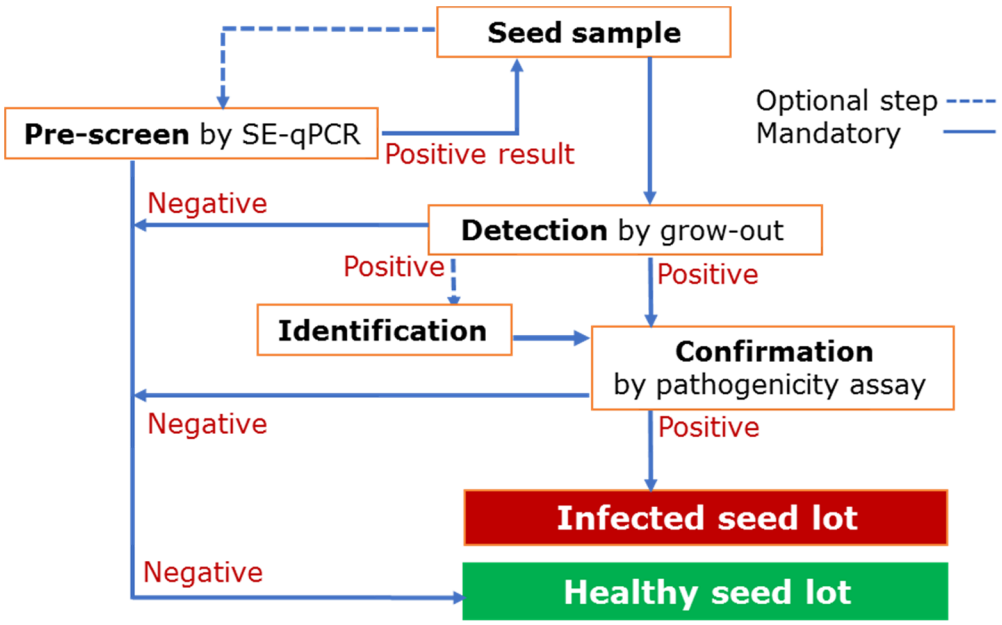
Method for the Detection of *Acidovorax citrulli* in seed of Cucurbit crops

Crops:	All Cucurbits, including, but not limited to: Watermelon (*Citrullus lanatus* var. *lanatus*) Cantaloupe (*Cucumis melo* var. *cantalupensis*) Melon (*Cucumis melo)* Squash (*Cucurbita pepo*) Cucumber (*Cucumis sativus*) Pumpkin (*Cucurbita maxima, Cucurbita moschata*) Gourd (*Lagenaria siceraria*)
Pathogen:	*Acidovorax citrulli*
Date:	August 2018

Principle

Detection of infectious *Acidovorax citrulli* bacteria on cucurbits seedlings by growing out seeds under environmental conditions highly conducive to producing disease symptoms. Isolates are recovered from symptomatic seedlings and identified, then confirmed with a pathogenicity assay.

The qPCR assay can be used as a pre-screen on a seed extract. If *A. citrulli* is not detected, the test is complete and the seed lot is considered healthy. As qPCR may detect both viable and non-viable bacteria, and non-target bacteria, a positive qPCR is followed by the grow-out to determine the presence of infectious *A. citrulli*.



Note: The protocols for pre-screening seed lots, detecting the target pathogen and confirming its viability and pathogenicity have been combined, as together they form a method for the detection of *A. citrulli* in seed of Cucurbit crops.

Method Validation

The grow-out has been in use by the industry for many years. For more information about validation data, contact NSHS (nshs@iastate.edu).

The seed extract qPCR (or SE-PCR) has been validated by seven independent laboratories using blended naturally infected seed samples through an ISHI-Veg comparative test. Seed from the following cucurbit crops were used during validation: melon (*Cucumis melo),* watermelon (*Citrullus lanatus)*, squash *(Cucurbita pepo*) and root stock squash (*Cucurbita maxima).* The validation report for this assay can be made available upon request to the ISF Secretariat.

Both assays have been approved by the US National Seed Health System (NSHS) as Standard A methods (see http://seedhealth.org/seed-health-testing-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 is provided such that it accommodates modular assay components. DNA isolation, positive extraction control selection, qPCR reaction mixture, cycling parameters, multiplex reactions and evaluation specifications (threshold/cut-offs) are variables that may differ between testing laboratories.

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, *viz*. 100% detection of *Acidovorax citrulli* across triplicate 5,000 seed subsamples in all qPCR reactions where each 45 mL aliquot contains ~12 CFU/mL. Or in a 5,000 seed sub-sample to which 250 mL buffer is added, 100% detection of *Acidovorax citrulli* in all qPCR reactions from triplicate samples of relevant crops, where each sample is spiked with ~3000 cells of *Acidovorax citrulli* is required (see **Materials and Reagents** section D.2).

Method Execution

To ensure process standardization and valid results, it is recommended that the best practices developed by ISHI-Veg be followed (see http://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-method-development/).

In line with the guidance provided in ISF's view on indirect seed health tests (see http://www.worldseed.org/resources/position-papers/#specifc-technical-subjects), if a sample is determined qPCR positive, the seed lot is considered to be *suspect*. A final decision on the status of the seed lot can be taken only after a test confirms the presence of viable *Acidovorax citrulli* in the sample and its pathogenicity.

Sample and sub-sample size

For the grow-out, the recommended *minimum* sample size is 10,000 seeds and can be up to 30,000 seeds per lot.

For the SE-qPCR the recommended sample size is 30,000 seeds with a *maximum* sub-sample size of 5,000 seeds. If the SE-qPCR is a positive and a confirmatory test is conducted, a new sample must be drawn for the grow-out.

Protocol for detecting *Acidovorax citrulli* in seed of Cucurbit crops

I. Detection and Confirmation

These protocols can also be found on the website of the National Seed Health System, see www.seedhealth.org >> Methods >> Seed Health Testing Methods >> Cucurbit >> Cb 1.1 *Acidovorax avenae* ssp. *citrulli* – Grow-out (http://seedhealth.org/cb1-1/).

1. Greenhouse Preparation

Clean greenhouse floors, walls, benches and equipment and sanitize with a known bactericide before each test is initiated in the greenhouse.

2. Planting seeds

2.1. Plant seed into any common, sanitized greenhouse potting mixture and maintain under conditions optimal for seed germination until seedling emergence.

- ❑ Seedling density should be such to allow unrestricted seedling development for a period of three weeks.

2.2. Plant a seed sample known to be infected with *A. citrulli* (or Ac) as a check on the test conditions and subsequent disease development. Maintain the positive check in an isolated area of the greenhouse.

3. Greenhouse conditions

3.1. Maintain relative humidity at 70% or higher, from the time seedlings emerge to final reading. Relative humidity should not be lower than 50% for more than 12 h.

3.2. Maintain greenhouse temperatures between 24°C (74°F) and 35°C (95°F) from seedling emergence to final inspection. Temperatures should not be out of this range for more than 12 h. The preferred temperature is 29°C (85°F).

3.3. Record temperature and relative humidity of the greenhouses, preferably above plant canopy for the duration of the test.

3.4. Supplement light to 12 hours per day if necessary.

4. Evaluation Procedures

4.1. Initiate seedling inspection once the cotyledons begin expanding and continue evaluations on a daily basis until final evaluation. This inspection should include the positive controls. Inspect the positive controls last and do not handle them. If positive controls do not show symptoms of the disease then the grow-out test is not valid. See below for pictures of typical disease symptoms on seedlings.

- ❑ Avoid contact with the seedlings until the final inspection.
- ❑ Remove symptomatic seedlings as soon as possible. Conduct a diagnostic test as described under section 4.2.2 and confirm the identity of the causal agent.
- ❑ Final confirmation of suspect plants is done using a pathogenicity assay.

4.2. At 18 days, or when the cotyledons are fully expanded and first true leaves are expanding, conduct a thorough final inspection of all of the seedlings. Seedling inspection should also be conducted immediately upon finding symptomatic seedlings and confirming the identity of the causal agent.

4.2.1. Inspect the seedlings individually.

- ❑ Only technicians trained in *A. citrulli* detection should conduct inspections. Additionally, color photographs of disease symptoms on seedlings should be

provided as a reference.

- ❑ Seedlings should be inspected under natural light in a well–lit area.
- ❑ Gloves should be worn when handling seedlings and changed between each seed lot.

4.2.2. Symptomatic seedlings can be identified by field test strips, ELISA, or PCR testing that is specific for *A. citrulli* by isolating the bacterium onto YDC, or semi-selective media such as King's Medium B (King et al., 1954. J. Lab. Clin. Med. 44:301) and Modified Tween. Colony morphology on the various media are as follows:

YDC: Ac colonies appear tannish, domed and round. Colonies range in size from 1 mm to 3 mm after 2 – 3 days incubation at 28°C.

Modified Tween: Ac colonies appear grayish, flat, round to oval, with a crystallized zone surrounding the colonies;

Kings B: Ac colonies are non - fluorescent, cream colored and slightly domed. Colonies remain constricted up to 5 days.

4.2.3. In the pathogenicity assay, suspect bacteria isolated from selective medium should be transferred to YDC. Each isolate should be inoculated onto watermelon or melon seedlings at the expanding cotyledon stage. Inoculations can be made by inserting a sterile toothpick into the suspect colony and inserting the toothpick into the growing point of the plant. Several single colonies of each suspect isolate should be inoculated. Plants should be kept under high humidity for 48 to 96 hours. Water soaked lesions will be visible within 24 to 96 hours.

5. Minimizing the risk of cross-contamination

5.1. Maintain adequate spacing (60 cm) between different seed lots placed in the same greenhouse. Barriers that can be disinfested or easily removed and disposed can also give adequate protection. The barriers should be 60 cm to 90 cm tall.

5.2. Strict rules and effective chemical treatments should be enforced to keep insects, spiders, rodents and any other known and potential vectors of *A. citrulli* out of the greenhouse.

5.3. Avoid manual manipulation of the seedlings during the grow-out except as necessary for inspecting individual seedlings during final inspection.

- ❑ The only exception to this is the contact necessary to remove the symptomatic seedlings for tissue testing.

5.4. Minimize splashing and run-off during watering, especially between different seed lots in the same greenhouse.

5.5. Remove all *A. citrulli* infected samples from greenhouse and immediately dispose of tissue appropriately.

6. Medium formulation: YDC (Yeast extract - dextrose - CaCO3 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 1.0 L final volume, autoclave.

7. Diagnostic photos

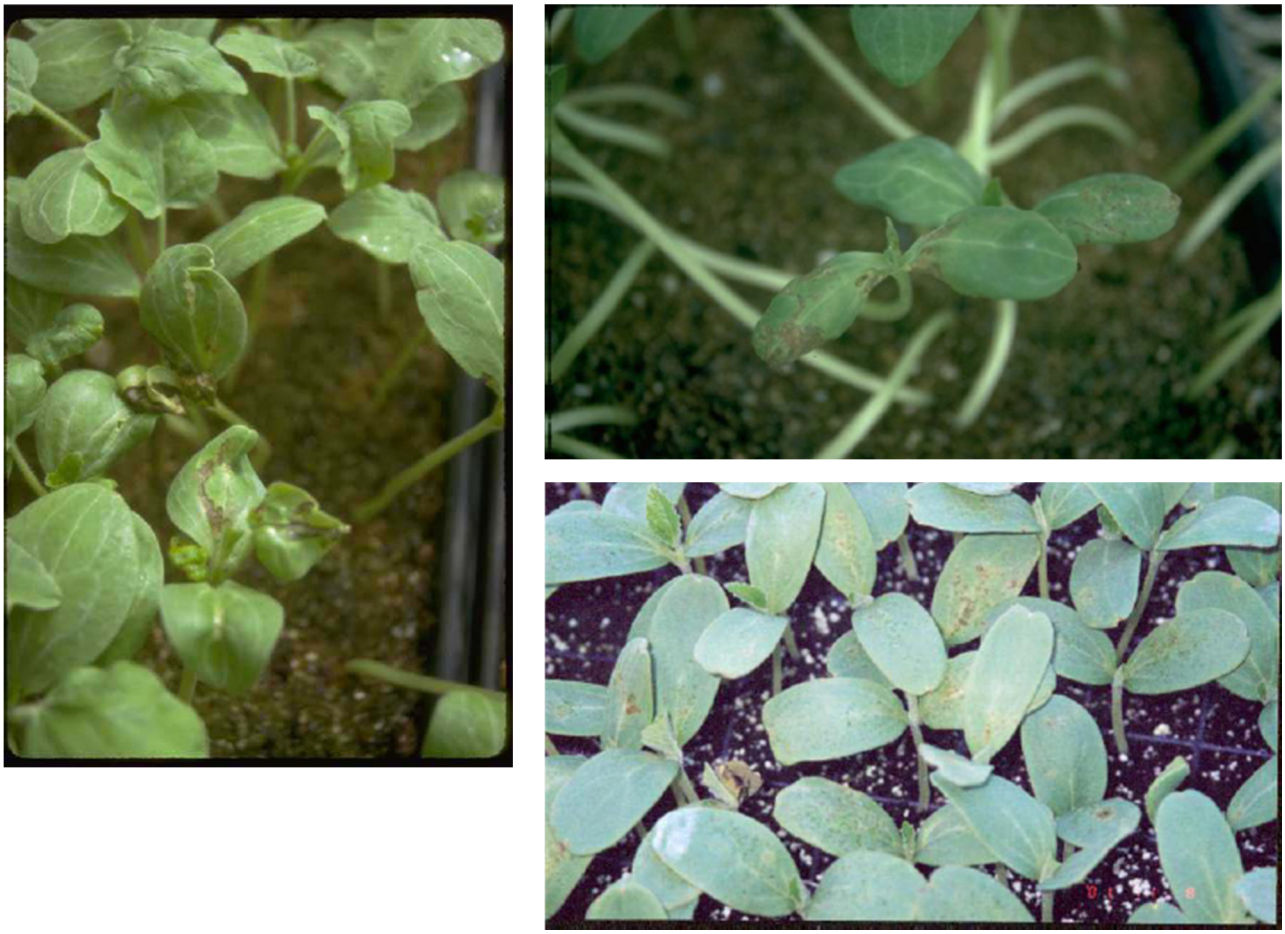


Figure 1: Typical symptoms of the disease at the seedling stage

II Pre-screen

1. Extraction of Bacteria from the Seed

1.1 Place seed sub-samples in a container appropriate for seed and buffer volume. Add the extraction buffer to each sub-sample at a ratio of 2.0 ml buffer per 1.0 g of seed (v:w) (see **Materials and Reagents** section A.2). Due to buffer absorbance by the seeds, sub-samples of large seed (e.g. squash) may require a slightly increased buffer to seed ratio (up to 2.5 ml per 1.0 g 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)**	***Acidovorax cattleyae* (Acat)**
Isolate	ATCC® 11633™ (deposited as *Xanthomonas vesicatoria* (Xe)	ATCC® 33619™
Concentration	OD_{600}= 0.100×10^{-3}	OD_{600}= 0.100, 1:50 dilution
Volume	100 µL spike/25mL extraction buffer (see **Materials and Reagents** section D.1)	5 µL spike/25 mL extraction buffer (see **Materials and Reagents** section D.1)

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 sub-sample (seed known to be free of Ac and the PEC target).

1.3.1 PPC spike recommendation:

	***Acidovorax citrulli* (Ac)**
Isolate	ATCC® 29625™
Concentration	OD_{600}= 0.100×10^{-3}
Volume	100µL Ac spike/25mL extraction buffer (see **Materials and Reagents** section D.2)

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

1.4.1 NPC options:

i. A sub-sample containing a typical volume of extraction buffer

ii. A sub-sample containing seed known to be free of Ac target and a volume of extraction buffer 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 (~120 rpm).

2. Collection of Target Bacteria by Differential Centrifugation

2.1 Transfer 45 mL 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 45 mL.

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

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

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

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 (see **Materials and Reagents** section C.1) to the pellet and re-suspend.

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

2.4.1.4 Centrifuge the suspension for 10 minutes at 1800 RCF.

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

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

3. DNA Isolation

3.1 Recommended DNA isolation kits (see **Materials and Reagents** section D.4):

3.1.1 MoBio PowerFood

3.1.2 Machery-Nagel NucleoSpin Plant II

3.1.3 Sbeadex Maxi Plant Kit

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

qPCR Reaction Mixture for Contig 21 Assay

Component	Final Concentration	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
qPCR Cocktail	-	20.00
Template DNA	-	5.00
qPCR Reaction	-	25.00

qPCR Cycling Parameters for Contig 21 Assay

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

qPCR Reaction Mixture for Contig 22 Assay

Component	Final Concentration	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
qPCR Cocktail	-	20.00
Template DNA	-	5.00
qPCR Reaction	-	25.00

qPCR Cycling Parameters for Contig 22 Assay

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

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

qPCR Reaction Mixture for ZUP (Ac) Assay

Component	Final Concentration	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)	0.20 µM	0.05
Template (total)		5.00

qPCR Cycling Parameters for ZUP (Ac)

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

4.2 Recommended PEC qPCR assays:

4.2.1 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

qPCR Reaction Mixture for PEC-Xv Assay

Component	Final Concentration	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
qPCR Cocktail	-	20.00
Template DNA	-	5.00
qPCR Reaction	-	25.00

qPCR Cycling Parameters for PEC–Xv Assay

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

4.2.2 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

qPCR Reaction Mixture for PEC-ZUP (Acat) Assay

Component	Final Concentration	Volume (µL) in 25 µL
Sterile MilliQ water	-	7.25
qPCR master mix (2x)	1x	12.50
ZUP 2791 (100 pmol/µl)	0.40 µM	0.10
ZUP 2792 (100 pmol/µl)	0.40 µM	0.10
ZUP 2566 (100 pmol/µl)	0.20 µM	0.05
Template (total)		5.00

qPCR Cycling Parameters for PEC –ZUP (Acat) Assay

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

4.2.3 Multiplex qPCR assays:

qPCR Reaction Mixture for ZUP (Ac/Acat) Assay (multiplex)

Component	Final Concentration	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)	0.20 µM	0.05
ZUP 2566 (100 pmol/µl)	0.20 µM	0.05
Template (total)		5.00

4.2.4 A Positive Amplification Control (PAC) is required.

4.2.5 PAC options

4.2.5.1 PAC DNA template loaded into separate qPCR reactions; Ac and PEC DNA at a recommended concentration of 2 pg/ul

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

4.3.1 NTC options

i. Molecular grade nuclease-free water

ii. $T_{10}E_{0.1}$ pH 8.0 (see **Materials and Reagents** section C.2)

iii. DNA isolation kit elution buffer

5. Interpretation of results

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 11462-1:2001 Guidelines 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).

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

5.2 A sample is considered qPCR positive (suspect) for Ac if one or more sub-samples show detection of Ac DNA by one or more Ac primer sets. It is the responsibility of the testing laboratory to determine threshold/cut-off 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 cut-off that reflects the increase in sensitivity and potential background noise based on the copy number of the target.

- ❑ **If a sample is qPCR positive (Ac suspect), the detection and confirmation test in Section 1 must be performed to reach a final conclusion about the sample and seed lot (See ISF's view on indirect seed health tests** http://www.worldseed.org/resources/position-papers/#specifc-technical-subjects).

Materials and Reagents

A. Buffer Preparations

A.1 **5x PBS** (Note: 5x is given as example; PBS buffer can be prepared at alternate concentrations)

Sequence	Ingredient	Amount	Unit
1	Sodium chloride (NaCl)	40.0	g
2	Di-sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)[1]	14.5	g
3	Potassium dihydrogen phosphate (KH2PO4)	1.0	g
4	Potassium chloride (KCl)	1.0	g
5	RO/DI water[2]	800	mL

[1] 14.5g Na2HPO4.12H2O can be substituted with 5.76 g of Na_2HPO_4 or 7.20 g $Na_2HPO_4.2H_2O$

[2] Adjust final volume to 1 L

Adjust pH of the final solution to 7.4 with NaOH or HCl, if necessary. Autoclave.

A.2 **Extraction buffer**

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

[1] 5x is given as an example; extraction buffer can be prepared from alternate concentrations. Prepare directly before use. Final solution is 1xPBS buffer+0.05% Tween 20.

B. Media Preparations

B.1 **YDC (Yeast extract - dextrose - CaCO3 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 1.0 L final volume, autoclave.

B.2 **Modified Tween Agar**

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	**Amount per liter**
Cycloheximide[1]	50 mg
Carbenicillin[1]	50 mg

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

B.3 **Cycloheximide (50 mg/ml)**

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

B.4 **Carbenicillin (50 mg/ml)**

Add 9.5 mL of MQ H_20 to a sterile tube. In a chemical hood add 500mg of Carbenicillin to this tube and invert to resuspend. Adjust volume to 10 mL with MQ H_20 and filter sterilize with a 32 mm 0.2 um Supor Membrane Syringe Filter (ref # 4652) connected to a 10 mL syringe (BD 301604) into another sterile tube.

C. Reagent/Solution Preparations

C.1 **Sorbitol solution**

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	8.88	mL

C.2 **$T_{10}E_{0.1}$ pH 8.0**

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

C.3 **Nutrient Broth**

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

Autoclave before use.

C.4 **Liquid KB**

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

Autoclave before use.

Suggested qPCR Mastermix

i. ABI TaqMan® master mix (2x)

ii. Quanta PerfeCTa Multiplex qPCR ToughMix

D. Recommended Control Preparations

D.1 **Bacterial Suspension – Positive Extraction Control (PEC)**

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.

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*: $OD_{600}=0.100\times10^{-3}$.

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	Sequence	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.

D.2 **Bacterial Suspension – Positive Process Control (PPC)**

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 OD600=0.100. Perform three (3), ten-fold (1:10) serial dilutions to prepare the spike solution of Ac: OD600=0.100×10-3.

One mL of Ac OD600=0.100×10-3 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.

D.3 **Standardized DNA – Positive Amplification Control (PAC)**

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.

D.4 **Recommended DNA Isolation Kit Modifications**

MoBio PowerFood Kit

- Garnet bead tube is modified from provided 0.5mL to 2.0mL screwcap tube.
- EDTA is added to solution PF6 for DNA storage stability at a concentration of 0.1mM.

Machery-Nagel NucleoSpin Plant II Kit

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

- lysis buffer composition: 480 µl PL1 + 20 µl of a 10 mg/ml proteinase K stock solution
- 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

II 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

Sbeadex Maxi Plant Kit

- No recommended modifications