Protocol for the detection of bacterial ring rot (*Clavibacter michiganensis* subsp. *sepedonicus*) from potato tubers

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I. Sample Preparation (completed by the submitter after receiving instructions from Plant Pathology Laboratory staff; complete submitter instructions are included on page 7).

1. Tubers can be collected two different ways: 1) randomly select the required number of tubers (usually 4,600) at harvest (preferred); 2) for tubers in storage, randomly select tubers from across the top of the entire pile. Place tubers in groups of 200 (+/- 10) per subsample (for a set of 4,600 tubers, therefore there will be 23 subsamples).
2. Tuber core samples are taken from each of the subsamples using a 13 mm melon baller. **Cores must be taken from the stolon end and be spherical or conical**. To prepare the coring tool, follow these steps:
    a. Wash the melon baller first with soap and water, then disinfect by dipping the METAL portion of the tool in 95% alcohol and flame the tool.
    b. Allow the ethanol to completely burn off before using. Repeat this process between each seed lot, but not between each subsample within each seed lot.
3. Place the cores from the 200 tuber subsample in a 1 quart Ziploc bag and seal. Make sure each bag is labeled with seed lot information and subsample number.
4. Core samples should be kept in the refrigerator at 4° C (39°F) and brought to the Plant Pathology Lab as soon as possible but by 7 days following coring.

II. Preparation of Tuber Cores for Overnight Soaking.

1. The area where tuber cores are processed is thoroughly cleaned between seed lots and sprayed with 70% ethanol, then allowed to dry.
2. Each subsample is placed in a metal colander which has also been cleaned and disinfected with 70% ethanol.
3. The cores are rinsed with tap water to remove the soil, and then rinsed 3 times with sterile distilled water.
4. Each subset is placed in a new 18 oz plastic cup and sterile distilled water is added just until the cores are covered.
5. Each cup is covered with aluminum foil and shaken on a platform shaker at 100 rpm overnight.

III. Preparation of Samples for Storage and PCR Testing.

1. The next morning, remove 1 ml of the soakate from each subsample and place in a sterile 1.5 ml tube. Label with PCE number. Samples can now be stored in the freezer (-20° C) or proceed directly to PCR.

IV. Preparation of Samples for Real-Time PCR testing.

Two different PCR protocols are used, and a third one is used if necessary (in case of any ambiguous results). The CelA Real-Time PCR protocol is performed first, after which a second duplex Real-Time PCR protocol is performed (Mills Cms72a and Cms50 primer sets).

In preparation for the PCRs, set up a set of serial dilutions of each subsamples: a non-diluted sample, a 1/10 dilution, and a 1/100 dilution. Three reactions are performed for each sub samples in each test. Protocols are included below.

CelA Protocol

Seed-lot Identification		
Date:	Time:	Operator:
Primer Set: CelA		

Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Master Mix component	Volume per Rxn	# Rxns	Total volume	✓ when done
20 µM Forward Primer	0.5			
20 µM Reverse Primer	0.5			
20 µM Probe	0.25			
Water	9.25			
2X Brilliant II Buffer	12.5			
DNA	2.0			
Total rxn volume	25.0			

Primer set	**CelA**
Forward Primer Sequence	TCTCTCAGTCATTGTAAGATGAT
Reverse Primer Sequence	ATTCGACCGCTCTCAAA
Probe Sequence	TTCGGGCTTCAGGAGTGCGTGT
Cycling Parameters	Step One: 95 C for 10 min Step Two, 40 cycles: 95 C, 30 sec 63 C, 45 sec 72 C, 30 sec
Expected amplicon size	149 bp
Expected melting temperature	~86-88C

Mills Protocol

Seed-lot Identification		
Date:	Time:	Operator:
Primer Set: Mills Cms 50, 72a		

Plate Layout

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

Master Mix component	Volume per Rxn	# Rxns	Total volume
20 µM Cms 50 Forward Primer	0.9		
20 µM Cms 50 Reverse Primer	0.9		
20 µM Cms72a Forward Primer	0.9		
20 µM Cms72a Reverse Primer	0.9		
20 µM Probe Cms50	0.25		
20 µM Probe Cms72a	0.35		

Water	6.3		
2X Brilliant II Buffer	12.5		
DNA	2.0		
Total rxn volume	25.0		

Primer set	**Mills Cms50**	**Mills Cms72a***
Forward Primer Sequence	GAGCGCGATAGAAGAGGAACTC	CTACTTTCGCGGTAAGCAGTT
Reverse Primer Sequence	CCTGAGCAACGACAAGAAAAATATG	GCAAGAATTTCGCTGCTATCC
Probe Sequence	TGAAGATGCGACATGGCTCCTCGGT	GATCGTGAATCCGAGACACGGTGACC
Cycling Parameters	Step One: 95 C for 10 min Step Two, 40 cycles: 95 C, 10 sec 60 C, 45 sec 72 C, 10 sec	
Expected amplicon size	192 bp	213 bp

V. Interpretation of Results (modified from *Protocol for the detection of Clavibacter michiganensis subsp. sepedonicus,the bacterial ring rot pathogen, in potato* by K. Kinzer., personal communication, 2013, NDSU Plant Pathology Department).

A positive reaction with real-time PCR is indicated by fluorescence that is greater than background fluorescence. The lowest number of PCR cycles (among dilutions) at which the amount of fluorescence exceeds the background fluorescence is reported, and this value is referred here as the Ct value.

CelA Interpretation

- NEGATIVE RESULT: For Ct values of 0.00 and ≥39, the amount of fluorescence did not exceed the fluorescence threshold, and the pathogen was not detected.
- POSITIVE RESULT: For Ct values ≤ 30.0 cycles, the pathogen was detected (a POSITIVE result).

Mills Interpretation-Secondary Testing

- Secondary Test: the results of the CelA test results must be verified, regardless of results of the first test??? by re-testing via PCR with a second primer/probe set (Mills primers/probes must be used for the re-test).

    - NEGATIVE RESULT: Negative results occur when one PCR test (CelA) detects the pathogen within 30 cycles but the Mills protocol does not.
    - POSITIVE RESULT: For Ct values ≤ 30.0 cycles, the pathogen was detected (a POSITIVE result).

SYBR Green Melting Curve Testing

- MELT TEMPERATURE ANALYSIS REQUIRED: If the results of the Mills protocol show that Ct values are <39, then the sample must be subjected to MELT TEMPERATURE ANALYSIS, using SYBR green with the CelA primers (excluding probes, which are not necessary for this assay).
    - NEGATIVE RESULT: If MELT CURVE ANALYSIS shows that melting temperature is not consistent with that of the positive controls
    - POSITIVE RESULT: If MELT CURVE ANALYSIS shows that melting temperature is consistent with the melting temperature of the positive controls

Melting Curve Protocol

Seed-lot Identification		
Date:	Time:	Operator:

Plate Layout

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

Master Mix component	Volume per Rxn	# Rxns	Total volume	✓ when done
20 µM Forward Primer	0.5			
20 µM Reverse Primer	0.5			
Water	7.0			
SYBR Green SSo MM	10			
DNA	2.0			
Total rxn volume	20.0			

Primer set	**CelA**
Forward Primer Sequence	TCTCTCAGTCATTGTAAGATGAT
Reverse Primer Sequence	ATTCGACCGCTCTCAAA
Cycling Parameters	Step One: 95 C for 10 min Step Two, 40 cycles: 95 C, 30 sec 63 C, 45 sec 72 C, 30 sec
Expected amplicon size	149 bp
Expected melting temperature	~86-88C

References:

1. Gudmestad, N. C., Anderson, N. R., Kinzer, K., Mallik, I., and Pasche, J. S. 2009. A Real-Time PCR Assay for the Detection of Clavibacter michiganensis subsp. sepedonicus Based on the Cellulase A Gene Sequence [electronic resource]. Plant disease: an international journal of applied plant pathology 93:649-659.
2. Mills, D., Russell, B. W., and Hanus, J. W. 1997. Specific detection of Clavibacter michiganensis subsp. sepedonicus by amplification of three unique DNA sequences isolated by subtraction hybridization. Phytopathology 87:853-861.

Directions given to submitters:

Instructions for Preparation of Potato Tuber Samples and Submission Procedures for Bacterial Ring Rot Testing

Robert Cating, Ph.D., Plant Pathology Diagnostician and Lab Manager, Hermiston Agricultural Research and Extension Center, Hermiston, Oregon 97838. 541-567-8321. Email Robert.cating@oregonstate.edu

1. **In order to process samples as quickly as possible, call the Plant Pathology Lab (541-567-8321 Ext. 120) or email Robert (Robert.cating@oregonstate.edu) prior to sending and/or coring to schedule a time to bring tuber samples for bacterial ring rot testing to the lab. If you need to ship, call the lab for information.**

2. Randomly select the required number of tubers (usually 4,600) best collected randomly at harvest but can come from across the top of the entire pile and place them in **groups of 200** in a plastic container lined with a new garbage bag. For example, a sample of 4,600 tubers would require 23 subsamples of 200 tubers. Be aware that tuber samples that are less than 4600 will not generally be adequate to test a seed lot for BRR

3. Tuber core samples are then taken from each of the subsamples using a 13mm melon baller.

    a. Wash the melon baller first with soap and water and then disinfect the melon baller by dipping the METAL portion of the tool in 95-100% alcohol and pass through a flame. Allow the alcohol to completely burn off before using. If this is not an option, disinfect the tool with a standard commercial disinfectant, replacing the disinfectant as needed. **Repeat this process between each seed lot but not between each subsample of each seed lot.**

    b. When the tool is ready to use, take a sample from each tuber at the stolon (stem) end containing as much of the vascular ring as possible. **Melon ballers should NOT be larger than 13mm**, but can be as small as 10 mm.

4. Place the cores from the 200 tuber sub sample in a 1 quart plastic Ziploc bag and seal. Make sure each bag is labeled with seed lot information and sub sample number.

5. The garbage bag can now be removed, closed, and labeled. It is recommended that tuber samples are kept until all testing is complete. Core samples should be stored in the refrigerator at 4ºC (39ºF) and brought to the laboratory as soon as possible. Again schedule submission with the plant pathology laboratory. Cores should **NOT** be stored for more than a few days before submission.

If you have questions about taking samples or need assistance, please contact the OSU Hermiston Plant Pathology Lab at the number above.