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A real-time PCR assay for the identification of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* isolates

Validation report, February 2019

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INTRODUCTION

Common bacterial blight can cause significant losses in beans (*Phaseolus vulgaris*) in tropical, subtropical and temperate climates. Its wide distribution, capacity to reduce yield and the lack of efficient treatment measures, in addition to the fact that it uses seeds as a means of dispersal and survival, make *Xanthomonas axonopodis* pv. *phaseoli* (Xap) one of the most economically important pathogens affecting beans worldwide.

Increasing knowledge of the genetic sequences of the bacteria that cause common bacterial blight as well as sequence information from related pathovars has led to regular changes in taxonomical classification. Most recently Constantin et al. (2016) changed the classification: the non-fuscous genetic lineages GL2 and GL3 of *Xanthomonas axonopodis* pv. *phaseoli* together with the fuscans-piment producing lineage, *X. axonopodis* pv. *phaseoli* var. *fuscans* are grouped in a single taxon, *X. citri* pv. *fuscans*. The genetic lineage previously described as *X. axonopodis* pv. *phaseoli* GL1 is now classed as *X. phaseoli* pv. *phaseoli*.

Although the causal agents of common bacterial blight can be differentiated into different taxonomic groups, it is not possible to differentiate them based on symptoms under natural conditions and they are usually grouped together for regulatory purposes. However, a differentiation can be made on isolates according to the production or absence of production of the fuscans pigment on culture media. The nomenclature used in this test plan corresponds to the names used in the current ISTA rule and those commonly used in international phytosanitary terminology.

Detection in seed

The current ISTA rule 7-021 version 3.1 (ISTA, 2017) is derived from the validation studies carried out between 2003 and 2011, in collaboration with ISHI-Veg (Grimault et al., 2012). For routine testing of bean seed a combination of two complementary semi-selective media, Milk Tween agar (MT) and *X. campestris* pv. *phaseoli* agar (XCP1) is used, followed by two possible options for the identification of suspect colonies: either a pathogenicity assay or a gel-based PCR test with Audy et al. (1994) primers.

The ISHI-Veg *Best Practices for PCR Assays in Seed Health Tests* (https://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-method-development/) indicates that an internal amplification control (IAC) is essential for isolate identification methods. The current ISTA method does not describe the use of an IAC.

A TaqMan assay based on the sequence amplified by the Audy et al. (1994) primers was developed and validated. In addition, a TaqMan assay based on the Wu et al. (2008) primers and probes was added as an IAC. The use of a TaqMan assay facilitates the interpretation of two parallel reactions in the same tube (duplex reaction) with the use of distinct fluorophores for the Xap probe and the IAC probe. In addition, by using a closed tube real-time PCR assay, the risks of cross-contamination in routine application are reduced when compared with a gel-based PCR assay. In contrast to gel-based methods, real-time PCR does not require the use of Ethidium Bromide. The

development and initial validation of this assay is described in a separate report available from the ISF secretariat (Baldwin, 2017).

OBJECTIVES

The objective of this project is to develop a method that detects and identifies *Xanthomonas axonopodis* pv. *phaseoli* (Xap) and *X. axonopodis* pv. *phaseoli* var. *fuscans* (Xapf) on bean seeds, and includes a pathogenicity assay (see the complete workflow in Figure 1) essential for confirming the presence of Xap/Xapf and declaring a final positive test result. With the addition of a real-time PCR identification assay containing an IAC, the method is ready to be internationally accepted as a reference and an industry standard.

The method has been validated based on the performance characteristics identified by ISHI-Veg (see Appendix A).

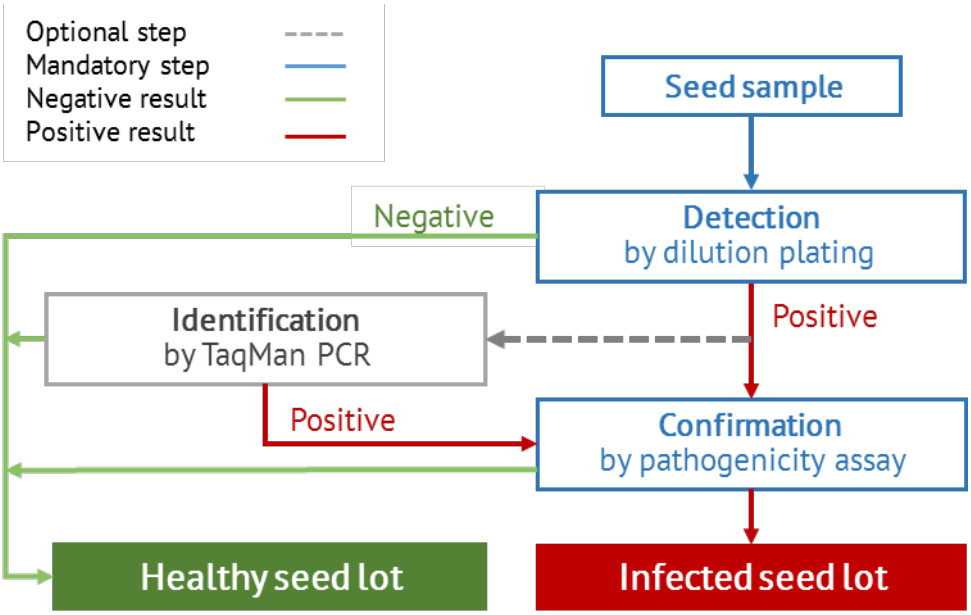

Figure 1. Workflow for the detection of Xap and Xapf on Bean seeds

METHOD VALIDATION

I. Analytical specificity

*The ability of an assay to detect the targeted pathogen (**inclusivity**) while excluding non-target organisms (**exclusivity**).*

I.1 Validation of the Xap TaqMan assay without an IAC

Initial validation of the specificity of the Xap TaqMan assay was done without an IAC in one laboratory (Vilmorin SA) on a collection of 15 Xap look-alike (on YDC) isolates from bean seeds previously having been identified as being PCR-negative, 7 other *Xanthomonas* pathogens and 25 Xap isolates from the Vilmorin collection previously identified by PCR. The isolate suspensions were prepared according to the procedure described for suspect isolates in the ISTA 7-021 rule. Two look-alike strains showed Cq values between 35-40 cycles, with the application of a Cq 35 cut-off, these real-time PCR results demonstrate the 100% **exclusivity** of the Xap TaqMan assay on this isolate collection. With the application of the same Cq 35 cut-off, all the Xap isolates were correctly identified (100% **inclusivity**). (Table 1).

Table 1. Xap TaqMan assay results on a collection of Xap and look-alike isolates

N°	Isolate ID	Cq value
1	Xap look-alike 69186.0	37.54
2	Xap look-alike 69914.1.3	NA
3	Xap look-alike 69201.4.0	NA
4	Xap look-alike 69253.5.1	NA
5	Xap look-alike 69276.10.0	NA
6	Xap look-alike 69193.2.0	NA
7	Xap look-alike 69189.2	NA
8	Xap look-alike 69208.6.1	NA
9	Xap look-alike 69270.0.2	NA
10	Xap look-alike 69176.1.1	39.74
11	Xap look-alike 69284.6.0.1	NA
12	Xap look-alike 77037.4.0	NA
13	Xap look-alike 69297.0	NA
14	Xap look-alike 69311.0	NA
15	Xap look-alike 70357.5.0	NA
16	CFBP Xe 6864	NA
17	CFBP Xp 7293	NA
18	CFBP Xv 4645	NA
19	CFBP Xg 6822	NA
20	CFBP Xcr 5829	NA
21	Xhc 539	NA
22	Xcc 645.2	NA
23	Xap CFBP 6546	16.52
24	Xap J95292 6.1.1	14.65
25	Xap 1008019	15.62
26	Xap 1007014	14.86
27	Xap 1008017	13.54
28	Xap J81747.1.2	15.54
29	Xap I70296.1.0	13.99
30	Xap i72066.3	13.50
31	Xap 717626.1	17.62
32	Xap 45.5	14.13
33	Xap J426542.2	13.78
34	Xap 716806.1	15.34
35	Xap 15.1p	13.88
36	Xap 7.1 p	14.37
37	Xap 59102.2	12.92
38	Xap 3.1 p	13.63
39	Xap 8.2 p	17.87
40	Xap 4.2 p	12.90
41	Xap 277401	13.72
42	Xap 58.6 d2b	14.18
43	Xap 20.1 p	14.03
44	Xap 17.1 p	13.85
45	Xap 106	19.60
46	Xap 107	13.89
47	Xap 108	14.99
NTC	Negative control	NA

NA = No Amplification; Xe, *X. euvesicatoria*; Xp, *X. perforans* ; Xv, *X. vesicatoria* ; Xg, *X. gardneri*, Xcr, *X. c. pv. raphani*; Xcc, *X. c. pv. campestris*. Cq cut-off value <35 for Xap. Target isolates are highlighted in yellow.

I.2 Validation of the Xap TaqMan assay with an IAC

Further validation of analytical specificity was done with the TaqMan assay including an IAC (PCR method described in the Section V. Repeatability and Reproducibility) on a 60 isolate DNA extract collection characterised and tested in a previous ISTA/ISHI comparative test (Grimault et al, 2012) and stored in the GEVES laboratory.

Results obtained with the duplex TaqMan reactions are presented in Table 2. Samples X1 to X30 are target Xap DNA extracts. All but one of the target extracts were detected at fewer than 20 cycles (Cq < 20). The extract X9 was only detected with the Xap TaqMan assay at Cq 34.9. The DNA extract X9 was also not detected with the current ISTA 7-021 PCR assay. The DNA extract X24 was not detected with ISTA 7-021 PCR assay but is detected with the Xap TaqMan assay. However, in the ISTA validation report it is indicated that extracts X9 and X24 were excluded from the analysis because "*X9 was not pure and there was a mistake during DNA preparation with X24*" (Grimault et al. 2012). Therefore the isolate X9 was also excluded from the analysis below.

Samples X31-X60 are non-target DNA extracts, X31 is a *X. axonopodis* pv. *dieffenbachiae* isolate also detected with the Audy primers of the ISTA 7-021 method (Grimault et al. 2012). Several of the non-target extracts show a late amplification with Cq values between 30-35. Traces of amplifications were also observed in the comparative test on some of the non-target DNA extracts (Appendix B), this may be due to non-specific amplification or traces of contamination in the extract solutions. The 14 non-target isolates showing late amplification results were re-tested on fresh isolate cultures. None of the non-target isolates were amplified with the Xap TaqMan assay (Table 3). The Xcc and Xcv isolates were not retested, other data show that the Xap TaqMan assay did not amplify other isolates from these species (Table 1). These amplifications were also likely due to traces of cross-contamination.

The analytical specificity was calculated according to the calculations below:

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

Inclusivity = ΣPA/(ΣPA+ΣND) × 100

Exclusivity = ΣNA/(ΣNA+ΣPD) × 100

Analytical specificity = (ΣNA+ΣPA)/(ΣPA+ΣNA+ΣPD+ΣND) × 100

	Expected result + (target)	**Expected result - (non target)**
Obtained result +	29*	1
Obtained result -	0	29

Inclusivity

$$= \frac{29}{29} \times 100 = 100\%$$

Accuracy

$$= \frac{(29 + 29)}{(29 + 29 + 1 + 0)} \times 100 = 98.3\%$$

Exclusivity

$$= \frac{29}{(29 + 1)} \times 100 = 96.6\%$$

Table 2. Cq values obtained with the Xap TaqMan assay on the comparative test DNA collection

Name	Identity	Xap TaqMan Assay	
		Xap	Wu
X1	Xap	16.04	16.82
X2	Xap	13.98	15.77
X3	Xap	14.16	16.08
X4	Xap	15.06	17.54
X5	Xap	17.66	16.73
X6	Xap	17.04	18.75
X7	Xap	15.90	16.86
X8	Xap	16.88	17.17
X9	Xap	34.93	18.61
X10	Xap	15.20	15.60
X11	Xap	15.86	15.33
X12	Xap	16.94	15.55
X13	Xap	15.43	17.74
X14	Xap	15.87	16.73
X15	Xap	14.50	16.83
X16	Xap	17.13	16.28
X17	Xap	16.06	15.88
X18	Xap	15.99	15.85
X19	Xap	16.17	16.42
X20	Xap	16.52	15.67
X21	Xap	13.85	16.45
X22	Xap	14.16	17.71
X23	Xap	14.28	15.66
X24	Xap	15.17	15.39
X25	Xap	14.62	15.72
X26	Xap	13.93	15.36
X27	Xap	15.71	17.00
X28	Xap	14.12	16.84
X29	Xap	15.65	15.78
X30	Xap	14.57	16.38
X31	Non-target	17.12	17.20
X32	Non-target	32.25	15.66

Name	Identity	Xap TaqMan Assay	
		Xap	Wu
X33	Non-target	32.18	15.28
X34	Non-target	34.76	15.89
X35	Non-target	NA	15.79
X36	Non-target	NA	16.95
X37	Non-target	32.97	17.21
X38	Non-target	32.53	17.77
X39	Non-target	33.42	16.86
X40	Non-target	33.89	16.24
X41	Non-target	NA	13.89
X42	Non-target	NA	14.89
X43	Non-target	NA	14.85
X44	Non-target	32.20	15.83
X45	Non-target	34.54	15.62
X46	Non-target	33.06	16.27
X47	Non-target	34.08	15.75
X48	Non-target	31.80	16.14
X49	Non-target	NA	15.53
X50	Non-target	NA	15.23
X51	Non-target	NA	15.94
X52	Non-target	NA	16.19
X53	Non-target	NA	14.91
X54	Non-target	NA	14.95
X55	Non-target	NA	15.63
X56	Non-target	NA	16.10
X57	Non-target	NA	16.49
X58	Non-target	NA	16.59
X59	Non-target	34.78	16.18
X60	Non-target	31.72	15.85
Xap	Non-target	14.26	15.72
Xcc	Non-target	29.80	15.23
Xcv	Non-target	33.74	16.69
Water	NTC	NA	33.73

NA= No amplification, Cq values from the Audy assay on non-target isolates amplified are highlighted in yellow. Non-target amplification (*X. axonopodis pv. dieffenbachiae*) highlighted in red.

Table 3. Cq values obtained with the Xap TaqMan assay on fresh isolates

Name	Audy-Wu Duplex (25µl)	
	Audy	Wu
X32	NA	16.58
X33	NA	15.93
X34	NA	16.15
X37	NA	15.19
X38	NA	22.40
X39	NA	16.47
X40	NA	17.17
X44	NA	17.66

Name	Audy-Wu Duplex (25µl)	
	Audy	Wu
X45	NA	17.44
X46	NA	17.88
X47	NA	16.73
X48	NA	18.92
X59	NA	20.18
X60	NA	18.37
Xap	19.93	19.32
Water	NA	30.71

NA= No amplification

I.3 Conclusion

Excluding the isolate X9 which was indicated to be an impure isolate in the 2011 validation report, **the inclusivity of the Xap TaqMan including the IAC assay was calculated to be 100%. The exclusivity of the assay was calculated to be 96.6%** due to the positive reaction with a *X. axonopodis* pv. *dieffenbachiae* isolate.

As observed with other assays using the Wu IAC, Cq values were observed for this TaqMan probe in the NTC control. One possible explanation is the presence in the mastermix of residual bacterial DNA from the bacteria used to produce the Taq polymerase.

II. Analytical sensitivity

The smallest amount of the target pathogen that can be detected.

The Xap TaqMan assay is destined to be used for identification purposes on pure isolate suspensions which are prepared at a recommended concentration. Therefore, it is not necessary to validate the analytical sensitivity of the method. However, experiments were done to validate that the assay functions correctly on a dilution series of isolate suspensions around the recommended concentration.

The current ISTA rule makes the following instruction about preparing isolate suspensions for PCR identification: *Make a slightly turbid cell suspension at 10^7 CFU/mL (OD600 nm approximately 0.05) in 1.0 mL sterile distilled/deionised water.*

Two Xap isolates from the GEVES collection were prepared as a highly charged suspension (D0), then serially diluted (D1-D4). Absorbance of each dilution was measured by optical density (OD 600 nm). Each dilution was treated by heating at 95°C for 5min and then tested with both the current ISTA gel-based PCR assay and the Xap TaqMan assay (Table 4).

Table 4. Identification on diluted isolate suspensions

Isolate	Method	Dilution series				
		D0	D1	D2	D3	D4
Xap 195	Visual	Very turbid		Very slightly turbid		
	OD 600 nm	1.730	0.223	0.024	0.003	0.001
	TaqMan Xap (Cq)	11.39	14.35	19.45	23.45	27.51
	TaqMan Wu (Cq)	12.88	15.71	20.96	23.89	24.83
	Gel-based PCR ISTA 7-021	Visible band @ 800bp	Visible band @ 800bp	Visible band @ 800bp	Negative	Negative
Xap 197	Visual	Very turbid		Very slightly turbid		
	OD 600 nm	1.652	0.199	0.023	0.003	0.003
	TaqMan Xap (Cq)	11.42	15.86	20.28	25.04	27.16
	TaqMan Wu (Cq)	11.47	15.60	19.73	23.89	24.78
	Gel-based PCR ISTA 7-021	Visible band @ 800bp	Visible band @ 800bp	Visible band @ 800bp	Negative	Negative

The ISTA gel-based assay and the Xap TaqMan assay correctly identify bacterial suspensions at optical densities above (0.199/0.223) and below (0.024/0.023) the recommended optical density of 0.05. Both assays performed correctly at much higher concentrations. In these experiments the

Xap TaqMan assay correctly identified target isolates in higher dilutions (D3, D4) which were negative with the ISTA gel-based method. Therefore, the current recommended suspension at approximately 0.05 (OD 600 nm) is satisfactory for both assays.

III. Selectivity

The effect of different matrices on the ability of the method to detect the target pathogen.

The Xap TaqMan assay is destined to be used on a single type of matrix: pure suspect bacterial isolate suspensions for identification. No other matrices were tested or validated in these studies.

IV. Robustness of the Xap TaqMan Assay in several laboratories

Ability to not vary according to small variations of parameters in the method.

The performance of the Xap TaqMan assay was tested on a set of three target and three non-target boiled control isolates prepared according to the ISTA 7-021 method and sent to participating laboratories. Although different equipment and mastermixes were used in each laboratory, thermal cycling conditions could be adapted in all of them.

The Wu IAC gave positive results in all the reactions. Two laboratories observed significantly lower Cq values of 24.8 and 24.7 in their NTC/NAC control reactions with the Wu assay. One hypothesis is that these Cq values are due to residual bacterial DNA in the mastermixes used, however these data do not provide proof of this hypothesis (Table 5). In conclusion, all isolates were identified as expected (as either target or non-target isolates) therefore, the method performed correctly despite the varying reaction conditions.

Table 5. Cq values obtained in each laboratory (A-H) with the Xap TaqMan assay

Lab	Xap 1		Xap 2		Xap 3		non-Xap 1		non-Xap 2		non-Xap 3		NTC/NAC	
	Audy	Wu	Audy	Wu	Audy	Wu	Audy	Wu	Audy	Wu	Audy	Wu	Audy	Wu
A	17.5	18.2	17.8	19.2	18.4	20.5	NA	17.6	NA	16.3	NA	17.9	NA	36.1
B	16.9	16.5	18.5	18.6	17.7	17.9	NA	17.4	NA	14.0	NA	16.6	NA	24.8
C	17.6	18.8	17.5	19.5	18.3	20.2	NA*	18.1	NA*	17.5	NA*	18.5	NA	>35
D	17.9	17.5	17.8	17.9	18.3	18.5	NA	16.8	NA	16.9	NA	17.1	NA	33.5
E	15.2	19.0	16.1	21.8	17.7	21.7	NA	18.7	NA	15.9	NA	19.9	NA	33.4
F	18.6	18.8	18.8	19.8	18.4	18.6	NA	17.5	NA	18.5	NA	18.2	NA	34.8
G	18.0	18.2	19.1	19.8	20.8	22.1	NA	18.9	NA	18.9	NA	18.0	NA	33.3
H	14.8	15.5	15.1	16.4	15.0	16.2	NA	15.4	NA	14.2	NA	16.3	NA	24.7

*Cq value with a non-typical curve

Lab PCR Mastermix: A) TaqMan Fast Universal PCR; B) Gene expression; C) Light cycler 480 probe; D) Quanta PerfeCTa Multiplex qPCR ToughMix; E) TaqMan Universal MasterMix II; F) Sso Advance Universal Probes Supermix; G) IDT PrimeTime Gene Expression; H) Quanta PerfeCTa Multiplex qPCR ToughMix

V. Repeatability and Reproducibility

Repeatability represents the degree of similarity in results of replicates of the same seed lots when the method is performed with minimal variations in a single laboratory.

Reproducibility represents the degree of similarity in results when the method is performed across laboratories with replicates of the same subsamples.

An ISHI-Veg/ISTA comparative test on 30 target and 30 non-target isolates was planned to validate the intralaboratory repeatability and the interlaboratory reproducibility of the Xap TaqMan assay. Eight laboratories, including the organiser, participated in the comparative test in 2018. The participating laboratories had to be experienced with seed health testing and molecular bacterial testing, in particular *Xanthomonas axonopodis* pv. *phaseoli* (Xap) and *X. axonopodis* pv. *phaseoli* var. *fuscans* (Xapf).

The comparative test was done with the TaqMan assay on the same set of target and non-target isolates in each participating laboratory. The isolates were characterized with both the TaqMan assay and the Audy gel-based PCR assay as described in the current ISTA method prior to the start of the CT by the organizing laboratory.

V.1 Materials and Methods

Samples for PCR constituted suspensions of dead bacteria (OD_{600} nm approximately 0.05, 10^7-10^8 CFU mL^{-1}) destroyed by heating at 95°C for 5 min. Each participating laboratory received 60 randomly coded samples of suspensions of dead bacteria from pure cultures of 30 Xap and 30 non-target isolates (other *Xanthomonas* isolates and look-alike saprophytes from bean seeds). In addition, suspensions of dead bacteria from pure cultures of one positive Xap isolate and one non-target isolate were identified to the participants as the positive process control (PPC) and the negative process control (NPC) respectively. Each participant was requested to test a positive amplification control (PAC – Xap DNA extract or isolate) from their own laboratory and a Non-Template Control (NTC).

Material supplied by each laboratory included: reagents for real-time PCR, sterile tubes, micropipettes with sterile filtered tips and the real-time PCR equipment.

Samples were all sent by courier service at ambient temperature from the GEVES laboratory on the 09 July 2018 and were received by laboratories at different dates (10 July - 6 August 2018) depending on distance and custom delays.

Method for PCR testing

Each sample was tested in duplicate PCR reactions.

The PCR mastermix Applied Universal TaqMan Mastermix II was used during the development of the assay. Each participant was, nevertheless, free to use another PCR mastermix but needed to ensure prior to participation in the CT that it was suitable for the TaqMan assay. In addition, the participant could choose to use other dyes or quenchers for the probes and change the overall reaction volume. Reaction mixture and conditions, however, had to be checked and/or optimized within each laboratory before the comparative test.

The TaqMan PCR assay for identifying Xap/ Xapf isolates

PCR primers and probes are described in Table 6. The PCR cycling conditions are described in

Table 7 and the PCR reactions should be prepared according to Table 8.

Table 6. PCR primers and probes

Primer Name	Sequence (5'-3')	5' Modification	3' Modification	Reference
AuF1	ACGGCCGGCGTCTTGTCTCT			Baldwin, 2017
AuR1	GCCGAGGTCCGCGAGATTCT			
Au1FAM	CGTCTCTGGCTTGACTGCGGTCGC	FAM	BHQ1	
WuF	CAACGCGAAGAACCTTACC			Wu, 2008
WuR	ACGTCATCCCCACCTTCC			
WuPr1	ACGACAACCATGCACCACCTG	Yakima Yellow	QSY	
WuPr2	ACGACAGCCATGCAGCACCT	Yakima Yellow	QSY	

Table 7. PCR cycling program

Temperature	Time	Cycles
95°C	10 min	1
95°C	15 sec	40
60°C	1 min	

Table 8. Example of a PCR reaction composition

Reagent	Units	Initial concentration	Final concentration	Volume
Water		-	-	2.8
TaqMan® Universal MasterMix II		2x	1x	12.5
AuF1	µM	10	0.4	1
AuR1	µM	10	0.4	1
Au1FAM	µM	10	0.08	0.2
WuF	µM	10	0.4	1
WuR	µM	10	0.4	1
WuPr1	µM	10	0.1	0.25
WuPr2	µM	10	0.1	0.25
Volume mix	µL	-	-	20
Sample	µL	-	-	5
Total Volume	µL	-	-	25

V.2 Reporting results

The participants reported quantitative (Cq values) as well as qualitative (positive/negative) results for each subsample and each primer set, according to the instructions in the test plan. Statistical analysis was performed on the qualitative data with the application cut-off value of Cq 35 (Cq≤35 = positive; Cq>35 = negative) on the quantitative Cq values obtained from the real-time PCR reactions.

Samples were scored in relation to the Cq value determined for the Non-Template Control (NTC). As the Wu assay detects microbial DNA present in the PCR mastermixes which could lead to Cq values in the NTC. Some late amplifications were obtained with the Xap/Xapf specific TaqMan PCR when validating specificity of the method on non- Xap/Xapf isolate suspensions at Cq > 35,

specificity was 100% with a cut-off at 35 (Baldwin, 2017). Therefore, a Cq cut-off of 35 was also applied to the suspect isolates. The interpretation of PCR results as used in the test is presented in Table 9.

Table 9. Interpretation of PCR results

Xap/Xapf TaqMan	Wu	qPCR Result	Interpretation
Cq ⩽ 35		Expected result for Xap/Xapf	Positive PCR result
Cq > 35 or ND	Cq ⩽ 35	Expected result for a non-Xap/Xapf isolate	Negative PCR result
Cq >35 or ND	Cq > 35 or ND	Amplification control failure	Invalid result, repeat PCR

A negative identification result could only be concluded if a PCR product detected with the Wu IAC assay was at least 3.3 Cq's lower than the Wu assay Cq value obtained on the NTC and no products were amplified with the Xap/Xapf specific assay at < 35 in both replicates.

A positive Xap/Xapf-specific result was concluded if a PCR product was detected with the Xap/Xapf specific assay at Cq < 35 in both replicates. If a different result was obtained in the duplicate, the samples should be retested in new duplicate PCR reactions. In this repeat PCR, a positive Xap/Xapf-specific result was concluded if a PCR product was detected with the Xap/Xapf specific assay at Cq < 35 in either one or both replicates. No interpretation of the Wu Cq value was necessary in the case of a positive Xap/Xapf result.

The data recorded in the record sheet provided were

- results of the PCR tests by indicating the Cq value obtained from each replicate and writing a conclusion for each sample as "+" if positive or "-" if negative for each primer set column.
- the date, the make and model of PCR machine and the PCR mix supplier for each PCR
- if possible, the quantification curve analysis

V.3 Results

Homogeneity of samples

The sample sets were prepared as aliquots from the same tubes of dead bacterial suspensions which had been previously tested with the ISTA 7-021 PCR method (Table 10). These results were used to identify the expected result for each sample indicated in Table 12. Each tube was homogenized with a vortex before being pipetted into multiple aliquots. Therefore, no homogeneity tests were performed.

Stability of samples

The organizing laboratory tested a set of samples stored at -20°C with the ISTA 7-021 PCR method after the conclusion of testing by all other participating laboratories. The results were compared with the initial tests (Table 10). The stability of samples was assessed by comparing the results from these two tests.

All 30 target Xap isolates tested positive with the ISTA 7-021 PCR method before the start of the comparative test and after other laboratories had completed the test. Amongst the 30 non-target isolates, all were negative before the start of testing. Stability testing after the conclusion of the comparative test revealed two bands on the gel from samples 20 and 23, which were expected

to be negative (Table 10). Unstable possibly due to cross-contamination, they were excluded from the analysis of assay performance (repeatability and reproducibility).

Table 10. Results of initial characterization and stability tests of the test samples with ISTA gel-based PCR

Sample	ISTA gel PCR start	ISTA gel PCR finish	Sample	ISTA gel PCR start	ISTA gel PCR finish	Sample	ISTA gel PCR start	ISTA gel PCR finish
1	-	-	21	-	-	41	+	+
2	-	-	22	-	-	42	+	+
3	-	-	23	-	+	43	+	+
4	-	-	24	-	-	44	+	+
5	-	-	25	-	-	45	+	+
6	-	-	26	-	-	46	+	+
7	-	-	27	-	-	47	+	+
8	-	-	28	-	-	48	+	+
9	-	-	29	-	-	49	+	+
10	-	-	30	-	-	50	+	+
11	-	-	31	+	+	51	+	+
12	-	-	32	+	+	52	+	+
13	-	-	33	+	+	53	+	+
14	-	-	34	+	+	54	+	+
15	-	-	35	+	+	55	+	+
16	-	-	36	+	+	56	+	+
17	-	-	37	+	+	57	+	+
18	-	-	38	+	+	58	+	+
19	-	-	39	+	+	59	+	+
20	-	+	40	+	+	60	+	+

Sample 1-30 are non-targets, and 31-60 are targets. - = negative; + = positive. Unstable samples are highlighted.

Performance of the assay

Results obtained by the participating laboratories are presented in Table 11. Several laboratories reported amplification control failures on some samples with a negative Xap result and a negative Wu result; these results are indicated as ND in Table 11. These amplification control failures were planned for in the interpretation of the test plan (Table 9), *viz*. in routine application of the method the PCR and/or suspension preparation would be repeated and in the case of a repetitive ND results a suspect isolate could be tested with the pathogenicity test. The exact cause of these ND results cannot be concluded from these results, but the effect of transport at ambient temperature for a duration of several days may be a factor. The ND results, therefore, were treated as missing values as it was not possible to give a result on the sample.

One laboratory (E) also gave a false positive result on one sample (isolate 16). The unique occurrence of false positive result in a single laboratory may have been due to a cross-contamination problem rather than a lack of Xap TaqMan assay specificity, but without proof, this result has been included in the specificity calculations.

Table 11. Qualitative results for all samples in each participating laboratory

		Results obtained by participating laboratories							
Isolate	**Expected**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
1	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-
3	-	-	ND (Wu Ct>35)[b]	-	ND (Wu negative)[b]	-	ND (Wu negative)[b]	ND (Wu negative)[b]	ND (Wu negative)[b]
4	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	+ (Cq25/29)[c]	-	-	-
17	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-
20[a]	-	+ (Cq 34)	-	+ (Cq34/37)	-	+ (Cq31/34)	-	+ (Cq34/34)	-
21	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-
23[a]	-	+ (Cq 32)	+ (Cq 32/34)	+ (Cq31/33)	+ (Cq33/33)	+ (Cq32/34)	+ (Ct30/22)	+ (Cq34/34)	-
24	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-
31	+	+	+	+	+	+	+	+	+
32	+	+	+	+	+	+	+	+	+
33	+	+	+	+	+	+	+	+	+
34	+	+	+	+	+	+	+	Tube lost	+
35	+	+	+	+	+	+	+	+	+
36	+	+	+	+	+	+	+	+	+
37	+	+	+	+	+	+	+	+	ND (Wu negative)[b]
38	+	+	+	+	+	+	+	+	+
39	+	+	+	+	ND (Wu negative)[b]	+	ND (Wu negative)[b]	ND (Wu negative)[b]	ND (Wu negative)[b]
40	+	+	+	+	+	+	+	+	+

		Results obtained by participating laboratories							
Isolate	**Expected**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
41	+	+	+	+	+	+	ND (Wu negative)[b]	ND (Wu negative)[b]	ND (Wu negative)[b]
42	+	+	+	+	+	+	+	+	+
43	+	+	+	+	+	+	ND (Wu negative)[b]	ND (Wu negative)[b]	ND (Wu negative)[b]
44	+	+	+	+	+	+	+	+	+
45	+	+	+	+	+	+	+	+	+
46	+	+	+	+	+	+	+	+	+
47	+	+	+	+	+	+	+	+	+
48	+	+	+	+	+	+	+	+	+
49	+	+	+	+	+	+	+	+	+
50	+	+	+	+	+	+	+	+	ND (Wu negative)[b]
51	+	+	+	+	+	+	+	+	+
52	+	+	+	+	+	+	+	+	+
53	+	+	+	+	+	+	+	+	+
54	+	+	+	+	+	+	+	+	+
55	+	+	+	ND (Wu negative)[b]	ND (Wu negative)[b]	+	ND (Wu negative)[b]	ND (Wu negative)[b]	ND (Wu negative)[b]
56	+	+	+	+	+	+	ND (Wu negative)[b]	+	ND (Wu negative)[b]
57	+	+	+	+	+	+	+	+	+
58	+	+	+	+	+	+	+	+	+
59	+	+	+	+	+	+	+	+	+
60	+	+	+	+	+	+	+	+	+
PPC	+	+	+	+	+	+	+	+	+
NPC	-	-	-	-	-	-	-	-	-
PAC	+	+	+	+	+	+	+	+	+
NTC	-	-	-	-	-	-	-	-	-

[a] These samples were unstable in stability tests and were excluded from further analysis; [b] ND result, indicating an amplification control failure; [c] False positive result

Inclusivity (i.e. diagnostic sensitivity), exclusivity (diagnostic specificity) and analytical specificity (accuracy) of the PCR assay (Table 12) were calculated according to the mathematical formulas:

$$\text{Inclusivity (\%)} = \Sigma PA / (\Sigma PA+\Sigma ND) \times 100$$

$$\text{Exclusivity (\%)} = \Sigma NA / (\Sigma NA+\Sigma PD) \times 100$$

$$\text{Analytical specificity (\%)} = (\Sigma NA+\Sigma PA) / (\Sigma PA+\Sigma NA+\Sigma PD+\Sigma ND) \times 100$$

PA = positive agreement, ND = negative deviation, NA = negative agreement and PD = positive deviation.

Table 12. Performance criteria for each laboratory

Laboratory	A	B	C	D	E	F	G	H	TOTAL
PA	30	30	29	28	30	25	25	23	220
NA	28	27	28	27	27	27	27	27	218
PD	0	0	0	0	1	0	0	0	1
ND	0	0	0	0	0	0	0	0	0
Inclusivity %	100	100	100	100	100	100	100	100	100
Exclusivity %	100	100	100	100	96.4	100	100	100	99.5
Analytical specificity %	100	100	100	100	98.3	100	100	100	99.8

PA = positive agreement, ND = negative deviation, NA = negative agreement and PD = positive deviation.

Concordance (reproducibility of qualitative data) was evaluated separately on non-target and target isolates using the method developed by Langton et al. (2002) (Tables 13 and 14).

Table 13. Concordance (reproducibility of qualitative data) on non-target isolates

Laboratory	Number of samples	positives		Estimate	Bootstrap s.e.	Bootstrap 95% limits lower	upper
1	28	28	Within lab pairs	2889			
2	27	27	Within lab matched pairs	2862			
3	28	28	Accordance (propn)	0,991	0,0088	0,9722222	1
4	27	27	Accordance (percentage)	99,1%	0,88%	97,22%	100,00%
5	28	27	Total pairs	23871			
6	27	27	Total matched pairs	23653			
7	27	27	Between pairings	20982			
8	27	27	same between	20791			
9			Concordance (propn)	0,991	0,0085	0,9730802	1
10			Concordance (percentage)	99,1%	0,85%	97,31%	100,00%
11			COR	0,97		0,9629158	1
12			Above results are based on 5000 bootstrap samples of 8 labs using representative method				

Table 14. Concordance (reproducibility of qualitative data) on target isolates

Laboratory	Number of samples	positives		Estimate	Bootstrap s.e.	Bootstrap 95% limits lower	upper
1	30	30	Within lab pairs	2942			
2	30	30	Within lab matched pairs	2942			
3	29	29	Accordance (propn)	1,000	0,0000	1	1
4	28	28	Accordance (percentage)	100,0%	0,00%	100,00%	100,00%
5	30	30	Total pairs	24090			
6	25	25	Total matched pairs	24090			
7	25	25	Between pairings	21148			
8	23	23	same between	21148			
9			Concordance (propn)	1,000	0,0000	1	1
10			Concordance (percentage)	100,0%	0,00%	100,00%	100,00%
11			COR	1,00		1	1
12			Above results are based on 5000 bootstrap samples of 8 labs using representative method				

The Xap TaqMan assay was reproducible on Xap isolates, with concordance values of 100%. The Xap TaqMan assay was slightly less reproducible on negative isolates, with concordance values of 99.1%. In all participating laboratories, on all isolates analysed, there were no deviations between the two Xap TaqMan assay repetitions (Appendix C), therefore the repeatability of the Xap TaqMan assay in this comparative test was 100%.

CONCLUSION

The validation data presented in this report shows that the Xap TaqMan assay is fit for purpose as an isolate identification assay. The IAC control ensures that a negative result cannot be concluded from the PCR assay when there is a reaction failure. When the reaction functioned correctly, all the target isolates were correctly identified as positive (inclusivity = 100%). When the reaction functioned correctly, the correct identification of non-target isolates was demonstrated to be less than 100% (exclusivity = 99.5%) due to a false positive result on sample 16 in laboratory E. This value is similar to the exclusivity calculated in analytical specificity experiments on the 60 isolate DNA extract collection from the previous ISTA/ISHI comparative test (exclusivity = 96.6%) This underlines that the pathogenicity assay is essential for confirming a PCR positive result on suspect isolates.

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Appendix A: ISHI-Veg Method Performance Characteristics

Version 1, May 2018

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: Results for Specificity

Comparison of the Xap Taqman-Wu duplex results with the 2011 comparative test results (Grimault et al. 2012)

Name	Type	Audy-Wu duplex		Audy 2011					
		Audy	Wu	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6
X1	Xap	16.0	16.8	1	1	1	1	1	1
X2	Xap var. fuscans	14.0	15.8	1	1	1	1	1	1
X3	Xap var. fuscans	14.2	16.1	1	1	1	1	1	1
X4	Xap var. fuscans	15.1	17.5	1	1	1	1	1	1
X5	Xap	17.7	16.7	1	1	1	1	1	1
X6	Xap	17.0	18.8	1	1	1	1	1	1
X7	Xap var. fuscans	15.9	16.9	1	1	1	1	1	1
X8	Xap	16.9	17.2	1	1	1	1	1	1
X9	Xap	34.9	18.6	0	0	0	0	0	0
X10	Xap	15.2	15.6	1	1	1	1	1	1
X11	Xap	15.9	15.3	1	1	1	1	1	1
X12	Xap	16.9	15.6	1	1	1	1	1	1
X13	Xap	15.4	17.7	1	1	1	1	1	1
X14	Xap	15.9	16.7	1	1	1	1	1	1
X15	Xap	14.5	16.8	1	1	1	1	1	1
X16	Xap	17.1	16.3	1	1	1	1	1	1
X17	Xap	16.1	15.9	1	1	1	1	1	1
X18	Xap	16.0	15.9	1	1	1	1	1	1
X19	Xap	16.2	16.4	1	1	1	1	1	1
X20	Xap	16.5	15.7	1	1	1	1	1	1
X21	Xap var. fuscans	13.9	16.5	1	1	1	1	1	1
X22	Xap var. fuscans	14.2	17.7	1	1	1	1	1	1
X23	Xap var. fuscans	14.3	15.7	1	1	1	1	1	1
X24	Xap var. fuscans	15.2	15.4	0	0	0	0	0	0
X25	Xap var. fuscans	14.6	15.7	1	1	1	1	1	1
X26	Xap var. fuscans	13.9	15.4	1	1	1	1	1	1
X27	Xap var. fuscans	15.7	17.0	1	1	1	1	1	1
X28	Xap var. fuscans	14.1	16.8	1	1	1	1	1	1
X29	Xap var. fuscans	15.7	15.8	1	1	1	1	1	1
X30	Xap var. fuscans	14.6	16.4	1	1	1	1	1	1
X31	Non-target	17.1	17.2	1	1	1	1	1	1
X32	Non-target	32.3	15.7	0	trace	0	0	0	0
X33	Non-target	32.2	15.3	0	trace	0	0	0	0
X34	Non-target	34.8	15.9	0	0	0	0	0	0
X35	Non-target	NA	15.8	0	0	0	0	0	0
X36	Non-target	NA	17.0	0	0	0	0	0	0
X37	Non-target	33.0	17.2	0	0	0	0	0	0
X38	Non-target	32.5	17.8	0	0	0	0	0	0

Name	Type	Audy-Wu duplex		Audy 2011					
		Audy	Wu	Lab1	Lab2	Lab3	Lab4	Lab5	Lab6
X39	Non-target	33.4	16.9	0	trace	0	0	0	0
X40	Non-target	33.9	16.2	0	0	0	0	0	0
X41	Non-target	NA	13.9	0	0	0	0	0	0
X42	Non-target	NA	14.9	0	0	0	0	X	0
X43	Non-target	NA	14.9	0	0	0	0	0	0
X44	Non-target	32.2	15.8	0	trace	0	0	0	0
X45	Non-target	34.5	15.6	0	0	0	0	0	0
X46	Non-target	33.1	16.3	0	trace	0	0	0	0
X47	Non-target	34.1	15.8	0	trace	0	0	0	0
X48	Non-target	31.8	16.0	0	trace	0	0	0	0
X49	Non-target	NA	15.5	0	0	0	0	0	0
X50	Non-target	NA	15.2	0	0	0	0	0	0
X51	Non-target	NA	15.9	0	0	0	0	0	0
X52	Non-target	NA	16.2	0	0	0	0	0	0
X53	Non-target	NA	14.9	0	0	0	0	0	0
X54	Non-target	NA	15.0	0	0	0	0	0	0
X55	Non-target	NA	15.6	0	0	0	0	0	0
X56	Non-target	NA	16.1	0	0	0	0	0	0
X57	Non-target	NA	16.5	0	0	0	0	0	0
X58	Non-target	NA	16.6	0	0	0	0	0	0
X59	Non-target	34.8	16.2	0	0	0	0	0	0
X60	Non-target	31.7	15.9	0	0	0	0	0	0
T+Xap	Xap	14.3	15.7	1	1	1	1	1	1
T-Xcc	Unknown	29.8*	15.2	0	trace	0	0	0	0

NA = No amplification; 0 = negative result; 1 = positive result; trace = traces of amplicon observed; Amplifications with the Audy assay on non-target isolates are highlighted in yellow and were negative in repeat PCRs as indicated in the report.

* The Xcc isolate was not retested, other data show that the Xap TaqMan assay did not amplify other isolates from this species (see Table 1). Amplification was likely due to traces of cross-contamination.

APPENDIX C: Comparative Test Raw Data

Table C1. Cq values for Xap Taqman Assay for each laboratory

Isolate	Replicate	Expected result	Laboratory							
			A	B	C	D	E	F	G	H
1	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
2	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
3	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
4	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
5	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
6	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
7	I	-	39.18	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
8	I	-	-	-	-	-	-	-	-	-
	II	-	35.28	-	-	-	-	-	-	-
9	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
10	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
11	I	-	-	-	-	-	36.14	-	-	-
	II	-	-	-	38.19	-	35.81	-	-	-
12	I	-	-	-	-	-	36.76	-	-	-
	II	-	-	38.71	-	-	-	-	-	-
13	I	-	38.49	37.42	-	39.63	-	-	39.44	-
	II	-	-	39.08	-	-	-	-	-	-
14	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
15	I	-	-	-	-	-	35.41	-	-	-
	II	-	-	-	-	-	38.41	-	-	-
16	I	-	-	-	-	-	24.87	-	-	-
	II	-	-	-	-	39.33	28.95	-	-	-
17	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
18	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
19	I	-	-	-	38.79	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
20	I	-	34.35	35.89	34.46	36.23	31.22	>35	34.95	-
	II	-	33.4	35.17	37.12	35.89	34.85	>35	34.77	-
21	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-

Isolate	Replicate	Expected result	Laboratory							
			A	B	C	D	E	F	G	H
22	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
23	I	-	32.44	33.97	30.83	33.46	32.07	30	34.65	39.74
	II	-	33.27	32.22	33.38	33.68	34.32	22	34.23	39.8
24	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
25	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
26	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
27	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
28	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
29	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
30	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
31	I	+	17.89	19.16	17.25	18.67	14.75	19	19.79	20.22
	II	+	18.43	19.04	17.27	18.63	17.74	19	20.06	20.98
32	I	+	18.44	19.34	17.42	19.99	17.40	19	19.34	22.57
	II	+	18.71	19.32	17.43	19.67	18.88	19	19.61	22.22
33	I	+	15.9	16.98	15.32	17.43	16.46	17	18.06	18.87
	II	+	16.46	16.92	15.19	17.39	16.93	17	18.11	19.06
34	I	+	17.99	18.88	16.85	19.16	18.15	19	Tube lost	22.46
	II	+	14.35	18.8	17.24	19.12	18.44	19	Tube lost	22.96
35	I	+	16.91	17.62	16.21	17.5	13.70	18	18.16	19.4
	II	+	18.24	17.46	16.11	17.29	15.41	18	18.15	19.36
36	I	+	17.76	18.53	16.86	19.94	16.40	19	19.32	22.27
	II	+	13.59	18.38	16.77	19.14	15.57	19	19.43	22.47
37	I	+	12.92	14.01	12.49	13.98	11.14	22	17.13	-
	II	+	17.74	13.72	12.72	14.04	12.26	22	17.39	-
38	I	+	12.9	13.75	12.11	14.12	11.49	13	14.02	14.89
	II	+	13.82	13.84	12.1	14.14	12.10	13	13.92	14.63
39	I	+	12.77	19.96	12.47	-	19.91	-	-	-
	II	+	18.25	20.96	12.75	-	26.73	-	-	-
40	I	+	13.46	14.41	13.24	14.62	11.18	14	14.70	22.37
	II	+	15.78	13.97	13.25	14.3	13.02	14	15.07	21.31
41	I	+	13.34	20.57	13.87	28.71	18.97	-	-	-
	II	+	13.59	20.46	14.2	29.08	21.88	-	-	-
42	I	+	13.05	13.76	12.26	14.19	11.18	13	13.94	15.94
	II	+	16.07	13.65	11.94	14.19	12.19	13	13.89	16.06
43	I	+	12.91	17.33	13.01	23.64	15.19	-	36.56	-
	II	+	13.48	17.34	13.16	21.99	17.06	-	36.83	-

Isolate	Replicate	Expected result	Laboratory							
			A	B	C	D	E	F	G	H
44	I	+	14.9	17.09	14.71	17.02	14.24	16	16.77	18.95
	II	+	14.38	17.01	14.58	17.14	15.57	16	17.07	18.6
45	I	+	15.52	17.02	14.73	16.24	15.82	16	17.19	19.34
	II	+	13.41	16.98	14.56	16.08	15.53	16	17.32	19.22
46	I	+	13.85	15.69	13.72	16.44	16.48	18	17.35	29.49
	II	+	15.11	15.66	13.85	16.55	18.08	20	17.02	30.09
47	I	+	14.38	15.86	14.15	15.74	13.97	16	16.72	18.63
	II	+	14.38	15.72	13.96	15.6	14.55	16	16.49	18.94
48	I	+	14.8	15.96	14.37	16.85	13.82	16	16.92	18.46
	II	+	15.49	15.95	14.28	16.4	14.59	16	16.91	18.01
49	I	+	15.31	16.59	14.51	17.04	13.46	16	17.18	18.35
	II	+	15.89	16.61	14.9	17	14.91	17	17.24	18.49
50	I	+	13.98	19.25	15.94	18.95	15.45	28	22.30	-
	II	+	16.71	19.28	15.96	18.84	17.69	25	22.44	-
51	I	+	13.67	15.61	13.43	16.58	14.82	16	16.94	19.14
	II	+	15.09	15.08	13.37	16.58	16.11	16	16.91	19.23
52	I	+	15.49	16.97	15.36	17.56	15.20	17	17.69	18.82
	II	+	16.13	16.88	15.27	17.54	15.90	16	17.33	18.35
53	I	+	14.7	15.97	14.45	15.51	12.42	16	16.69	17.95
	II	+	14.38	15.71	14.26	15.34	14.57	16	16.81	17.91
54	I	+	13.92	18.3	14.56	18.25	16.55	18	19.53	28
	II	+	15.66	18.25	14.83	18.35	17.63	18	19.47	28.08
55	I	+	14.29	23.63	-	-	27.46	-	-	-
	II	+	16.47	24.18	42.55	-	34.02	-	-	-
56	I	+	14.25	19.48	16	19.86	19.97	>35	26.05	-
	II	+	16.08	19.12	15.65	19.93	20.24	>35	26.31	-
57	I	+	16.03	17.34	15.09	16.54	13.79	17	17.86	20.29
	II	+	15.22	17.2	15.15	16.52	15.61	17	17.60	20.45
58	I	+	15.16	16.89	15.26	18.72	14.80	18	17.71	25.39
	II	+	17.59	16.67	15.06	18.59	16.52	18	17.81	25.22
59	I	+	15.03	16.77	14.36	16.2	14.41	16	17.36	18.31
	II	+	14.67	16.74	14.44	16.25	15.26	16	17.44	18.26
60	I	+	15.64	16.36	14.86	16.99	14.47	16	16.97	19.06
	II	+	15.38	16.4	15.1	17.06	15.12	16	17.16	18.82
NPC	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
NTC	I	-	-	-	-	-	-	-	-	-
	II	-	-	-	-	-	-	-	-	-
PAC	I	+	Missing	22.75	14.7	20.24	13.52	17	Missing	20.05
	II	+	Missing	22.99	14.8	20.26	13.75	17	Missing	20.25
PPC	I	+	18.07	18.02	16.04	18.63	16.07	18	18.73	21.07
	II	+	18.03	17.97	16.17	18.49	16.56	18	18.58	20.45

NPC: Negative Process Control; NTC: Negative Template Control; PAC: Positive Amplification Control; PPC: Positive Process Control ; - = Negative; + = Positive

Table C2. Cq values for Wu Taqman Assay for each laboratory

Isolate	Replicate	Expected result	Laboratory							
			A	B	C	D	E	F	G	H
1	I	-	15.35	18.09	17.18	17.29	15.58	19	18.54	19.76
	II	-	15.66	18.16	16.84	17.08	16.00	19	18.65	19.96
2	I	-	16.74	20.24	16.98	17.59	16.88	19	18.07	20.64
	II	-	16.61	20.09	16.52	17.48	16.43	19	18.17	20.48
3	I	-	13.97	36.24	13.8	-	21.03	-	-	-
	II	-	13.8	35	13.31	-	20.84	-	-	-
4	I	-	19.4	15.9	15.09	14.86	13.10	17	15.87	17.29
	II	-	19.63	15.94	14.5	14.55	13.66	17	15.91	17.66
5	I	-	14	16.21	14.6	15.7	14.35	17	15.55	20.69
	II	-	14.24	16.09	14.05	15.61	13.87	17	15.95	20.76
6	I	-	17.43	13.89	13.23	13.26	12.34	16	14.98	15.44
	II	-	16.87	13.24	12.84	13.26	12.04	15	14.99	15.42
7	I	-	17.01	15.66	12.88	12.74	11.89	15	13.84	15.1
	II	-	13.76	15.33	12.29	12.79	11.79	14	13.96	15.1
8	I	-	15.24	17.23	15.66	15.69	12.93	18	17.13	18.86
	II	-	16.49	17.18	14.88	15.59	13.57	18	17.05	19.11
9	I	-	17.02	16.08	14.88	14.7	13.23	18	15.32	17.81
	II	-	16.76	16.08	14.52	14.46	13.72	17	15.93	17.84
10	I	-	15	18.18	15.36	14.84	13.68	17	16.20	17.49
	II	-	15.29	18.05	14.62	14.9	14.07	17	16.33	17.49
11	I	-	15.7	27.84	21.98	23.16	21.47	30	27.11	27.16
	II	-	14.54	27.67	21.46	23.48	20.90	30	27.06	27.01
12	I	-	15.58	16.04	15.08	15.49	13.61	17	16.17	17.33
	II	-	15.79	15.86	14.55	15.47	14.06	17	16.38	16.94
13	I	-	13.89	18.54	16.6	16.54	15.80	19	17.96	19.55
	II	-	18.06	18.49	16.18	16.45	15.73	19	18.05	19.75
14	I	-	15.89	17.35	16.48	16.4	15.76	18	17.58	17.81
	II	-	16.12	17.41	15.8	16.3	15.25	18	17.66	17.99
15	I	-	15.61	18.06	15.66	15.61	12.64	18	17.10	19.98
	II	-	17.73	18.01	15.49	15.24	15.08	18	17.04	19.79
16	I	-	15.83	18.6	16.35	15.8	13.26	19	17.49	20.89
	II	-	15.06	18.53	15.81	15.72	14.95	19	17.35	20.51
17	I	-	15.12	16.66	15.28	15.43	13.83	17	16.21	17.33
	II	-	14.98	16.69	14.73	15.26	14.08	17	16.25	17.37
18	I	-	13.68	17.73	16.35	16.09	15.81	18	17.00	18.09
	II	-	19.1	17.87	15.7	16.05	12.62	18	17.05	18.41
19	I	-	17.69	21.46	15.93	15.9	13.31	19	17.89	20.45
	II	-	17.54	21.48	15.61	15.69	15.05	19	17.65	19.63
20	I	-	15.56	15.54	13.73	14.51	11.48	16	15.21	17.19
	II	-	16.28	14.85	13.1	14.41	12.97	16	15.18	17.26

Isolate	Replicate	Expected result	Laboratory							
			A	B	C	D	E	F	G	H
21	I	-	16.48	16.26	14.66	15.58	14.38	18	17.94	22.74
	II	-	16.28	15.85	14.35	15.43	15.02	18	17.43	22.86
22	I	-	19.38	15.75	14.44	15.04	14.09	16	15.64	16.75
	II	-	19.14	15.32	14.13	14.76	13.78	16	15.70	16.8
23	I	-	16.52	18.15	16.02	16.16	16.26	15	17.44	17.86
	II	-	15.76	17.35	15.53	16.03	15.56	18	17.65	18.05
24	I	-	14.8	15.6	14.36	16.76	14.01	17	16.24	18.86
	II	-	15	14.78	14.19	16.94	13.44	17	16.36	19.05
25	I	-	17.64	20.96	19.29	19.94	18.78	21	21.20	22.13
	II	-	17.3	20.8	18.69	20.05	18.09	21	20.92	21.61
26	I	-	16.69	22.45	19.07	18.55	18.48	22	20.64	23.18
	II	-	16.64	22.09	18.7	18.28	18.75	22	20.43	23.31
27	I	-	19.45	18.24	16.3	16.57	15.93	18	17.41	19.18
	II	-	13.91	18.18	15.79	16.58	15.37	18	17.28	18.98
28	I	-	14.49	19.37	17.31	17.3	16.77	19	18.23	18.18
	II	-	13.84	19.25	16.36	17.07	15.97	19	18.32	18.19
29	I	-	15.68	19.8	17.75	17.23	17.29	20	18.39	20.16
	II	-	16.3	19.72	17.47	17.26	17.03	19	18.38	19.51
30	I	-	16.46	22.46	20.14	20.36	18.08	23	21.76	25.7
	II	-	16	22.29	19.76	20.05	18.65	23	21.86	25.5
31	I	+	14.96	20.3	18.47	18.2	15.45	21	19.97	21.17
	II	+	15.74	20.18	17.95	18.27	17.17	21	20.09	20.7
32	I	+	24.49	20.52	18.74	19.15	18.00	21	19.59	22.4
	II	+	23.52	20.44	18.22	18.82	18.15	21	19.89	22.26
33	I	+	19.1	17.21	15.99	16.22	16.54	18	17.56	19.07
	II	+	14.68	17.27	15.43	15.99	15.91	18	17.71	18.94
34	I	+	14.09	19.81	17.99	18.06	18.22	20	Tube lost	21.96
	II	+	13.73	19.69	17.73	18	17.52	20	Tube lost	21.64
35	I	+	17.16	18.47	17.39	16.98	14.47	20	18.18	18.7
	II	+	16.92	18.34	16.7	16.81	14.84	19	18.10	18.69
36	I	+	14.18	19.6	18.41	19.08	17.26	21	19.75	22.59
	II	+	14.28	19.43	17.82	18.38	15.40	21	19.77	22.69
37	I	+	19.77	15.16	13.58	13.53	12.27	34	18.77	-
	II	+	19.06	15.06	13.35	13.63	12.13	32	18.98	-
38	I	+	15.97	14.55	13.01	13.24	12.21	15	13.98	15.46
	II	+	15.89	14.79	12.69	13.19	11.62	15	13.95	15.55
39	I	+	15.56	31.35	13.7	-	31.92	-	-	-
	II	+	15.34	34.23	13.49	-	33.91	-	-	-
40	I	+	17.29	15.36	14.07	14.09	11.97	16	14.61	28.79
	II	+	16.73	15.06	13.59	13.68	12.41	16	14.90	29.51
41	I	+	14.58	33.51	15.44	38.33	21.70	-	-	-
	II	+	15.59	34.62	15.31	38.03	26.31	-	-	-

Isolate	Replicate	Expected result	Laboratory							
			A	B	C	D	E	F	G	H
42	I	+	15.92	14.96	13.33	13.52	12.16	15	14.13	16.06
	II	+	15.62	14.79	12.56	13.49	12.00	15	14.23	16.59
43	I	+	20.79	23.1	14.26	27.56	18.98	-	-	-
	II	+	20.48	24.79	13.85	27.16	18.17	-	-	-
44	I	+	14.14	17.25	15.07	15.55	14.38	17	16.20	17.89
	II	+	16.87	17.18	14.53	15.56	14.75	17	16.54	17.69
45	I	+	15.71	18.22	16.08	16.03	16.49	18	17.32	19.84
	II	+	15.43	18.14	15.43	15.88	15.15	18	17.42	19.69
46	I	+	17.48	18.72	15.2	17.53	18.16	21	18.26	-
	II	+	17.14	18.66	14.69	17.67	18.45	24	18.07	-
47	I	+	14.39	16.63	15.3	15.31	14.68	18	16.90	18.51
	II	+	13.99	16.54	14.59	15.27	14.05	18	16.82	18.69
48	I	+	18.27	16.52	15.19	16	14.47	18	16.74	18.19
	II	+	18.53	16.65	14.76	15.75	14.08	17	16.79	18.07
49	I	+	15.88	17.55	15.64	15.93	14.16	18	17.18	18.92
	II	+	15.87	17.63	15.3	16	14.28	18	17.36	18.87
50	I	+	15.37	26.22	18.06	19.31	17.78	>35	24.13	-
	II	+	15.52	26.29	17.22	19.16	18.04	32	24.38	-
51	I	+	14.07	17	14.82	17.12	16.22	19	17.70	21.14
	II	+	14.17	16.52	14.29	17.12	16.47	19	17.68	21.16
52	I	+	18.03	16.48	15.43	15.39	15.02	17	16.46	17.02
	II	+	17.89	16.38	14.79	15.5	14.49	17	16.35	16.93
53	I	+	16.26	16.63	15.37	15.02	13.22	18	16.71	18.4
	II	+	16.24	16.38	14.75	14.94	14.09	18	16.79	18.47
54	I	+	16.22	22.63	16.19	18.42	18.55	22	21.41	31.92
	II	+	16.21	22.76	15.77	18.43	18.49	22	21.17	31.31
55	I	+	19.22	37.36	-	-	33.36	-	-	-
	II	+	16.22	39.38	-	-	-	-	-	-
56	I	+	17.11	26.2	18.36	22.05	22.30	-	29.57	-
	II	+	16.94	24.23	17.35	22.07	21.31	-	29.94	-
57	I	+	16.37	17.49	15.57	15.19	13.69	18	17.00	19.44
	II	+	22.86	17.4	14.93	15.17	14.37	18	16.90	19.75
58	I	+	16.33	17.27	16.07	18.63	15.18	20	17.13	26.34
	II	+	15.52	17.08	15.13	18.55	15.63	20	17.23	25.04
59	I	+	19.86	17.9	15.54	15.81	15.06	18	17.51	17.47
	II	+	19.26	17.91	15.18	15.94	15.01	18	17.53	19.01
60	I	+	16.93	16.69	15.55	15.74	14.66	18	16.67	18.8
	II	+	16.34	16.74	15.25	15.79	14.37	18	16.88	18.71
NPC	I	-	23.4	16.34	21.77	22.66	20.45	24	23.55	27.59
	II	-	16.25	16.5	21.59	22.59	19.89	24	23.90	27.48
NTC	I	-	25.8	35.87	30.73	34.28	21.33	18	34.11	34
	II	-	25.13	37.16	30.42	34.55	21.12	19	34.05	33.22

Isolate	Replicate	Expected result	Laboratory							
			A	B	C	D	E	F	G	H
PAC	I	+	Missing	23.11	15.23	20.28	14.50	18	Missing	19.02
	II	+	Missing	23.33	15.11	20.27	13.80	19	Missing	19.36
PPC	I	+	18.07	18.46	17.03	17.31	16.14	19	18.35	20.22
	II	+	20.44	18.49	16.51	17.29	15.69	19	18.26	20.2

NPC: Negative Process Control; NTC: Negative Template Control; PAC: Positive Amplification Control; PPC: Positive Process Control; - = Negative; + = Positive.

APPENDIX D: References

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