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Seed Extract and qPCR Method for the Detection of *Acidovorax citrulli* on Cucurbit Seeds

Validation report, February 2017

ISHI International Technical Group Cucurbits - K. Kleinhesselink, Monsanto, USA

ISHI VALIDATION REPORTS

This ISHI validation study has been conducted to determine the fitness of the described method for its intended purpose according to common practices in effect at the time.

DISCLAIMER

ISF cannot guarantee that laboratories following the protocol described herewith will obtain similar results. Many factors, such as staff skills, laboratory equipment and conditions, reagents and sampling methods can influence the results. Consequently, in case of any litigation ISF will not accept any liability on the use of these tests.

Published by:

International Seed Federation (ISF)

Reposoir 7, 1260 Nyon, Switzerland

Developed by ISHI

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Proposal for a Seed Extract qPCR Method for the Detection of *Acidovorax citrulli* on Cucurbit Seeds

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INTRODUCTION

Acidovorax citrulli (Ac), a seed-borne bacterial pathogen, is the causal agent of bacterial fruit blotch (BFB) and has emerged as a threat to cucurbit production. Strains of Ac can infect several species in the family of Cucurbitaceae, including watermelon, melon, cucumber, squash and pumpkin, and significant losses have been reported in melon and watermelon. Plants infected with Ac may show various symptoms in different plant species and typically start with irregular water-soaked lesions on cotyledon leaves, and turn into necrotic lesions, which can extend into stem or true leaves, in severe cases, seedling collapse and die. As the disease progress, symptoms on fruits include water-soaking lesions, cracking in the rind and internal rotting flesh resulting fruit rot. The infection severity is depending on several factors, such as the amount of inoculum, virulence level of the strains, environmental conditions and host plants. The level of infection can range from severe to very mild or asymptomatic, and that may escape from pathogen detection via visualization in grow-out assays. Since 1990s, BFB has been rapidly spread globally and to date, BFB outbreaks have been reported worldwide, including the Americas, Asia, Africa, Australia, Europe, the Middle and Far East (Burdman and Walcott, 2012). Contaminated seeds were known as primary inoculum source to cause widespread of BFB and recently, symptomatic cotyledons were identified as the main source for secondary spread in nurseries (Chalupowicz et al., 2015). Since there are no commercial cultivars with resistance to BFB, the effective management for BFB requires a range of approaches, including exclusion with zero tolerance for Ac in cucurbit seeds. Thus, the seed health testing with robust and effective detection techniques is critical for disease management.

Currently, one of the standard methods with approval from USDA National Seed Health System for detecting Ac is seedling grow-out assays, which is also widely used within seed industry for seed health testing. The method requires 10,000-30,000 seeds per seed lot to be planted in sanitized greenhouse under controlled conditions for symptom development. These seedlings are maintained and visually inspected for BFB-like symptoms continuously for up to 21 days after planting. Suspected seedlings are collected and followed by PCR assays to confirm the presence of Ac. Subsequently, isolation of Ac from the PCR-positive seedlings and following plant inoculation with recovered isolates are necessary to fulfill Koch's postulates. The detection method is simple to conduct and is capable of determining viability and virulence of the Ac isolates. However, this method features lower specificity due to the potential complications with other biotic or abiotic induced symptoms and false negative results from very mild or asymptomatic infections, which could lead to BFB occurrence later in the field with favorable conditions. In addition, the bioassay method is labor, time and space-intensive, and requires knowledge and experience to recognize the characteristics of signs and disease symptoms for routine testing application.

With the increasing demands in cucurbit seed production and zero tolerance of Ac presence in seeds, the need of a rapid, sensitive and robust detection method for Ac testing is highly desired. Techniques with advanced PCR development have become preferred tools to detect and identify plant pathogenic bacteria and offer many advantages over grow-out bioassays and serological methods. Efforts to develop molecular methods to detect *Ac* from seed samples directly have been undergone across several private sectors and associations. Since then, multiple testing methods have been developed and with an effort driven by the International Seed Health Initiative Vegetable (ISHI-Veg) group, seed companies gathered from around the world to work in partnership in the development of a standardized direct qPCR method for the detection of Ac. Prior to method validation a comparison of existing methods was completed and key steps harmonized based on comparative testing and other considerations (Annex C).

OBJECTIVE

To align and optimize the current National Seed Health System (NSHS) accredited *Ac* direct qPCR methods and generate a standardized ISHI reference method by defining the intended purpose of assay, performance parameters and method acceptance criteria, in collaboration with industry partners. The optimized method will be validated against the defined acceptance criteria and submitted to NSHS for accreditation as a performance based method.

INTENDED PURPOSE OF ASSAY

To determine the presence of *Acidovorax citrulli* (*Ac*) DNA in cucurbit seed by a method consisting of bacterial extraction, DNA isolation and detection by qPCR assays.

Ac Method Validation Performance Targets

Performance Parameter		Acceptance Criteria
Sensitivity	**Limit of Detection (LOD)**	Any detection of *Ac* at the 5K subsample level where subsamples contain an *Ac* spike equal to or less than the LOD of NSHS method Cb1.4 (337 CFU]
	Limit of Quantification (LOQ)	Detection of *Ac* across all PCR replicates within a 5K subsample level where subsamples contain an *Ac* spike one order of magnitude greater than the LOD of the NSHS method Cb1.4 (3,370 CFU).
Specificity	**Inclusivity**	Two qPCR assays, each are inclusive of 100% of *Ac* isolates tested. No false negative result is obtained.
	Exclusivity	Two qPCR assays with a primary consideration for inclusivity that detect (i.e., cross react with) a minimal number of non-*Ac* isolates. Tolerance of false positive results is determined by application / risk assessment.
	Selectivity	All qPCR assays detect genomic DNA within their respective operational ranges with a mean amplification efficiency between 90-100%, R^2=0.99 in the presence of a representative level of interferences.
Accuracy	**Trueness: Bias**	Characterize a reference standard and evaluate the level of bias across various cucurbit crops.
	Precision: Repeatability	Under repeatable conditions, standard deviation (σ) of the reference standard in all qPCR assays should be within σ = 0.37 Ct.
	Precision: Reproducibility	Under reproducible conditions, standard deviation (σ) of the reference standard in all qPCR assays should be within σ = 1.11 Ct and for the total method with a coefficient of variance (CV) < 3%.
Robustness		Comparable detection of *Ac* is observed with variations to the process including but not limited to DNA isolation kits, PCR chemistries and qPCR instruments. Method deviations should be recorded and method performance evaluated against the acceptance criteria above.

METHOD VALIDATION

Sensitivity

Method sensitivity was determined by evaluating the limit of detection (LOD) and limit of quantification (LOQ). These parameters were assessed at two separate levels. First, the sensitivity of the qPCR assays was examined using standardized reference DNA isolated from strains of *Acidovorax citrulli (Ac)* and *Xanthomonas vesicatoria (Xv)*. Following the assessment of the qPCR assays, the sensitivity of the total method was evaluated, using a standardized spike of *Ac* and *Xv* cells into a variety of cucurbit samples.

qPCR Assay Sensitivity

Evaluate qPCR assay LOD and LOQ using standardized reference DNA.

Experiment Method:

DNA was isolated from *Ac* and *Xv* bacterial isolates using the Qiagen DNeasy Blood & Tissue Kit (with RNase A treatment) according to manufacturer's instructions. The DNA concentration was then measured with a Qubit fluorometer, using the dsDNA BR Assay Kit following manufacturer's instructions and each sample was standardized to 4ng/µL. The 4ng/µL standardized DNA solutions were then combined in equal parts to create a stock solution of 2ng/µL *Ac* and 2ng/ µL *Xv*, respectively. The mixed DNA stock solution was then serially diluted, resulting in DNA templates ranging from 200pg/µL to 2fg/µL. For each, 5µL of the DNA dilution was used as a template for singleplex qPCR reactions using the Contig 21, Contig 22 and *Xv* qPCR assays. Assays were run in triplicate.

Results:

Total Target DNA	Contig 21	Contig 22	*Xv*
1ng	18.761	18.701	18.133
	18.647	18.618	18.093
	18.705	18.648	17.989
Ct Mean	18.704	18.656	18.072
Ct SD	0.057	0.042	0.074
100pg	22.242	22.159	22.775
	22.384	21.971	22.835
	22.323	21.982	22.943
Ct Mean	22.316	22.037	22.851
Ct SD	0.071	0.106	0.085
10pg	25.728	25.451	26.944
	25.313	25.850	27.488
	25.760	25.485	27.162
Ct Mean	25.600	25.595	27.198
Ct SD	0.249	0.221	0.274
1pg	29.440	28.934	30.736
	30.300	28.817	30.810
	29.000	29.172	30.645
Ct Mean	29.580	28.974	30.730
Ct SD	0.661	0.181	0.083
100fg	31.968	32.816	33.347
	32.546	32.369	34.976
	32.837	32.606	33.651
Ct Mean	32.450	32.597	33.991
Ct SD	0.442	0.224	0.866
10fg	35.774	35.883	-
	34.253	34.998	-
	36.132	35.854	34.19
Ct Mean	35.386	35.578	N/A
Ct SD	0.998	0.503	N/A

Table 1: Sensitivity of each qPCR assay.

The MIQE for guideline for qPCR assays guideline states:

> "Typically, sensitivity is expressed as the *limit of detection* (LOD), which is the concentration that can be detected with reasonable certainty (95% probability is commonly used) with a given analytical procedure. The most sensitive LOD theoretically possible is 3 copies per PCR *(28)*, assuming a Poisson distribution, a 95% chance of including at least 1 copy in the PCR, and single-copy detection" (Bustin, 2009).

In this experiment Contig 21, Contig 22, and *Xv* qPCR assays detected the corresponding targets at DNA concentrations as low as 10fg. This indicates that both the LOD and LOQ of the *Ac* qPCR assays are equal to or less than 10fg of DNA. To further understand the biological relevance of detection at a target DNA concentration of 10fg, the copy number of *Ac* genome present was calculated based on the genome size of 5,352,772 bp, using the New England BioLabs NEBioCalculator (http://nebiocalculator.neb.com/#!/dsdnaamt). Based on these calculations, 10fg of genomic DNA is equivalent to 1.82 copies of *Ac* (1 *Ac* genome copy = 5.49fg DNA). Genome copy number of *Xv* in 10fg DNA was calculated using the calculator above based on the genome size of 5,178,450 bp, equating to 1.88 copies per 10fg (1 *Xv* genome copy = 5.31fg). The results indicate that the above qPCR assays are able to detect their target DNA consistently at very low levels.

The positive amplification control (PAC), otherwise known as a qPCR positive control, is required at a concentration that shows consistent performance and is equal to or greater than LOQ of the assays. Therefore, 10pg of purified target DNA was selected as a standardized reference for use as the PAC in the qPCR assays.

Ac direct qPCR method Sensitivity

Experiment I:

Evaluate the sensitivity of the method (i.e., the complete procedure including seed wash, DNA isolation, and qPCR testing) for detection of *Ac* and *Xv* in various cucurbit seed samples. Determine the appropriate spike concentrations for the positive process control (PPC) and the positive extraction control (PEC).

Experiment Method:

A stock cell suspension of *Ac* was prepared in PBS buffer from an overnight incubation of *Ac* inoculated nutrient broth. *Ac* concentration was adjusted with a spectrophotometer to an absorbance of OD_{600}=0.100. A stock cell suspension of *Xv* was prepared by suspending cells from a culture grown on YDC media in PBS buffer; this suspension was also adjusted to an absorbance of OD_{600}=0.100. Ten-fold dilution series of the stock cell suspensions were performed to generate spike solutions ranging from $OD_{600}=0.100\times10^{-1}$ - 0.100×10^{-7} for *Ac* and *Xv*.

Seed wash samples were prepared by obtaining melon, watermelon and squash seeds known to be free of *Ac* and *Xv*. Seven samples, each containing 5,000 seeds, were prepared per crop. Buffer was added to each seed sample using at a ratio of 2mL buffer per 1g seed. For each bacterial concentration produced above, one seed sample was spiked with 1mL of the diluted suspension of each pathogen per 250mL of seed wash buffer. This resulted in the generation of 7 seed samples spiked with equivalent *Ac* and *Xv* suspensions ranging from $OD_{600}=0.100\times10^{-1}$ - 0.100×10^{-7}. DNA was isolated from a single 45mL aliquot per sample and tested by Contig 21, Contig 22, and *Xv* qPCR assays in duplicate following the '*Ac* direct qPCR method' (Annex D).

Results:

	Contig 21			Contig 22			*Xv*		
Concentration of spike solutions	Melon	Watermelon	Squash	Melon	Watermelon	Squash	Melon	Watermelon	Squash
$OD_{600}=0.100x10^{-1}$	18.810	18.515	20.007	18.940	19.149	20.567	19.264	18.315	18.838
	18.612	18.354	20.195	18.772	19.135	20.536	19.241	18.136	18.895
$OD_{600}=0.100x10^{-2}$	22.514	21.776	23.633	22.306	22.458	23.968	22.686	21.893	22.383
	22.298	21.898	23.601	22.493	22.547	23.926	22.665	21.964	22.424
$OD_{600}=0.100x10^{-3}$	25.467	24.834	27.418	25.574	25.556	27.936	25.849	25.154	26.390
	25.503	24.783	27.215	25.547	25.677	27.547	25.907	24.914	26.465
$OD_{600}=0.100x10^{-4}$	29.790	29.452	29.954	29.736	29.924	31.022	30.061	28.401	29.575
	29.534	28.869	30.501	29.596	29.611	30.988	30.069	28.464	29.594
$OD_{600}=0.100x10^{-5}$	32.829	31.728	34.707	32.904	32.194	33.949	34.518	32.000	32.571
	32.562	31.798	33.688	33.175	32.302	-	32.829	30.826	32.602
$OD_{600}=0.100x10^{-6}$	34.808	-	-	35.687	-	-	-	33.920	34.299
	-	35.880	-	34.926	34.905	36.096	-	32.495	34.486
$OD_{600}=0.100x10^{-7}$	-	-	-	-	-	-	-	34.535	34.724
	-	-	-	-	-	-	-	33.376	34.993

Table 2: The sensitivity of '*Ac* direct qPCR method' across various cucurbit crop species

For the method, *Ac* detection is defined as a positive result generated by one or both of the *Ac* qPCR assays (Contig 21 and Contig 22). The LOQ of the method for the detection of *Ac* was shown to be at a concentration of $OD_{600} = 0.100x10^{-5}$ in a sample of 5,000 seeds across the crop species evaluated. The LOD of the method for the detection of *Ac* was shown to be at a concentration of $OD_{600} = 0.100x10^{-6}$ in a sample of 5,000 seeds across the crop species evaluated. It appears that crop species may have a slight affect on *Ac* detection as evaluated by comparing relative Ct values across spike dilutions and crop species; however, the method sensitivity was not different between species when samples are analyzed as described in the method.

There appears to be a crop species affect on the LOD of the method for the detection of *Xv*. For melon, the LOD is at a concentration of $OD_{600} = 0.100x10^{-5}$ in a sample of 5,000 seeds. For watermelon and squash, the LOD is $OD_{600} = 0.100x10^{-7}$ in a sample of 5,000 seeds. Given that *Xv* will be used as a PEC in a fixed concentration, this issue will be resolved by selecting a spike concentration that results in consistent detection irrespective of crop species.

This experiment also demonstrated that the addition of *Xv* as a PEC does not interfere with the ability of the method to detect *Ac*, and supports the process control spike concentrations at $OD_{600} = 0.100x10^{-3}$ for both *Ac* (PPC) and *Xv* (PEC) across the crop species tested.

Experiment II:

Evaluate the LOQ and LOD of the method (complete procedure including seed wash, DNA isolation, and qPCR testing) for the detection of *Ac* in a reference seed sample. Note that the PEC (*Xv)* is not examined for sensitivity, as it is used at a known and fixed concentration greater than the LOQ of the Xv assay using this method.

Experiment Method:

Four replicate melon samples, containing 5,000 seeds each, from a seed lot known to be free of *Ac* used in this experiment. A stock cell suspension of *Ac* corresponding to $OD_{600}=0.100$ was prepared then serially diluted to generate spike solutions ranging from $OD_{600}=0.100\times10^{-1}$ - 0.100×10^{-6}. For spike concentrations $OD_{600}=0.100\times10^{-3}$ - 0.100×10^{-6}, 1mL per 250mL extraction buffer was spiked into a corresponding 5,000 seed sample and processed as described in the '*Ac* direct qPCR method' (Annex D). DNA was isolated from three-45mL replicate aliquots from each sample and tested by Contig 21 and Contig 22 qPCR assays in triplicate.

Results:

5,000 Seed Sample	Concentration of *Ac* Spike Solution	Aliquot (45mL)	Contig 21	Contig 22	*Ac* Detected?
1	$OD_{600}=0.100x10^{-3}$	A	26.132	25.692	Yes
			25.864	25.908	
			25.903	26.038	
		B	25.889	26.201	Yes
			26.353	26.001	
			26.015	26.227	
		C	25.821	25.989	Yes
			25.948	26.127	
			25.997	26.200	
		Ct Mean	25.991	26.043	-
		Ct σ	0.164	0.172	
2	$OD_{600}=0.100x10^{-4}$	A	29.664	29.262	Yes
			29.650	29.789	
			29.320	29.442	
		B	29.289	29.664	Yes
			29.131	29.299	
			29.320	29.622	
		C	28.841	29.499	Yes
			29.292	29.141	
			29.485	29.411	
		Ct Mean	29.332	29.459	-
		Ct σ	0.255	0.208	
3	$OD_{600}=0.100x10^{-5}$	A	32.562	32.665	Yes
			33.284	33.102	
			32.555	33.694	
		B	32.609	32.666	Yes
			33.384	32.467	
			31.930	32.138	
		C	32.446	33.548	Yes
			32.446	33.241	
			33.177	33.557	
		Ct Mean	32.710	33.009	-
		Ct σ	0.475	0.549	
4	$OD_{600}=0.100x10^{-6}$	A	35.442	-	Yes
			-	37.870	
			-	-	
		B	-	35.954	Yes
			35.658	35.726	
			-	-	
		C	-	36.302	Yes
			36.209	36.298	
			-	35.814	
		Ct Mean	35.770	36.327	-
		Ct σ	0.395	0.793	

Table 3: The sensitivity of *Ac* direct qPCR method on reference seed material

The lowest concentration of *Ac* that showed consistent detection in every qPCR replicate under repeatable conditions (performed within one lab on one seed sample per concentration with the same reagents on the same day and by the same technician) by both Contig 21 and Contig 22 assays was at a spike concentration of $OD_{600}=0.100x10^{-5}$. This preliminary LOQ will be confirmed by evaluating detection of this *Ac* spike concentration under reproducible (multiple seed samples, reagent sets, days, etc.) conditions.

Given that at least one of the triplicate PCR reactions produced a Ct value by Contig 21 or Contig 22 assays, the lowest concentration of *Ac* that was detected under repeatable conditions was at a spike concentration of $OD_{600} = 0.100x10^{-6}$. This preliminary LOD will be confirmed by evaluating detection of this this *Ac* spike concentration under reproducible conditions.

Experiment III:

Evaluate the LOD and LOQ of the method (complete procedure including seed wash, DNA isolation, and qPCR testing) for detection of *Ac* under reproducible conditions (4 technicians, 2 dates, different reagent sets).

Experiment Method:

Twelve replicate samples of 5,000 seeds from a melon seed lot known to be free of *Ac* and *Xv* were used in this experiment. Stock cell suspensions for *Ac* and *Xv* were prepared and each was adjusted to an absorbance of OD_{600}=0.100. The stock *Ac* cell suspension was serially diluted to generate spike solutions ranging from $OD_{600}=0.100\times10^{-1}$ - 0.100×10^{-6}. 250mL of buffer was added to seed samples and spiked with 1mL of the *Ac* suspensions of $OD_{600}=0.100x10^{-3}$, $x10^{-5}$ and $x10^{-6}$ producing 4 replicate samples at each concentration The stock *Xv* cell suspension was serially diluted to generate spike solutions ranging from $OD_{600}=0.100\times10^{-1}$ - 0.100×10^{-3} Then 1mL of *Xv* $OD_{600}=0.100x10^{-3}$ was spiked into each sample as a PEC. Replicate spiked samples were given to different technicians who processed the samples as described in the '*Ac* direct qPCR method' Annex D) with the exception that two-45mL aliquots were analyzed instead of one Extracted DNA was tested by Contig 21, Contig 22 and *Xv* qPCR assays in duplicate. The above process was repeated on a subsequent day using fresh preparations of cell suspensions and different lots of reagents for DNA isolation.

Results:

Concentration of *Ac* spike solution	Concentration of *Xv* spike solution	Technician	Aliquot	Day 1			Day 2		
				Contig 21	Contig 22	*Xv*	Contig 21	Contig 22	*Xv*
$OD_{600}=0.100x10^{-3}$	$OD_{600}=0.100x10^{-3}$	A	1	25.506	26.090	26.645	25.451	25.300	26.722
				25.393	25.630	26.819	25.322	25.413	26.668
			2	25.228	25.754	26.621	25.524	25.506	26.856
				25.000	25.647	26.410	25.556	25.599	26.925
		B	1	25.150	25.885	26.680	25.246	25.449	26.854
				25.007	25.650	26.695	25.306	25.405	26.851
			2	25.204	25.877	26.587	25.309	25.335	27.202
				25.145	25.781	26.547	25.179	25.368	27.235
		C	1	25.570	25.420	26.531	25.449	25.520	26.931
				25.266	25.506	26.571	25.433	25.338	26.886
			2	25.245	25.640	26.315	25.479	25.637	26.878
				25.195	25.837	26.345	25.455	25.508	26.977
		D	1	25.539	25.640	26.579	24.660	25.563	26.662
				25.377	25.680	26.506	25.444	25.435	26.637
			2	25.227	25.700	26.594	25.182	25.451	26.809
				25.214	25.627	26.724	25.299	25.455	26.866
		CT Mean		25.267	25.710	26.573	25.331	25.455	26.872
		CT σ		0.170	0.160	0.134	0.213	0.096	0.169
$OD_{600}=0.100x10^{-5}$	$OD_{600}=0.100x10^{-3}$	A	1	31.898	32.801	26.630	32.521	32.258	26.547
				31.560	32.963	26.765	31.671	32.279	26.630
			2	31.838	32.755	26.666	33.676	32.179	26.556
				31.657	32.295	26.586	32.512	32.497	26.593
		B	1	32.790	32.626	26.724	32.431	31.576	26.823
				32.214	33.249	26.495	31.958	32.187	26.944
			2	32.010	32.660	26.642	31.967	31.717	26.873
				31labora.801	32.677	26.627	32.390	31.714	26.870
		C	1	31.717	32.261	26.911	32.706	32.824	26.742
				31.860	32.787	26.835	32.385	32.505	26.286
			2	32.614	31.756	26.904	32.114	31.942	26.975
				31.896	31.739	26.735	32.417	31.801	26.984
		D	1	31.722	32.418	27.183	32.952	32.772	26.907
				32.640	33.787	26.961	31.670	32.160	26.784
			2	31.959	32.834	27.049	32.146	32.277	26.663
				32.009	33.352	26.834	32.753	32.250	26.857
		CT Mean		32.012	32.685	26.784	32.392	32.184	26.752
		CT σ		0.368	0.534	0.184	0.501	0.365	0.193
$OD_{600}=0.100x10^{-6}$	$OD_{600}=0.100x10^{-3}$	A	1	34.474	36.323	26.979	34.555	36.352	26.884
				35.974	37.975	26.639	35.899	35.835	26.809
			2	34.877	34.673	26.876	35.708	34.239	26.906
				34.297	35.442	26.751	-	34.850	26.968
		B	1	35.753	35.168	26.885	35.521	-	27.107
				34.586	35.926	27.009	34.417	35.661	26.878
			2	34.835	37.666	26.825	39.483	34.166	27.095
				34.300	34.389	26.671	35.802	34.916	26.945
		C	1	-	36.436	27.114	36.244	34.967	27.129
				34.548	36.214	26.892	-	34.834	26.663
			2	34.341	36.402	27.132	-	34.811	27.113
				34.598	35.149	27.007	34.726	34.815	26.864
		D	1	35.827	34.223	26.988	36.012	36.057	27.035
				-	34.768	26.848	35.852	36.158	26.758
			2	34.920	35.394	26.862	35.048	36.575	26.818
				-	35.379	26.887	-	36.107	26.893
		CT Mean		34.872	35.720	26.898	35.901	35.370	26.942
		CT σ		0.596	1.078	0.139	1.476	0.768	0.155

Table 4: Sensitivity of *Ac* direct qPCR method on reference seed material under reproducible conditions

Similar to the results for repeatability, the lowest concentration of *Ac* that showed consistent detection in every qPCR replicate under reproducible conditions by both Contig 21 and Contig 22 assays was at a spike concentration of $OD_{600}=0.100x10^{-5}$. The lowest concentration of *Ac* that showed consistent detection in each DNA isolation under reproducible conditions by both Contig 21 and Contig 22 assays was at a spike concentration of $OD_{600} = 0.100x10^{-6}$. Based on historical data, generated by routine plating of the PPC in the NSHS Cb1.4 method for the detection of *Ac*, 200 µL of an *Ac* $OD_{600}=0.100x10^{-6}$ bacterial suspension contains an average of 47 CFU (Annex A). Therefore, 1mL of an *Ac* $OD_{600}=0.100x10^{-6}$ suspension contains approximately 237 CFU.

Method Sensitivity Conclusions

The lowest spike concentration of *Ac* that resulted in consistent detection in every qPCR replicate for the total method under reproducible conditions by the Contig 21 and Contig 22 assays was $OD_{600}=0.100x10^{-5}$. This concentration corresponds to a pathogen load of 2,370 CFU *Ac* per 5,000 seed. This value represents the LOQ of the method.

The lowest concentration of *Ac* that resulted in detection from each 5,000 seed sample (i.e., at least one PCR reaction is positive) under reproducible conditions by both Contig 21 or Contig 22 assays was at a spike concentration of $OD_{600} = 0.100x10^{-6}$, which corresponds to a pathogen load of 237 CFU *Ac*.

These results demonstrate that the LOD of this method is 237 CFU *Ac* per 5,000 seeds, which is comparable to the sensitivity indicated in the validation of the NSHS Cb1.4 method for the detection of *Ac* (LOD=337 CFU per 5,000 seeds).

Specificity

Method specificity was determined by evaluating the inclusivity, exclusivity and selectivity of the assay.

Inclusivity/Exclusivity

Experiment Method:

The vegetable seed industry undertook a collaborative effort to determine acceptable qPCR assays for the detection of *Ac*. Monsanto prepared a standardized batch of qPCR reagents including mastermix, molecular grade nuclease-free water, and the primer-probe sets for Contig 21, Contig 22, and ZUP (*Ac*) qPCR assays. The reagents were distributed to participating labs of Bayer Crop Science, HM-Clause, Rijk Zwaan, and Syngenta. Five laboratories in total participated in the isolate library screening; each company evaluated the provided qPCR assays against their respective *Ac* and select non-*Ac* bacterial isolate collections. Reference Annex E for qPCR reaction preparation and cycling parameters.

Results:

	Total Isolates Tested	Detected by Contig 21	Detected by Contig 22	Detected by ZUP (Ac)
Ac Isolates	517	517	517	517
Ac related and non-*Ac*	52	0	2	18

Table 6: *Ac* isolate collection screening summary. Total data set is presented in Annex A, Section 1.

The three qPCR assays evaluated accurately detected 100% of the *Ac* isolates evaluated across all participating laboratories. The ZUP (*Ac*) qPCR assay had the highest incidence of cross-reactions observed with non-*Ac* isolates (34%), as compared to the Contig 21 or Contig 22 qPCR assays (0% and 4%, respectively) for the isolates included in the panel.

It is recommended that seed testing laboratories evaluate their required parameters for assay performance (i.e., the acceptable false positive rate) and select at least 2 of these 3 primers for routine testing use.

Selectivity

Selectivity experiments examined the ability of the qPCR assays to detect their intended target in the presence of interferences, such as those produced by a sample matrix.

Experiment Method:

Method selectivity was examined by obtaining melon, watermelon and squash seed samples known to be free of *Ac*. Four samples of 5,000 seed per species were prepared and processed by the *Ac* direct qPCR method (Annex D) to generate representative seed sample matrices.

A bulk qPCR reaction mix was prepared as described in Annex E with the modification that the total volume of water per reaction was reduced by 5µL. Per previous method details standardized DNA solutions of *Ac* and *Xv* were generated. DNA template solutions ranged from 2ng/µL to 20fg/µL.

qPCR reactions were prepared containing 15µL of reaction mix, 5µL DNA template, and 5µL seed sample matrix. All combinations of DNA template concentration and sample matrix were tested in triplicate by the Contig 21, Contig 22 and *Xv* qPCR assays to produce a qPCR standard curve for each sample matrix and qPCR assay.

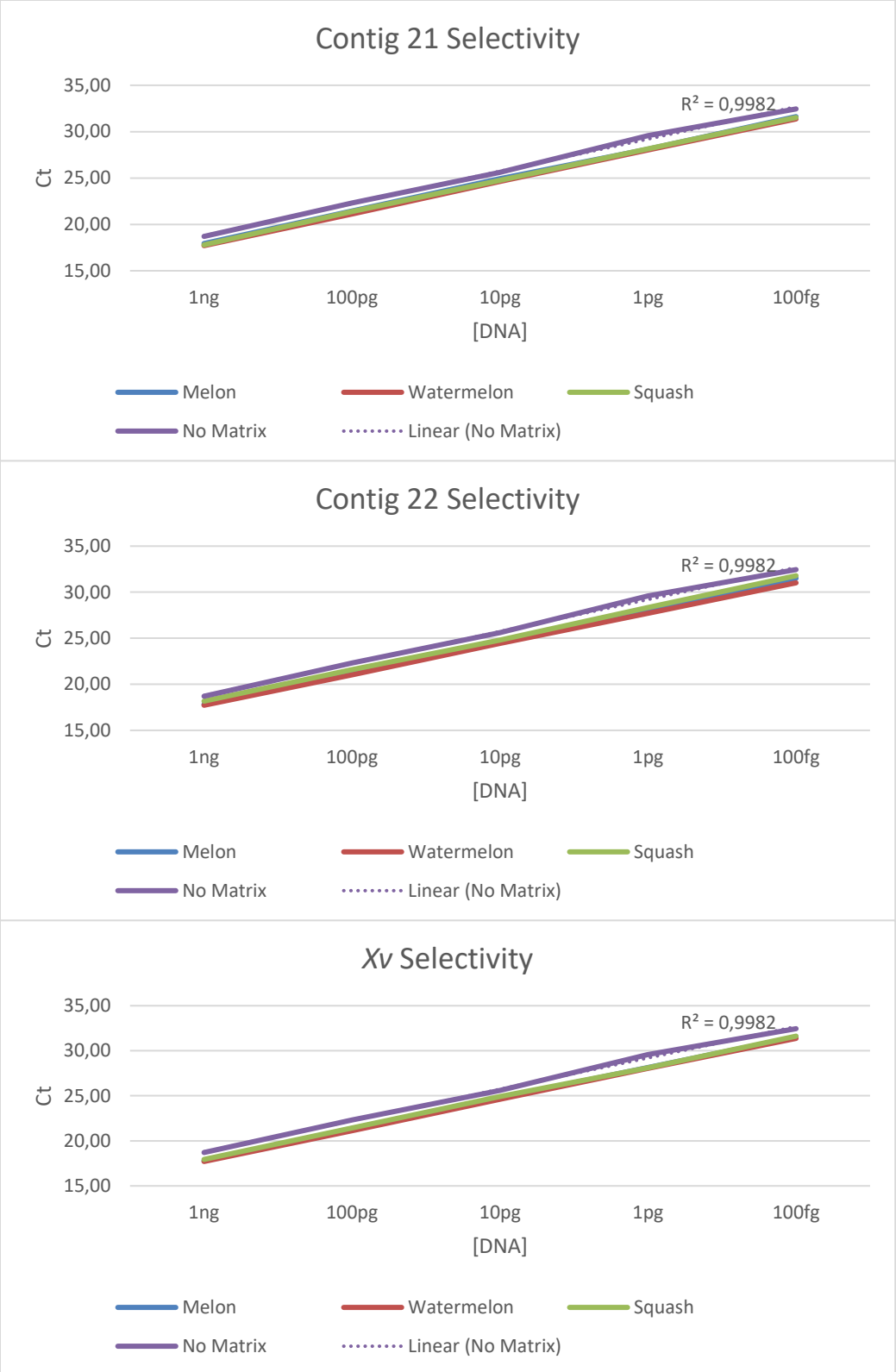

Figure 1: qPCR assay efficiency in the presence of various crop species matrix

The amplification efficiency was estimated through a linear regression of the dilution curve. In the presence of multiple cucurbit sample matrices, the Contig 21, Contig 22 and *Xv* qPCR assays consistently performed with a mean amplification efficiency between 90-100%. All amplification efficiency values generated are within the acceptable range, given in the qPCR assay validation requirements demonstrating a high degree of selectivity and negligible affect of sample matrix on qPCR assay performance.

Accuracy

Method accuracy was determined by evaluating the trueness and precision of the assay (ISO 5725-1:1994).

Trueness

Trueness (i.e. bias) was evaluated to determine the difference between a standardized reference sample and experimental sample results across a variety of cucurbit crop species.

Experiment I: Reference Sample Characterization

To determine the baseline Ct values associated with the PEC and PPC controls from a standard reference sample.

Experiment Method:

Six replicate samples of 5,000 melon seeds obtained from a lot known to be free of *Ac* used in this experiment. Stock cell suspensions for *Ac* and *Xv* were prepared and used as described previously for PEC and PPC. Samples were processed as described in the '*Ac* direct qPCR method' (Annex D). Prior to incubation, *Ac* $OD_{600}=0.100\times10^{-3}$ and *Xv* $OD_{600}=0.100x10^{-3}$ were spiked into each sample at a ratio of 1mL per 250mL extraction buffer. Total DNA was isolated from three-45mL replicate aliquots from each sample and tested by Contig 21, Contig 22 and *Xv* qPCR assays, in duplicate. Ct values were analyzed across qPCR assays. Means and standard deviations were generated. Ct ranges were calculated by (mean ± (3 X standard deviation of the mean) expected Ct values of PEC and PPC used in routine testing.

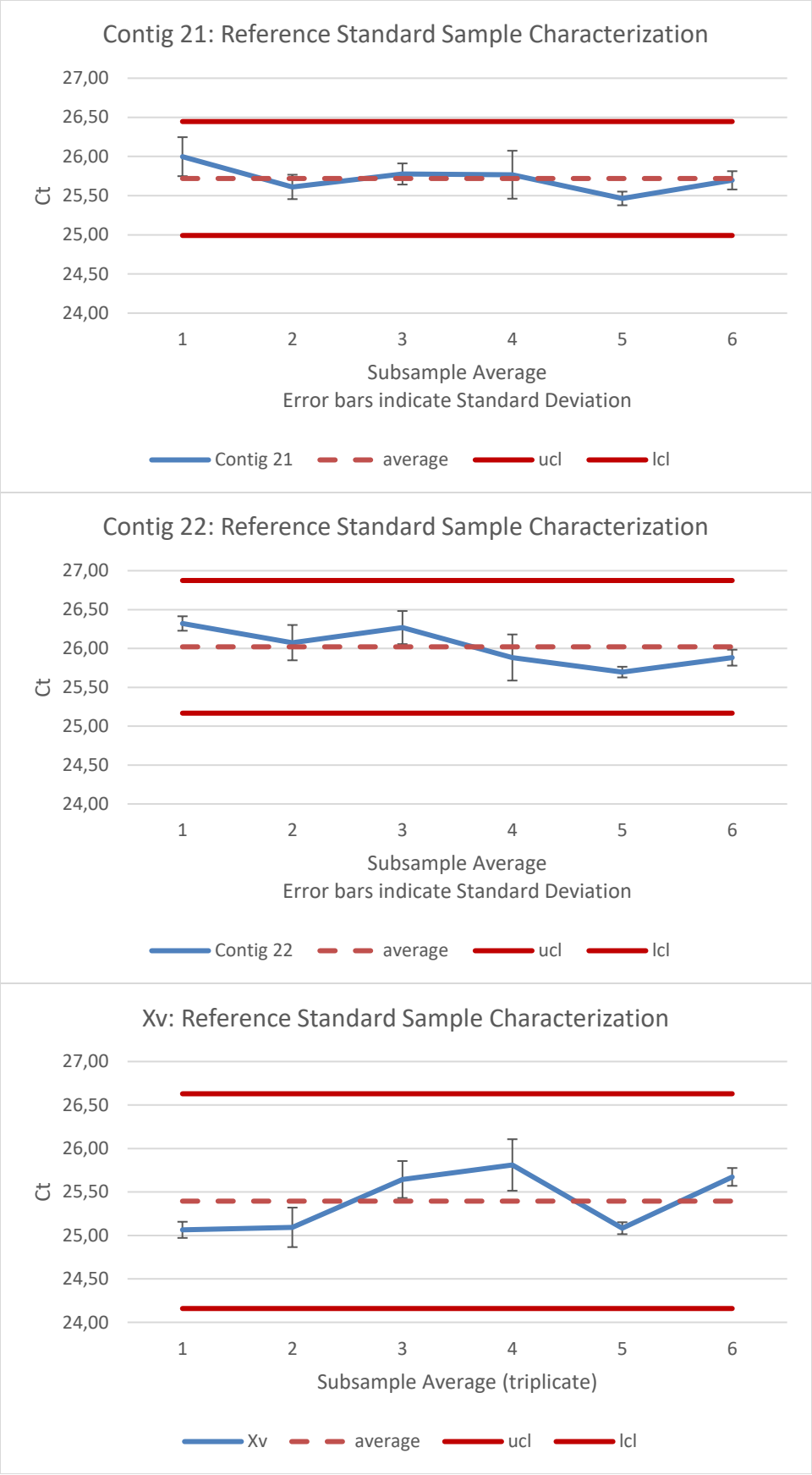

Figure 2: Characterization of reference standard and expected ranges

From this experiment the sample to sample variances of the PEC and the PPC were evaluated and upper and lower control limits were calculated as $\bar{x}\pm3\sigma$ (Figure 2). This range represents a 99.7% normal distribution probability of Ct values when using fixed PEC and PPC spike concentrations across subsamples from a single seed batch. This seed and the associated ranges were used in the following crop species bias experiment.

Experiment II: Crop Species Bias

Experiment Method:

To determine the method bias of other crop species against the standard reference sample, various cucurbit crop species were evaluated. Three melon, nine watermelon and twelve squash lots known to be free of *Ac* and Xv were obtained and 5,000 seed samples were prepared from each lot. Watermelon samples were selected based on historical test results of "inconclusive by PEC inhibition", suggesting high levels of matrix effect. Stock cell suspensions for *Ac* and *Xv* were prepared as previously described for the PPC and the PEC. Samples were processed as described in the *Ac* direct qPCR method (Annex D). Prior to incubation, *Ac* $OD_{600}=0.100\times10^{-3}$ and *Xv* $OD_{600}=0.100x10^{-3}$ were spiked into each sample at a ratio of 1mL per 250mL extraction buffer. Total DNA was isolated from three-45mL replicate aliquots from each sample and tested by Contig 21, Contig 22 and *Xv* qPCR assays, in duplicate.

Results:

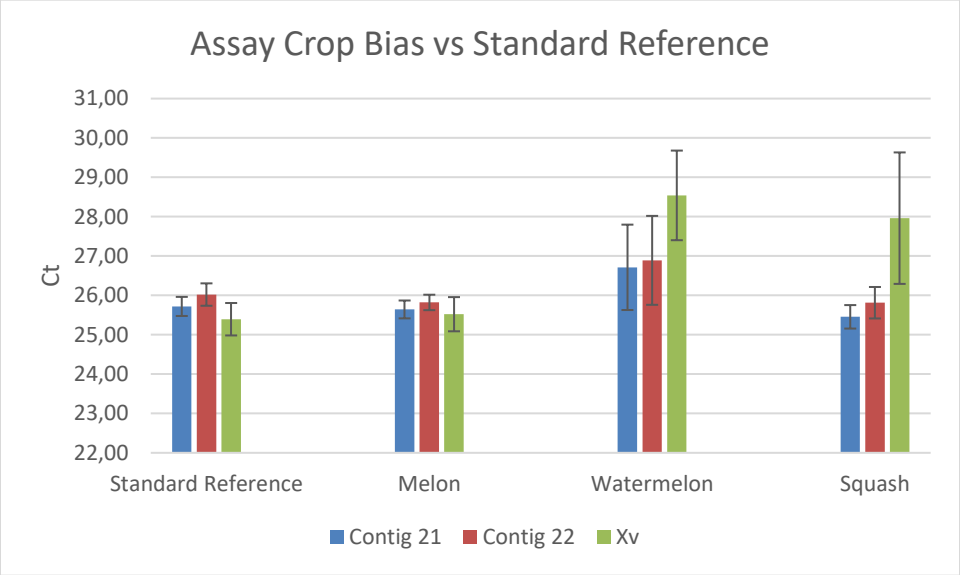

Figure 3: Sample Matrix Bias. The standard deviation between varieties within a crop is represented by the error bars.

Bias experiment data was evaluated at the crop species level, averaging the values across varieties within each crop species respectively. Bias data tables used in generating the above figure can be found in Annex A, Section 2.

The Contig 21 and Contig 22 qPCR assays yielded Ct values in melon and squash samples within the expected ranges defined in figure 2 demonstrating little bias based on sample matrix. Contig 21 and Contig 22 produced Ct values outside the ranges defined in figure 2 for watermelon test samples demonstrating a higher degree of bias based on sample matrix. This difference is attributed to the specific watermelon sample matrix which historically produced inhibitory effects on target recovery and detection of PECs. As the selectivity experiment showed negligible variance in Ct due to matrix effect in the qPCR assay, the crop bias is likely due to a component in the target recovery process such as lysis efficiency. As shown in both squash and watermelon samples the PEC (*Xv*) assay is more greatly affected by sample matrix than the *Ac* target assays. As one of the purposes of the PEC is to monitor qPCR inhibition caused by sample matrix the data supports the PEC selection

based on the assumption that the PEC assay is more sensitive to inhibitory compounds than the *Ac* target assays and therefore any inhibition of the target assays will be identified.

Given the differences in seed physiology between the crop species and historical testing experiences, the bias was expected; therefore, it was concluded that PPC and PEC control range limits must be set per crop species in each testing laboratory (ISO 11462-1: 7.2 Definition of process targets and limits).

Samples with PEC Ct values outside the expected range are to be reported as inconclusive, and further investigation for the sample is required.

Precision

Method precision was determined by evaluating the degree of variance present in processing standardized reference material under repeatable and reproducible conditions.

Repeatable conditions represent the most fundamental level of short-term inherent variability of a process. Under repeatable conditions, a single technician processes replicates of a reference sample, using the same reagents and equipment, over a short duration of time.

Reproducible conditions represent the long-term inherent variability of a process. Under reproducible conditions, multiple technicians process multiple samples of reference material, using different reagent batches and equipment, over a larger interval of time.

qPCR Assay Precision

The precision of the Contig 21, Contig 22, and *Xv* qPCR assays were evaluated using the qPCR positive amplification control (PAC).

Experiment Method:

qPCR assay precision under repeatable conditions was evaluated by preparing singleplex qPCR reactions for *Ac* and *Xv* assays using a mixed DNA template with a concentration of 10pg per reaction per target (5µL DNA template at 2pg *Ac* DNA and 2pg *Xv* DNA per µL, Annex E). Precision was evaluated across 30 reactions per qPCR assay.

qPCR assay precision under reproducible conditions was evaluated by compiling PAC values generated during method validation (n=20). The PAC is defined as singleplex qPCR reactions for *Ac* and *Xv* assays using a mixed DNA template with a concentration of 10pg per reaction per target (5µL DNA template at 2pg *Ac* DNA and 2pg *Xv* DNA per µL, Annex E). The compiled data was produced over multiple days, by different technicians, using different batches of reagents run on multiple qPCR instruments.

Results:

qPCR Assay Precision Repeatability			
Reaction	Contig 21	Contig 22	Xcv
1	24.945	25.157	25.164
2	24.748	24.926	25.195
3	24.901	25.014	25.182
4	25.024	24.875	25.172
5	24.896	24.984	25.177
6	25.179	24.929	25.072
7	24.768	24.858	25.108
8	24.947	24.932	24.859
9	25.188	24.801	25.261
10	24.849	24.884	25.102
11	25.177	25.113	25.140
12	25.167	24.936	25.232
13	25.020	24.850	25.164
14	25.238	24.899	25.212
15	25.207	24.948	25.170
16	25.452	24.801	25.127
17	25.340	25.000	25.289
18	25.207	25.142	25.316
19	25.411	24.975	25.131
20	25.332	25.150	25.215
21	25.162	24.855	25.154
22	25.242	24.857	25.217
23	25.171	24.943	25.227
24	25.270	24.919	25.132
25	25.007	24.971	25.187
26	25.179	24.931	25.093
27	25.263	24.865	24.953
28	25.330	25.134	25.183
29	25.254	25.234	25.138
30	24.965	24.954	25.207

qPCR Assay Precision Reproducibility			
Reaction	Contig 21	Contig 22	Xcv
1	24.967	24.995	25.365
2	24.947	24.952	25.292
3	26.287	25.189	25.211
4	25.750	25.053	25.344
5	25.395	24.950	25.400
6	25.832	24.851	25.368
7	25.394	26.001	25.917
8	25.316	26.309	25.785
9	24.733	24.800	25.276
10	24.775	24.710	25.181
11	24.854	24.835	25.576
12	24.811	24.835	25.475
13	24.971	24.976	25.716
14	24.891	25.200	25.794
15	24.753	24.806	25.626
16	24.763	24.714	24.954
17	24.744	24.507	25.092
18	25.728	25.451	26.944
19	25.313	25.850	26.488
20	25.760	25.485	26.162

	Contig 21	Contig 22	Xv
CT Mean	25.128	24.961	25.159
CT σ	0.186	0.113	0.089
LCL	24.569	24.623	24.892
UCL	25.687	25.300	25.427

	Contig 21	Contig 22	Xv
CT Mean	25.199	25.123	25.598
CT σ	0.465	0.471	0.488
LCL	23.803	23.710	24.133
UCL	26.595	26.537	27.063

Table 10: qPCR assay precision under repeatable and reproducible conditions.

At a standard reference DNA concentration of 2pg/µL, the qPCR data demonstrates that the Contig 21, Contig 22, and *Xv* qPCR assays are within the precision parameters given in Table 1 ($\sigma < 0.37$ Ct under repeatable conditions, $\sigma < 1.11$ CT under reproducible conditions). This low degree of variance within the qPCR assays indicates a high degree of reliability and supports their use in the total method.

'*Ac* direct qPCR method' Precision

The precision of the method was examined by measuring the Ct variance of the positive process control (PPC) and positive extraction control (PEC) using a standard reference sample under both repeatable and reproducible conditions.

Experiment Method:

Precision Repeatability (Day 1)

Six replicate samples of 5,000 melon seeds were prepared and used in this experiment. Stock cell suspensions for *Ac* and *Xv* were prepared as previously described to generate spike solutions ranging from $OD_{600}=0.100\times10^{-1}$ - 0.100×10^{-3} for each pathogen. Samples were processed as described in the '*Ac* direct qPCR method' (Annex D). Prior to incubation, *Ac* $OD_{600}=0.100\times10^{-3}$ and *Xv* $OD_{600}=0.100\text{x}10^{-3}$ were spiked into each sample at a ratio of 1mL per 250mL extraction buffer. Total DNA was isolated from three-45mL replicate aliquots from each sample and tested by Contig 21, Contig 22 and *Xv* qPCR assays, in duplicate.

Precision Reproducibility (Day 2)

The above process was repeated on a subsequent day using fresh preparations of cell suspensions and different lots of reagents for DNA isolation. To capture the maximum variance, each aliquot from a given sample was processed by a different technician on Day 2. Data was pooled over both days to calculate reproducibility.

Results:

	Day 1				Day 2			
Reference Seed Sample	Aliquot	Contig 21	Contig 22	*Xv*	Aliquot	Contig 21	Contig 22	*Xv*
1	A	25.837	26.146	25.146	A	25.981	26.365	24.909
		25.821	26.396	24.643		26.153	26.599	25.318
	B	25.856	26.380	25.307	B	26.430	26.693	25.822
		25.836	26.293	24.882		26.360	26.708	25.562
	C	26.341	26.344	25.414	C	25.565	25.715	24.435
		26.296	26.366	24.991		25.659	25.973	24.805
	Ct Mean	25.998	26.321	25.064	Ct Mean	26.025	26.342	25.142
	Ct σ	0.249	0.093	0.284	Ct σ	0.358	0.413	0.517
2	A	25.681	26.186	25.348	A	25.339	25.937	24.826
		25.752	26.245	25.164		25.361	26.169	25.216
	B	25.457	25.757	25.010	B	24.974	25.747	25.106
		25.375	25.824	24.809		25.156	25.866	24.965
	C	25.688	26.295	24.930	C	25.363	25.963	24.964
		25.717	26.141	25.294		25.239	26.151	24.969
	Ct Mean	25.612	26.075	25.093	Ct Mean	25.239	25.972	25.008
	Ct σ	0.156	0.227	0.212	Ct σ	0.153	0.164	0.135
3	A	25.591	26.386	25.793	A	25.438	25.761	25.256
		25.744	26.229	25.684		25.473	25.826	24.989
	B	25.880	26.293	25.909	B	25.185	25.539	24.798
		25.978	26.596	25.678		25.346	25.494	24.501
	C	25.749	25.980	25.487	C	25.009	25.298	24.542
		25.719	26.128	25.306		24.871	25.274	24.498
	Ct Mean	25.777	26.269	25.643	Ct Mean	25.220	25.532	24.764
	Ct σ	0.135	0.213	0.216	Ct σ	0.243	0.229	0.311
4	A	25.489	25.633	25.612	A	25.552	25.609	24.635
		25.531	25.684	25.824		25.269	25.537	24.590
	B	25.627	25.745	25.903	B	25.277	25.464	24.556
		25.649	25.732	25.710		25.029	25.469	24.473
	C	26.138	26.354	25.797	C	25.179	25.453	23.853
		26.172	26.154	26.014		25.210	25.483	23.996
	Ct Mean	25.768	25.884	25.810	Ct Mean	25.253	25.502	24.351
	Ct σ	0.306	0.296	0.141	Ct σ	0.172	0.060	0.337
5	A	25.542	25.643	25.315	A	25.534	25.817	24.916
		25.505	25.730	25.306		25.513	25.626	24.939
	B	25.314	25.594	25.212	B	25.376	25.544	24.858
		25.409	25.690	25.009		25.199	25.322	24.871
	C	25.488	25.736	25.337	C	25.525	25.951	24.908
		25.529	25.783	24.316		25.523	25.748	24.975
	Ct Mean	25.464	25.696	25.083	Ct Mean	25.445	25.668	24.911
	Ct σ	0.087	0.069	0.395	Ct σ	0.134	0.222	0.043
6	A	25.534	25.738	25.494	A	25.231	25.580	25.307
		25.568	25.792	25.639		25.220	25.573	25.110
	B	25.740	25.866	25.197	B	25.942	26.490	26.017
		25.823	25.973	25.651		25.995	26.335	26.222
	C	25.743	25.922	25.918	C	25.851	26.222	25.967
		25.768	25.999	26.136		25.804	26.388	25.564
	Ct Mean	25.696	25.882	25.672	Ct Mean	25.674	26.098	25.698
	Ct σ	0.117	0.103	0.327	Ct σ	0.354	0.413	0.439

Table 11: *'Ac* direct qPCR method' precision under repeatable and reproducible conditions

Precision	Repeatability			Reproducibility					
	Day 1			Day 2			Day 1&2		
	Contig 21	Contig 22	*Xv*	Contig 21	Contig 22	*Xv*	Contig 21	Contig 22	*Xv*
Reference Seed Average	25.716	26.022	25.386	25.469	25.856	24.980	25.599	25.936	25.193
Reference Seed σ	0.240	0.281	0.409	0.374	0.402	0.511	0.336	0.357	0.509
Sample to Sample σ	0.179	0.244	0.349	0.320	0.339	0.445	0.278	0.295	0.439
Coefficient of Variation (CV)	0.93%	1.08%	1.61%	1.47%	1.56%	2.05%	1.31%	1.37%	2.02%

Table 12: Method precision summary

Control Range	Contig 21	Contig 22	*Xv*
3σ	1.0077	1.0695	1.5273
LCL	24.591	24.866	23.665
UCL	26.606	27.005	26.720

Table 13: Calculation of PPC and PEC control ranges for positive control samples

Method precision under repeatable conditions was calculated using data from the standard reference seed characterization experiment performed in the trueness study (Table 12, Day 1). As expected, there was an increase in variation as compared to the qPCR assay precision; however, standard deviation was well within precision target given in method validation criteria. The experiment was repeated by a different technician, producing a second set of repeatability data (Day 2), which also demonstrated performance within the method validation criteria requirements.

Method precision under reproducible conditions was calculated by evaluating the combined data sets of days 1 and 2. To evaluate method precision, the standard deviation across all samples (reference seed σ) was calculated and standard deviations between samples were compared (sample to sample σ). The assay demonstrates exceptional precision with coefficient of variation (CV) values at or below 2.05% for all assays indicating that the method is robust and can lead to consistent detection although lab conditions may vary.

Using the reproducibility data generated in this experiment, upper and lower control limits were calculated as $\bar{x} \pm 3\sigma$. This range represents the 99.7% normal distribution probability of Ct values when using a reference standardized spike and seed matrix under routine laboratory testing conditions. Associated control ranges are to be used in the routine application of this method as an expected range for the PPC.

Detection of *Ac* in Naturally Infected Seed Across Various Crops

To verify the ability of the method to detect seeds naturally infected with *Ac*, infected cucurbit seeds were tested at the recommended sample size.

Experiment:

Naturally infected seeds of melon, watermelon and squash were obtained at 30,000 seeds per sample. These samples were previously identified as naturally infected with *Ac* by the current NSHS accredited methods (Cb 1.1 *Acidovorax avenae* ssp. *citrulli* – Cucurbit and Cb1.4 method for the detection of *Ac*). Samples were batched into 5,000 seed subsamples, and tested as described in Annex D.

Results:

Crop Species	5K seed subsample	Contig 21	Contig 22	*Xv*
Melon	1	30.921	30.984	28.444
		30.894	31.149	28.517
	2	30.448	29.667	28.048
		29.830	29.490	27.377
	3	28.193	28.195	28.212
		28.436	28.239	28.153
	4	29.674	29.737	27.842
		30.173	29.809	28.114
	5	28.817	28.816	28.113
		28.628	28.370	27.967
	6	29.374	29.002	27.668
		29.362	29.233	27.680
Watermelon	1	18.508	18.368	27.470
		18.490	18.576	27.481
	2	18.519	18.561	27.653
		18.626	18.653	27.611
	3	19.007	18.674	27.701
		18.896	18.737	27.706
	4	18.404	18.198	27.681
		18.429	18.250	27.651
	5	18.923	18.677	27.589
		18.794	18.712	27.731
	6	18.985	19.293	27.502
		18.896	18.843	27.399
Squash	1	37.158	36.628	27.235
		35.701	-	27.304
	2	37.182	34.700	27.333
		34.429	34.848	27.416
	3	34.530	37.563	27.280
		-	-	27.500
	4	36.147	35.408	27.441
		35.612	-	27.317
	5	36.140	-	27.723
		-	36.919	27.639
	6	35.755	36.452	27.275
		-	-	27.171

Table 18: *Ac* direct qPCR method demonstrating detection of naturally infected seed.

The method resulted in detection of *Ac* in all naturally infected seed samples demonstrating that the method is fit for the intended purpose across the crop species evaluated. With the inclusion of a PEC (and associated ranges) the data demonstrates negligible difference in target detection across the crop species evaluated. Routine application of a PEC supports adoption of the method across various cucurbit crops as issues with sample loss or inhibition would be readily identified and the sample moved to secondary testing.

Interlaboratory Reproducibility

Experiment Method:

To determine interlaboratory reproducibility of the *Ac* direct qPCR method, Monsanto and HM-Clause participated in a comparative test. Naturally infected samples of cucurbit seed with various infection levels were distributed to the participating laboratories (Table 19). Samples were processed by the *Ac* direct qPCR method (Annex D). Total DNA was isolated from each sample and tested by Contig 21, Contig 22 and Xv qPCR assays in duplicate by different technicians, using different batches of reagents and processed on different days in different laboratories. This experiment was performed twice in each laboratory.

Results:

		Lab 1						Lab 2					
		Contig 21 CT		Contig 22 CT		*Xv* CT		Contig 21 CT		Contig 22 CT		*Xv* CT	
Sample	Variety	*Run 1*	*Run 2*	*Run 1*	*Run 2*	*Run 1*	*Run 2*	*Run 1*	*Run 2*	*Run 1*	*Run 2*	*Run 1*	*Run 2*
1	Watermelon 1	24.702	23.246	24.670	23.407	26.326	26.369	26.87	27.11	26.70	27.36	26.35	26.78
		24.685	23.298	24.775	23.465	26.240	26.274	26.50	26.94	26.50	27.21	26.88	26.67
2	Watermelon 1	23.464	24.268	23.444	24.487	25.794	26.845	25.62	26.63	25.62	27.08	26.45	26.31
		23.303	24.320	23.409	24.512	25.781	26.880	25.67	26.71	25.72	27.07	26.43	26.75
3	Watermelon 1	24.599	23.516	24.706	23.599	25.841	26.868	26.34	26.69	26.32	26.96	25.56	26.37
		24.619	23.532	24.611	23.212	25.865	26.893	26.33	26.51	26.28	26.90	26.40	26.08
4	Melon 1	24.340	24.148	27.566	25.544	-	31.446	23.45	27.29	23.76	27.06	26.97	32.62
		24.725	24.192	26.942	26.108	-	31.781	23.50	27.30	23.78	28.06	27.10	30.39
5	Melon 1	24.292	25.326	25.963	26.579	29.172	32.481	24.34	30.77	25.30	31.04	28.09	33.93
		24.512	25.558	27.274	26.548	30.192	32.468	24.39	29.97	25.02	31.66	28.35	33.60
6	Melon 1	21.617	24.353	23.742	25.832	29.163	31.792	26.77	25.04	27.81	27.64	31.06	33.25
		21.675	24.444	23.367	26.104	29.315	31.464	25.67	25.89	27.07	26.94	30.15	32.95
0% INF	Melon 2	-	35.567	-	36.947	26.695	28.129	-	-	-	-	27.10	26.75
		-	-	-	-	26.774	28.067	-	-	-	-	27.75	26.60
10% INF	Melon 2/3 Blend	26.467	25.803	26.275	26.203	26.444	28.344	22.25	28.76	22.51	29.15	27.10	26.81
		26.312	25.806	26.382	26.121	26.471	27.806	22.57	28.43	22.45	29.03	27.05	26.74
20% INF	Melon 2/3 Blend	25.733	25.380	25.821	25.574	26.848	27.848	28.65	27.77	28.63	28.26	26.44	27.21
		25.582	25.257	25.785	25.615	26.590	28.068	30.61	27.58	28.59	28.21	25.70	27.10
10	Melon 3	20.316	19.517	20.445	19.595	27.676	27.911	22.75	23.03	22.83	23.54	26.21	27.27
		20.252	19.455	20.315	19.566	27.558	28.069	22.84	23.09	22.83	23.56	25.79	27.37
11	Watermelon 2	17.262	16.829	17.275	16.923	25.890	26.988	21.05	20.03	20.40	20.56	26.23	26.23
		17.130	16.834	17.163	16.982	25.784	26.933	20.18	20.19	20.39	20.61	25.95	26.40
12	Squash 1	26.852	25.596	26.939	25.389	26.516	28.007	28.59	27.08	28.37	27.34	26.12	26.52
		26.953	25.183	26.960	25.264	26.464	28.085	28.27	27.16	28.55	27.39	24.99	26.35
NPC	Melon 2	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-
PPC	Melon 2	25.765	25.811	25.783	26.163	26.570	27.732	26.34	26.52	26.98	27.15	26.29	26.87
		25.617	25.976	25.784	26.470	26.529	27.761	26.51	26.50	27.14	27.07	25.82	26.61

Table 19: Interlaboratory recovery and detection of *Ac* on naturally infected seed by the *Ac* direct qPCR method.
*Lab 1, sample 4: run 1 was not spiked with *Xv*.

The data demonstrates a high degree of interlaboratory reproducibility of a fixed method with 100% detection of naturally infested *Ac* in both laboratories across all positive samples, including multiple crop species and infection levels.

Melon 1 (samples 4-6) was a phenotypically atypical variety of melon seed. The seeds were very large with abnormal buffer absorption characteristics. Although the Ct values for the PEC are higher than expected, both laboratories demonstrate similar levels of detection sensitivity and precision for both *Ac* and the PEC target. Similar to the results of the ISHI comparative test (Table 17), samples characterized as negative produced low levels of detection with non-replicated high Ct values near the LOD of the method.

Robustness

Method Comparative LOD Evaluation

An interlaboratory comparative test was performed to demonstrate that various combinations of DNA isolation methods and PCR chemistries can produce equivalent recovery and detection of *Ac* by meeting the minimum requirements of the method acceptance criteria.

Experiment Method

Four industry laboratories, Rijk Zwaan, HM-Clause, Monsanto and Syngenta, participated in an interlaboratory comparative LOD study. Nine samples consisting of 5,000 melon seed each taken from a seed lot known to be free of *Ac* were distributed by Monsanto to participating laboratories. A stock cell suspension for *Ac* corresponding to OD_{600}=0.100 was prepared by each laboratory. A ten-fold dilution series of the stock cell suspension was performed to generate spike solutions ranging from $OD_{600}=0.100\times10^{-5}$ - 0.100×10^{-7}. For each spike concentration, 1mL of *Ac* was spiked into three corresponding 5,000 seed samples. All samples were processed as described in Annex E with qPCR reactions run in triplicate. Each company isolated DNA using various kits including MoBio PowerFood, Qiagen DNEasy Blood and Tissue, Machery Nagel Nucleo Spin II, and Sbeadex Maxi Plant processed with method variants including automated platforms (e.g. KingFisher). Each company then tested isolated DNA in triplicate by at least two validated *Ac* qPCR assays using various qPCR protocols including different mastermix formulations, multiplex reactions and qPCR instruments.

*Note that lab 2 processed two samples per spike concentration and ran qPCR reactions in duplicate.

Results:

Ac OD_{600}=0.100	Lab 1 MoBio PowerFood	Lab 1 Qiagen DNEasy BT	Lab 2 MoBio PowerFood	Lab 3 Machery Nagel NucleoSpin II	Lab 4 Sbeadex Maxi Plant
10^-5 (LOQ)	3 of 3	3 of 3	2 of 2	3 of 3	3 of 3
10^-6 (LOD)	3 of 3	2 of 3	2 of 2	3 of 3	2 of 3
10^-7	0 of 3	0 of 3	2 of 2	1 of 3	3 of 3

Table 14: Summary of *Ac* detection per sample by laboratory and DNA isolation method.

The full data set in presented in Annex A, Section 3. The data demonstrates that even with variations to the DNA extraction method and PCR chemistries, the method is robust enough for different laboratories to detect *Ac* in samples spiked with bacterial concentrations representing the LOQ and LOD. DNA detection becomes more varied as each respective method approaches its LOD; however, all laboratories consistently detected *Ac* at or before the limits stated in the method acceptance criteria. This data supports recommendations made in the proposed protocol (Method for the Detection of *Acidovorax citrulli* DNA on cucurbit seed; Annex E) in relation to method flexibility.

ISHI Method Comparative Test:
Interlaboratory Test of a Flexible Molecular Method for the Detection of *Ac*.

An interlaboratory comparative test was performed using seeds known to be naturally infested with *Ac*.to demonstrate that various combinations of DNA isolation methods and PCR chemistries lead to recovery and detection of *Ac*.

Experiment Method:

Reference CT Test plan, Annex F.

Comparative test sample characterization results

Sample ID	Crop	5K Seed Subsample	Contig 21	Contig 22	ZUP(*Ac*)	*Xv*	ZUP(Ac)	ZUP (*Acat*)
742	Melon	1	-	-	-	26.94	-	24.485
742	Melon		-	-	-	26.65	-	25.978
742	Melon	2	-	-	-	26.62	-	24.533
742	Melon		-	-	-	26.78	-	25.334
742	Melon	3	-	-	-	26.60	N/A	N/A
742	Melon		-	-	-	26.70	N/A	N/A
742	Melon	4	-	-	-	26.36	N/A	N/A
742	Melon		-	-	-	26.28	N/A	N/A
742	Melon	5	-	-	-	26.73	N/A	N/A
742	Melon		-	-	-	26.76	N/A	N/A
742	Melon	6	-	-	-	26.81	N/A	N/A
742	Melon		-	-	-	26.89	N/A	N/A
661	Watermelon	1	-	-	-	26.59	-	22.447
661	Watermelon		-	-	-	26.71	-	23.494
661	Watermelon	2	-	-	-	26.40	-	23.077
661	Watermelon		-	-	-	26.47	-	22.521
661	Watermelon	3	-	-	-	26.35	N/A	N/A
661	Watermelon		-	-	-	26.42	N/A	N/A
661	Watermelon	4	-	-	-	26.28	N/A	N/A
661	Watermelon		-	-	-	26.53	N/A	N/A
661	Watermelon	5	-	-	-	26.70	N/A	N/A
661	Watermelon		-	-	-	26.93	N/A	N/A
661	Watermelon	6	-	-	-	26.33	N/A	N/A
661	Watermelon		-	-	-	26.47	N/A	N/A
PPC	Melon	1	24.74	24.98	22.34	26.58	23.951	23.449
PPC	Melon		24.60	25.13	22.19	26.60	22.941	23.445
NPC	Melon	1	-	-	-	-	-	-
NPC	Melon		-	-	-	-	-	-
PAC (10pg)		-	27.76	28.16	26.53	29.00	28.786	29.192
PAC (10pg)		-	27.66	28.46	26.22	28.88	N/A	N/A
NTC		-	-	-	-	-	-	-
NTC		-	-	-	-	-	N/A	N/A

Table 15: Characterization of *Ac* negative seed lots used for the comparative test.

Sample ID	Crop	5K Seed Subsample	Contig 21	Contig 22	ZUP(*Ac*)	*Xv*	ZUP(Ac)	ZUP (*Acat*)
161	Melon	1	20.55	21.48	21.66	27.08	25.516	27.112
161	Melon		20.55	21.47	21.50	27.09	23.976	25.716
161	Melon	2	19.95	20.95	21.57	27.04	25.733	27.606
161	Melon		19.99	20.93	21.61	27.02	23.048	24.944
161	Melon	3	20.50	21.45	21.36	26.85	N/A	N/A
161	Melon		20.63	21.45	21.35	26.84	N/A	N/A
161	Melon	4	19.97	20.73	20.69	26.92	N/A	N/A
161	Melon		19.96	20.78	20.67	26.99	N/A	N/A
161	Melon	5	20.62	21.48	21.81	27.09	N/A	N/A
161	Melon		20.66	21.49	21.72	27.04	N/A	N/A
161	Melon	6	20.47	21.14	21.37	26.95	N/A	N/A
161	Melon		20.51	21.01	21.37	26.92	N/A	N/A
280	Melon	1	21.69	22.34	22.68	27.03	24.366	25.043
280	Melon		21.71	22.22	22.68	26.91	23.161	22.116
280	Melon	2	21.26	21.84	22.33	26.72	24.708	25.906
280	Melon		21.29	21.86	22.38	26.62	25.047	25.061
280	Melon	3	20.21	20.72	21.09	26.89	N/A	N/A
280	Melon		20.26	20.62	21.15	26.88	N/A	N/A
280	Melon	4	21.30	21.79	22.62	26.56	N/A	N/A
280	Melon		21.30	21.81	22.65	26.56	N/A	N/A
280	Melon	5	21.73	22.20	22.84	27.17	N/A	N/A
280	Melon		21.67	22.18	23.06	27.32	N/A	N/A
280	Melon	6	20.83	21.22	21.35	27.54	N/A	N/A
280	Melon		20.83	21.25	21.24	27.60	N/A	N/A
354	Melon	1	29.51	29.70	30.63	27.12	31.559	22.530
354	Melon		29.27	30.52	30.72	26.95	32.321	23.477
354	Melon	2	28.67	29.14	29.39	27.08	31.938	22.376
354	Melon		28.56	29.18	29.47	27.09	31.203	22.166
354	Melon	3	29.47	29.89	30.64	27.18	N/A	N/A
354	Melon		29.52	29.96	30.56	27.16	N/A	N/A
354	Melon	4	28.62	29.33	30.10	27.38	N/A	N/A
354	Melon		28.69	28.95	30.06	27.10	N/A	N/A
354	Melon	5	29.87	30.34	30.72	27.03	N/A	N/A
354	Melon		29.81	30.38	30.79	26.99	N/A	N/A
354	Melon	6	29.19	29.72	30.32	26.88	N/A	N/A
354	Melon		29.23	29.82	30.39	26.82	N/A	N/A
301	Watermelon	1	19.16	19.52	19.89	26.97	21.520	23.929
301	Watermelon		18.92	19.51	19.65	26.96	22.816	27.198
301	Watermelon	2	19.22	19.68	20.29	26.81	20.385	23.121
301	Watermelon		19.17	19.71	20.07	26.98	22.156	25.181
301	Watermelon	3	18.15	18.79	18.83	26.66	N/A	N/A
301	Watermelon		18.44	18.77	18.96	26.67	N/A	N/A
301	Watermelon	4	18.95	19.35	19.40	27.06	N/A	N/A
301	Watermelon		18.81	19.30	19.25	26.95	N/A	N/A
301	Watermelon	5	18.92	19.42	19.34	27.04	N/A	N/A
301	Watermelon		18.84	19.38	19.44	27.06	N/A	N/A
301	Watermelon	6	18.75	19.18	19.59	26.68	N/A	N/A
301	Watermelon		18.80	19.26	19.72	26.63	N/A	N/A

Table 16: Characterization of *Ac* positive seed lots used for the comparative test.

Seed lots naturally infested with *Ac* were characterized using 30,000 seed following the *Ac* direct qPCR method (Annex D). The experiment was repeated using 10,000 seeds substituting the PEC *Xv* with *Acat* to verify functionality of the alternate PEC on the selected seed lots.

Results:

Infection Level	Sample ID	Crop Species	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6a	Lab 6b	Lab 7	Stability Check (Lab 1)
High +	161	Melon	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
High +	161	Melon	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
High +	280	Melon	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
High +	280	Melon	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Moderate +	354	Melon	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Moderate +	354	Melon	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Moderate +	354	Melon	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Moderate +	354	Melon	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Moderate +	354	Melon	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Moderate +	354	Melon	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
High +	301	Watermelon	Positive	Positive	Positive	Positive	Positive	Inhibited	Positive	Positive	Positive
High +	301	Watermelon	Positive	Positive	Positive	Positive	Positive	Inhibited	Positive	Positive	Positive
High +	301	Watermelon	Positive	Positive	Positive	Positive	Positive	Inhibited	Positive	Positive	Positive
High +	301	Watermelon	Positive	Positive	Positive	Positive	Positive	Inhibited	Positive	Positive	Positive
Negative	742	Melon	Inconclusive	Negative	Negative	Positive	Negative	Negative	Negative	Inconclusive	Negative
Negative	742	Melon	Negative	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative
Negative	661	Watermelon	Negative	Negative	Negative	Positive	Negative	Inhibited	Negative	Negative	Negative
Negative	661	Watermelon	Negative	Negative	Negative	Positive	Positive	Inhibited	Inconclusive	Negative	Negative
(+) Process Control	742	Melon	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
(-) Process Control	742	Melon	Negative	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative
Ac (+) PCR Control		-	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
PEC (+) PCR Control		-	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Positive	Positive
NTC (-) PCR Control		-	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
DNA Isolation			MoBio PowerFood	MoBio PowerFood	Machery Nagel Nucleo Spin plant II	Sbeadex Maxi Plant	Machery Nagel Nucleo Spin plant II	TRIsure	TRIsure	Sbeadex maxi kit LGC Genomics	MoBio PowerFood
qPCR MasterMix			ABI Taqman	ABI Taqman	Quanta PerfeCTa Multiplex qPCR Tough Mix	Quanta PerfeCTa Multiplex qPCR Tough Mix	Quanta PerfeCTa Multiplex qPCR Tough Mix	SensiFAST Probe Lo-ROX	SensiFAST Probe Lo-ROX	Quanta PerfeCTa Multiplex qPCR Tough Mix	ABI Taqman
qPCR Plex			single	Single	multi	multi	multi	single	single	multi	single

Table 17: Summaries of method details and sample results of the ISHI comparative test.

Method Control Evaluation

Positive Process Control	qPCR Ct replicated by each of the *Ac* detection assays (within expected range*)
Positive Extraction Control	qPCR Ct replicated by the PEC detection assays (within expected range*)
Negative Process Control	No amplification by Ac detection assay, or Ct value exceeds negative cut-off value
Amplification Control	qPCR Ct replicated by respective detection assays (within expected range*)
Non-Template Control	No amplification by any detection assay, or Ct value exceeds negative cut-off

Sample Evaluation

Positive	qPCR Ct are replicated by any of the *Ac* detection assays
Negative	No amplification by Ac detection assay, or Ct value exceeds negative cut-off
Inconclusive	Non-replicated amplification present near the LOD
Inhibited	PEC shows a significant shift in Ct value (outside of expected range*)

Table 18: CT Result analysis scoring criteria.
*Expected range is determined per laboratory

For results to be considered valid, all controls should perform as expected. Laboratory 4 had a positive detection in the negative process control. Therefore, data from Laboratory 4 was excluded from subsequent analysis. Laboratory 6 did not detect the PEC in the standardized control DNA distributed to participating labs (PEC(+) PCR Control). This is due to the use of an alternative PEC qPCR assay that does not detect the particular *Xanthomonas* isolate used to generate the PEC(+) PCR Control distributed to the participating laboratories. However, the PEC qPCR assay selected by Laboratory 6 does detect the *Xanthomonas* strain used to spike seed samples as an PEC and therefore detection in seed samples was not affected as demonstrated by the Positive Process Control data above. Data from Laboratory 6 was included in further analysis.

All participating laboratories found *Ac* present in all expected positive samples. There were no false negatives. This is a very important attribute of a routine testing assay. It is more impressive considering that there were different DNA extraction kits, mastermixes, thermocyclers, etc. used in the testing. However, with the advances in recent years in off-the-shelf nucleic acid kits towards improved DNA quality and yields, it is not unexpected. These results show that equivalent detection of *Ac* in naturally infested seed samples can occur with method variation across multiple laboratories and supports a flexible approach to method execution.

Laboratory 6 conducted the method both with and without an inhibitor removal step. The results indicate that the inhibitor removal step may be required based on crop species and the DNA isolation chemistry selected by a given laboratory. The need for a supplemental inhibitor removal step may be identified through the monitoring of the performance of process controls.

Several of the samples characterized as negative did result in the generation of high Ct values resulting in positive or inconclusive results. These are considered false positive detects. These data points may be attributed to several different factors such as a low level of dead *Ac* cells in the negative seed batch selected, a low level of cross-contamination within a laboratory, or cross reactions with one of the qPCR assays used (Table 6) If this were to happen with routine samples, the next step is to perform a bioassay, such as a grow-out, to permit resolution of the infection rate.

SUMMARY AND CONCLUSIONS

The goal of standardizing seed industry testing methods for the detection of *Ac* presented many challenges and opportunities. Working collaboratively to optimize the existing NSHS accredited methods produced an improved and industry aligned protocol, which was submitted to the ISHI-Veg group for adoption as a standard reference method. During this project, it was realized that some degree of method flexibility can be highly beneficial to the method alignment process. It allows laboratories to more accurately state their current methods for testing and also allows for the continuous improvement of the method moving forward. The idea of flexibility in the method is made possible by the concept of performance based testing. To ensure the quality and accuracy of a test result, the intended purpose of the assay, performance parameters and acceptance criteria replace strict, step-by-step instruction methods. Performance based methods guarantee quality and accuracy of the test results, instead of requiring that each step of the process be done in a specific way. The result of defining these parameters and criteria was a flexible reference method that provided testing guidance capable of tightly controlling some method components while allowing flexibility for others.

As new seed-borne pathogen tests continue to be developed, the system for alignment, validation and accreditation should be adjusted to meet the opportunities of next generations testing platforms. The experiments performed in this study demonstrate that testing methods can be developed that deliver valid results while allowing for some process flexibility. An increase in specificity and sensitivity has been shown, as compared to the existing Monsanto method (NSHS Cb1.4). Method optimizations have decreased complexity of the testing process and expanded testing to include squash seed varieties. Finally, the multi-company collaborative data supports method flexibility. Consistent results were achieved with variable centrifugation speeds, DNA isolation kits and PCR chemistries; providing confidence in the quality and accuracy of the method. To ensure optimal method performance, process controls are included to monitor data quality across the stated method variations and crop species in scope. The validation data clearly demonstrates sensitive, specific, accurate and reproducible detection of *Ac* DNA in cucurbit seed.

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APPENDIX: *Ac* Direct qPCR Method Validation.
Annex A.
Experimental References

Section 1: Sensitivity – Experiment III Historical Spike Plating

Date	Average CFU	Date	Average CFU	Date	Average CFU	Date	Average CFU
05.30.12	40	07.05.12	62	09.07.12	62	10.25.12	37
06.01.12	34	07.06.12	72	09.11.12	43	11.01.12	51
06.05.12	44	07.09.12	63	09.12.12	37	11.08.12	41
06.06.12	48	07.10.12	61	09.13.12	47	11.13.12	29
06.07.12	43	07.12.12	47	09.14.12	53	11.14.12	37
06.08.12	48	07.13.12	86	09.18.12	52	11.15.12	35
06.12.12	44	07.17.12	52	09.25.12	36	11.19.12	44
06.13.12	22	07.18.12	62	09.26.12	37	11.21.12	35
06.14.12	49	07.19.12	32	09.27.12	39	11.28.12	53
06.15.12	48	07.20.12	31	10.02.12	49	11.29.12	43
06.19.12	66	07.24.12	63	10.04.12	30	11.30.12	48
06.20.12	39	07.26.12	66	10.09.12	57	12.4.12	43
06.21.12	58	07.27.12	46	10.10.12	30	12.5.12	42
06.22.12	52	07.31.12	48	10.12.12	44	12.6.12	38
06.26.12	38	08.01.12	40	10.16.12	49	12.7.12	35
06.27.12	53	08.02.12	61	10.17.12	42	12.11.12	29
06.28.12	60	08.09.12	46	10.18.12	39	12.12.12	47
06.29.12	64	08.14.12	48	10.19.12	43	12.13.12	41
06.30.12	77	08.15.12	57	10.23.12	52	Average	47.33333
07.03.12	68	09.05.12	43	10.24.12	42	Stdev	11.98448

Table 5: Average CFU recovery by plating 200µL of OD_{600}=0.100x10^{-6} *Ac* cell suspension.

Section 2: Specificity – Inclusivity/Exclusivity

Summary of data from evaluations of selected qPCR assays against *Ac* and related species collections across several industry laboratories.
Isolates were verified as *Ac* by various assessments (e.g., morphology, biochemical) including pathogenicity tests.

Note: Monsanto screening of Zup(Ac) primer set was completed at a later date with an increased concentration of *Ac* DNA as compared with the initial screening of the Contig 21, Contig 22 primer sets (1pg vs. 100pg). This data is used to demonstrate isolate coverage only; conclusions of qPCR assays sensitivity cannot be drawn from this data set.

Isolate Library	Isolate ID	Contig 21	Contig 22	Zup (Ac)
Syngenta	4000	26.76	27.02	24.27
Syngenta	4000	27.49	26.88	25.33
Syngenta	4001	27.19	26.64	25.30
Syngenta	4032	26.59	26.64	23.14
Syngenta	4033	27.06	27.19	22.84
Syngenta	4034	27.15	27.04	22.46
Syngenta	4226	26.77	26.64	22.70
Syngenta	4226	27.12	26.40	23.57
Syngenta	4227	27.75	27.09	24.16
Syngenta	4244	27.20	27.23	22.88
Syngenta	4244	27.44	26.79	23.78
Syngenta	4245	25.69	25.15	21.53
Syngenta	4250	25.07	25.01	20.80
Syngenta	4254	25.37	25.55	21.33
Syngenta	4264	25.21	25.30	21.13
Syngenta	4264	25.52	24.76	21.74
Syngenta	4265	25.47	24.78	21.75
Syngenta	4266	25.83	25.91	21.59
Syngenta	4266	26.50	25.94	22.73
Syngenta	4267	27.07	26.38	23.20
Syngenta	4268	25.84	25.27	21.56
Syngenta	4287	25.29	25.31	21.10
Syngenta	4287	25.94	25.34	22.04

Isolate Library	Isolate ID	Contig 21	Contig 22	Zup (Ac)
Syngenta	4288	25.33	24.74	21.42
Syngenta	4377	25.27	25.90	20.66
Syngenta	4378	25.54	25.54	21.28
Syngenta	4509	25.48	25.41	21.21
Syngenta	4509	26.17	25.42	22.21
Syngenta	4510	26.44	25.68	22.43
Syngenta	4521	24.87	25.16	20.73
Syngenta	4522	25.15	25.17	20.84
Syngenta	4523	25.24	25.31	21.06
Syngenta	4532	24.94	24.96	20.20
Syngenta	4539	25.64	25.73	21.09
Syngenta	4540	25.01	25.02	20.44
Syngenta	4541	27.14	27.12	23.10
Syngenta	4542	25.11	25.09	21.15
Syngenta	4543	26.13	26.34	22.21
Syngenta	4544	26.23	26.38	21.81
Syngenta	4569	25.99	26.01	21.31
Syngenta	4570	25.47	25.38	21.13
Syngenta	4571	25.36	25.40	20.85
Syngenta	4572	26.39	26.41	21.86
Syngenta	4576	25.44	25.55	21.36
Syngenta	4577	23.98	23.98	19.39
Syngenta	4578	24.04	24.02	19.26

Isolate Library	Isolate ID	Contig 21	Contig 22	Zup (Ac)
Syngenta	4579	24.24	24.12	19.80
Syngenta	4580	24.88	24.77	20.48
Syngenta	4606	26.23	26.06	21.90
Syngenta	4607	26.18	25.97	22.02
Syngenta	4608	26.12	25.99	21.68
Syngenta	4609	27.01	26.82	22.58
Syngenta	4610	26.96	26.56	22.54
Syngenta	4611	26.42	26.27	22.06
Syngenta	4612	29.10	29.05	25.65
Syngenta	4630	26.05	25.93	21.23
Syngenta	4631	26.16	26.07	21.43
Syngenta	4632	26.32	26.19	22.14
Syngenta	4633	26.12	26.04	21.91
Syngenta	4657	26.35	26.26	21.74
Syngenta	4657	26.27	25.69	22.19
Syngenta	4658	27.08	26.40	22.87
Syngenta	4659	24.56	24.57	19.97
Syngenta	4659	25.08	24.61	21.07
Syngenta	4660	25.74	25.22	21.68
Syngenta	4661	27.92	27.73	23.71
Syngenta	4661	26.06	25.15	22.07
Syngenta	4662	25.71	25.03	21.86
Syngenta	4663	25.52	25.67	21.05
Syngenta	4664	26.86	26.67	22.10
Syngenta	4664	25.92	25.18	21.44
Syngenta	4665	25.56	24.95	21.21
Syngenta	4683	25.58	25.35	21.11
Syngenta	4693	26.61	26.40	22.12
Syngenta	4694	27.50	27.17	23.11
Syngenta	4695	26.64	26.33	22.22
Syngenta	4708	26.25	26.02	21.54
Syngenta	4709	28.91	28.39	24.75
Syngenta	4710	26.29	26.17	22.08
Syngenta	4711	27.35	27.09	22.84
Syngenta	4712	27.94	27.08	24.32
Syngenta	4713	25.86	24.90	21.74
Syngenta	4718	26.06	25.17	21.60
Syngenta	4719	26.22	25.25	22.23
Syngenta	4720	25.58	24.53	21.54
Syngenta	4721	25.43	24.49	21.19
Syngenta	4722	25.10	24.16	20.56
Syngenta	4723	25.35	24.54	20.85
Syngenta	4808	26.68	26.02	22.60
Syngenta	4851	24.49	23.70	20.31
HM Clause	A13	23.15	22.64	20.40
HM Clause	A23	23.34	23.06	20.60
HM Clause	A24	22.92	22.44	20.10
HM Clause	A25	22.63	22.23	19.84
HM Clause	A26	23.15	22.67	20.27
HM Clause	A27	22.67	22.42	20.02
HM Clause	A28	22.89	22.37	20.16
HM Clause	A33	22.71	22.38	20.49
HM Clause	A34	23.50	22.94	20.69
HM Clause	A73	22.89	22.23	20.28
HM Clause	A76	22.93	22.23	20.30
HM Clause	A118	22.35	21.86	19.77
HM Clause	A122	22.95	22.34	20.11
Monsanto	*Ac* - 1	22.34	28.51	19.00
Monsanto	*Ac* - 2	29.78	28.72	18.72
Monsanto	*Ac* - 3	28.70	28.50	18.26
Monsanto	*Ac* - 4	26.75	29.46	18.70
Monsanto	*Ac* - 5	27.74	28.42	18.89

Isolate Library	Isolate ID	Contig 21	Contig 22	Zup (Ac)
Monsanto	*Ac* - 6	31.46	29.10	18.11
Monsanto	*Ac* - 7	30.86	29.09	19.13
Monsanto	*Ac* - 8	29.57	28.22	18.44
Monsanto	*Ac* - 9	29.39	28.88	18.65
Monsanto	*Ac* - 10	29.71	28.78	18.08
Monsanto	*Ac* - 12	31.33	27.97	17.60
Monsanto	*Ac* - 13	29.61	28.29	17.94
Monsanto	*Ac* - 14	29.08	28.91	18.51
Monsanto	*Ac* - 15	30.48	27.89	18.02
Monsanto	*Ac* - 16	29.45	28.86	18.96
Monsanto	*Ac* - 17	31.83	27.80	18.57
Monsanto	*Ac* - 18	30.65	28.10	18.11
Monsanto	*Ac* - 19	31.36	29.98	18.26
Monsanto	*Ac* - 20	30.61	28.75	18.61
Monsanto	*Ac* - 21	30.56	28.84	18.26
Monsanto	*Ac* - 22	30.18	28.59	19.32
Monsanto	*Ac* - 23	29.17	28.10	18.97
Monsanto	*Ac* - 24	29.28	27.71	18.53
Monsanto	*Ac* - 25	29.18	26.69	18.90
Monsanto	*Ac* - 26	30.40	28.18	18.14
Monsanto	*Ac* - 27	28.35	28.22	18.89
Monsanto	*Ac* - 28	24.74	28.63	18.93
Monsanto	*Ac* - 29	25.57	28.29	18.50
Monsanto	*Ac* - 30	29.91	27.93	17.98
Monsanto	*Ac* - 31	27.55	28.41	19.20
Monsanto	*Ac* - 32	30.16	28.44	18.40
Monsanto	*Ac* - 33	28.12	28.19	19.12
Monsanto	*Ac* - 34	29.22	28.82	18.40
Monsanto	*Ac* - 35	30.51	29.11	17.94
Monsanto	*Ac* - 36	29.57	29.20	18.60
Monsanto	*Ac* - 38	30.15	28.69	18.31
Monsanto	*Ac* - 66	28.84	29.16	18.80
Monsanto	*Ac* - 67	24.21	28.63	18.86
Monsanto	*Ac* - 68	26.70	27.91	18.71
Monsanto	*Ac* - 74	29.55	29.16	18.87
Monsanto	*Ac* - 76	29.67	27.71	18.68
Monsanto	*Ac* - 91	30.46	28.12	18.61
Monsanto	*Ac* - 98	28.91	27.85	18.68
Monsanto	*Ac* - 114	30.09	28.55	18.43
Monsanto	*Ac* - 155	29.54	28.09	18.38
Monsanto	*Ac* - 162	30.03	28.82	18.45
Monsanto	*Ac* - 167	29.73	29.03	18.33
Monsanto	*Ac* - 173	30.21	28.48	18.22
Monsanto	*Ac* - 174	29.74	28.97	18.97
Monsanto	*Ac* - 175	29.26	28.95	18.49
Monsanto	*Ac* - 181	29.13	28.18	18.53
Monsanto	*Ac* - 185	30.24	28.93	18.06
Monsanto	*Ac* - 186	27.88	27.62	18.36
Monsanto	*Ac* - 187	29.43	27.62	18.17
Monsanto	*Ac* - 188	24.57	27.95	18.47
Monsanto	*Ac* - 190	28.87	27.47	18.47
Monsanto	*Ac* - 191	29.26	29.05	18.96
Monsanto	*Ac* - 192	30.04	28.79	17.48
Monsanto	*Ac* - 193	29.49	28.82	18.15
Monsanto	*Ac* - 194	29.13	27.66	18.66
Monsanto	*Ac* - 195	31.12	28.72	18.94
Monsanto	*Ac* - 196	30.00	28.74	18.73
Monsanto	*Ac* - 197	30.26	28.46	18.72
Monsanto	*Ac* - 198	28.75	28.46	18.55
Monsanto	*Ac* - 199	30.10	28.48	19.09
Monsanto	*Ac* - 208	29.73	27.98	18.34
Monsanto	*Ac* - 213	29.56	28.45	18.99

Isolate Library	Isolate ID	Contig 21	Contig 22	Zup (Ac)
Monsanto	*Ac* - 214	29.04	28.40	19.02
Monsanto	*Ac* - 215	28.69	28.05	18.35
Monsanto	*Ac* - 216	29.95	28.11	18.97
Monsanto	*Ac* - 217	30.21	29.21	18.92
Monsanto	*Ac* - 218	30.84	27.79	17.98
Monsanto	*Ac* - 219	30.40	28.89	18.95
Monsanto	*Ac* - 220	29.96	29.37	19.01
Monsanto	*Ac* - 221	29.51	28.14	18.31
Monsanto	*Ac* - 222	28.44	28.60	18.33
Monsanto	*Ac* - 223	29.62	27.71	18.21
Monsanto	*Ac* - 224	29.92	29.04	18.92
Monsanto	*Ac* - 225	30.25	27.59	17.76
Monsanto	*Ac* - 226	30.19	28.36	17.66
Monsanto	*Ac* - 227	29.59	28.42	17.99
Monsanto	*Ac* - 228	30.14	28.22	18.85
Monsanto	*Ac* - 229	30.76	28.08	18.01
Monsanto	*Ac* - 230	28.30	28.11	18.51
Monsanto	*Ac* - 231	29.61	27.09	18.69
Monsanto	*Ac* - 232	29.83	27.79	18.91
Monsanto	*Ac* - 233	30.91	28.87	18.59
Monsanto	*Ac* - 234	29.25	27.77	18.68
Monsanto	*Ac* - 235	30.62	27.77	17.91
Monsanto	*Ac* - 236	28.81	27.25	18.04
Monsanto	*Ac* - 237	30.18	28.52	18.20
Monsanto	*Ac* - 238	32.17	28.48	18.14
Monsanto	*Ac* - 239	31.30	28.02	18.60
Monsanto	*Ac* - 240	30.33	28.87	18.74
Monsanto	*Ac* - 241	28.67	28.41	18.98
Monsanto	*Ac* - 242	29.02	27.92	18.63
Monsanto	*Ac* - 243	29.90	28.13	19.08
Monsanto	*Ac* - 245	28.74	27.79	18.96
Monsanto	*Ac* - 246	29.23	27.52	18.69
Monsanto	*Ac* - 247	30.04	27.90	18.90
Monsanto	*Ac* - 248	29.74	28.55	18.29
Monsanto	*Ac* - 249	31.69	28.82	18.38
Monsanto	*Ac* - 250	31.11	28.48	18.70
Monsanto	*Ac* - 251	30.29	29.16	18.20
Monsanto	*Ac* - 252	28.96	29.70	17.73
Monsanto	*Ac* - 253	30.80	29.00	18.39
Monsanto	*Ac* - 254	31.42	28.34	18.55
Monsanto	*Ac* - 255	31.99	29.23	18.00
Monsanto	*Ac* - 256	29.37	28.88	18.33
Monsanto	*Ac* - 257	26.25	27.80	18.33
Monsanto	*Ac* - 258	28.57	28.96	18.80
Monsanto	*Ac* - 259	30.14	28.29	18.93
Monsanto	*Ac* - 260	26.61	28.55	19.26
Monsanto	*Ac* - 261	31.81	28.29	18.40
Monsanto	*Ac* - 264	29.35	27.59	19.15
Monsanto	*Ac* - 266	30.99	28.35	18.92
Monsanto	*Ac* - 267	30.61	28.78	18.15
Monsanto	*Ac* - 268	30.59	29.74	17.90
Monsanto	*Ac* - 269	31.62	29.14	18.47
Monsanto	*Ac* - 270	30.90	28.88	18.19
Monsanto	*Ac* - 271	31.28	28.38	18.25
Monsanto	*Ac* - 272	31.70	29.29	18.33
Monsanto	*Ac* - 273	29.79	29.66	18.97
Monsanto	*Ac* - 274	29.95	28.92	17.95
Monsanto	*Ac* - 275	30.56	27.50	18.03
Monsanto	*Ac* - 276	30.37	28.01	17.73
Monsanto	*Ac* - 277	29.90	29.79	18.59
Monsanto	*Ac* - 278	30.40	29.35	18.55
Monsanto	*Ac* - 279	31.72	27.76	18.93

Isolate Library	Isolate ID	Contig 21	Contig 22	Zup (Ac)
Monsanto	*Ac* - 280	29.76	28.64	18.57
Monsanto	*Ac* - 281	27.16	28.48	18.62
Monsanto	*Ac* - 282	28.56	28.65	18.74
Monsanto	*Ac* - 283	30.40	29.02	18.17
Monsanto	*Ac* - 284	30.46	29.05	18.50
Monsanto	*Ac* - 285	29.44	28.08	18.17
Monsanto	*Ac* - 286	28.88	28.82	18.08
Monsanto	*Ac* - 287	30.16	28.28	18.36
Monsanto	*Ac* - 288	30.25	29.09	18.77
Monsanto	*Ac* - 289	30.08	28.77	18.27
Monsanto	*Ac* - 290	30.84	26.71	18.42
Monsanto	*Ac* - 291	30.24	28.79	18.22
Monsanto	*Ac* - 292	29.83	28.76	18.46
Monsanto	*Ac* - 293	29.87	27.15	18.23
Monsanto	*Ac* - 294	29.49	28.54	18.16
Monsanto	*Ac* - 295	27.83	27.39	17.99
Monsanto	*Ac* - 296	29.79	28.31	18.07
Monsanto	*Ac* - 297	29.67	27.65	18.38
Monsanto	*Ac* - 298	30.27	28.93	18.06
Monsanto	*Ac* - 299	29.28	28.96	17.98
Monsanto	*Ac* - 300	30.41	28.64	17.80
Monsanto	*Ac* - 301	29.44	29.30	18.49
Monsanto	*Ac* - 302	31.44	28.55	18.85
Monsanto	*Ac* - 303	29.16	28.90	18.35
Monsanto	*Ac* - 304	29.97	29.82	18.18
Monsanto	*Ac* - 305	29.55	29.57	18.32
Monsanto	*Ac* - 306	29.84	27.91	18.48
Monsanto	*Ac* - 307	30.23	28.70	19.55
Monsanto	*Ac* - 308	29.73	29.06	18.85
Monsanto	*Ac* - 309	28.63	28.74	18.30
Monsanto	*Ac* - 310	28.81	28.53	18.28
Monsanto	*Ac* - 311	28.85	28.71	18.46
Monsanto	*Ac* - 313	29.13	27.95	18.21
Monsanto	*Ac* - 314	29.45	29.27	18.23
Monsanto	*Ac* - 315	29.82	28.36	18.15
Monsanto	*Ac* - 316	29.97	27.74	18.11
Monsanto	*Ac* - 317	29.75	29.40	18.45
Monsanto	*Ac* - 318	30.31	29.04	18.46
Monsanto	*Ac* - 319	29.78	29.17	18.06
Monsanto	*Ac* - 320	28.97	27.14	17.63
Monsanto	*Ac* - 321	28.72	29.09	17.99
Monsanto	*Ac* - 322	29.11	29.10	17.88
Monsanto	*Ac* - 323	28.19	27.44	17.46
Monsanto	*Ac* - 324	27.35	26.51	17.96
Monsanto	*Ac* - 325	29.31	29.55	17.67
Monsanto	*Ac* - 326	28.52	28.88	17.55
Monsanto	*Ac* - 327	28.97	27.53	18.37
Monsanto	*Ac* - 328	28.62	28.21	18.06
Monsanto	*Ac* - 329	28.16	28.12	17.75
Monsanto	*Ac* - 330	29.12	28.68	17.85
Monsanto	*Ac* - 331	29.12	28.68	17.59
Monsanto	*Ac* - 332	30.12	28.93	18.92
Monsanto	*Ac* - 333	28.76	29.27	17.58
Monsanto	*Ac* - 334	29.26	28.06	17.69
Monsanto	*Ac* - 335	28.58	28.32	17.71
Monsanto	*Ac* - 336	28.48	27.99	18.08
Monsanto	*Ac* - 337	29.16	28.80	17.50
Monsanto	*Ac* - 338	29.25	29.15	18.14
Monsanto	*Ac* - 339	29.08	29.11	17.92
Monsanto	*Ac* - 340	29.35	25.75	17.09
Monsanto	*Ac* - 341	28.75	28.49	17.93
Monsanto	*Ac* - 342	27.74	27.67	18.17

Isolate Library	Isolate ID	Contig 21	Contig 22	Zup (Ac)
Monsanto	*Ac* - 343	29.04	28.53	18.02
Monsanto	*Ac* - 344	29.13	27.59	18.23
Monsanto	*Ac* - 345	29.82	28.57	17.94
Monsanto	*Ac* - 346	30.81	28.79	18.43
Monsanto	*Ac* - 347	29.29	29.21	17.78
Monsanto	*Ac* - 348	28.67	28.64	17.83
Monsanto	*Ac* - 349	29.65	28.86	17.86
Monsanto	*Ac* - 350	29.17	29.21	17.58
Monsanto	*Ac* - 351	28.51	28.62	17.91
Monsanto	*Ac* - 352	29.67	28.14	18.51
Monsanto	*Ac* - 353	28.14	27.87	17.90
Monsanto	*Ac* - 354	30.19	29.30	18.66
Monsanto	*Ac* - 355	28.70	28.16	17.45
Monsanto	*Ac* - 356	28.89	28.59	18.14
Monsanto	*Ac* - 357	30.90	28.38	17.90
Monsanto	*Ac* - 358	29.82	28.40	17.80
Monsanto	*Ac* - 359	29.17	27.66	18.20
Monsanto	*Ac* - 360	29.81	27.65	18.15
Monsanto	*Ac* - 361	28.78	28.51	17.91
Monsanto	*Ac* - 362	30.19	28.39	18.64
Monsanto	*Ac* - 363	27.80	28.62	19.02
Monsanto	*Ac* - 364	29.21	29.00	19.23
Monsanto	*Ac* - 365	28.45	29.11	19.08
Monsanto	*Ac* - 366	29.90	27.66	18.56
Monsanto	*Ac* - 367	29.80	29.12	18.92
Monsanto	*Ac* - 368	29.80	28.13	18.44
Monsanto	*Ac* - 369	29.23	29.06	18.92
Monsanto	*Ac* - 370	29.71	29.21	17.95
Monsanto	*Ac* - 371	30.38	28.66	18.58
Monsanto	*Ac* - 372	30.70	28.66	18.12
Monsanto	*Ac* - 373	30.15	25.66	17.31
Monsanto	*Ac* - 374	29.78	27.15	18.88
Monsanto	*Ac* - 375	27.65	27.26	18.35
Monsanto	*Ac* - 376	30.51	28.13	19.24
Monsanto	*Ac* - 377	29.80	27.80	19.08
Monsanto	*Ac* - 378	29.17	28.00	19.27
Monsanto	*Ac* - 379	30.23	28.39	19.04
Monsanto	*Ac* - 380	32.17	32.01	19.17
Monsanto	*Ac* - 381	30.12	29.41	19.05
Monsanto	*Ac* - 382	29.44	30.58	19.52
Monsanto	*Ac* - 383	29.54	29.17	19.10
Monsanto	*Ac* - 384	28.30	28.98	19.42
Monsanto	*Ac* - 385	29.48	28.08	18.14
Monsanto	*Ac* - 386	30.67	28.86	18.18
Monsanto	*Ac* - 387	29.16	29.48	18.99
Monsanto	*Ac* - 388	30.50	27.53	17.98
Monsanto	*Ac* - 389	29.86	28.63	18.66
Monsanto	*Ac* - 390	29.91	29.46	19.44
Monsanto	*Ac* - 391	30.08	29.66	19.48
Monsanto	*Ac* - 392	26.94	26.59	19.41
Monsanto	*Ac* - 393	28.34	28.66	19.31
Monsanto	*Ac* - 394	28.86	28.07	18.57
Monsanto	*Ac* - 395	30.31	28.43	18.89
Monsanto	*Ac* - 396	29.00	28.92	18.14
Monsanto	*Ac* - 397	29.49	29.00	17.64
Monsanto	*Ac* - 398	28.43	28.45	18.20
Monsanto	*Ac* - 399	28.59	28.41	18.10
Monsanto	*Ac* - 400	28.21	28.31	18.03
Monsanto	*Ac* - 418	29.41	28.15	17.51
Monsanto	*Ac* - 419	29.00	27.91	18.69
Monsanto	*Ac* - 420	28.20	27.24	18.11
Monsanto	*Ac* - 421	27.99	28.59	17.56

Isolate Library	Isolate ID	Contig 21	Contig 22	Zup (Ac)
Monsanto	*Ac* - 422	29.44	28.43	17.56
Monsanto	*Ac* - 423	28.05	28.36	17.95
Monsanto	*Ac* - 424	29.84	27.46	18.64
Monsanto	*Ac* - 425	28.53	28.95	17.88
Monsanto	*Ac* - 426	28.73	28.31	18.13
Monsanto	*Ac* - 427	30.88	28.85	18.77
Monsanto	*Ac* - 428	30.87	29.84	18.51
Monsanto	*Ac* - 429	28.51	28.56	17.86
Monsanto	*Ac* - 430	28.95	27.97	17.88
Monsanto	*Ac* - 437	27.65	28.24	17.99
Monsanto	*Ac* - 438	28.04	28.12	18.42
Monsanto	*Ac* - 439	28.42	28.67	17.70
Monsanto	*Ac* - 440	28.96	28.36	17.69
Monsanto	*Ac* - 441	35.65	27.14	17.72
Monsanto	*Ac* - 442	29.12	28.42	18.14
Monsanto	*Ac* - 443	29.00	28.60	17.60
Monsanto	*Ac* - 444	29.46	28.36	17.29
Monsanto	*Ac* - 445	29.72	28.27	18.66
Monsanto	*Ac* - 446	30.76	28.73	17.79
Monsanto	*Ac* - 447	30.76	27.08	18.93
Monsanto	*Ac* - 448	31.26	28.01	18.16
Monsanto	*Ac* - 449	30.28	27.33	18.01
Monsanto	*Ac* - 450	30.68	27.68	17.98
Monsanto	*Ac* - 451	29.07	27.96	17.59
Monsanto	*Ac* - 452	28.66	28.65	17.77
Monsanto	*Ac* - 453	29.06	28.55	17.69
Monsanto	*Ac* - 454	29.03	28.44	17.93
Monsanto	*Ac* - 455	28.65	28.95	17.66
Monsanto	*Ac* - 456	28.57	28.68	18.76
Monsanto	*Ac* - 457	31.43	27.09	18.26
Monsanto	*Ac* - 458	30.97	28.65	17.92
Monsanto	*Ac* - 459	31.37	28.58	18.77
Monsanto	*Ac* - 460	31.61	28.40	18.08
Monsanto	*Ac* - 461	30.86	28.66	18.23
Monsanto	*Ac* - 462	31.50	28.04	18.23
Monsanto	*Ac* - 463	30.14	27.75	18.25
Monsanto	*Ac* - 464	31.00	28.03	17.76
Monsanto	*Ac* - 465	31.39	27.96	17.64
Monsanto	*Ac* - 466	31.05	28.00	17.92
Monsanto	*Ac* - 467	31.65	26.34	18.19
Monsanto	*Ac* - 468	31.38	28.18	18.18
Monsanto	*Ac* - 469	31.54	27.63	18.36
Monsanto	*Ac* - 470	30.84	29.26	18.45
Monsanto	*Ac* - 471	31.09	28.24	18.30
Monsanto	*Ac* - 472	30.18	28.90	18.93
Monsanto	*Ac* - 473	30.54	29.07	18.82
Monsanto	*Ac* - 474	27.81	27.39	19.20
Monsanto	*Ac* - 475	31.03	27.86	17.96
Monsanto	*Ac* - 476	30.68	28.41	18.99
Monsanto	*Ac* - 477	28.43	28.07	18.24
Monsanto	*Ac* - 478	30.72	26.93	18.40
Monsanto	*Ac* - 479	30.64	28.00	18.20
Monsanto	*Ac* - 480	30.32	27.09	18.22
Monsanto	*Ac* - 481	30.52	28.71	18.03
Monsanto	*Ac* - 482	28.86	27.63	18.22
Monsanto	*Ac* - 483	31.40	27.87	18.17
Monsanto	*Ac* - 484	29.95	27.68	17.90
Monsanto	*Ac* - JC I	30.37	27.67	17.73
Monsanto	*Ac* - JC II	30.29	27.65	17.65
Monsanto	*Ac* - F09-274-1	30.70	27.12	18.24
Monsanto	*Ac* - F09-265-1	31.02	28.29	17.92
Bayer CropScience	134	24.12	24.01	21.15

Isolate Library	Isolate ID	Contig 21	Contig 22	Zup (Ac)
Bayer CropScience	135	24.83	24.63	23.10
Bayer CropScience	156	24.12	23.88	21.39
Bayer CropScience	160	23.82	23.74	20.49
Bayer CropScience	161	24.05	23.99	20.88
Bayer CropScience	162	24.44	24.25	21.53
Bayer CropScience	163	24.71	24.37	22.00
Bayer CropScience	204	25.24	24.92	23.29
Bayer CropScience	205	25.18	24.86	23.17
Bayer CropScience	227	23.92	24.36	22.76
Bayer CropScience	228	23.03	23.53	21.75
Bayer CropScience	468	24.11	30.42	22.94
Bayer CropScience	908	26.58	25.98	23.99
Bayer CropScience	909	26.26	25.96	24.75
Bayer CropScience	926	25.46	24.96	23.89
Bayer CropScience	927	25.93	25.36	23.80
Bayer CropScience	931	23.56	23.58	20.47
Bayer CropScience	932	23.97	23.98	21.05
Bayer CropScience	943	24.97	24.82	22.42
Bayer CropScience	949	26.63	25.84	24.31
Bayer CropScience	951	24.46	24.09	21.71
Bayer CropScience	952	23.59	23.77	20.62
Bayer CropScience	953	23.76	24.09	20.67
Bayer CropScience	954	24.83	24.91	21.84
Bayer CropScience	955	24.73	24.89	22.01
Bayer CropScience	956	24.04	24.23	21.23
Bayer CropScience	957	24.40	24.60	21.93
Bayer CropScience	958	25.22	25.25	23.25
Bayer CropScience	959	24.51	24.28	23.80
Bayer CropScience	960	25.40	25.37	22.49
Bayer CropScience	975	25.34	25.29	22.76
Bayer CropScience	976	24.30	24.25	21.40
Bayer CropScience	989	25.38	25.30	23.28
Bayer CropScience	990	23.06	23.08	20.07
Bayer CropScience	995	23.81	23.91	20.83
Bayer CropScience	997	24.03	24.19	21.32
Bayer CropScience	998	24.22	24.21	21.48
Bayer CropScience	999	23.72	23.73	20.77
Bayer CropScience	5637	23.75	23.79	20.75
Rijk Zwaan	1	27.30	27.13	22.69
Rijk Zwaan	2	20.00	20.73	15.86
Rijk Zwaan	3	19.87	20.43	15.57
Rijk Zwaan	4	21.90	20.39	15.94
Rijk Zwaan	5	20.03	20.13	15.24
Rijk Zwaan	6	20.38	20.18	15.45
Rijk Zwaan	7	20.37	20.10	14.89
Rijk Zwaan	8	21.02	21.31	16.11
Rijk Zwaan	9	20.27	20.29	15.58
Rijk Zwaan	10	20.39	20.45	15.92
Rijk Zwaan	11	19.10	19.57	14.56
Rijk Zwaan	12	20.58	20.43	15.14

Isolate Library	Isolate ID	Contig 21	Contig 22	Zup (Ac)
Rijk Zwaan	13	19.63	20.01	15.18
Rijk Zwaan	14	21.25	21.16	16.28
Rijk Zwaan	15	20.83	20.49	15.88
Rijk Zwaan	16	20.11	20.17	15.44
Rijk Zwaan	17	21.98	21.55	16.78
Rijk Zwaan	18	21.45	21.81	16.19
Rijk Zwaan	19	20.27	20.15	15.70
Rijk Zwaan	20	20.67	20.40	16.28
Rijk Zwaan	21	21.40	20.78	15.91
Rijk Zwaan	22	18.94	19.30	14.32
Rijk Zwaan	23	22.22	20.03	15.09
Rijk Zwaan	24	20.03	20.55	15.62
Rijk Zwaan	25	22.20	21.37	16.12
Rijk Zwaan	27	21.76	21.28	16.66
Rijk Zwaan	28	21.36	21.15	16.91
Rijk Zwaan	30	20.67	20.34	15.10
Rijk Zwaan	31	20.02	19.93	15.02
Rijk Zwaan	32	26.08	25.77	22.06
Rijk Zwaan	33	19.81	20.20	15.46
Rijk Zwaan	34	19.34	19.36	14.72
Rijk Zwaan	35	21.48	21.84	16.51
Rijk Zwaan	37	23.16	23.04	18.40
Rijk Zwaan	38	19.60	19.83	15.43
Rijk Zwaan	39	20.62	20.52	15.83
Rijk Zwaan	42	20.79	20.50	16.24
Rijk Zwaan	43	20.55	20.36	16.45
Rijk Zwaan	44	21.62	21.36	16.39
Rijk Zwaan	45	20.23	20.29	16.22
Rijk Zwaan	46	20.41	20.12	15.17
Rijk Zwaan	47	20.94	20.40	15.77
Rijk Zwaan	48	20.83	20.96	16.71
Rijk Zwaan	53	19.31	19.45	14.25
Rijk Zwaan	55	20.04	20.21	15.14
Rijk Zwaan	57	19.05	19.61	14.26
Rijk Zwaan	58	19.51	19.11	14.20
Rijk Zwaan	59	18.74	19.16	13.67
Rijk Zwaan	60	20.24	19.26	14.44
Rijk Zwaan	61	19.98	19.88	14.75
Rijk Zwaan	62	20.71	20.39	16.16
Rijk Zwaan	63	20.01	19.72	15.08
Rijk Zwaan	64	19.59	20.03	15.71
Rijk Zwaan	65	20.28	20.08	15.83
Rijk Zwaan	66	20.89	20.80	16.46
Rijk Zwaan	67	19.07	19.51	15.57
Rijk Zwaan	68	20.66	20.88	15.89
Rijk Zwaan	69	20.50	20.03	15.28
Rijk Zwaan	70	20.24	20.28	15.44
Rijk Zwaan	71	19.62	19.15	14.25
Rijk Zwaan	72	20.50	20.95	15.58

Non-*Ac*, and *Ac*-related Isolate Data

Isolate Library	Isolate ID	Confirmed *Ac* Pathogenicity	Species	Contig 21	Contig 22	SynZup
Syngenta	4251	No	Undetermined "*Ac*-Like"	-	-	26.65
Syngenta	4507	No	Undetermined "*Ac*-Like"	-	-	-
Syngenta	4508	No	Undetermined "*Ac*-Like"	-	-	-
Syngenta	4591	No	Undetermined	-	-	25.49
Syngenta	4592	No	Undetermined	-	-	25.16
Syngenta	4593	No	Undetermined	-	-	24.81
Syngenta	4594	No	Undetermined	-	-	26.15
Syngenta	4595	No	Undetermined	-	-	39.39
Syngenta	4596	No	Undetermined	-	-	23.06
Syngenta	4597	No	Undetermined	-	-	-
Syngenta	4598	No	Undetermined	-	-	-
Syngenta	4599	No	Undetermined	-	-	-
Syngenta	4600	No	Undetermined	-	-	25.62
Syngenta	4601	No	Undetermined	-	-	24.11
Syngenta	4602	No	Undetermined	-	-	25.18
Syngenta	4603	No	Undetermined	-	-	-
Syngenta	4604	No	Undetermined	-	-	-
Syngenta	4605	No	Undetermined; "*Ac*-Like"	-	36.83	28.35
Syngenta	4852	No	Undetermined	-	-	-
HM Clause	A42	No	"Cmm-like"	-	-	-
HM Clause	A43	No	"Cmm-like"	-	-	-
HM Clause	A44	No	"Cmm-like"	-	-	-
HM Clause	A45	No	"Cmm-like"	-	-	-
HM Clause	A46	No	Clavibacter michiganensis michiganensis	-	-	-
HM Clause	*Xv*	No	Xanthomonas campestris vesicatoria	-	-	-
HM Clause	Xcc	No	Xanthomonas campestris campestris	-	-	-
HM Clause	Pst	No	Pseudomonas syringae tomato	-	-	-
Monsanto	*Ac* – 244	No	Undetermined; "*Ac*-Like"	-	-	20.50
Bayer CropScience	130	No	Acidovorax spp.	-	-	-
Bayer CropScience	137	No	Acidovorax spp.	-	-	-
Bayer CropScience	148	No	Acidovorax spp.	-	-	-
Bayer CropScience	427	No	Undetermined "*Ac*-Like"	-	-	-
Bayer CropScience	941	No	Undetermined "*Ac*-Like"	-	-	38.08
Bayer CropScience	977	No	Undetermined "*Ac*-Like"	-	-	-
Bayer CropScience	271	No	Pseudomonas syringae lachrymans	-	-	36.95
Bayer CropScience	283	No	Pseudomonas syringae lachrymans	-	-	-
Bayer CropScience	415	No	Pseudomonas syringae lachrymans	-	38.03	37.43
Bayer CropScience	913	No	Pseudomonas syringae lachrymans	-	-	37.83
Bayer CropScience	966	No	Pseudomonas syringae lachrymans	-	-	-
Bayer CropScience	136	No	Acidovorax facilis	-	-	-
Bayer CropScience	138	No	Acidovorax konjaci	-	-	-
Bayer CropScience	139	No	Acidovorax cattleyae	-	-	37.77
Bayer CropScience	143	No	Acidovorax valerianellae	-	-	-
Bayer CropScience	145	No	Acidovorax cattleyae	-	-	-
Bayer CropScience	150	No	Acidovorax valerianellae	-	-	-
Bayer CropScience	154	No	Acidovorax valerianellae	-	-	-
Bayer CropScience	925	No	Acidovorax valerianellae	-	-	-
Bayer CropScience	973	No	Acidovorax valerianellae	-	-	-
Rijk Zwaan	101	No	Acidovorax cattleyae	-	-	-
Rijk Zwaan	102	No	Acidovorax cattleyae	-	-	-
Rijk Zwaan	103	No	Acidovorax cattleyae	-	-	24.57
Rijk Zwaan	104	No	Acidovorax cattleyae	-	-	-

Section 3: Selectivity

	Contig 21			Contig 22			*Xv*		
Total *Ac* DNA	Melon matrix	Watermelon matrix	Squash matrix	Melon matrix	Watermelon matrix	Squash matrix	Melon matrix	Watermelon matrix	Squash matrix
10ng	14.267	14.520	14.550	14.611	14.457	14.811	14.479	14.556	14.369
	14.369	14.556	14.479	14.434	14.408	14.643	14.550	14.520	14.267
	14.759	14.579	14.554	14.513	14.376	14.755	14.554	14.579	14.759
1ng	17.702	17.737	17.669	17.852	17.716	18.206	17.669	17.737	17.702
	17.649	17.655	17.834	17.836	17.671	18.181	17.834	17.655	17.649
	18.458	17.737	17.871	17.765	17.780	18.130	17.871	17.737	18.458
100pg	21.134	20.933	21.481	21.314	20.958	21.634	21.312	21.265	22.157
	21.032	21.185	21.338	21.336	21.128	21.561	21.338	21.185	21.032
	22.157	21.265	21.312	21.201	21.013	21.562	21.481	20.933	21.134
10pg	24.557	24.682	24.807	24.525	24.438	24.793	24.651	24.509	25.853
	24.355	24.691	24.813	24.599	24.458	24.850	24.807	24.682	24.557
	25.853	24.509	24.651	24.650	24.450	24.770	24.813	24.691	24.355
1pg	27.857	27.928	28.467	28.013	27.799	28.231	27.978	28.223	27.755
	27.755	28.223	27.978	27.912	27.790	28.407	28.012	27.953	28.686
	28.686	27.953	28.012	27.827	27.564	28.399	28.467	27.928	27.857
100fg	31.711	31.645	31.583	31.848	31.149	31.840	31.461	30.894	32.424
	30.736	31.574	31.521	31.365	30.574	31.617	31.521	31.574	30.736
	32.424	30.894	31.461	31.330	31.326	31.900	31.583	31.645	31.711

Mean Amplification Efficiency			
Matrix Type	Contig 21	Contig 22	*Xv*
None	98.217 %	96.103 %	96.964 %
Melon	97.399 %	97.319 %	97.093 %
Watermelon	98.377 %	99.830 %	98.101 %
Squash	97.685 %	99.862 %	96.160 %

Table 7: Matrix standard curve data and mean amplification efficiency of qPCR assays for *Ac* and *Xv* with respect to crop species.

Section 4: Trueness – Reference Sample Characterization

Reference Seed Sample	Aliquot	Contig 21	Contig 22	*Xv*
1	A	25.837	26.146	25.146
		25.821	26.396	24.643
	B	25.856	26.380	25.307
		25.836	26.293	24.882
	C	26.341	26.344	25.414
		26.296	26.366	24.991
	Ct Mean	25.998	26.321	25.064
	Ct σ	0.249	0.093	0.284
2	A	25.681	26.186	25.348
		25.752	26.245	25.164
	B	25.457	25.757	25.010
		25.375	25.824	24.809
	C	25.688	26.295	24.930
		25.717	26.141	25.294
	Ct Mean	25.612	26.075	25.093
	Ct σ	0.156	0.227	0.212
3	A	25.591	26.386	25.793
		25.744	26.229	25.684
	B	25.880	26.293	25.909
		25.978	26.596	25.678
	C	25.749	25.980	25.487
		25.719	26.128	25.306
	Ct Mean	25.777	26.269	25.643
	Ct σ	0.135	0.213	0.216
4	A	25.489	25.633	25.612

		25.531	25.684	25.824
	B	25.627	25.745	25.903
		25.649	25.732	25.710
	C	26.138	26.354	25.797
		26.172	26.154	26.014
	Ct Mean	25.768	25.884	25.810
	Ct σ	0.306	0.296	0.141
5	A	25.542	25.643	25.315
		25.505	25.730	25.306
	B	25.314	25.594	25.212
		25.409	25.690	25.009
	C	25.488	25.736	25.337
		25.529	25.783	24.316
	Ct Mean	25.464	25.696	25.083
	Ct σ	0.087	0.069	0.395
6	A	25.534	25.738	25.494
		25.568	25.792	25.639
	B	25.740	25.866	25.197
		25.823	25.973	25.651
	C	25.743	25.922	25.918
		25.768	25.999	26.136
	Ct Mean	25.696	25.882	25.672
	Ct σ	0.117	0.103	0.327

Table 8: Characterization of a standard reference sample spiked with *Ac* and *Xv*

Section 5: Trueness – Crop Species Bias

Samples with the same starting number are from the same 5000 seed sample. Decimal place number indicates the aliquot taken from the sample. PCR were run in duplicate.

Melon			
Sample	**Contig 21 CT**	**Contig 22 CT**	**Xcv CT**
1.1	25.489	25.633	25.612
	25.531	25.684	25.824
1.2	25.627	25.745	25.903
	25.649	25.732	25.710
1.3	26.138	26.354	25.797
	26.172	26.154	26.014
CT Mean	25.768	25.884	25.810
CT σ	0.306	0.296	0.141
2.1	25.542	25.643	25.315
	25.505	25.730	25.306
2.2	25.314	25.594	25.212
	25.409	25.690	25.009
2.3	25.488	25.736	25.337
	25.529	25.783	24.316
CT Mean	25.464	25.696	25.083
CT σ	0.087	0.069	0.395
3.1	25.534	25.738	25.494
	25.568	25.792	25.639
3.2	25.740	25.866	25.197
	25.823	25.973	25.651
3.3	25.743	25.922	25.918
	25.768	25.999	26.136
CT Mean	25.696	25.882	25.672
CT σ	0.117	0.103	0.327

Watermelon			
Sample	Contig 21 CT	Contig 22 CT	Xcv CT
4.1	26.266	26.788	27.809
	26.434	26.607	27.839
4.2	25.996	26.204	27.714
	26.160	26.200	27.446
4.3	25.868	26.150	27.473
	25.841	26.110	27.661
CT Mean	26.094	26.343	27.657
CT σ	0.234	0.282	0.166
5.1	26.698	26.767	27.963
	26.891	26.696	28.094
5.2	26.617	26.557	28.462
	26.548	26.663	27.977
5.3	26.209	26.194	27.756
	26.326	26.469	27.600
CT Mean	26.548	26.558	27.975
CT σ	0.249	0.207	0.297
6.1	26.782	26.627	29.090
	26.585	26.655	29.052
6.2	26.403	26.652	28.850
	26.498	26.824	28.609
6.3	26.270	26.482	28.627
	26.191	26.636	28.422
CT Mean	26.455	26.646	28.775
CT σ	0.216	0.109	0.267
7.1	27.196	27.392	28.738
	27.335	27.556	28.860
7.2	26.928	27.186	28.673
	26.896	27.204	28.756
7.3	26.877	27.138	28.705
	27.021	27.246	29.005
CT Mean	27.042	27.287	28.790
CT σ	0.185	0.158	0.123
8.1	26.701	26.921	28.104
	27.205	27.167	28.200
8.2	26.717	27.147	28.816
	26.800	26.930	28.672
8.3	31.587	31.961	33.245
	31.599	32.167	33.317
CT Mean	28.435	28.715	30.059
CT σ	2.453	2.597	2.510
9.1	27.169	27.358	28.798
	27.210	27.393	28.821
9.2	26.547	26.399	28.140
	26.866	26.447	28.333
9.3	26.603	26.526	27.656
	26.414	26.571	27.961
CT Mean	26.802	26.782	28.285
CT σ	0.335	0.463	0.464
10.1	26.854	26.948	28.625
	27.300	27.367	28.765
10.2	26.623	26.813	28.176
	26.803	26.681	28.514
10.3	25.957	26.239	27.853
	26.107	26.468	27.822
CT Mean	26.607	26.753	28.293
CT σ	0.500	0.392	0.403
11.1	26.871	27.214	29.306
	27.263	27.480	29.494
11.2	25.823	26.510	28.955
	26.007	26.510	29.322
11.3	27.213	26.935	30.161
	27.007	27.468	29.687
CT Mean	26.697	27.020	29.487
CT σ	0.625	0.442	0.409
12.1	25.923	26.282	27.739
	26.167	26.169	27.663
12.2	25.653	25.877	27.481
	25.453	25.914	27.676
12.3	25.540	25.569	27.287
	25.658	25.606	27.400
CT Mean	25.732	25.903	27.541
CT σ	0.265	0.288	0.179

Squash			
Sample	Contig 21 CT	Contig 22 CT	Xcv CT
13.1	25.837	26.146	25.146
	25.821	26.396	24.643
13.2	25.856	26.380	25.307
	25.836	26.293	24.882
13.3	26.341	26.344	25.414
	26.296	26.366	24.991
CT Mean	25.998	26.321	25.064
CT σ	0.249	0.093	0.284
14.1	25.681	26.186	25.348
	25.752	26.245	25.164
14.2	25.457	25.757	25.010
	25.375	25.824	24.809
14.3	25.688	26.295	24.930
	25.717	26.141	25.294
CT Mean	25.612	26.075	25.093
CT σ	0.156	0.227	0.212
15.1	25.591	26.386	25.793
	25.744	26.229	25.684
15.2	25.880	26.293	25.909
	25.978	26.596	25.678
15.3	25.749	25.980	25.487
	25.719	26.128	25.306
CT Mean	25.777	26.269	25.643
CT σ	0.135	0.213	0.216
16.1	25.817	25.893	28.376
	25.685	25.919	28.946
16.2	24.791	25.007	28.012
	24.880	25.020	27.965
16.3	24.941	25.317	28.273
	25.201	25.364	28.373
CT Mean	25.219	25.420	28.324
CT σ	0.436	0.404	0.352
17.1	25.419	25.525	28.343
	25.471	25.788	28.340
17.2	25.310	25.600	28.269
	25.360	25.728	28.353
17.3	25.412	25.652	28.170
	25.471	25.721	28.309
CT Mean	25.407	25.669	28.297
CT σ	0.063	0.096	0.070
18.1	25.559	25.730	28.159
	25.581	25.765	28.463
18.2	25.560	25.711	28.412
	25.552	25.643	28.382
18.3	25.625	25.770	28.511
	25.561	25.756	28.567
CT Mean	25.573	25.729	28.416
CT σ	0.027	0.048	0.142
19.1	25.423	25.647	29.262
	25.409	25.527	29.458
19.2	25.324	25.507	29.201
	25.339	25.435	29.303
19.3	25.368	25.502	29.269
	25.417	25.241	28.976
CT Mean	25.380	25.476	29.245
CT σ	0.042	0.134	0.157
20.1	25.313	25.596	29.620
	25.330	25.502	29.357
20.2	25.148	25.555	29.097
	25.147	25.315	29.039
20.3	24.953	25.264	28.911
	24.880	25.298	28.972
CT Mean	25.128	25.422	29.166
CT σ	0.183	0.146	0.271
21.1	25.360	25.522	28.598
	25.316	25.591	28.733
21.2	25.284	25.538	28.762
	25.157	25.506	28.650
21.3	25.231	25.448	28.786
	25.142	25.392	28.562
CT Mean	25.248	25.499	28.682
CT σ	0.087	0.070	0.092
22.1	25.543	26.447	29.969
	25.686	26.455	29.692
22.2	25.600	26.362	29.763
	25.526	26.411	29.772
22.3	25.233	26.156	29.130
	25.306	26.032	29.055
CT Mean	25.482	26.310	29.563
CT σ	0.176	0.175	0.377
23.1	25.171	25.989	28.751
	25.135	26.258	28.593
23.2	25.259	26.332	28.885
	25.212	26.233	28.994
23.3	25.394	25.528	29.398
	25.420	25.543	29.293
CT Mean	25.265	25.980	28.986
CT σ	0.118	0.363	0.311
24.1	25.294	25.510	29.663
	25.389	25.543	29.492
24.2	-	-	-
	-	-	-
24.3	25.275	25.480	29.576
	25.344	25.392	29.728
CT Mean	25.326	25.481	29.615
CT σ	0.051	0.065	0.103

Annex B

Comparative Experimental Data

Section 1: Robustness - Method Comparative LOD Evaluation

Four labs participated in the study in which 3-5000 seed samples were spiked with differing concentrations of *Ac* (OD_{600} = 0.100 X 10^{-5} to 10^{-7}). Samples were then processed per the routine lab protocol for Ac direct PCR (Annex E). Note: lack of detection is represented by a hyphen (-). Samples not tested are represented by N/A.

Lab 1 MoBio LOD	Contig 21					
Ac OD_{600}=0.100	5K Sub 1	5K Sub 2	5K Sub 3	CT Mean	CT σ	Detection
10^-5	32.562	32.609	32.446	32.710	0.475	3 of 3
10^-5	33.284	33.384	32.446			
10^-5	32.555	31.930	33.177			
10^-6	35.442	-	-	35.770	0.395	1 of 3
10^-6	-	35.658	36.209			
10^-6	-	-	-			
10^-7	-	-	-	-	-	0 of 3
10^-7	-	-	-			
10^-7	-	-	-			

Lab 2 MoBio LOD	Contig 21					
Ac OD_{600}=0.100	5K Sub 1	5K Sub 2	5K Sub 3	CT Mean	CT σ	Detection
10^-5	29.880	30.320	N/A	30.290	0.531	2 of 2
10^-5	29.930	31.030	N/A			
10^-5	N/A	N/A	N/A			
10^-6	33.350	33.18	N/A	33.480	0.262	2 of 2
10^-6	33.750	33.640	N/A			
10^-6	N/A	N/A	N/A			
10^-7	38.420	36.77	N/A	38.018	0.888	2 of 2
10^-7	38.820	38.060	N/A			
10^-7	N/A	N/A	N/A			

Lab 1 Qiagen LOD	Contig 21					
Ac OD_{600}=0.100	5K Sub 1	5K Sub 2	5K Sub 3	CT Mean	CT σ	Detection
10^-5	28.903	28.820	36.227	31.334	3.667	3 of 3
10^-5	28.900	28.993	36.497			
10^-5	28.882	28.853	35.935			
10^-6	-	-	-	35.907	N/A	1 of 3
10^-6	-	-	-			
10^-6	35.907	-	-			
10^-7	-	-	-	N/A	N/A	0 of 3
10^-7	-	-	-			
10^-7	-	-	-			

Lab 3 LOD	Contig 21					
Ac OD_{600}=0.100	5K Sub 1	5K Sub 2	5K Sub 3	CT Mean	CT σ	Detection
10^-5	37.500	37.600	37.400	36.788	0.799	3 of 3
10^-5	37.360	36.850	35.800			
10^-5	36.010	-	35.780			
10^-6	37.390	-	-	38.287	0.876	2 of 3
10^-6	-	-	-			
10^-6	38.330	-	39.140			

10^-7	-	-	-	N/A	N/A	0 of 3
10^-7	-	-	-			
10^-7	-	-	-			

Lab 4 LOD	Contig 21					
Ac OD_{600}=0.100	5K Sub 1	5K Sub 2	5K Sub 3	CT Mean	CT σ	Detection
10^-5	37.890	-	-	37.854	0.785	3 of 3
10^-5	36.690	37.770	37.570			
10^-5	38.100	39.320	37.640			
10^-6	-	-	-	N/A	N/A	0 of 3
10^-6	-	-	-			
10^-6	-	-	-			
10^-7	-	-	-	N/A	N/A	0 of 3
10^-7	-	-	-			
10^-7	-	-	-			

Lab 1 MoBio LOD	Contig 22					
Ac OD_{600}=0.100	5K Sub 1	5K Sub 2	5K Sub 3	CT Mean	CT σ	Detection
10^-5	32.665	32.666	33.548	33.009	0.549	3 of 3
10^-5	33.102	32.467	33.241			
10^-5	33.694	32.138	33.557			
10^-6	-	35.954	36.302	36.327	0.793	3 of 3
10^-6	37.870	35.726	36.298			
10^-6	-	-	35.814			
10^-7	-	-	-	-	-	0 of 3
10^-7	-	-	-			
10^-7	-	-	-			

Lab 2 MoBio LOD	Contig 22					
Ac OD_{600}=0.100	5K Sub 1	5K Sub 2	5K Sub 3	CT Mean	CT σ	Detection
10^-5	30.340	30.400	N/A	30.383	0.202	2 of 2
10^-5	30.150	30.640	N/A			
10^-5	N/A	N/A	N/A			
10^-6	34.160	34	N/A	34.235	0.641	2 of 2
10^-6	35.140	33.640	N/A			
10^-6	N/A	N/A	N/A			
10^-7	39.030	37.280	N/A	38.190	1.077	2 of 2
10^-7	39.210	37.240	N/A			
10^-7	N/A	N/A	N/A			

Lab 1 Qiagen LOD	Contig 22					
Ac OD_{600}=0.100	5K Sub 1	5K Sub 2	5K Sub 3	CT Mean	CT σ	Detection
10^-5	29.253	29.380	36.932	32.406	4.002	3 of 3
10^-5	29.510	29.323	37.899			
10^-5	30.146	-	36.808			
10^-6	-	-	-	36.838	N/A	2 of 3
10^-6	36.826	36.850	-			
10^-6	-	-	-			
10^-7	-	-	-	N/A	N/A	0 of 3
10^-7	-	-	-			
10^-7	-	-	-			

Lab 3 LOD	Contig 22					
Ac OD_{600}=0.100	5K Sub 1	5K Sub 2	5K Sub 3	CT Mean	CT σ	Detection
10^-5	35.070	34.280	34.070	34.940	0.695	3 of 3
10^-5	35.810	35.190	34.750			
10^-5	36.070	35.000	34.220			

10^-6	-	-	-	36.610	0.636	2 of 3
10^-6	-	-	-			
10^-6	37.060	36.160	-			
10^-7	-	-	-	37.750	N/A	1 of 3
10^-7	-	-	-			
10^-7	37.750	-	-			

Lab 4 LOD	**Contig 22**					
Ac OD_{600}**=0.100**	**5K Sub 1**	**5K Sub 2**	**5K Sub 3**	**CT Mean**	**CT σ**	**Detection**
10^-5	35.750	N/A	35.160	37.151	1.292	3 of 3
10^-5	38.790	37.570	37.340			
10^-5	37.210	38.240	N/A			
10^-6	-	-	-	37.190	N/A	1 of 3
10^-6	-	-	-			
10^-6	-	-	37.190			
10^-7	-	-	-	38.120	N/A	1 of 3
10^-7	-	-	-			
10^-7	-	38.12	-			

Lab 3 LOD	**Zup (Ct Cutoff = 35)**					
Ac OD_{600}**=0.100**	**5K Sub 1**	**5K Sub 2**	**5K Sub 3**	**CT Mean**	**CT σ**	**Detection**
10^-5	31.550	31.390	31.060	31.589	0.463	3 of 3
10^-5	31.420	31.350	31.130			
10^-5	32.460	31.810	32.130			
10^-6	-	[35.050]	[35.720]	34.043	0.535	3 of 3
10^-6	33.710	33.360	33.740			
10^-6	34.340	34.280	34.830			
10^-7	[35.100]	[36.230]	[36.270]	34.890	N/A	1 of 3
10^-7	34.890	[36.690]	-			
10^-7	-	[37.740]	-			

Lab 4 LOD	**Zup (Ct Cutoff = 35)**					
Ac OD_{600}**=0.100**	**5K Sub 1**	**5K Sub 2**	**5K Sub 3**	**CT Mean**	**CT σ**	**Detection**
10^-5	33.870	31.330	33.050	32.568	1.062	3 of 3
10^-5	33.220	33.150	32.760			
10^-5	31.960	30.570	33.200			
10^-6	[36.200]	-	[36.010]	34.230	N/A	1 of 3
10^-6	[35.310]	-	[37.050]			
10^-6	-	34.230	[38.650]			
10^-7	33.420	34.140	33.690	33.750	0.364	2 of 3
10^-7	[35.410]	-	[35.870]			
10^-7	[36.400]	[35.240]	[37.940]			

Section 2: Robustness - ISHI Method Comparative Test

				Testing Laboratory: 1				Testing Laboratory: 2			Testing Laboratory: 3			
				Target Pathogen Assay (Ct)			IPC Assay (Ct)	Target Pathogen Assay (Ct)		IPC Assay (Ct)	Target Pathogen Assay (Ct)			IPC Assay (Ct)
Infection Level	SAMPLE ID	CT Sample ID - PCR REP	Crop Species	Contig 21	Contig 22	Zup (Ac)	Xcv	Contig 21	Contig 22	Xcv	Contig 21	Contig 22	Zup (Ac)	Zup (Acat)
High +	161	1 - a	Melon	21.37	22.01	22.41	26.46	23.40	23.58	28.37	33.12	32.90	28.39	29.44
High +	161	1 - b	Melon	21.24	21.93	22.46	26.40	22.63	23.48	28.42	33.30	32.47	28.39	30.11
High +	161	2 - a	Melon	20.67	21.66	22.14	26.26	22.72	23.24	27.34	33.96	33.13	29.39	29.6
High +	161	2 - b	Melon	20.72	21.70	22.12	26.38	22.63	23.26	28.04	33.87	33.06	29.25	30.75
High +	280	3 - a	Melon	20.35	20.71	20.96	26.19	23.60	24.91	28.66	27.93	27.03	23.84	30.49
High +	280	3 - b	Melon	20.48	20.74	21.04	26.26	24.09	25.02	28.60	27.88	26.81	23.84	29.98
High +	280	4 - a	Melon	20.01	20.29	20.60	26.19	23.97	25.12	28.89	26.24	25.15	22.48	29.97
High +	280	4 - b	Melon	19.97	20.24	20.65	26.25	24.92	24.30	28.74	26.00	25.23	22.22	29.97
Moderate +	354	5 - a	Melon	27.63	27.65	27.37	26.43	31.58	31.27	28.15	34.36	33.05	29.48	29.75
Moderate +	354	5 - b	Melon	27.56	27.54	27.50	26.33	31.65	31.33	28.37	34.37	33.82	29.52	29.99
Moderate +	354	6 - a	Melon	29.79	29.92	30.02	26.52	31.42	31.50	28.14	35.00	35.47	31.55	29.9
Moderate +	354	6 - b	Melon	29.92	29.76	30.14	26.52	31.48	32.15	28.29	35.63	35.47	31.48	29.63
Moderate +	354	7 - a	Melon	29.92	30.58	30.76	26.42	28.92	29.19	29.06	N/A	36.25	32.14	30.46
Moderate +	354	7 - b	Melon	30.14	30.19	30.60	26.42	28.94	29.35	29.06	36.86	36.09	32.04	29.74
Moderate +	354	8 - a	Melon	30.56	30.81	30.76	26.25	28.91	30.12	28.25	35.71	34.97	31.85	30.68
Moderate +	354	8 - b	Melon	30.54	30.61	30.93	26.28	28.46	28.89	28.21	36.44	34.89	33.05	30.13
Moderate +	354	9 - a	Melon	31.41	31.21	31.20	26.58	31.43	31.34	28.81	34.21	35.99	31.85	30.48
Moderate +	354	9 - b	Melon	30.91	31.31	31.14	26.56	31.32	31.49	28.63	35.59	36.38	31.53	30.63
Moderate +	354	10 - a	Melon	26.68	26.99	27.15	26.47	31.33	31.54	28.36	36.23	36.56	33.57	29.67
Moderate +	354	10 - b	Melon	26.70	26.94	27.10	26.40	32.07	31.87	28.44	36.80	36.30	34.54	30.79
High +	301	11 - a	Watermelon	19.32	19.54	20.19	26.24	20.41	21.20	28.79	22.51	22.08	18.81	30.46
High +	301	11 - b	Watermelon	19.29	19.51	20.08	26.23	20.51	21.19	28.60	22.52	22.13	18.88	29.43
High +	301	12 - a	Watermelon	19.40	19.61	20.21	26.25	20.60	21.35	28.93	22.45	21.85	18.66	29.44
High +	301	12 - b	Watermelon	19.31	19.64	20.20	26.17	20.75	21.42	28.80	22.43	21.99	18.60	29.51
High +	301	13 - a	Watermelon	18.81	18.96	19.07	26.09	20.89	21.30	28.33	22.65	21.94	19.12	30.42
High +	301	13 - b	Watermelon	18.85	18.93	19.13	25.98	21.01	21.35	28.58	22.52	21.87	19.21	29.44
High +	301	14 - a	Watermelon	19.28	19.44	20.16	26.16	21.05	21.56	28.56	23.32	22.78	19.81	30.19
High +	301	14 - b	Watermelon	19.29	19.46	20.17	26.15	20.81	21.43	28.64	23.27	22.76	19.81	29.68
None	742	15 - a	Melon	-	-	-	25.81	-	-	27.49	-	-	-	29.96
None	742	15 - b	Melon	-	-	-	26.02	-	-	27.56	-	-	-	30.63
None	742	16 - a	Melon	37.17	-	39.14	25.99	-	-	27.92	-	-	-	29.54
None	742	16 - b	Melon	-	-	39.22	26.07	-	-	27.83	-	-	-	29.9
None	661	17 - a	Watermelon	-	-	-	26.60	-	-	27.82	-	-	-	29.81
None	661	17 - b	Watermelon	-	-	-	26.56	-	-	27.85	-	-	-	30.73
None	661	18 - a	Watermelon	-	-	-	26.13	-	-	27.79	-	-	-	29.84
None	661	18 - b	Watermelon	-	-	-	26.21	-	-	27.78	-	-	-	29.91
None	742	19 (+) Process Control - a	Melon	25.42	25.49	22.77	25.97	26.49	27.18	27.84	33.89	32.79	27.32	30.22
None	742	19 (+) Process Control - b	Melon	25.38	25.49	22.74	26.09	26.43	27.20	28.00	33.22	33.17	27.48	29.87
None	742	20 (-) Process Control - a	Melon	-	-	-	-	-	-	-	-	-	-	-
None	742	20 (-) Process Control - b	Melon	-	-	-	-	-	-	-	-	-	-	-
N/A	-	Ac (+) PCR Control - a	N/A	28.23	28.26	25.59	-	26.91	27.36	-	30.35	29.06	25.03	-
N/A	-	Ac (+) PCR Control - b	N/A	28.23	28.35	25.64	-	27.08	27.43	-	30.47	29.74	25.41	-
N/A	-	IPC (+) PCR Control - a	N/A	-	-	-	28.32	-	-	27.21	-	-	-	27.67
N/A	-	IPC (+) PCR Control - b	N/A	-	-	-	28.29	-	-	27.25	-	-	-	27.44
N/A	-	NTC (-) PCR Control - a	N/A	-	-	-	-	-	-	-	-	-	-	-
N/A	-	NTC (-) PCR Control - b	N/A	-	-	-	-	-	-	-	-	-	-	-

				Testing Laboratory: 4			Testing Laboratory: 5				Testing Laboratory: 6a		
				Target Pathogen Assay (Ct)		IPC Assay (Ct)	Target Pathogen Assay (Ct)			IPC Assay (Ct)	Target Pathogen Assay (Ct)		IPC Assay (Ct)
Infection Level	SAMPLE ID	CT Sample ID - PCR REP	Crop Species	Contig 22	Zup (Ac)	Zup (Acat)	Contig 21	Contig 22	Zup (Ac)	Zup (Acat)	Contig 21	Contig 22	Xcv
High +	161	1 - a	Melon	21.00	20.18	29.7910614	23.97	22.78	20.73	26.2	31.25	30.67	34.03
High +	161	1 - b	Melon	20.85	20.00	29.7273865	24.00	22.81	21.08	26.35	29.64	29.32	36.77
High +	161	2 - a	Melon	20.50	19.60	29.535511	23.07	22.04	20.25	25.55	28.16	34.61	28.61
High +	161	2 - b	Melon	20.22	19.25	28.8598728	23.14	22.08	20.32	25.5	28.45	32.00	34.84
High +	280	3 - a	Melon	22.73	22.47	30.6234894	22.71	21.26	19.17	25.61	23.66	23.62	27.61
High +	280	3 - b	Melon	22.87	22.42	30.298748	22.48	21.64	19.14	25.45	23.96	23.53	27.57
High +	280	4 - a	Melon	20.00	19.38	29.7970161	23.39	22.24	20.18	25.28	21.66	22.06	27.10
High +	280	4 - b	Melon	19.97	19.29	29.574831	23.44	22.32	20.24	25.42	23.06	22.25	28.75
Moderate +	354	5 - a	Melon	30.60	30.20	31.4344139	37.33	36.12	33.15	29.47	34.88	35.28	27.73
Moderate +	354	5 - b	Melon	30.30	29.76	31.1776104	35.80	34.79	33.38	29.9	31.15	32.85	30.95
Moderate +	354	6 - a	Melon	30.15	29.47	30.9231949	37.68	35.47	33.28	29.28	31.79	30.16	30.56
Moderate +	354	6 - b	Melon	30.17	29.59	31.1724854	36.76	36.41	33.34	28.96	33.90	31.06	26.74
Moderate +	354	7 - a	Melon	30.32	30.11	31.0969753	32.65	31.75	28.91	26.07	31.68	32.72	27.19
Moderate +	354	7 - b	Melon	30.76	30.16	30.8379974	33.09	N/A	29.23	27.49	32.59	31.71	27.01
Moderate +	354	8 - a	Melon	30.35	30.00	30.58675	36.44	35.20	32.09	28.06	33.11	34.05	31.50
Moderate +	354	8 - b	Melon	30.45	29.91	30.5240364	36.36	34.68	31.91	27.74	36.86	-	31.63
Moderate +	354	9 - a	Melon	30.40	29.88	31.8226223	36.27	34.27	33.39	29.8	33.71	30.21	32.08
Moderate +	354	9 - b	Melon	30.28	29.83	30.6562595	35.51	34.45	32.90	30.41	32.96	34.52	32.16
Moderate +	354	10 - a	Melon	22.78	21.79	30.3492336	35.08	N/A	32.54	30.17	33.31	35.14	31.09
Moderate +	354	10 - b	Melon	22.79	21.58	30.1829567	37.49	N/A	32.66	30.31	35.30	34.95	33.50
High +	301	11 - a	Watermelon	18.94	19.08	29.1410713	23.19	21.92	20.38	25.87	-	-	-
High +	301	11 - b	Watermelon	18.83	18.94	28.9849987	23.12	21.82	20.37	26.07	-	-	-
High +	301	12 - a	Watermelon	18.51	18.58	28.9155693	23.07	21.85	20.21	25.61	-	-	-
High +	301	12 - b	Watermelon	18.52	18.73	28.9340744	23.35	21.25	20.24	25.86	-	-	-
High +	301	13 - a	Watermelon	18.43	18.27	28.8632412	23.17	21.52	20.21	26.05	-	-	-
High +	301	13 - b	Watermelon	18.59	18.49	28.5520515	23.42	23.00	20.35	26.21	-	-	-
High +	301	14 - a	Watermelon	18.26	18.51	28.4432755	23.82	22.70	20.44	26	-	-	-
High +	301	14 - b	Watermelon	18.31	18.55	28.697567	23.65	21.77	20.47	26.13	-	-	-
None	742	15 - a	Melon	27.16	27.46	28.9197292	-	-	-	26.57	-	-	28.14
None	742	15 - b	Melon	27.13	27.43	28.7855606	-	-	-	26.56	-	-	28.45
None	742	16 - a	Melon	-	-	28.9972343	-	36.50	34.25	25.83	-	-	28.42
None	742	16 - b	Melon	-	36.39	28.6413574	37.22	36.45	34.08	25.59	-	-	28.22
None	661	17 - a	Watermelon	35.82	34.36	29.4934082	-	-	-	24.27	-	-	-
None	661	17 - b	Watermelon	36.43	36.46	29.2234955	-	-	-	24.64	-	-	-
None	661	18 - a	Watermelon	33.81	33.55	29.3838062	-	39.30	-	25.18	-	-	-
None	661	18 - b	Watermelon	33.70	33.20	28.9813061	-	36.81	-	24.84	-	-	-
None	742	19 (+) Process Control - a	Melon	31.47	28.61	29.4224281	29.82	27.54	23.11	23.25	27.54	29.40	26.92
None	742	19 (+) Process Control - b	Melon	31.61	28.82	29.314085	30.01	27.54	23.43	23.54	29.74	29.26	27.37
None	742	20 (-) Process Control - a	Melon	30.58	30.81	-	-	-	39.65	24.68	-	-	-
None	742	20 (-) Process Control - b	Melon	30.94	30.80	-	-	-	39.89	24.61	-	-	-
N/A	-	Ac (+) PCR Control - a	N/A	26.02	22.76	-	29.67	27.72	23.14	-	29.51	28.31	-
N/A	-	Ac (+) PCR Control - b	N/A	26.00	22.84	-	29.61	27.65	N/A	N/A	28.96	28.32	-
N/A	-	IPC (+) PCR Control - a	N/A	-	-	26.8771229	-	-	-	27.67	-	-	-
N/A	-	IPC (+) PCR Control - b	N/A	-	-	27.2410202	-	-	N/A	N/A	-	-	-
N/A	-	NTC (-) PCR Control - a	N/A	-	-	-	-	-	-	-	-	-	-
N/A	-	NTC (-) PCR Control - b	N/A	-	-	-	-	-	-	-	-	-	-

				Testing Laboratory: 6b			Testing Laboratory: 7					Sample Stability Check			
				Target Pathogen Assay (Ct)		IPC Assay (Ct)	Target Pathogen Assay (Ct)			IPC Assay (Ct)		Target Pathogen Assay (Ct)			IPC Assay (Ct)
Infection Level	SAMPLE ID	CT Sample ID - PCR REP	Crop Species	Contig 21	Contig 22	Xcv	Contig 21	Contig 22	Zup (Ac)	Zup (Acat)		Contig 21	Contig 22	Zup (Ac)	Xcv
High +	161	1 - a	Melon	30.60	31.69	32.81	19.65	20.23	18.77	28.07		20.40	20.60	18.71	26.7227
High +	161	1 - b	Melon	33.57	34.53	31.85	20.12	20.56	18.79	28.06		20.33	20.53	18.39	26.6882
High +	161	2 - a	Melon	28.73	29.73	30.32	20.31	21.00	19.52	27.13		21.72	21.90	19.94	26.8545
High +	161	2 - b	Melon	27.47	31.69	34.93	20.44	20.94	19.40	27.13		21.66	21.84	19.90	26.9402
High +	280	3 - a	Melon	22.53	22.23	29.22	17.53	18.05	16.68	22.87		20.59	20.58	18.88	26.5264
High +	280	3 - b	Melon	22.60	22.35	30.17	17.49	18.00	16.62	22.6		20.64	20.66	18.90	26.5587
High +	280	4 - a	Melon	24.80	23.62	28.38	18.25	18.81	17.47	22.26		20.74	20.82	19.12	26.9125
High +	280	4 - b	Melon	24.76	24.02	30	18.29	18.76	17.45	22.46		20.81	20.82	19.06	26.885
Moderate +	354	5 - a	Melon	35.47	35.23	29.72	28.00	27.83	26.80	23.33		30.64	30.86	28.85	26.7755
Moderate +	354	5 - b	Melon	36.49	33.91	29.86	28.04	27.99	27.06	23.4		30.58	30.18	29.02	26.9239
Moderate +	354	6 - a	Melon	33.43	33.77	28.15	27.90	28.25	27.42	23.23		28.52	28.45	26.81	26.7947
Moderate +	354	6 - b	Melon	34.00	34.01	27.62	28.13	28.30	27.50	23.25		28.66	28.53	26.79	26.8412
Moderate +	354	7 - a	Melon	32.45	32.11	25.81	26.05	26.60	24.49	22.95		29.30	29.21	27.21	26.6003
Moderate +	354	7 - b	Melon	32.96	32.55	26.03	26.00	26.22	24.51	22.95		28.97	29.21	27.23	26.7018
Moderate +	354	8 - a	Melon	34.90	34.12	26.62	26.86	26.78	25.37	23.13		29.01	28.94	27.13	26.8229
Moderate +	354	8 - b	Melon	35.57	34.11	26.35	26.90	27.07	26.00	23		28.94	29.17	27.12	26.7355
Moderate +	354	9 - a	Melon	36.51	35.37	29.67	24.64	24.68	23.46	24.09		30.51	30.70	29.14	26.7597
Moderate +	354	9 - b	Melon	-	-	30.01	24.64	24.71	23.39	24.15		30.90	30.55	29.05	26.7265
Moderate +	354	10 - a	Melon	33.36	36.09	28.33	26.87	26.23	25.21	23.56		30.26	30.66	28.56	26.7768
Moderate +	354	10 - b	Melon	-	35.08	30.19	26.73	26.83	25.36	23.89		29.96	29.97	28.58	26.7629
High +	301	11 - a	Watermelon	21.68	21.18	30.46	17.06	17.69	16.48	22.05		18.98	19.16	17.21	26.7864
High +	301	11 - b	Watermelon	21.56	21.16	30.23	17.11	17.66	16.48	22.17		19.02	19.30	17.19	26.7898
High +	301	12 - a	Watermelon	20.18	20.34	28.2	17.90	18.13	17.06	22.79		18.86	18.97	17.19	26.7119
High +	301	12 - b	Watermelon	19.92	20.32	27.67	17.89	18.13	17.28	22.54		18.90	19.09	17.21	26.6345
High +	301	13 - a	Watermelon	-	20.13	29.13	16.47	17.12	16.07	22.09		18.84	18.97	17.23	27.0977
High +	301	13 - b	Watermelon	19.95	20.09	28.68	16.41	17.16	16.30	22.16		18.83	18.95	17.24	27.2264
High +	301	14 - a	Watermelon	19.87	19.77	29.95	16.98	17.19	16.57	22.19		19.11	19.27	17.33	26.7848
High +	301	14 - b	Watermelon	20.12	20.05	30.05	17.04	17.30	16.62	22.15		19.16	19.21	17.37	26.8983
None	742	15 - a	Melon	-	-	29.77	-	36.35	38.57	22.26		-	-	-	26.5319
None	742	15 - b	Melon	-	-	29.41	-	-	37.94	22.27		-	-	-	26.5207
None	742	16 - a	Melon	-	-	32.58	-	-	37.84	22.17		-	-	-	26.6612
None	742	16 - b	Melon	-	-	32.6	-	-	37.31	22.13		-	-	-	26.788
None	661	17 - a	Watermelon	-	-	30.56	-	-	-	22.09		-	-	-	26.5108
None	661	17 - b	Watermelon	-	-	36.06	-	-	-	22.1		-	-	-	26.4546
None	661	18 - a	Watermelon	-	36.16	27.53	-	-	-	22.28		-	-	-	26.4661
None	661	18 - b	Watermelon	-	-	28.86	-	-	-	22.26		-	-	-	26.4175
None	742	19 (+) Process Control - a	Melon	32.61	30.35	28.89	29.01	26.73	24.01	22.02		25.38	25.34	22.09	26.56
None	742	19 (+) Process Control - b	Melon	31.57	30.18	30.10	28.92	26.18	23.90	22.02		25.36	25.40	22.05	26.57
None	742	20 (-) Process Control - a	Melon	-	-	-	-	-	38.13	21.9		-	-	-	-
None	742	20 (-) Process Control - b	Melon	-	-	-	-	-	38.13	21.85		-	-	-	-
N/A	-	Ac (+) PCR Control - a	N/A	30.05	28.91	-	29.78	27.62	24.11	-		25.01	24.98	21.65	-
N/A	-	Ac (+) PCR Control - b	N/A	29.80	27.55	-	29.77	27.26	24.14	-		24.98	24.87	21.64	-
N/A	-	IPC (+) PCR Control - a	N/A	-	-	-	-	-	-	28.7		-	-	-	24.88
N/A	-	IPC (+) PCR Control - b	N/A	-	-	-	-	-	-	28.52		-	-	-	24.86
N/A	-	NTC (-) PCR Control - a	N/A	-	-	-	-	-	-	-		-	-	-	-
N/A	-	NTC (-) PCR Control - b	N/A	-	-	-	-	-	-	-		-	-	-	-

Annex C.

NSHS Accepted Procedures

qPCR Assay Guidelines

Variable	MVS	Syngenta	Outcome	Justification
DNA dilution	1:50 dilution	No Dilution	No dilution	Removal of inhibitors upstream, dilution not required; ↑ sensitivity
qPCR chemistry	ABI TaqMan universal UNG (2x)	iQ Supermix (2x)	Laboratory's choice	Both master mix formulas are valid
qPCR primers	Contig 21, MonZup	SynZup	Contig 21, Contig 22, SynZup (Select 2 of 3)	All primers accepted per collaborative screen. Laboratory chooses any 2 of 3.
Fixed "delta-RN" threshold	Fixed	Auto call	Fixed recommended	Reduced run variation
Number of cycles	40 (C21,22)	35 (SynZup)	Primer Specific	Cycle number determined in primer validation

Bacterial Isolation Guidelines

Variable	MVS	Syngenta	Outcome	Justification
Buffer composition	PABST	PBS-Tween	PBS-Tween	comparative data
Buffer volume	fixed	variable	variable	validation data
Incubation time	1 Hr	20-24 Hrs	1 Hr	comparative data
Incubation shake	150 rpm	100-120 rpm	range 100-150	RPM range is aligned
Aliquot size	250 mL	10mL	45mL	comparative data
Centrifugation - pellet debris	5 mins @ 960 RCF	5 mins @ 1000 RCF	5 mins @ 1000 RCF	RCF is aligned
Centrifugation + inhibitor removal	15 mins @ 4800 RCF + PVPP	15 mins @ 3200 RCF + Sorbitol	Optional	Inhibition is monitored by PEC
Centrifugation - pellet bacteria	15 mins @ 6950 RCF	10 mins @ 3200 RCF	Recommended minimum-10 mins @ 3200 RCF	Available data indicates 10 min @ 3200 RCF is sufficient to pellet target

Annex D.

***Ac* direct qPCR Protocol**

1.0 PURPOSE

To detect the presence of *Acidovorax avenae* ssp. *citrulli* on cucurbit seed by direct qPCR assay.

2.0 SCOPE

Monsanto Quality Assurance- Woodland Seed Health Testing Lab

3.0 MATERIALS AND METHODS

4.1 EQUIPMENT AND MATERIALS

4.1.1 Recommended Equipment

Equipment	Equipment
micro pipettes (1-1,000µL)	Fiberlite F14-6x250y
laminar flow hood	Fiberlite F14-14x50cy
Spectrophotometer	pipette aid
incubator/Shaker (37	micro centrifuge
Incubator(28	PF vortex genie 2 adapter
Vortex	microtube heat block
balance (Res 0.01g, Cap 600g)	PF vacuum manifold
fume Hood	vacuum system
pH Meter	-20°C Freezer
milli Q Water System	8x channel EDP-3
table shaker	Life Technologies ViiA 7
seed wash draining rack	Autorep-E
Autoclave	PCR plate centrifuge
floor centrifuge	96 Well cold block
4ºC Refrigerator	

4.1.2 Recommended Labware

Labware	Labware
Cuvettes	Sterile scalpel
inoculation loop 10µL	2L waste beakers
inoculation loop 1µL	1.7mL and 2.0mL microtubes
Cell spreader	Disposable Funnels
15mL Snap Cap Tube	50mL tube racks
50mL sterile centrifuge tubes	Miracloth®
20-1000µL filtered pipette tips	Serological pipette (assorted)
10L Polypropylene Carboy	2mL MoBio micro centrifuge tubes
Stir Rod	PowerFood Microbead matrix(MoBio kit)
Stir Plate (2x)	Filter Column (MoBio kit)
Graduated Cylinders (assorted)	2mL PF Microtube (MoBio kit)
Reagent weigh boat (assorted)	MoBio spin filter vacuum manifold adapters
microtube rack	Lukerlock stop caulks
10x 12 Specimen bags	Sterile 96-well Nunc plate
Sterile Autorep-E tips (5mL, 12.5mL)	96-well ViiA 7 real-time PCR plate
sterilite 16qt bins	optically clear plate seals
autoclave pan (assorted)	5mL falcon snap cap tubes
250mL polypropylene bottles	

4.1.3 Reagents

Reagents	Reagents
1x Phos-Tween	70% EtOH
Nutrient Broth	10% Clorox Bleach
YDC Media	Polyvinylpolypyrrolidone (PVPP)
Nutrient Agar	PowerFood Solutions 1-6 (MoBio kit)
Modified Tween Agar for BFB	Omnipur sterile nuclease free water
20x Phos-Tween	100% EtOH
MQ H2O	5M HCl
Ascorbic Acid	Contig 21 primer/probe

Saturated NaOH
Potassium Disulfite
TaqMan Universal PCR MM no ampErase UNG (2x)
Tris-EDTA (T10E0.1)
Zup primer/probe
Xv primer/probe

4.2 METHOD

4.2.1 Preparations Prior to Starting the Assay

1. Sample preparation:
   i. Prepare required number of specimen buckets, label each with subsample ID.
   ii. Prepare required number of seeds (by weight), in specimen buckets, for each subsample. Record weights on coversheet (00-SH-25-028).

2. Materials preparation:

   Reference document 00-SH-25-030 Assay for BFB PCR Reagent Preparation, for required reagents.

   Maintain the following bacterial cultures so that they are available prior to *Ac* and *Xv* stock suspension preparations:

   i. 2-7 day culture of *Ac* on modified Tween agar for BFB.
   ii. 2-3 day culture of *Xv* on YDC agar.

   Maintain the following bacterial spike suspensions at 4°C

   i. 1-21 day old suspension of *Ac*, OD_{600}= 0.100. This is the *Ac* stock suspension.
   ii. 1-21 day old suspension of *Xv*, OD_{600}= 0.100. This is the *Xv* stock suspension.

3. Labware preparation:
   i. Labeled sets of tubes for all subsamples as follows:

      -Two sets of 50mL conical tubes.
      -Three sets of 2.0mL microcentrifuge tubes
      -One set of MoBio MicroBead tubes
      -One set of MoBio spin filter columns
      -One set of MoBio 2.0mL elution tubes

4. DNA extraction preparation:
   i. Set a 2.0mL microtube heat block to 65°C.
   ii. Place solutions PF1 and PF3 into a 55°C water bath. Allow reagents to preheat for at least 10 minutes prior to use (reagents should be used while still warm).
   iii. Prepare MoBio vacuum manifold with spin filter adapters and columns prior to step 4.3.3.vi.

4.2.2 Seed Wash

1. Assay preparation and seed wash

   i. Reference worksheet 00-SH-00-000 Assay for BFB PCR Reagent Preparation: Modified PBST buffer for *Ac* PCR. Mix on a magnetic stir plate using a magnetic stir rod until buffer is homogeneous.

   ii. Retrieve the *Ac* stock suspension from a 4°C incubator. Vortex to mix, and perform three ten-fold serial dilutions in PBST buffer. The third dilution should correspond to a cell concentration of $OD_{600}= 0.100\times10^{-3}$ in a final volume of 10mL. This is the *Ac* spike.

   iii. Retrieve the *Xv* stock suspension from a 4°C incubator. Vortex to mix, and perform three ten-fold serial dilutions in PBST buffer. The third dilution should correspond to a cell concentration of $OD_{600}= 0.100\times10^{-3}$ in a final volume of 50mL. This is the *Xv* spike.

   iv. Dispense 2mL of PBST buffer per gram of seed into all subsamples and controls. Refer to coversheet (00-SH-00-000) for subsample-specific buffer volumes.

v. Add 1mL of *Xv* spike per 250mL of PBST buffer present to all subsamples and the PPC. Refer to coversheet (00-SH-00-000) for subsample-specific spike volumes.

vi. Add 1mL of *Ac* spike per 250mL of PBST buffer present to the PPC. Refer to coversheet (00-SH-00-000) for subsample-specific spike volumes.

vii. Shake all subsamples and controls at 125rpm for one hour at room temperate.

2. Bacterial isolation

i. Aspirate a 45mL aliquot of seed rinsate from each subsample, and transfer to a clean 50mL conical tube. Centrifuge subsamples and controls for five minutes at 1000RCF.

ii. Transfer the supernatant to a clean 50mL conical tube, discard the pellet. Centrifuge subsamples and controls for 15 minutes at 6950RCF.

iii. Carefully pour off the supernatant until only the pellet and approximately 1mL of supernatant remain. Preserve as much of the pellet as possible.

iv. Resuspend the pellet in the remaining supernatant, and transfer the suspension to a clean 2.0mL microcentrifuge tube. Centrifuge subsamples and controls for two minutes at 16,000RCF.

v. Aspirate and discard the supernatant, while preserving as much of the pellet as possible. Proceed with DNA extraction.

4.2.3 DNA Extraction

1. MoBio PowerFood Microbial DNA Isolation

i. Remove solution PF1 from 55°C water bath, and invert to mix. Add 450µL of solution PF1 into each of the 2.0mL microcentrifuge tubes containing a pellet from the seed wash centrifugation process.

ii. Re-suspend the pellet, then transfer to the corresponding MO BIO MicroBead screw cap microcentrifuge tube. Incubate at 65°C for 10 minutes.

iii. After incubation, transfer subsamples from 65°C heat block to MO BIO vortex adapter. Vortex at maximum speed for 10 minutes. Remove subsamples from vortex adapter and centrifuge at 13,000RCF for 1 minute.

iv. Add 100µL of solution PF2 to a new set of 2.0mL microcentrifuge tubes. Remove subsamples from centrifuge. Avoiding the pellet, transfer supernatant into corresponding 2mL microcentrifuge tube containing PF2. Discard the screw cap tube. Vortex each subsample for approximately 5 seconds. Incubate subsamples at 4°C for 5 minutes; then centrifuge at 13,000 RCF for one (1) minute.

v. Remove solution PF3 from 55°C water bath, and invert to mix. Add 900µL of solution PF3 to a new set of 2.0mL microcentrifuge tubes. Remove subsamples from centrifuge. Transfer supernatant into corresponding 2.0mL microcentrifuge tube containing PF3 and discard the tube containing the PF2 pellet. Vortex each subsample for 5 seconds.

vi. Ensure that all unused ports are closed on the vacuum manifold and then turn on vacuum source.

vii. Transfer each subsample step wise into the corresponding spin filter column under vacuum. Allow to drain through, and repeat until total volume has passed through the column.

Note: If a spin filter column becomes clogged on the vacuum manifold, it can be processed by centrifugation. Turn off vacuum source and equalize negative internal manifold pressure by opening an unused port. Remove the affected spin filter column and place in a clean MO BIO 2mL microcentrifuge tube. Centrifuge the tube at 13,000RCF for one minute. Discard the flow through and repeat if necessary. Remove the column from the tube and return it to the vacuum manifold.

viii. After all subsamples have completely passed through spin filter columns, turn off vacuum source and equalize negative manifold pressure by opening an unused port. Once vented, close the port.

ix. Dispense 850µL of 200-proof ethanol into each spin filter column. Turn on the vacuum source and allow ethanol to pass through the column.

x. Shake solution PF4 vigorously to mix, dispense 650µL of solution PF4 into each spin filter column, and allow solution to pass through column completely. Continue to pull vacuum through the filter for one additional minute.

xi. Dispense 650µL of solution PF5 into each spin filter column and allow solution to pass through column completely. Continue to pull vacuum through the filter for one additional minute.

xii. Turn off vacuum source and equalize negative manifold pressure by opening an unused port. Once vented, close the port.

xiii. Transfer spin filter columns from vacuum manifold to clean MO BIO 2mL microcentrifuge tubes and centrifuge for two minutes at 13,000 RCF.

xiv. Remove tubes from centrifuge and transfer spin filter columns to clean MO BIO 2mL microcentrifuge tubes. Add 100µL of solution MON-PF6 to the center of each spin filter column. Centrifuge at 13,000RCF for one minute.

xv. Remove samples from centrifuge and verify that eluent is present in the microcentrifuge tube. Discard spin filter columns and store extracted DNA at -20°C.

2. DNA Extraction Cleanup:

i. In the general lab area, wipe down work area used during the seed wash process with 70% ethanol.

ii. MO BIO Adapters:
   a. Under vacuum, rinse mini spin filter adapters with MQ water followed by 70% ethanol. Place spin filter adapters in a 100mL Pyrex autoclave bottle and rinse for two minutes under RO/DI water.
   b. Adjust volume of water in bottle to the 100mL mark. Label autoclave tape with the date and affix to bottle. Set the adapters aside to soak overnight.
   c. After the overnight soak, autoclave for 30 minutes.
   d. After autoclaving, rinse adapters for two minutes under RO/DI water; drain.
   e. Dry the adapters in a designated clean area.
   f. After drying, store the adapters in a 100mL Pyrex bottle.

iii. MO BIO Manifold:
   a. In a fume hood, remove drain plug from vacuum manifold and collect waste reagents into designated waste container. Manifold is stored in a fume hood with the drain plug out.
   b. Collect all disposable plastics used in the extraction process and store in fume hood overnight. Discard using the appropriate waste stream.

4.2.4 Real-time PCR Assay

1. qPCR plate loading

Reference 00-SH-25-030 to prepare the 2pg/µL genomic DNA standard.

Note: Steps 3-5 can be completed using STAR method "BFB PCR plate load v2.0.

2. qPCR cycling parameters
Contig 21, Contig 22, and *Xv* qPCR assays:

Step 1: 10 min- 95°C
Step 2: 15 sec- 95°C
Step 3: 60 sec- 60°C
(Repeat steps 2 and 3 for a total of 40 cycles)

4.3 INTERPRETATION OF RESULTS

4.3.1 Assay Control Parameters

In order to be considered a valid assay, the following control parameters must be met:

1. Threshold is set to corresponding ΔR_n values:

Primer Set	ΔR_n
Contig 21	0.0302
Contig 22	0.0302
Xv	0.0490

2. PCR positive control range:

Primer Set	Ct Range
Contig 21	24.852-26.348
Contig 22	24.932-26.259
Xv	26.377-28.019

3. Positive Process Control (PPC) range:

Primer Set	Ct Range
Contig 21	24.624-27.116
Contig 22	24.362-27.802
Xv	24.514-29.032

4. Negative Process Control (NPC) and PCR negative control (NTC) value:

Primer Set	Ct Range
Contig 21	Undetermined
Contig 22	Undetermined
Xv	Undetermined

5. Positive Extraction Control (PEC) Range:

Crop Species	*Xv* Ct Range
Melon	24.218-26.824
Watermelon	25.125-31.955
Squash	22.952-32.972

4.4 RESULTS REPORTING

After control parameters are verified and the test is confirmed valid, samples can be associated with the following results:

PASS: No *Ac* detected in sample; *Xv* internal control within range.

FINV: 1. *Ac* detected in sample.
2. *Xv* internal control is not within range

A sample with FINV (further investigation) result requires additional testing by a valid *Ac* detection method which can discriminate biological significance (i.e. bioassay/grow out method).

Annex E.

Method for the Detection of *Acidovorax citrulli* DNA on cucurbit seed

Crops: Melon (*Cucumis melo)*
Watermelon (*Citrullus lanatus)*
Squash *(Cucurbita pepo*)
Root Stock Squash (*Cucurbita maxima)*

Pathogen: *Acidovorax citrulli*

Revision history: Version 1.0 - October 2016

Sample and sub-sample size

The recommended sample size is 30,000 seeds; with a maximum sub-sample size of 5,000 seeds.

Principle

- Extraction and collection of *Acidovorax citrulli* (*Ac*) bacteria from seed
- Isolation of *Ac* DNA from extracted bacteria
- qPCR Assay of isolated DNA
- Confirmation of suspect samples by *Ac* grow out assay

  Ac grow out assay: NSHS Vegetable Crop Methods

  Cb1.1 Acidovorax avenae ssp citrulli – Cucurbit

  http://seedhealth.org/seed-health-methods

Restrictions on use

- This test method is suitable for untreated seed. The method may also be suitable for seed treated with some physical and/or chemical processes. It is the responsibility of the testing laboratory to verify that treatment processes do not present an antagonistic effect to method utility.

- Guidance for molecular testing methods are provided such that they accommodate modular assay components. DNA isolation, PEC selection, qPCR reaction mixture, cycling parameters, multiplex reactions and evaluation specifications (threshold/cutoffs) are variables that may differ between testing laboratories. Method guidelines allow for this flexibility; however, it is the responsibility of the testing laboratory to verify that the selected combination of assay components meet the following minimum recovery and detection parameter:

  100% detection of *Ac* across triplicate 5,000 seed subsamples in all qPCR reactions where each 45mL aliquot contains ~12CFU/mL.

  Example: for a 5,000 seed sample to which 250mL buffer is added, 100% detection of *Ac* in all qPCR reactions from triplicate samples of relevant crops, where each sample is spiked with ~3000 cells of *Ac*. (reference appendix 4.2.1)

It is strongly recommended following the best practices described by ISHI-Veg for the reliable use of molecular methods in seed health testing to ensure process standardization and valid results.

ISHI-Veg "Best Practices for Molecular Techniques" available on ISHI website
http://www.worldseed.org

Method Description

1 Extraction of Bacteria From the Seed

1.1 Place seed sub-samples in a container appropriate for seed and buffer volume. Add PBS-Tween buffer to each sub-sample at a ratio of 2.0ml buffer per 1.0g of seed (v:w) (appendix - *Ac* PCR: 1.2 Extraction Buffer (PBS-Tween)). Due to buffer absorbance by the seeds, sub-samples with large seed (e.g., squash) may require a slightly increased buffer to seed ratio (up to 2.5ml per 1.0g of seed).

1.2 A positive extraction control (PEC) spike is required. Each sub-sample being evaluated for *Ac* is spiked with the PEC and the spike volume is adjusted to a standardized concentration (i.e. added at a fixed ratio to the required extraction buffer volume). Suspension may be prepared daily or in bulk (stored at -80°C in a glycerol solution).

1.2.1 PEC spike recommendations:

Xanthomonas euvesicatoria (*Xe*)*:* Jones et al. ATCC® 11633™ (deposited as X*anthomonas vesicatoria* (*Xv*)) is a recommended isolate for the *Xv* PEC.

Spike concentration: OD_{600}= 0.100×10^{-3}
Spike volume: 100µL spike/25mL PBS-Tween
(appendix - *Ac* PCR: 4.1.1 PEC-*Xv*)

Acidovorax cattleyae (*Acat*): Schaad et al. ATCC® 33619™ is a recommended isolate for the *Acat* PEC.

Spike concentration: OD_{600}= 0.100, 1:50 dilution
Spike volume: 5µL spike/25mL PBS-Tween
(appendix - *Ac* PCR: 4.1.2 PEC-*Acat*)

1.3 A Positive Process Control (PPC) sub-sample is required. It is recommended that both the PPC and the PEC spikes be added to the PPC subsample (seed known to be free of *Ac* and the PEC target).

1.3.1 PPC spike recommendation:

Acidovorax citrulli (*Ac*): Schaad et al. ATCC® 29625™ is a recommended isolate.

Spike concentration: OD_{600}= 0.100×10^{-3}
Spike volume: 100µL Ac spike/25mL PBS-Tween
(appendix - *Ac* PCR: 4.2.1 PPC-*Ac*)

1.4 A Negative Process Control (NPC) sub-sample is required.

1.4.1 NPC options:

- A sub-sample containing a typical volume of PBS-Tween
- A sub-sample containing seed known to be free of *Ac* target and a volume of PBS-Tween based on the ratio provided in 1.1.

1.5 Incubate all sub-samples and controls on an orbital shaker for 1-2 hours at room temperature (~23°C); at a speed sufficient to agitate sample (~120rpm).

2 Collection of Target Bacteria by Differential Centrifugation

2.1 Transfer 45mL of seed rinsate per 5,000 seed sub-sample into a 50mL centrifugation tube.

Note: If multiple containers were used to incubate a sub-sample, equal volumes of rinsate must be collected from each container to generate a total volume of 45mL.

2.2 Centrifuge sub-samples for 5 minutes at 1000RCF.

2.3 Decant the supernatant into a new 50mL centrifugation tube and discard pellet.

2.4 Centrifuge supernatant for a minimum of 15 minutes at 3200RCF.

2.4.1 Optional: If additional PCR inhibitor removal is desired, a sorbitol treatment can be used at this point.

2.4.1.1 Decant supernatant from step 2.4

2.4.1.2 Add 1mL of sorbitol solution (appendix - *Ac* PCR: 3.1 Sorbitol Solution) to the pellet and resuspend.

2.4.1.3 Incubate suspension for 20-60 minutes at room temperature.

2.4.1.4 Centrifuge the suspension for 10 minutes at 1800RCF.

2.5 Carefully decant and dispose of the supernatant while preserving as much of the pellet as possible.

2.6 Add a volume of PBS buffer sufficient to resuspend the pellet to achieve the input volume recommended by the DNA isolation kit which will be used, typically 0.5-2.0mL.

3 DNA Isolation

3.1 Recommended DNA isolation kits:

3.1.1 MoBio PowerFood

3.1.2 Machery-Nagel NucleoSpin Plant II

3.1.3 Sbeadex Maxi Plant Kit

Reference appendix 5 for suggested modifications to DNA isolations kits.

4 qPCR Assay

A minimum of two of the three industry validated *Ac* qPCR assays must be used in conjunction with a PEC qPCR assay.

4.1 *Ac* qPCR assays:

4.1.1 Contig 21 qPCR assay

Primer/Probe Sequences for Contig 21 Assay

Contig 21	Target	Label	Sequence 5'→ 3'	quencher	# bases
Aac F1	*Ac*	-	ACC gAA CAg AgA gTA ATT CTC AAA gAC	-	27
Aac R1	*Ac*	-	gAg CgT gAT ggC CAA TgC	-	18
Aac P1	*Ac*	6FAM	CAT CgC TTg AgC AgC AA	MGBNFQ	17

PCR Reaction Mixture for Contig 21 Assay

Component	[Final]	Volume (µL)
Sterile Milli Q water	-	6.988
Contig 21 Forward (100 pmol/µl)	0.90 µM	0.225
Contig 21 Reverse (100 pmol/µl)	0.90 µM	0.225
Contig 21 Probe (100 pmol/µl)	0.250 µM	0.062
qPCR master mix (2x)	-	12.50
PCR Cocktail	-	20.00
Template DNA	-	5.00
PCR Reaction	-	25.00

PCR Cycling Parameters for Contig 21 Assay

Step 1:	10mins	95°C	enzyme activation
Step 2:	15 sec	95°C	denaturation
Step 3:	60 sec	60°C	annealing/elongation
Step 4:	40 cycles; steps 2 & 3	-	ramp rate 1.6°C/sec
Step 5:	10 sec	40°C	end run

4.1.2 Contig 22 qPCR assay

Primer/Probe Sequences for Contig 22 Assay

Contig 22	Target	Label	Sequence 5'→3'	quencher	# bases
Aac F2	*Ac*	-	gAA AgT ggT TgT TCT ggT gAT CAA	-	24
Aac R2	*Ac*	-	TTC ggA ggA CTC ggg ATT T	-	19
Aac P2	*Ac*	6FAM	ATg gTC TgC gAg CCA g	MGBNFQ	16

PCR Reaction Mixture for Contig 22 Assay

Component	[Final]	Volume (µL)
Sterile Milli Q water	-	6.988
Contig 22 Forward (100 pmol/µl)	0.90 µM	0.225
Contig 22 Reverse (100 pmol/µl)	0.90 µM	0.225
Contig 22 Probe (100 pmol/µl)	0.250 µM	0.062
qPCR master mix (2x)	-	12.50
PCR Cocktail	-	20.00
Template DNA	-	5.00
PCR Reaction	-	25.00

PCR Cycling Parameters for Contig 22 Assay

Step 1:	10mins	95°C	enzyme activation
Step 2:	15 sec	95°C	denaturation
Step 3:	60 sec	60°C	annealing/elongation
Step 4:	40 cycles; steps 2 & 3	-	ramp rate 1.6°C/sec
Step 5:	10 sec	40°C	end run

4.1.3 ZUP (*Ac*) qPCR assay

Primer/Probe Sequences for ZUP (*Ac*) Assay

ZUP	Target	Label	Sequence 5'→ 3'	quencher	# bases
2549	*Ac*	-	gAg TCT CAC gAg gTT gTT	-	18
2550	*Ac*	-	gAC CCT ACg AAA gCT CAg	-	18
2551	*Ac*	6FAM	TgC AgC CCT TCA TTg ACg g	BHQ1	19

PCR Reaction Mixture for ZUP (*Ac*) Assay

Component	[Final]	Volume (µL) in 25 µL
Sterile MilliQ water		7.25
qPCR master mix (2x)	1x	12.50
ZUP 2549 (100 pmol/µl)	0.40 µM	0.10
ZUP 2550 (100 pmol/µl)	0.40 µM	0.10
ZUP 2551 (100 pmol/µl)	0.20µM	0.05
Template (total)		5.00

PCR Cycling Parameters for ZUP (*Ac*)

Step 1:	10mins	95°C	enzyme activation
Step 2:	15 sec	95°C	Denaturation
Step 3:	45 sec	60°C	annealing/elongation
Step 4:	40 cycles; steps 2 & 3	-	ramp rate 1.0°C/sec

4.2 Recommended PEC qPCR assays:

4.2.1.1 PEC –*Xv* qPCR assay

Primer/Probe Sequences for PEC-*Xv* Assay

Xv	Target	Label	Sequence 5'→ 3'	quencher	# bases
Xcv F1	*Xv*	-	CCT CgA Tgg gCA CCT gAT T	-	19
Xcv R1	*Xv*	-	CgT CgA TTg CCg ggT ACT	-	18
Xcv P1	*Xv*	6FAM	ATC gCg gCC AAg AA	MGBNFQ	14

PCR Reaction Mixture for PEC-*Xv* Assay

Component	[Final]	Volume (µL)
Sterile Milli Q water	-	6.988
Xcv Forward (100 pmol/µl)	0.90 µM	0.225
Xcv Reverse (100 pmol/µl)	0.90 µM	0.225
Xcv Probe (100 pmol/µl)	0.250 µM	0.062
qPCR master mix (2x)	1x	12.50
PCR Cocktail	-	20.00
Template DNA	-	5.00
PCR Reaction	-	25.00

PCR Cycling Parameters for PEC-*Xv* Assay

Step 1:	10mins	95°C	enzyme activation
Step 2:	15 sec	95°C	Denaturation
Step 3:	60 sec	60°C	annealing/elongation
Step 4:	40 cycles; steps 2 & 3	-	ramp rate 1.6°C/sec
Step 5:	10 sec	40°C	end run

4.2.1.2 PEC – ZUP (*Acat*) qPCR assay

Primer/Probe Sequences for PEC-ZUP(*Acat*) Assay

ZUP	Target	Label	Sequence 5'→ 3'	quencher	# bases
2791	*Acat*	-	TgT AgC gAT CCT TCA CAA g	-	19
2792	*Acat*	-	TgT CgA TAg ATg CTC ACA AT	-	20
2566	*Acat*	VIC	CTT gCT CTg CTT CTC TAT CAC g	BHQ1	22

PCR Reaction Mixture for PEC-ZUP (*Acat*) Assay

Component	[Final]	Volume (µL) in 25 µL
Sterile MilliQ water	-	7.25
qPCR master mix (2x)	1x	12.50
ZUP 2564 (100 pmol/µl)	0.40 µM	0.10
ZUP 2565 (100 pmol/µl)	0.40 µM	0.10
ZUP 2566 (100 pmol/µl)	0.20µM	0.05
Template (total)	-	5.00

PCR Cycling Parameters for PEC –ZUP (*Acat*) Assay

Step 1:	10mins	95°C	enzyme activation
Step 2:	15 sec	95°C	denaturation
Step 3:	45 sec	60°C	annealing/elongation
Step 4:	40 cycles; steps 2 & 3	-	ramp rate 1.6°C/sec

4.3 Multiplex qPCR assays:

PCR Reaction Mixture for ZUP (*Ac/Acat*) Assay (multiplex)

Component	[Final]	Volume (µL) in 25 µL
Sterile MilliQ water		7
qPCR master mix (2x)	1x	12.50
ZUP 2549 (100 pmol/µl)	0.40 µM	0.10
ZUP 2550 (100 pmol/µl)	0.40 µM	0.10
ZUP 2791 (100 pmol/µl)	0.40 µM	0.10
ZUP 2792 (100 pmol/µl)	0.40 µM	0.10
ZUP 2551 (100 pmol/µl)	0.20µM	0.05
ZUP 2566 (100 pmol/µl)	0.20µM	0.05
Template (total)		5.00

4.4 A Positive Amplification Control (PAC) is required.

4.4.1 PAC options

4.4.1.1 PAC DNA template loaded into separate PCR reactions.
- *Ac* and PEC DNA at a recommended concentration of 2pg/ul

4.5 A non-template control (NTC) is required.

4.5.1 NTC options
- Molecular grade nuclease-free water
- $T_{10}E_{0.1}$ pH 8.0 (appendix - *Ac* PCR: 3.2 $T_{10}E_{0.1}$ pH 8.0)
- DNA isolation kit elution buffer

5 Results Interpretation

5.1 PEC/PPC/PAC/AIC must fall within their expected ranges for the test to be considered valid. Control ranges are determined per laboratory. Note PEC control range limits must be set per crop species in each testing laboratory.

5.1.1 Recommended range determination:

ISO 11462-1:2001Guidelines for implementation of statistical process control (SPC) – Part 1: Elements of SPC. Geneva, Switzerland: International Organization for Standardization (ISO).

ISO 11462-2:2010 Guidelines for implementation of statistical process control (SPC) – Part 2: Catalogue of tools and techniques. Geneva, Switzerland: International Organization for Standardization (ISO).

5.2 NPC/NTC must be negative (no detection of *Ac*) for the test to be considered valid.

5.3 A sample is considered qPCR positive (suspect) for *Ac* if one or more subsamples show detection of *Ac* DNA by one or more *Ac* primer sets. It is the responsibility of the testing laboratory to determine threshold/cutoff values, such that they meet the performance-based acceptance criteria stated in the restrictions on use section of this method. Assays which target multi-copy loci (ZUP) may need a CT cutoff that reflects the increase in sensitivity and potential background noise based on the copy number of the target.

5.4 If a sample is determined qPCR positive (*Ac* suspect), a biological test such as a grow-out assay must be performed to reach a final conclusion regarding the sample.

Ac grow out assay: NSHS Vegetable Crop Methods.

Cb1.1 Acidovorax avenae ssp citrulli – Cucurbit

http://seedhealth.org/seed-health-methods

Appendix - Ac PCR

1. Buffer Preparations

1.1. PBS Buffer – Ac PCR

5x PBS - Ac PCR

Sequence	Ingredient	Amount	Unit
1	Sodium chloride (NaCl)	40.0	g
2	Di-sodium hydrogen phosphate dodecahydrate (Na2HPO4.12H2O)	14.5	g
3	Potassium dihydrogen phosphate (KH2PO4)	1.0	g
4	Potassium chloride (KCl)	1.0	g
5	RO/DI water	800	mL

- Adjust pH to 7.4 with NaOH or HCl (if necessary),
- adjust final volume to 1L
- Autoclave.

Note:

5x is given as example; PBS buffer can be prepared at alternate concentrations.

14.5g Na2HPO4.12H2O can be substituted with:

- 5.76g Na2HPO4
- 7.20g Na2HPO4.2H2O

1.2. Extraction Buffer (PBS-Tween) – Ac PCR

Extraction buffer - Ac PCR

Sequence	Ingredient	Amount	Unit
1	5x PBS - Ac PCR	200.0	mL
2	RO/DI water	800	mL
3	Tween 20	0.5	mL

- Prepare directly before use.

Note:

5x is given as an example; extraction buffer can be prepared from alternate concentrations. Final solution is 1xPBS-Ac PCR buffer+0.05% Tween 20.

2. Media Preparations

2.1. YDC (Yeast extract - dextrose - $CaCO_3$ Agar)

Compound	Amount per liter
Yeast extract	10.0 g
D-glucose (dextrose)	20.0 g
$CaCO_3$	20.0 g
Agar	15.0 g

-Add water to 1.0L final volume, autoclave.

2.2. Modified Tween Agar - *Ac* PCR

Compound	Amount per liter
RO/DI water	970 mL
Agar	15.0 g
Peptone	5.0 g
$CaCl_2 \cdot 2H_2O$ (99%)	0.25 g
Tween 80	10.0 mL
Berberine (hemisulfate salt)	0.2 g
1% Methyl Violet B	1.0 mL
Antibiotics	
Cycloheximide[1]	50 mg
Carbenicillin[1]	50 mg

[1] Add after autoclaving at a temperature below 60°C

2.2.1. Cycloheximide (50mg/ml)

2.2.1.1. Add 9.5mL 40% MeOH to a sterile tube. In a chemical hood add 500mg of Cycloheximide to this tube and invert to resuspend. Adjust volume to 10mL with 40% MeOH and filter sterilize with a 32mm 0.2um Supor Membrane Syringe Filter (ref # 4652) connected to a 10mL syringe (BD 301604) into another sterile tube.

2.2.2. Carbenicillin (50mg/ml)

2.2.2.1. Add 9.5mL of MQ H20 to a sterile tube. In a chemical hood add 500mg of Carbenicillin to this tube and invert to resuspend. Adjust volume to 10mL with MQ H20 and filter sterilize with a 32mm 0.2um Supor Membrane Syringe Filter (ref # 4652) connected to a 10mL syringe (BD 301604) into another sterile tube.

3. Reagent/Solution Preparations

3.1. Sorbitol Solution

Sorbitol solution - Ac PCR

Sequence	Ingredient	Amount	Unit
1	D-sorbitol	0.62	G
2	1 M Tris-HCl	1.0	mL
3	0,5 M EDTA pH 8,0	100.0	µL
4	β-mercapto-ethanol (98%)	20.0	µL
5	RO/DI water, adjust final volume to	10	mL

3.2. $T_{10}E_{0.1}$ pH 8.0

$T_{10}E_{0.1}$ pH 8.0 - Ac PCR

Sequence	Ingredient	Amount	Unit
1	Molecular grade nuclease-free water	990	mL
2	1M Tris pH 8	10.0	mL
3	500mM EDTA pH 8	200.0	µL

3.3. Nutrient Broth

Nutrient Broth -Ac PCR

Sequence	Ingredient	Amount	Unit
1	Nutrient Broth	8	g
2	RO/DI water	1.0	L

- Autoclave before use.

3.4. Liquid King's B medium

Liquid KB -Ac PCR

Sequence	Ingredient	Amount	Unit
1	Proteose pepton No3 from Difco	20	g
2	Glycerol	7.95	mL
	MgSO4.7H2O	1.5	g
	K2HPO4	1.5	g
	RO/DI Water	970	mL

- Autoclave before use.

3.5. Suggested qPCR Mastermix

3.5.1. ABI TaqMan® master mix (2x)

3.5.2. Quanta PerfeCTa Multiplex qPCR ToughMix

4. Recommended Control Preparations

4.1. Bacterial Suspension – Positive Extraction Control

Bacterial suspensions may be used freshly prepared or following proper preservation and storage. It is recommended that the Ct values are monitored using a control chart to ensure there is no drift or degradation over time for all controls subject to storage. Examples of freshly prepared and glycerol preserved control preparations are given below.

4.1.1. PEC-*Xv*

Use a 2-3 day old culture of *Xv* on YDC agar media. Prepare a cell suspension of this culture corresponding to OD_{600}=0.100. Perform three (3), ten-fold (1:10) serial dilutions to prepare the spike solution *Xv*: $OD_{600}=0.100\times10^{-3}$.

4.1.2. PEC-Acat

Prepare a 10 ml KB culture suspension in a new 50 ml reaction tube by inoculating *A. cattleyae* (Acat, ZUM3739) from a KB agar plate not older than 10 days. Incubate the culture suspension at 27°C on a shaker overnight. Determine the optical density of the overnight culture at 600 nm using a photometer set to "absorbance". Dilute the overnight culture to OD 600nm = 0.100 using sterile saline. Prepare the Acat glycerol stock according to following table:

A. cattleyae (Acat) ZUM 3739 glycerol stock solution

Part	Seq	Ingredient	Amount	Unit
A	1	15 v/v % glycerol[1]	49	ml
A	2	Acat O/N culture adjusted to OD_{600}= 0.1	1	ml

[1] Sterilized by autoclaving

Mix the preparation well by inverting the tube 10 times. Prepare aliquots of the glycerol stock in 1.5 ml reaction tubes and store at -80°C. Once frozen, thaw for use only, do not re-freeze.

4.2. Bacterial Suspension – Positive Process Control

4.2.1. PPC-Ac

Inoculate a tube containing 9mL nutrient broth with a 1ul loop of *Ac*. Cap tube loosely and place in a tube rack at an approximate 45 degree angle. Incubate/shake overnight (~18 hours) at 200RPM, 37°C. After incubation period, prepare a cell suspension of this culture corresponding to OD_{600}=0.100. Perform three (3), ten-fold (1:10) serial dilutions to prepare the spike solution of *Ac*: $OD_{600}=0.100\times10^{-3}$.

One mL of *Ac* $OD_{600}=0.100\times10^{-3}$ bacterial suspension is approximately equal to 3000 cells, and can be used as a spike to evaluate laboratory performance of the method against the minimum recovery and detection parameter stated in the restrictions on use section of this document.

4.3. Standardized DNA – Positive Amplification Control

4.3.1. PAC-Ac, *Xv*, *Acat*

Inoculate a tube containing 9mL nutrient broth with a 1ul loop of bacteria grown on solid media. Cap tube loosely, place in a rack at an approximate 45° angle, and shake overnight at 200RPM at 37°C. Process 1ml of resulting culture using Qiagen DNeasy Blood and Tissue Kit including an RNase-A digestion. Elute DNA with 200µL buffer AE. Quantify eluted DNA. Normalize to 2ng/µL stock concentration and 2pg/µL working concentration. PAC concentrations may vary per laboratory and should produce CT values less than the LOQ of the method.

5. Recommended DNA Isolation Kit Modifications

5.1. MoBio PowerFood Kit

5.1.1. Garnet bead tube is modified from provided 0.5mL to 2.0mL screwcap tube.
5.1.2. EDTA is added to solution PF6 for DNA storage stability. [EDTA=0.1mM]

5.2. Machery-Nagel NucleoSpin Plant II Kit

5.2.1. Modifications of Macherey-Nagel NucleoSpin® 96 Plant II (96 well format, ref # 740663)The vacuum manifold option of the protocol is followed with the following modifications:

- lysis buffer composition (480 µl PL1, 20 µl proteinase K (20 mg/ml stock, *no* RNase A)
- lysis buffer volume used for resuspending pellet 450 µl
- lysis incubation time 1 – 20 hr
- lysate clearing: 20 min centrifugation at 3200 g
- PW1 washing step is performed 2X
- Dry membranes for 15 min
- Only 1 DNA elution step
- Option to elute DNA not by vacuum but by centrifugation at 3200 g for 2 min

5.2.2. Modifications of Macherey-Nagel NucleoSpin® Plant II (single tube format, reference# 740770)

- lysis buffer composition (480 µl PL1, 20 µl proteinase K (20 mg/ml stock, *no* RNase A)
- lysis buffer volume used for resuspending pellet 450 µl
- lysis incubation time 1 – 20 hr
- lysate clearing: 5 min centrifugation at 19000 g
- centrifugation steps involving silica column performed at 19000 g
- PW1 washing step is performed 2X
- Only 1 elution step using 100 µl PE, pre-heated at 70°C

5.3. Sbeadex Maxi Plant Kit

5.3.1. No recommended modifications

Annex F.

ISHI-Veg Comparative Test Plan for the Detection of *Acidovorax citrulli* on Cucurbit Seeds by Direct qPCR Method

1. Organization and design

1.1. Test Organizer

Andrew Acas
Monsanto Vegetable Seed Co.
34737 State Highway 16
Woodland, CA 95695
U.S.A.
T: +1 530-406-6507
E: andrew.k.acas@monsanto.com

1.2. Pathogen

Acidovorax citrulli (Ac)

1.3. Crop(s)

Melon (*Cucumis melo)*
Watermelon (*Citrullus lanatus)*

1.4. Participating Laboratories: Criteria & Contact Information

Required Criteria: Participating laboratory must be experienced in seed health testing and molecular testing of bacterial plant pathogens.

Table of Participating Laboratories

Table 1. Participating laboratories.

Laboratory	Contact	Email	Remarks
Anove	Leandro de León	ldeleon@anove.es	
Bayer CS	Bart Geraats	bart.geraats@bayer.com	
HM Clause	Geeta Sanjeev	g.sanjeev@hmclause.com	
Monsanto	Kurt Kleinhesselink	kurt.kleinhesselink@monsanto.com	
Rijk Zwaan	Marjolein Spiekerman	m.spiekerman@rijkzwaan.nl	
Syngenta	Bert Woudt	bert.woudt@syngenta.com	
Naktuinbouw	Harrie Koenraadt	h.koenraadt@naktuinbouw.nl	

1.5. Timeline

Table 2. Time Schedule

Time	**Action**	**Assignee**	**Remarks**
April 2016	Draft test plan to ITG	K. Kleinhesselink	
May 2016	Sample preparation/distribution	K. Kleinhesselink	
June 2016	Testing samples – Data Return	All participants	
July 2016	Data analysis and report	K. Kleinhesselink	

2. Introduction and objective of comparative test (CT) plan

2.1. Introduction

2.2. Seed Health tests

2.3. Objective of comparative test

The objective of this comparative test is to verify the result of method validation, demonstrating under reproducible conditions consistent detection of *Ac* naturally infected seeds.

3. Materials and Methods

3.1. Seed samples and controls

Each participating laboratory will receive a set of 20 cucurbit seed samples. Each sample will contain 5,000 seeds.

One sample will be labeled "(+) Positive Control" and requires *Ac* AND PEC to be spiked into the sample.

One sample will be labeled "(-) Negative Control"; no spike shall be added to this sample.

18 "blind" samples remain to be evaluated for the presence of *Ac*. The set of 18 "blind" samples will consist of non-infected samples, samples with a moderate level of *Ac* and samples with a high level of *Ac*. Samples containing Ac originate from naturally infected seed lots. All seed samples will be randomly coded and their identity will only be known by the test organizer.

In addition to the samples, standardized reference DNA will be distributed to be used as a qPCR positive control (Ac, Xv and Acat @[2pg/μL]). Reference DNA serves to normalize differences between various qPCR instruments so that cycle threshold (Ct) values can be compared with a known degree of variance/bias.

It is important that the seed lots be well-characterized before the CT to have a good understanding of the expected results and subsequent analysis. This is achieved by following ISTA document" Guidelines for organizing and analyzing results of Proficiency tests (PT) and interlaboratory tests for validation of methods (CT)" (ISTA, 2013) where applicable. A homogeneity and stability test will be also performed by the test organizer before and after the CT of each seed lot following the above Guidelines.

3.2. Methods

Each laboratory will perform the direct qPCR method on each seed sample. The protocols and materials needed for the test are described in Ct plan appendix.

For the purposes of the CT, the Positive process control (Sample # 19) will be spiked with Both *Ac* and selected PEC.

Ac Spike Concentration: $OD_{600} = 0.100 \times 10^{-3}$

Ac Spike Volume: 1.27mL per 5,000 seed sample

PEC Concentration and Volume to be determined by each testing laboratory.

Time schedule for direct qPCR

DAY	ACTION	TIME NEEDED
0	Preparation of materials (buffers, labware, etc.)	3 h
1	Seed wash (soak and shake)	1 h
1	Concentration of bacteria	2-3 h
1	DNA isolation	Variable by method, ~3 h
1-2	PCR setup & runtime	3 h
1-2	Interpretation and reporting	3 h

3.3. Statistical analysis

Participants are to record the results of the direct qPCR method in the table below and return it to the test organizer. After compiling the results, analysis will be completed on a binary qualitative (positive/negative) level, per sample. The standardized reference PCR positive control will be evaluated at the cycle threshold (Ct) level to examine bias if necessary. The method of Langton *et al.* (2002) will be used to evaluate the accordance (repeatability of qualitative data) and concordance (reproducibility of qualitative data) of each method per contamination level.

3.4. Data capture form

Testing Laboratory:		**Target Pathogen Assay (Ct)**			**PEC Assay (Ct)**	
Sample ID - PCR REP	**Crop Species**	**Contig 21**	**Contig 22**	**Zup (Ac)**	**Xcv**	**Zup (Acat)**
1 - a						
1 - b						
2 - a						
2 - b						
3 - a						
3 - b						
4 - a						
4 - b						
5 - a						
5 - b						
6 - a						
6 - b						
7 - a						
7 - b						
8 - a						
8 - b						
9 - a						
9 - b						
10 - a						
10 - b						
11 - a						
11 - b						
12 - a						
12 - b						
13 - a						
13 - b						
14 - a						
14 - b						
15 - a						
15 - b						
16 - a						
16 - b						
17 - a						
17 - b						
18 - a						
18 - b						
19 (+) Process Control - a						
19 (+) Process Control - b						
20 (-) Process Control - a						
20 (-) Process Control - b						
Ac (+) PCR Control - a						
Ac (+) PCR Control - b						
PEC (+) PCR Control - a						
PEC (+) PCR Control - b						
NTC (-) PCR Control - a						
NTC (-) PCR Control - b						