International Seed Federation
Seed is Life

Detection of *Acidovorax citrulli* in Cucurbit Seeds by SE-qPCR

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Developed by ISHI-Veg

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Crop: Watermelon (*Citrullus lanatus var. lanatus*)
Melon (*Cucumis melo*)
Squash (*Cucurbita pepo*)
Root stock squash (*Cucurbita maxima*)

Pathogen: *Acidovorax citrulli*

Version: 2.0 (May 2021)

PRINCIPLE

Detection of infectious *Acidovorax citrulli* bacteria on cucurbits seeds is done by growing out seeds under environmental conditions highly conducive to producing disease symptoms followed by a biological assay to confirm the presence of infectious *A. citrulli*.

A seed extract qPCR assay (SE-qPCR) may be used as a pre-screen. The test is complete if no *A. citrulli* is detected and the seed lot is considered healthy. However, as qPCR detects both viable and non-viable bacterial DNA, a positive pre-screen SE-qPCR is followed by the grow-out to determine the presence of infectious *A. citrulli*.

The complete method process workflow is presented in Figure 1.

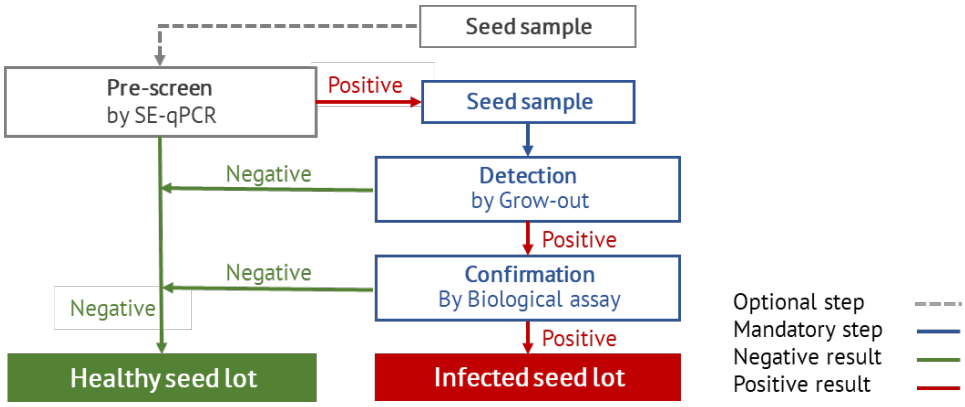

Figure 1. Method process workflow

METHOD VALIDATION

The seed extract qPCR (SE-qPCR) has been validated by seven independent laboratories using blended naturally infected seed samples through an ISHI-Veg comparative test (Kleinhesselink, 2017). 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.

The SE-qPCR has 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

Technical details on the reagents / material used in the validation study (e.g. supplier information) are provided in the protocol and the validation report. If material and consumables from different suppliers are used, it is necessary to verify their performance. For PCR methods, in-house method optimization is often necessary by changing certain parameters as described in Best Practices for PCR Assays in Seed Health Tests.

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 *A. 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 subsample to which 250 mL buffer is added, 100% detection of *A. citrulli* in all qPCR reactions from triplicate samples of relevant crops, where each sample is spiked with ~3000 cells of *A. citrulli* is required.

METHOD EXECUTION

To ensure process standardization and valid results, it is strongly recommended to follow the best practices described by ISHI-Veg.

In line with the guidance provided in ISF's view on indirect seed health tests, 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 *A. citrulli* in the sample and its pathogenicity.

SAMPLE AND SUBSAMPLE SIZE

The minimum recommended sample size is 10,000 seeds up to 30,000 seeds per lot, with a maximum subsample 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.

REVISION HISTORY

Version	Date	Changes (minor editorial changes not indicated)
1.0	July 2017	First version of the protocol.
1.1	August 2018	NSHS grow-out protocol v1.1 combined with the ISHI pre-screening protocol, as together they form a method for the detection of *A. citrulli* in seed of Cucurbit crops.
2.0	May 2021	Protocol presented in accordance with ISHI-Veg's protocol guidelines. Reference to grow-out protocols included in method description. Sample size adjusted to align with grow-out assay.

Protocol for detecting *Acidovorax citrulli* in Cucurbit Seeds

I. PRE-SCREEN BY SEED-EXTRACT qPCR (OPTIONAL STEP)

For PCR methods, in-house method optimization is often necessary by changing certain parameters as described in Best Practices for PCR Assays in Seed Health Tests.

Materials

- Container
- Orbital shaker
- Seed extraction buffer (Table I.1)
- 5x PBS (Table I.2)
- Sorbitol solution (Table I.3)
- $T_{10}E_{0.1}$ (Table I.4)
- Nutrient Broth (Table I.5)
- Liquid KB (Table I.6)
- Controls (Table I.7 to I.10)
- qPCR mix, primers (Table I.11) and equipment
- DNA isolation kit
- Centrifuge
- Lab disposables

Table I.1. Seed Extraction Buffer

Compound	Amount
5x PBS[a]	200.0 mL
RO/DI water	800 mL
Tween™ 20	0.5 mL

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

Table I.2. 5x PBS

Compound	Amount
Sodium chloride (NaCl)	40.0 g
Di-sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)[a]	14.5 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.0 g
Potassium chloride (KCl)	1.0 g
RO/DI water[b]	800 mL

[a] 14.5g $Na_2HPO_4.12H_2O$ can be substituted with 5.76 g of Na_2HPO_4 or 7.20 g $Na_2HPO_4.2H_2O$
[b] Adjust final volume to 1 L
Notes: Adjust pH of the final solution to 7.4 with NaOH or HCl, if necessary. Autoclave.

Table I.3. Sorbitol solution

Compound	Amount
D-sorbitol	0.62 g
1 M Tris-HCl pH 8	1.0 mL
0.5 M EDTA pH 8	100.0 µL
β-mercapto-ethanol (98%)	20.0 µL
RO/DI water	8.88 mL

Table I.4. $T_{10}E_{0.1}$ pH 8

Compound	Amount
Molecular grade nuclease-free water	990 mL
1 M Tris-HCl pH 8	10.0 mL
500 mM EDTA pH 8	200.0 µL

Table I.5. Nutrient Broth

Compound	Amount
Nutrient Broth	8 g
RO/DI water	1 L

Note: Autoclave before use

Table I.6. Liquid KB

Compound	Amount
Proteose pepton No3 from Difco	20 g
Glycerol	7.95 mL
$MgSO_4.7H_2O$	1.5 g
K_2HPO_4	1.5 g
RO/DI Water	970 mL

Note: Autoclave before use

Table I.7. Types of controls used

Control type	Description
Positive Extraction Control (PEC)	*A. cattleyae* OR *X. euvesicatoria* spike (Table I.8)
Internal Amplification Control (IAC)	*A. cattleyae* OR *X. euvesicatoria* spike (Table I.8)
Positive Process Control (PPC)	Seed extract from known *A. citrulli* infected seed (Table I.9)
Negative Process Control (NPC)	Extraction buffer
	Seed extract from seed known to be free of *A. citrulli* target
Positive Amplification Control (PAC)	*A. citrulli* / PEC DNA
Non Template Control (NTC)	Molecular grade nuclease-free water
	$T_{10}E_{0.1}$ pH 8
	DNA isolation kit elution buffer

Table I.8. PEC spike recommendations

	***Xanthomonas euvesicatoria* (Xe)**	***Acidovorax cattleyae* (Acat)**
Isolate	ATCC 11633™ (deposited as X. *vesicatoria*)	ATCC 33619™ or ZUM3739
Concentration	OD_{600}= 0.100×10^{-3}	OD_{600}= 0.100, 1:50 dilution
Volume	100 µL spike/25mL extraction buffer	5 µL spike/25 mL extraction buffer

Notes: Bacterial suspensions may be used freshly prepared or following proper preservation and storage. It is recommended that the Cq 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-Xe: Use a 2-3 day old culture of Xe on YDC agar media. Prepare a cell suspension of this culture corresponding to OD_{600}=0.100. Perform three, ten-fold (1:10) serial dilutions to prepare the spike solution Xe: OD_{600}=0.100×10^{-3}.

PEC-Acat: Prepare a 10 mL KB culture suspension in a new 50 mL reaction tube by inoculating *A. cattleyae* 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_{600}=0.100 using sterile saline. Prepare the *A. cattleyae* glycerol stock according to Table I.9. 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.

Table I.9. PEC-Acat glycerol stock solution

Compound	**Amount**
15 v/v % glycerol[a]	49 mL
Acat O/N culture adjusted to OD_{600}= 0.1	1 mL

[a] Sterilized by autoclaving

Table I.10. PPC spike recommendation

	***Acidovorax citrulli* (Ac)**
Isolate	ATCC 29625™
Concentration	OD_{600}= 0.100×10^{-3}
Volume	100 µL spike/25 mL extraction buffer

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

One mL of Ac OD_{600}=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.

Table I.11. PCR assays, primers and primer-sequences

PCR assay	Target	Primers	Primer sequences
Contig 21	Ac	Aac F1	5' – ACC GAA CAG AGA GTA ATT CTC AAA GAC– 3'
		Aac R1	5' – GAG CGT GAT GGC CAA TGC – 3'
		Aac P1	5' – 6FAM – CAT CGC TTG AGC AGC AA – MGBNFQ – 3'
Contig 22	Ac	Aac F2	5' – GAA AGT GGT TGT TCT GGT GAT CAA– 3'
		Aac R2	5' – TTC GGA GGA CTC GGG ATT – 3'
		Aac P2	5' – 6FAM – ATG GTC TGC GAG CCA G – MGBNFQ – 3'
ZUP	Ac	ZUP2549	5' – GAG TCT CAC GAG GTT GTT – 3'
		ZUP2550	5' – GAC CCT ACG AAA GCT CAG– 3'
		ZUP2551	5' – 6FAM – TGC AGC CCT TCA TTG ACG G– BHQ1 – 3'
PEC-Xe	Xe	Xcv F1	5' – CCT CGA TGG GCA CCT GAT T– 3'
		Xcv R1	5' – CGT CGA TTG CCG GGT ACT– 3'
		Xcv P1	5' – 6FAM – ATC GCG GCC AAG AA– MGBNFQ – 3'
PEC-ZUP	Acat	ZUP2791	5' – TGT AGC GAT CCT TCA CAA G– 3'
		ZUP2792	5' – TGT CGA TAG ATG CTC ACA AT– 3'
		ZUP2566	5' – VIC – CTT GCT CTG CTT CTC TAT CAC G– BHQ1 – 3'

1. Extraction of bacteria from the seed

1.1. Place seed subsamples in a container appropriate for seed and buffer volume.

1.2. Add the extraction buffer to each subsample at a ratio of 2.0 mL buffer per 1.0 g of seed (v:w). Due to buffer absorbance by the seeds, subsamples 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).

1.3. A positive extraction control (PEC) spike is required. Each subsample being evaluated for *A. citrulli* is spiked with the PEC and the spike volume is adjusted to a standardised concentration (i.e. added at a fixed ratio to the required extraction buffer volume). See Table I.8. Suspension may be prepared daily or in bulk (stored at -80 °C in a glycerol solution).

1.4. Both a Positive Process Control (PPC) and Negative Process Control (NPC) subsample are required (Table I.7 and I.10). It is recommended that both the PPC and the PEC spikes be added to the NPC subsample (seed known to be free of *A. citrulli* and the PEC target).

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

2. Collection of Target Bacteria by Differential Centrifugation

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

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

2.2. Centrifuge subsamples for 5 min 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 min at 3200 RCF.

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

2.4.1. Decant supernatant from step 2.4

2.4.2 Add 1 mL of sorbitol solution (Table I.3) to the pellet and re-suspend.

2.4.3 Incubate suspension for 20-60 min at room temperature.

2.4.4 Centrifuge the suspension for 10 min 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

The assay has been validated with the MoBio PowerFood, Machery-Nagel NucleoSpin Plant II and Sbeadex Maxi Plant kits. It is the responsibility of the user to check with an in-lab validation study if the sensitivity and selectivity of the assay is not influenced when using a different DNA isolation kit.

Recommended Modifications to the DNA kits tested:

i. MoBio PowerFood Kit

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

ii. 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 hours
- lysate clearing: 20 min centrifugation at 3200 g
- PW1 washing step is performed 2×
- 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 hours
- lysate clearing: 5 min centrifugation at 19000 g
- centrifugation steps involving silica column performed at 19000 g
- PW1 washing step is performed 2×
- Only 1 elution step using 100 µL PE, pre-heated at 70 °C

4. qPCR assay

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

The assay has been validated with the ABI TaqMan® master mix (2x) and Quanta perfecta Multiplex qPCR ToughMix. If different PCR mixtures and amplification programs are used, it is necessary to verify their performance.

4.1. For the Contig 21, Contig 22 and PEC – Xe qPCR assay: see the primer/probe sequences in Table I.11, the reaction mixture in Table I.12, and the cycling parameters in Table I.13.

Table I.12. PCR mix for the Contig 21, Contig 22 and PEC-Xe qPCR

Component	For 1 reaction (in µL)	Final Concentration
Sterile Milli Q water	6.988	
Forward primer (100 pmol/µL)	0.225	0.90 µM
Reverse primer (100 pmol/µL)	0.225	0.90 µM
Probe (100 pmol/µL)	0.062	0.250 µM
qPCR master mix (2×)	12.50	1×
Template DNA	5.00	
Total	**25.00**	

Table I.13. qPCR cycling parameters for the Contig 21, Contig 22 and PEC-Xe qPCR

Step	Temperature	Duration	Description
hold	95°C	10 min	Enzyme activation
40 cycles	95°C	15 sec	Denaturation
(ramp rate 1.6°C/sec)	60°C	60 sec	Annealing/elongation
hold	40°C	10 sec	End run

4.2. For the ZUP (Ac) and PEC – ZUP (Acat) qPCR assay: see the primer/probe sequences in Table I.11, the reaction mixture in Table I.14, and the cycling parameters in Table I.15.

Table I.14. PCR mix for the ZUP (Ac and Acat) qPCR

Component	For 1 reaction (in µL)	Final Concentration
Sterile Milli Q water	7.25	
ZUP 2549 / 2791 (100 pmol/µL)	0.10	0.40 µM
ZUP 2550 / 2792(100 pmol/µL)	0.10	0.40 µM
ZUP 2551 / 2566 (100 pmol/µL)	0.05	0.20 µM
qPCR master mix (2×)	12.50	1×
Template DNA	5.00	
Total	**25.00**	

Table I.15. qPCR cycling parameters for the ZUP (Ac and Acat) qPCR

Step	Temperature	Duration	Description
hold	95°C	10 min	Enzyme activation
40 cycles (ramp rate 1.6°C/sec)	95°C	15 sec	Denaturation
	60°C	45 sec	Annealing/elongation

4.3. For the multiplex ZUP qPCR assay: see the reaction mixture in Table I.16, and the cycling parameters in Table I.15.

Table I.16. PCR mix for the multiplex ZUP (Ac and Acat) qPCR

Component	For 1 reaction (in µL)	Final Concentration
Sterile Milli Q water	7.00	
ZUP 2549 (100 pmol/µL)	0.10	0.40 µM
ZUP 2550 (100 pmol/µL)	0.10	0.40 µM
ZUP 2791 (100 pmol/µL)	0.10	0.40 µM
ZUP 2792(100 pmol/µL)	0.10	0.40 µM
ZUP 2551 (100 pmol/µL)	0.05	0.20 µM
ZUP 2566 (100 pmol/µL)	0.05	0.20 µM
qPCR master mix (2×)	12.50	1×
Template DNA	5.00	
Total	**25.00**	

4.4. A Positive Amplification Control (PAC) and a non-template control (NTC) are required (Table I.7). Load PAC DNA template into separate qPCR reactions at a recommended concentration of 2 pg/µL.

Prepare the PAC-Ac, Xe, Acat as follows:

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

5. Interpretation of results

Cut-off values must be established by each laboratory for their positive and internal amplification controls (IAC) prior to the assay being used on routine samples. For recommendations on setting cut-off values, see Real-time PCR pre-screening in seed health methods.

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 Cq cut-off that reflects the increase in sensitivity and potential background noise based on the copy number of the target.

Test results are only valid when all included controls presented in Table I.7 give the expected result. NPC/NTC must be negative (no detection of *A. citrulli*) and PEC/PPC/PAC/IAC must fall within their expected ranges for the test to be considered valid. Control ranges are determined per laboratory. Note that PEC control range limits must be set per crop species in each testing laboratory.

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. 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.

5.2. If a sample is qPCR positive (Ac suspect), the detection, identification and confirmation grow-out assay, described in section II, must be performed to reach a final conclusion about the sample and seed lot (See ISF's view on indirect seed health tests).

II. DETECTION, IDENTIFICATION AND CONFIRMATION BY GROW-OUT ASSAY

- For melon seed, see the protocol for the Detection of *Acidovorax citrulli* in melon seed by sweat box grow-out on the ISF website.
- For all cucurbits, see version 1.1 of the *Acidovorax citrulli* grow-out protocol on the NSHS website.

REFERENCES

Kleinhesselink, K. (2017). Validation report: Seed Extract and qPCR Method for the Detection of *Acidovorax citrulli* on Cucurbit Seeds. International Seed Federation (ISF), Nyon, Switzerland.