnold (AN3, AN7) SYBR-green (*P. syringae* pv. *pisi* specific reaction mix)

Mix	Initial concentration	Volume (µL)
Water		1.5
Qiagen Quantitect SYBR 2x	2x	7.5
AN3/1	10 µM	1.0
AN3/2	10 µM	1.0
AN7/2	10 µM	1.0
Pspi3	10 µM	1.0
Heat killed bacterial suspension	10^{7}-10^{8} CFU/mL	2.0
Total		15.0

Table A4. Wu SYBR-green (Universal bacterial primer reaction mix)

Mix	Initial concentration	Volume (µL)
Water		3.5
Qiagen Quantitect SYBR 2x	2x	7.5
WuF	10 µM	1.0
WuR	10 µM	1.0
Heat killed bacterial suspension	10^{7}-10^{8} CFU/mL	2.0
Total		15.0

Table A5. Amplification program

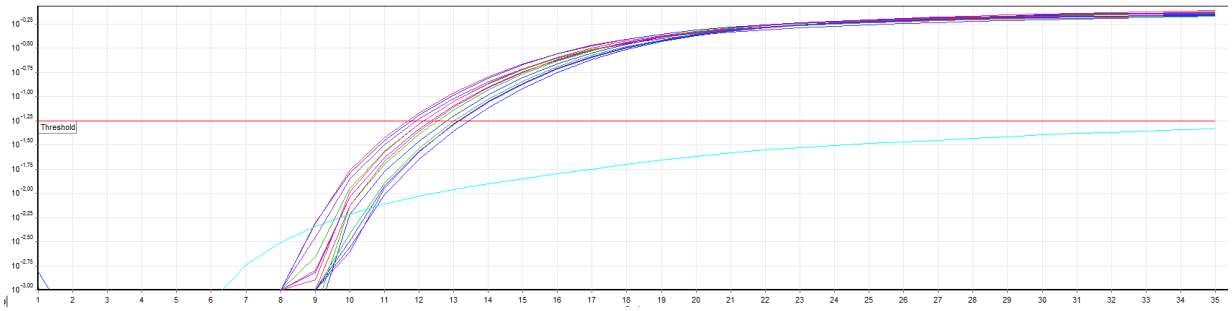
95°C	15 min	
94°C	10 sec	35 cycles
60°C	15 sec	
72°C	30 sec	
Melt Curve		72°C-95°C

Interpretation of PCR results

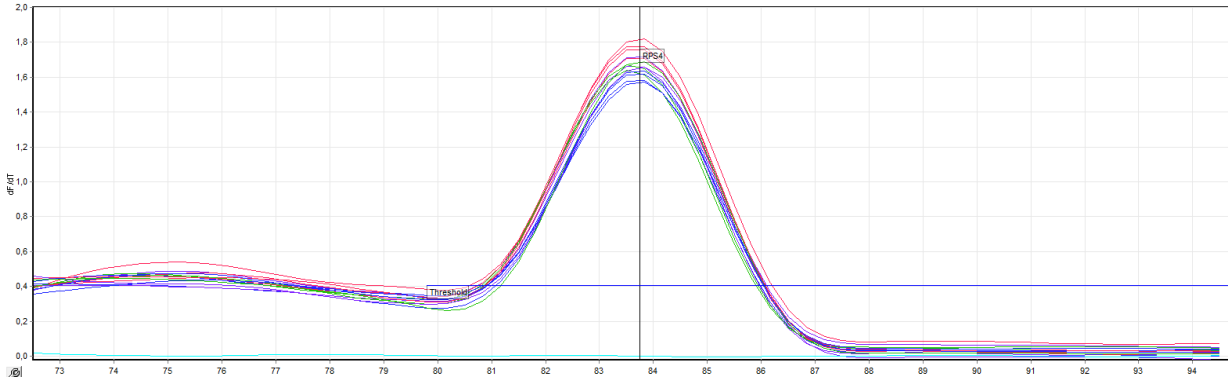
As the cut-off value is dependent on equipment, material and chemistry, it needs to be verified in each laboratory when implementing the test. The example below is with a Cq threshold of 35 cycles.

Example of real-time PCR result (using Qiagen Quantitect SYBR real-time PCR mix)

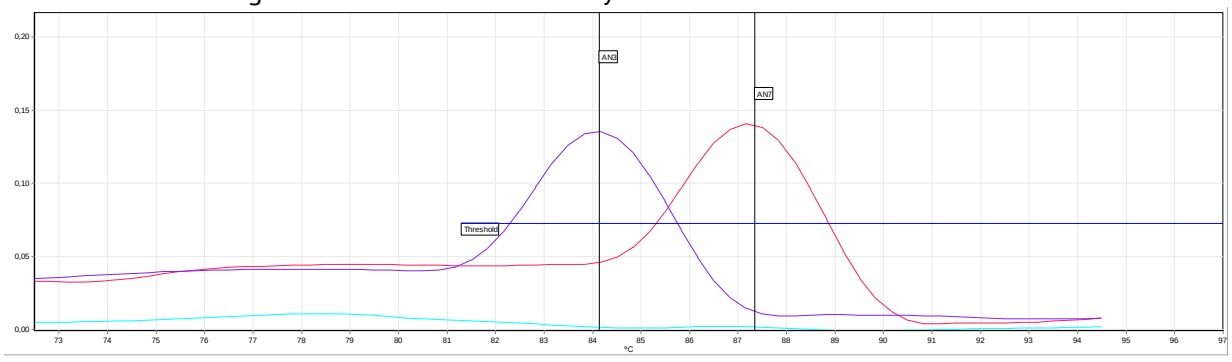
1. Quantification Curves: Threshold fixed manually in the exponential phase of amplification, above the background fluorescence.



2. Melt Curve analysis avrRps4: Threshold fixed above the non-specific decrease in fluorescence (dF/dT). The melt curve is used to identify the specific amplification products with reference to the positive control reaction (± 1.5°C).



3. Melt Curve analysis Arnold duplex: Threshold fixed above the non-specific decrease in fluorescence (dF/dT). The melt curve is used to identify the specific amplification products with reference to the positive control reactions (± 1.5°C). Either the AN3 or the AN7 primers amplify *Pseudomonas syringae* pv. *pisi* isolates. The two PCR products have different melt temperatures which can be distinguished in a melt curve analysis.



Presence of a *P. syringae* pv. *pisi* specific PCR product at the same melt temperature as the positive isolate control reaction (± 1.5°C), detected before the Cq threshold and in the absence of amplification in the negative control reactions leads to a positive result. A negative *Pseudomonas syringae* pv. *pisi* result on a suspect isolate suspension can only be validated if the amplification control reaction with the Wu primers is positive (see Table A6).

Table A6. Interpretation of PCR results

avrRps4	Arnold	Wu	qPCR Result	Interpretation
Cq ⩽ 35 Positive	Ct ⩽ 35 Positive		Expected result for Psp	Positive PCR result, pathogenicity assay needed for confirmation
Negative*	Negative*	Ct ⩽35 Positive	Expected result for a non-Psp isolate	Negative PCR identification, isolate is not Psp
Negative*	Negative*	Ct >35	Amplification control failure	Invalid result, repeat PCRs
Cq ⩽ 35 Positive	Negative*	Ct ⩽35 Positive	Discordant Psp specific amplification	Positive PCR result, pathogenicity assay needed for confirmation
Negative	Cq ⩽ 35 Positive	Ct ⩽35 Positive	Discordant Psp specific amplification	Positive PCR result, pathogenicity assay needed for confirmation

* Negative: Cq > 35 cycles or no amplification; or amplification of a PCR product with a melt curve temperature above or below 1.5°C compare to the positive control

Annex B. Characterization of isolates using AN3-AN7, avrRps4 and Wu primers.

Composition of the sample set used in the CT. Isolates found positive by PCR, negative in oxydase test and pathogenic are considered as *Pseudomonas syringae* pv. *pisi* strains (Psp). Other isolates are look-alike saprophytes (non-Psp). A negative PCR result is Cq > 35 cycles, or no amplification, or amplification of a PCR product before 35 cycles but with a melt curve temperature above or below 1.5°C compared to the positive control and a positive PCR result is amplification of a PCR product before 35 cycles with a melt curve temperature identical to the positive control (±1.5°C). PCR runs were performed on the same suspension but at different times (maximun 7 days between runs). The threshold is fixed above the background noise of fluorescence.

Code	Isolate names	AN3-AN7		avrRps4		Wu		Oxydase[1]	Pathogenicity[2]	Final result
		Cq	Melt °C	Cq	Melt °C	Cq	Melt °C			
1	1.1	UD	UD	UD	UD	17.75	84.8	Positive	Non-pathogenic	non-Psp
2	30	16.15	85.2 (AN7)	13.93	84	11.73	84.3	Negative	Pathogenic	Psp
3	L44260	16.38	85.2 (AN7)	14.29	84	14.51	84.5	Negative	Pathogenic	Psp
4	2.16	UD	UD	UD	UD	14.27	85.3	Positive	Non-pathogenic	non-Psp
5	3.4	UD	UD	UD	UD	14.39	84.7	Negative	Non-pathogenic	non-Psp
6	6	16.26	85.2 (AN7)	14.36	84	12.98	84.5	Negative	Pathogenic	Psp
7	3.14	30.95	75.5	UD	UD	12.78	84.5	Positive	Non-pathogenic	non-Psp
8	1.2	UD	UD	UD	UD	17.39	84.3	Positive	Non-pathogenic	non-Psp
9	24	15.73	85.2 (AN7)	13.56	84	11.3	84.3	Negative	Pathogenic	Psp
10	K55817	16.08	82.2 (AN3)	13.71	84	13.09	84.5	Negative	Pathogenic	Psp
11	2.3	30.18	75.3	32.87	77.2	22.63	83.8	Positive	Non-pathogenic	non-Psp
12	3.6	31	75.5	UD	UD	12.56	84.5	Positive	Non-pathogenic	non-Psp
13	11	15.92	85.2 (AN7)	13.75	84	13.56	84.5	Negative	Pathogenic	Psp
14	3.15	30.86	75.8	UD	UD	12.26	84.5	Positive	Non-pathogenic	non-Psp
15	L19.4.D1	15.89	85.2 (AN7)	13.89	84	10.49	84.3	Negative	Pathogenic	Psp
16	2.10	UD	75.2	UD	UD	17.59	85.3	Negative	Non-pathogenic	non-Psp
17	31	16.18	85.2 (AN7)	13.09	84	10.61	84.2	Negative	Pathogenic	Psp
18	7	16.07	85.3 (AN7)	14.21	84	13.24	84.5	Negative	Pathogenic	Psp

Code	Isolate names	AN3-AN7		avrRps4		Wu		Oxydase[1]	Pathogenicity[2]	Final result
		Cq	Melt °C	Cq	Melt °C	Cq	Melt °C			
19	2.4	**33.76**	**82.7 (AN3)**	UD	UD	12.46	84.5	Positive	Non-pathogenic	non-Psp
20	3.7	34.41	75	UD	UD	12.44	85.3	Negative	Non-pathogenic	non-Psp
21	5	16	85.2 (AN7)	14.22	84	12.81	84.5	Negative	Pathogenic	Psp
22	3.16	30.78	75.8	UD	UD	12.86	84.7	Positive	Non-pathogenic	non-Psp
23	1.2+K55817	13.05	82.0 (AN3)	NA	NA	11.38	84.3	Negative	NA	Psp
24	2.7	34.58	74.7	33.34	77.7	26.4	85.3	Negative	Non-pathogenic	non-Psp
25	18	16.24	82 (AN3)	14.32	84	10.85	84.3	Negative	Pathogenic	Psp
26	21	16.24	85.3 (AN7)	14.26	84	12.59	84.5	Negative	Pathogenic	Psp
27	2.18	UD	UD	UD	UD	16.42	84.2	Negative	Non-pathogenic	non-Psp
28	3.8	30.68	75.3	UD	UD	12.21	84.5	Positive	Non-pathogenic	non-Psp
29	9	16.02	85.2 (AN7)	14.19	84	12.77	84.5	Negative	Pathogenic	Psp
30	3.19	30.77	75.5	UD	UD	12.62	84.7	Positive	Non-pathogenic	non-Psp
31	2.10+18	13.46	82.2 (AN3)	NA	NA	11.84	84.5	Negative	NA	Psp
32	2.11	UD	UD	UD	UD	17.37	84.5	Negative	Non-pathogenic	non-Psp
33	25	15.88	85.2 (AN7)	13.54	83.8	11.11	84.3	Negative	Pathogenic	Psp
34	17	16.24	85.3 (AN7)	13.79	84	13.11	84.5	Negative	Pathogenic	Psp
35	2.17	UD	UD	UD	UD	18.22	84.5	Negative	Non-pathogenic	non-Psp
36	3.9	32.98	74.7	UD	UD	12.38	84.7	Positive	Non-pathogenic	non-Psp
37	15	16.25	85.2 (AN7)	13.77	84	13.93	84.5	Negative	Pathogenic	Psp
38	3.21	31.59	75.3	UD	UD	12.33	84.5	Positive	Non-pathogenic	non-Psp
39	2.17+L.1.1.D2	13.38	85.3 (AN7)	NA	NA	10.5	84.2	Negative	NA	Psp
40	2.23	30.9	75.2	UD	UD	14.56	85.5	Positive	Non-pathogenic	non-Psp
41	16	15.33	85.2 (AN7)	13.2	84	11.26	84.3	Negative	Pathogenic	Psp
42	8	16.13	85.2 (AN7)	13.62	84	11.89	84.5	Negative	Pathogenic	Psp
43	2.DO.2	UD	UD	UD	UD	12.82	84.5	Positive	Non-pathogenic	non-Psp
44	3.10	30.56	75.5	UD	UD	12.38	84.5	Positive	Non-pathogenic	non-Psp

Code	Isolate names	AN3-AN7		avrRps4		Wu		Oxydase[1]	Pathogenicity[2]	Final result
		Cq	Melt °C	Cq	Melt °C	Cq	Melt °C			
45	4	15.87	85.2 (AN7)	14.07	84	12.89	84.5	Negative	Pathogenic	Psp
46	3.24	UD	UD	32.75	76.7	16.47	84.7	Negative	Non-pathogenic	non-Psp
47	3.14+7	12.75	85.3 (AN7)	NA	NA	10.89	84.2	Negative	NA	Psp
48	2.13	UD	74.5	UD	UD	13.18	85.3	Positive	Non-pathogenic	non-Psp
49	17.5.D2	12.38	85.3 (AN7)	NA	NA	10.9	84.3	Negative	Pathogenic	Psp
50	23	16.12	85.2 (AN7)	13.78	84	12.8	84.5	Negative	Pathogenic	Psp
51	3.1	UD	UD	UD	UD	14.14	85	Negative	Non-pathogenic	non-Psp
52	3.11	30.85	75.5	31.12	79.2	12.25	84.5	Positive	Non-pathogenic	non-Psp
53	L.1.1.D2	15.65	85.2 (AN7)	13.61	84	12.82	84.5	Negative	Pathogenic*	Psp
54	1	16.15	85.2 (AN7)	13.72	84	13.5	84.5	Negative	Pathogenic	Psp
55	3.4+21	13.52	85.3 (AN7)	NA	NA	10.96	84.2	Negative	NA	Psp
56	2.15	UD	UD	UD	UD	17.74	84.5	Positive	Non-pathogenic	non-Psp
57	L30.5.D0	12.16	85.3 (AN7)	NA	NA	10.44	84.3	Negative	Pathogenic	Psp
58	28	15.79	85.2 (AN7)	13.42	84	12.61	84.5	Negative	Pathogenic	Psp
59	3.2	30.08	78.8	31.38	79	12.88	84.3	Negative	Non-pathogenic	non-Psp
60	3.12	29.82	75.7	UD	UD	12.88	84.7	Positive	Non-pathogenic	non-Psp
AN3 Control	K55817	16.43	82.2 (AN3)	14.43	83.8	13.09	84.5	Negative	Pathogenic*	Psp
AN7 Control	L44260	16.22	85.2 (AN7)	16.84	84	14.51	84.5	Negative	Pathogenic*	Psp
NTC	water	UD	UD	UD	UD	27.87	85.3	NA	Negative	Negative

NOTE: UD: undetermined Cq value that indicated a negative result (no amplification curve or amplification curve below the background noise of fluorescence). NA: missing data. NTC: Negative Template Control.

[1] To characterize isolates an oxydase-test was performed by the organizing lab using the ISTA method 7-029. Psp isolates are oxydase negative.

[2] Pathogenicity performed in multiple runs to construct the organizer's inhouse isolate database, not repeated within this CT except for 3 isolates previously characterized that were used as controls and are labelled using an "*" in the table. Thus, only PCR negative isolates were tested by a pathogenicity assay for characterization as the PCR positive strains were previously tested for their pathogenicity.

Annex C. Results of the test for stability of the samples.

Code	Isolate names	Expected result	AN3-AN7		avrRps4		Wu		Final result
			Cq	Melt °C	Cq	Melt °C	Cq	Melt °C	
1	1.1	Negative	UD	UD	UD	UD	17.08	84.5	Negative
2	30	Positive	13.92	85.2 (AN7)	11.97	83.7	13.43	84.3	Positive
3	L44260	Positive	15.01	85.0 (AN7)	13.30	83.5	14.55	84.2	Positive
4	2.16	Negative	UD	UD	UD	UD	11.39	85.3	Negative
5	3.4	Negative	UD	UD	UD	UD	12.24	84.5	Negative
6	6	Positive	14.87	85.0 (AN7)	13.00	83.5	14.58	84.3	Positive
7	3.14	Negative	**30.29**	**84.7**	UD	UD	11.83	84.3	**Positive**
8	1.2	Negative	UD	UD	UD	UD	16.58	84.0	Negative
9	24	Positive	13.77	85.0 (AN7)	11.72	83.7	13.33	84.2	Positive
10	K55817	Positive	14.12	82.0 (AN3)	12.74	83.7	14.24	84.3	Positive
11	2.3	Negative	UD	UD	UD	UD	20.73	83.5	Negative
12	3.6	Negative	UD	UD	UD	UD	14.56	84.5	Negative
13	11	Positive	14.76	85.0 (AN7)	12.44	83.7	14.22	84.3	Positive
14	3.15	Negative	UD	UD	UD	UD	13.83	84.5	Negative
15	L19.4.D1	Positive	14.37	85.0 (AN7)	12.20	83.8	13.99	84.3	Positive
16	2.10	Negative	UD	UD	UD	UD	16.39	85.2	Negative
17	31	Positive	15.40	85.0 (AN7)	12.50	83.7	14.80	84.3	Positive
18	7	Positive	16.04	85.0 (AN7)	14.02	83.7	15.17	84.3	Positive
19	2.4	Negative	UD	UD	UD	UD	12.40	84.3	Negative
20	3.7	Negative	UD	UD	UD	UD	12.76	85.0	Negative
21	5	Positive	15.64	85.0 (AN7)	13.14	83.7	14.62	84.3	Positive
22	3.16	Negative	UD	UD	UD	UD	14.15	84.5	Negative

Code	Isolate names	Expected result	AN3-AN7		avrRps4		Wu		Final result
			Cq	Melt °C	Cq	Melt °C	Cq	Melt °C	
23	1.2+K55817	Positive	15.26	81.8 (AN3)	13.60	83.7	14.81	84.2	Positive
24	2.7	Negative	UD	UD	UD	UD	23.96	85.0	Negative
25	18	Positive	13.10	82.0 (AN3)	11.66	83.7	12.77	84.3	Positive
26	21	Positive	15.61	85.0 (AN7)	13.79	83.5	15.26	84.2	Positive
27	2.18	Negative	UD	UD	UD	UD	16.89	84.2	Negative
28	3.8	Negative	UD	UD	UD	UD	13.32	84.5	Negative
29	9	Positive	15.01	85.0 (AN7)	12.96	83.5	14.53	84.2	Positive
30	3.19	Negative	UD	UD	UD	UD	13.00	84.3	Negative
31	2.10+18	Positive	13.72	82.0 (AN3)	11.98	83.7	13.40	84.7	Positive
32	2.11	Negative	UD	UD	UD	UD	17.63	84.2	Negative
33	25	Positive	13.22	85.2 (AN7)	11.42	83.8	13.13	84.3	Positive
34	17	Positive	15.01	85.2 (AN7)	12.89	83.7	15.00	84.3	Positive
35	2.17	Negative	UD	UD	UD	UD	17.69	84.2	Negative
36	3.9	Negative	UD	UD	UD	UD	12.97	84.5	Negative
37	15	Positive	12.66	85.2 (AN7)	11.66	83.7	12.52	84.3	Positive
38	3.21	Negative	UD	UD	UD	UD	13.71	84.5	Negative
39	2.17+L.1.1.D2	Positive	16.25	85.2 (AN7)	14.08	83.8	15.70	84.3	Positive
40	2.23	Negative	UD	UD	UD	UD	13.12	85.3	Negative
41	16	Positive	13.42	85.0 (AN7)	12.10	83.8	13.20	84.3	Positive
42	8	Positive	14.09	85.0 (AN7)	12.19	83.7	13.90	84.3	Positive
43	2.DO.2	Negative	UD	UD	UD	UD	11.25	84.3	Negative
44	3.10	Negative	UD	UD	UD	UD	13.97	84.3	Negative
45	4	Positive	13.62	85.2 (AN7)	11.37	83.7	13.10	84.2	Positive

Code	Isolate names	Expected result	AN3-AN7		avrRps4		Wu		Final result
			Cq	Melt °C	Cq	Melt °C	Cq	Melt °C	
46	3.24	Negative	UD	UD	UD	UD	18.21	84.3	Negative
47	3.14+7	Positive	15.68	85.2 (AN7)	14.14	83.7	13.20	84.3	Positive
48	2.13	Negative	UD	UD	UD	UD	12.09	85.2	Negative
49	17.5.D2	Positive	16.32	85.2 (AN7)	13.81	83.7	15.88	84.3	Positive
50	23	Positive	14.87	85.2 (AN7)	12.70	83.7	14.17	84.3	Positive
51	3.1	Negative	UD	UD	UD	UD	14.60	84.7	Negative
52	3.11	Negative	UD	UD	UD	UD	14.11	84.5	Negative
53	L.1.1.D2	Positive	16.77	85.0 (AN7)	14.17	83.7	16.18	84.3	Positive
54	1	Positive	13.92	85.0 (AN7)	12.15	83.7	13.37	84.3	Positive
55	3.4+21	Positive	17.77	85.0 (AN7)	14.95	83.7	13.95	84.5	Positive
56	2.15	Negative	UD	UD	UD	UD	18.15	84.3	Negative
57	L30.5.D0	Positive	15.53	85.0 (AN7)	13.06	83.7	14.48	84.3	Positive
58	28	Positive	14.95	85.2 (AN7)	12.43	83.7	14.34	84.3	Positive
59	3.2	Negative	UD	UD	UD	UD	13.70	84.0	Negative
60	3.12	Negative	UD	UD	UD	UD	13.52	84.5	Negative
AN3 Control	K55817	Positive	13.47	82.0 (AN3)	11.70	83.7	13.32	84.3	Positive
AN7 Control	L44260	Positive	15.68	85.0 (AN7)	13.77	83.7	15.61	84.3	Positive
NTC	water	Negative	UD	UD	UD	UD	UD	UD	Negative

NOTE: Blue cells indicate negative results, i.e. Cq greater than 35 cycles or no amplification; or amplification of a PCR product with a melt curve temperature above or below 1.5 °C compare to the positive control. Yellow cells indicate positive results, i.e. Cq value above threshold and specific melt temperature. The threshold is fixed above the background noise of fluorescence. UD: undetermined Cq value that indicated a negative result (no amplification curve or amplification curve bellow the background noise of fluorescence). PCR runs were performed separately on the same suspension and at the same day.

Annex D: Comparative Test Results

Code	Name	Expected results	AN3 and AN7					avrRps4					Wu				
			Lab A	Lab B	Lab C	Lab D	Lab E	Lab A	Lab B	Lab C	Lab D	Lab E	Lab A	Lab B	Lab C	Lab D	Lab E
1	1.1	negative	11.4	33.2	UD	UD	UD	UD	UD	UD	33.7	UD	11.5	17.0	22.7	NRE / 16.1	17.7
2	30	positive	12.0	12.5	13.8	13.9	18.9	10.5	11.9	13.2	11.1	17.2	12.4	11.7	19.9	11.0	16.3
3	L44260	positive	11.9	12.1	14.6	15.1	19.6	10.8	12.1	14.2	11.6	17.9	14.2	11.4	20.6	11.9	17.3
4	2.16	negative	UD	34.1	UD	UD	UD	UD	UD	UD	UD	UD	12.8	12.0	18.3	12.2	14.0
5	3.4	negative	UD	33.5	UD	UD	UD	UD	UD	UD	31.5	34.4	13.9	12.1	19.1	12.1	14.9
6	6	positive	12.4	13.5	15.0	15.3	19.4	11.3	13.7	13.3	13.8	17.7	10.7	12.0	21.1	11.1	17.4
7	3.14	negative	UD	33.2	UD	UD	UD	UD	UD	UD	32.3	35.0	11.0	12.7	18.1	11.7	15.0
8	1.2	negative	UD	23.1	UD	UD	UD	UD	UD	UD	UD	UD	11.8	14.9	22.7	6.6	16.5
9	24	positive	12.5	12.6	13.8	14.3	18.6	10.9	12.0	13.0	12.0	16.9	15.5	11.7	19.4	10.6	16.1
10	K55817	positive	12.4	13.1	13.0	15.9	18.0	11.4	12.1	13.9	12.4	17.1	11.4	11.6	20.4	UD / 14.4	16.2
11	2.3	negative	UD	33.4	UD	UD	UD	UD	UD	UD	UD	UD	13.9	21.2	26.8	14.1	15.5
12	3.6	negative	UD	32.4	UD	UD	UD	UD	UD	UD	31.1	34.3	12.2	12.0	18.7	11.1	15.6
13	11	positive	12.8	12.7	13.8	14.9	18.3	11.4	12.9	13.2	12.4	16.7	13.1	12.0	19.7	10.4	15.9
14	3.15	negative	UD	33.2	UD	UD	UD	UD	UD	UD	32.0	34.8	10.5	12.6	19.0	11.1	16.3
15	L19.4.D1	positive	12.9	12.9	14.7	14.8	19.2	12.2	13.5	13.1	12.0	18.1	12.2	11.9	20.4	10.3	16.7
16	2.10	negative	UD	33.9	UD	UD	UD	UD	UD	UD	UD	UD	14.4	14.9	23.3	15.8	19.9
17	31	positive	12.9	12.8	15.1	16.4	21.1	10.6	12.3	13.0	12.8	18.6	13.5	11.8	20.8	11.3	19.2
18	7	positive	13.1	13.2	14.3	15.7	19.3	12.2	13.5	13.9	11.7	18.0	12.6	11.9	20.1	10.6	17.1
19	2.4	negative	UD	30.1	UD	UD	UD	UD	UD	UD	**24.2***	28.7	13.8	11.4	18.8	10.8	14.9
20	3.7	negative	UD	33.2	UD	UD	UD	UD	UD	UD	33.5	35.6	12.2	11.0	18.2	10.7	15.0
21	5	positive	13.2	13.1	13.7	15.3	18.6	11.4	13.2	13.2	13.9	17.1	13.6	12.0	19.7	10.8	16.4
22	3.16	negative	UD	33.7	UD	UD	UD	UD	UD	UD	31.5	35.0	13.9	12.8	19.1	11.5	15.5

Code	Name	Expected results	AN3 and AN7					avrRps4					Wu				
			Lab A	Lab B	Lab C	Lab D	Lab E	Lab A	Lab B	Lab C	Lab D	Lab E	Lab A	Lab B	Lab C	Lab D	Lab E
23	1.2+K55817	positive	13.2	14.3	15.0	16.3	19.9	12.1	14.1	14.3	14.3	19.1	13.2	12.5	21.1	10.5	15.0
24	2.7	negative	UD	31.6	UD	UD	UD	UD	UD	UD	UD	UD	16.2	23.6	30.6	19.3	21.7
25	18	positive	12.3	13.0	12.7	15.1	17.6	11.3	12.7	12.8	12.7	16.4	12.5	11.7	19.1	13.8	15.3
26	21	positive	12.4	12.8	15.3	15.6	20.1	11.4	13.3	15.0	11.0	18.4	14.2	11.9	21.2	10.4	17.5
27	2.18	negative	UD	32.2	UD	UD	UD	UD	UD	UD	UD	UD	12.7	16.9	24.2	16.6	20.3
28	3.8	negative	UD	32.9	UD	UD	UD	UD	UD	UD	31.5	35.2	11.1	12.1	19.3	11.0	16.5
29	9	positive	12.3	13.2	14.9	15.1	19.6	11.5	13.3	13.7	11.5	17.8	19.4	11.9	21.0	12.1	17.4
30	3.19	negative	UD	UD	UD	UD	UD	UD	UD	UD	33.9	34.7	11.8	12.2	19.2	18.1	15.9
31	2.10+18	positive	13.8	14.8	13.7	16.2	18.7	12.8	14.2	13.0	13.4	17.7	16.1	12.8	20.3	12.8	16.1
32	2.11	negative	UD	34.9	UD	UD	UD	UD	UD	UD	UD	UD	10.5	14.9	24.5	15.6	20.6
33	25	positive	12.9	12.7	13.0	15.7	18.0	11.7	12.9	12.3	14.5	16.8	11.5	12.1	18.8	11.7	15.8
34	17	positive	13.1	12.9	14.8	15.3	19.3	12.1	13.2	14.7	13.7	17.7	14.4	12.1	21.3	10.1	17.2
35	2.17	negative	UD	34.0	UD	UD	UD	UD	UD	UD	UD	UD	11.5	16.0	24.5	16.4	20.7
36	3.9	negative	UD	30.9	UD	UD	UD	UD	UD	UD	32.7	35.4	13.4	11.7	18.8	11.6	15.9
37	15	positive	12.3	12.4	12.8	15.1	17.3	11.1	12.7	12.7	16.0	16.9	12.8	11.8	19.4	11.5	15.2
38	3.21	negative	UD	32.9	UD	UD	UD	UD	UD	UD	31.6	35.1	14.2	13.0	19.1	10.9	17.1
39	2.17+L.1.1.D2	positive	13.4	13.9	16.5	17.1	21.4	13.1	13.6	14.6	14.8	19.7	14.0	11.7	22.2	10.5	18.5
40	2.23	negative	UD	33.8	UD	UD	UD	UD	UD	UD	UD	UD	14.0	11.8	19.7	12.2	14.9
41	16	positive	12.5	12.4	13.5	15.5	18.4	**UD** / 15.5	13.0	13.6	13.1	17.4	14.1 / 14.9	11.9	19.6	10.9	16.2
42	8	positive	14.7	12.2	14.0	14.6	18.4	13.5	12.7	13.4	12.5	17.0	13.1	11.7	19.9	10.2	16.1
43	2.DO.2	negative	UD	31.3	UD	UD	34.3	UD	UD	UD	29.6	27.4	12.4	11.2	18.2	11.5	13.8
44	3.10	negative	UD	34.3	UD	UD	UD	UD	UD	UD	32.9	35.5	11.7	13.7	18.9	11.8	15.7
45	4	positive	11.7	12.7	13.5	14.3	18.1	10.9	12.8	12.0	12.3	16.5	13.1	12.0	19.1	10.9	15.3
46	3.24	negative	UD	32.8	UD	UD	UD	UD	UD	UD	UD	UD	12.9	16.2	21.9	13.2	18.4
47	3.14+7	positive	13.3	15.9	14.5	16.3	19.3	12.3	14.2	13.5	13.8	18.3	11.0	12.8	18.4	10.4	15.3

Code	Name	Expected results	AN3 and AN7					avrRps4					Wu				
			Lab A	Lab B	Lab C	Lab D	Lab E	Lab A	Lab B	Lab C	Lab D	Lab E	Lab A	Lab B	Lab C	Lab D	Lab E
48	2.13	**negative**	UD	31.6	UD	UD	UD	UD	UD	UD	UD	UD	12.7	12.5	18.3	12.0	14.6
49	17.5.D2	**positive**	12.9	13.1	15.0	15.0	19.7	11.1	13.3	13.6	14.5	18.4	16.7	12.2	21.6	10.1	17.3
50	23	**positive**	12.6	12.9	13.6	14.4	18.4	10.6	13.1	12.3	13.6	17.1	11.8	12.2	19.6	10.5	15.9
51	3.1	**negative**	UD	32.5	UD	UD	UD	UD	UD	UD	34.5	**34.1** / UD	16.6	11.9	20.1	12.2	16.7 / 13.4
52	3.11	**negative**	UD	32.4	UD	UD	UD	UD	UD	UD	31.8	34.5	13.0	13.2	18.9	11.1	15.8
53	L.1.1.D2	**positive**	12.5	13.2	15.2	15.4	20.2	11.1	13.4	13.7	13.9	18.6	11.6	12.1	21.5	10.5	18.0
54	1	**positive**	12.6	13.0	12.9	15.3	17.3	10.8	13.2	11.6	13.3	16.2	14.4	12.6	18.9	11.1	15.3
55	3.4+21	**positive**	14.0	14.6	16.3	15.8	20.9	13.3	14.3	14.3	14.9	19.7	11.4	12.5	19.5	10.4	15.9
56	2.15	**negative**	UD	32.1	UD	UD	UD	**34.7 / 24.3**	UD	UD	**26.2***	29.7	12.1 / 36.7	17.4	23.2	10.3	16.2
57	L30.5.D0	**positive**	13.0	12.7	14.1	16.3	19.0	12.6	13.4	12.8	13.3	17.5	10.9	12.3	19.9	10.9	16.6
58	28	**positive**	12.6	12.6	13.3	14.3	18.2	11.2	12.7	12.0	12.9	16.6	12.4	12.1	20.1	10.3	15.9
59	3.2	**negative**	UD	30.9	UD	UD	UD	UD	UD	UD	34.9	UD	14.4	12.1	19.9	11.1	17.0
60	3.12	**negative**	UD	30.8	UD	UD	UD	UD	UD	UD	31.7	35.0	11.8	12.9	18.2	11.2	15.3
AN3 Control	K55817	**positive**	14.5	14.5	13.2	16.3	18.3	13.0	13.8	12.6	14.6	17.6	12.5	13.0	20.2	11.4	16.4
AN7 Control	L44260	**positive**	13.6	13.7	15.5	16.0	20.4	11.7	13.1	14.5	14.0	19.8	11.6	12.1	21.7	10.9	18.6
Negative control		**negative**	UD	32.4	UD	UD	UD	UD	UD	UD	UD	UD	30.6	29.3	UD	24.7	UD

NOTE: Cq values from the first run are reported as well as those of the second run, if performed (Cq value first run / Cq value second run). Cq values in bold: false positive or false negative results. Cells in blue indicate a final negative result reported by participants, i.e. Cq value under threshold or with non-specific melt temperature; cells in yellow indicate positive results i.e. Cq value above threshold and specific melt temperature. The threshold is fixed by each lab above the background noise of fluorescence. UD: undetermined Cq value that indicated a negative result (no amplification curve). NRE: not readable. *Lab D after checking of the melting curve determined that the false positive (samples 19 and 56) were negative.