

MAINE SOP FOR THE DETECTION OF
Clavibacter michiganensis Subsp. Sepedonicus
In potato

Version: 4.0

Maine Potato Disease Testing Laboratory
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History of the Maine Potato Disease Testing Laboratory:

The Maine Potato Disease Testing Laboratory (MPDTL) is an APHIS-accredited laboratory which began in 2004 as a partnership between the University of Maine and the Maine State Department of Agriculture. In March of 2008 the Maine Potato Board provided a new space for the MPDTL at 744 Main Street in Presque Isle. On January 1, 2010, under agreement with the Maine Department of Agriculture, Conservation and Forestry, the Maine Potato Board began staffing the lab. In the summer of 2014, the lab moved to its new location in the basement of the Harley Welch Agriculture Building. The MPDTL is a secure facility set up for the purpose of testing seed potatoes for certification by the Maine Department of Agriculture, Conservation and Forestry.

Current use of the Maine Potato Disease Testing Laboratory:

Currently the Maine Potato Disease Testing Laboratory conducts all potato pathogen testing that relates to the production of certified seed potatoes. The lab staff works closely with the Maine Department of Agriculture, Conservation and Forestry, Division of Animal and Plant Health to ensure that the Seed Certification Rules are being followed where testing for potato pathogens is concerned.

Staff at the Maine Potato Disease Testing Laboratory:

Andrew Plant: Lab Manager (2017-Present)

M.S. Botany and Plant Pathology, 2005, University of Maine, Orono

B.S. Biology, 2002, University of Maine, Orono

Andrew has seventeen years of experience working in potato integrated pest management, pathology, and agronomy as an Associate Professor for the University of Maine Cooperative Extension. He has conducted or collaborated on numerous research projects spanning fields of agronomy, pathology, and entomology. Andrew brings experience utilizing ELISA protocols for plant pathogen detection, and conducting cPCR, qPCR, and RPA protocols for pathogen detection.

Shelby Nicak: Lab Technician (2018- Present)

A.S. Medical Laboratory Technology, 2012, River Valley Community College

B.S. Molecular Cellular Biology, 2009, University of Maine, Orono

Shelby brings 5+ years professionally as a QC/ Food Safety Manager providing internal auditing, quality control, HACCP training, and environmental testing. Her laboratory experience is extensive, including work while achieving her Associates and Bachelors degrees. Shelby has experience in ELISA protocols, RT-qPCR, and DNA extraction.

Larry Feinstein, PhD: Lab Consultant

Assistant Professor of Biology, University of Maine at Presque Isle (UMPI)

Ph.D. Microbial Ecology, 2012, Kent State University, Kent, Ohio

Dr. Feinstein works with the lab developing Standard Operating Procedures, providing recommendations for equipment purchases, and conducting MPDTL quality control testing at his UMPI laboratory. He has over 15 years of experience conducting research in the fields of plant

biology, environmental microbiology, infectious disease, soil science, and molecular biology. He has been trained in the molecular detection of potato pathogens at the Agricultural Certification Services lab in Fredericton, New Brunswick and is currently conducting research on pathogen dispersal with the University of Maine Cooperative Extension.

INTRODUCTION

The Maine Potato Disease Testing Laboratory is following protocols that were developed by the Canadian Food Inspection Agency, and North Dakota State University.

Bacterial Ring Rot (BRR) is a potato disease caused by the bacterium *Clavibacter michiganensis* subsp. *sepedonicus* [syn. *Corynebacterium sepedonicum*] (Cms). Seed Potato Certification Regulations specify a zero tolerance for this disease which means that a single infected plant or tuber found in a field, greenhouse or storage results in the loss of the total potato production of the farm unit as seed, and limits the options for disposal of the production to non-seed uses.

Potato plants infected with bacterial ring rot do not always exhibit visible symptoms in the field or in the tubers at harvest and in storage. However, such symptomless (or latent) infections can be detected by laboratory tests. Specifically, the tests are designed to detect the pathogen. Currently the enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay staining (IFA or IFAS) tests are two serological tests approved in Canada for testing for bacterial ring rot pathogen under the seed certification program. In Canada, the ELISA test is used to screen samples for possible infections and the IFA test is applied to only those samples that are positive in ELISA. Polymerase chain reaction is a molecular technique that has increased detection abilities (specificity and sensitivity) as compared to ELISA and IFA. Current commercially available ELISA antibodies have been shown to only work on mucoid strains of Cms (Gudmested et. al, 2009). Non-mucoid strains of Cms are known to exist and may go undetected in seed potato systems relying exclusively on ELISA-based testing protocols. IFA has been shown to detect both mucoid and non-mucoid strains of Cms, but is often considered laborious, tedious, and interpretation can be subjective (Smith et. al., 2008). Tests may be conducted on composite samples of stems or tubers. To maximize the probability of detecting the ring rot pathogen, CFIA requires that stems are sampled after at least 75% of the number of growing days has passed but before senescence takes place. Stems are sampled by removing 0.5-1.5 cm of tissue from each stem at soil level. Tubers are sampled at any time after harvest by removing a 0.5-1.0 gram core from the stolon attachment site to obtain the maximum amount of vascular tissue where the pathogen is found. For postharvest tuber testing of seed lots destined for export (to Canada), it is required that 1% of tubers from a seed lot be screened, with a minimum of 5 tubers and maximum of 400.

This lab will employ PCR testing as an initial screen, and utilize multiple PCR primers in conjunction with melt temperature analysis as its confirmatory tests. Three PCR primers and PCR melt temperature analysis will each serve as a separate molecular diagnostic test. The MPDTL will utilize the ELISA test as an alternate confirmatory step. ELISA results will not supersede a PCR result in confirmation of a positive. As stated above, ELISA is limited in its sensitivity (only detecting mucoid strains), and can lead to false negative results should a non-mucoid strain be present.

QUALITY ASSURANCE

1. Record keeping
2. Sample storage and tracking
3. Personnel training record
4. Proficiency testing
5. Equipment location list
6. Equipment maintenance and calibration record
7. Changing protocols and quality assurance procedures

1. Record keeping

All worksheets, forms, and information regarding bacterial ring rot testing should be written in black ink. Errors are crossed out, not deleted or erased, and the corrections written in next to the original. Duplicate forms documenting tuber and stem check-in are used: one copy is kept in the lab and one given to the grower for their records.

The lab will maintain one loose leaf binder with all protocols and quality assurance materials, and another loose leaf binder or binders with all sample records. Electronic copies of all sample records will also be kept and access to these records is restricted to lab-authorized personnel only. There is a third “working” protocol book with page protectors for reference while performing procedures. Other materials relating to that sample may also be included, such as print correspondence and summaries of phone conversations.

The sample records binder includes the following materials filed by BRR ID number:

Check-in form

Coating Plates with the Capture Antibody worksheet

Core processing worksheet

ELISA worksheet/loading diagram

ELISA plate reader printout

Copy of results report- Electronically Available

The sample records materials are stored in locked filing cabinets in Storage Room 1. Access to the locked location is limited to the Lab Technician at Maine Potato Seed Disease Testing Laboratory and his/her designated staff, and the Director of the Maine Department of Agriculture Division of Animal and Plant Health and his/her designated staff. Access is reviewed as personnel changes.

Records are kept for 7 years and then destroyed.

Copies of protocols are made available upon request.

2. Sample storage and tracking

All samples and their derivatives (tuber cores, tuber extracts, ELISA plates, cDNA, PCR products) are kept in a secure location. These locations will always be locked when bacterial ring rot sample derivatives are being stored.

Access to the secure location is limited to the Lab Technician at the Maine Potato Disease Testing Laboratory and her/his designated staff, and the Director of the Maine Department of Agriculture, Conservation and Forestry, Division Animal and Plant Health and her/his designated staff. Access is reviewed as personnel changes.

All sample derivatives are destroyed by autoclaving when they are no longer required (see summary table below). Tuber cores can be destroyed after a negative test result. Because ELISA plates fade quickly, the sample plates are not stored and can be destroyed after they are read in the plate reader. Only the reserved sample extract is kept for an extended period. If the sample is positive the extract is kept for 3 years; if the sample is negative the extract is kept until the end of the crop year following testing.

Sample or derivative	Storage	Security	Destroy by autoclaving
Cores	Stored until negative test	NA	Following negative test result
Reserved extract	Refrigerated until final PCR/ELISA results obtained, then preserved with glycerol & frozen at -20C	Locked	If positive, after 3 yrs. If negative, after end of crop yr. following testing
Aliquots A&B	Refrigerate until PCR/ELISA complete	Locked	After PCR is complete, in case repeat test is required.
ELISA plates	Not stored	None required	After reading of ELISA is complete

3. Personnel training record

All persons performing bacterial ring rot testing must be qualified to perform the testing procedures. Persons may qualify in one of two ways: either as a qualified trainer or as a trainee that has completed documented training with a trainer. A qualified trainer will have a total of three years of experience that combines education and/or work experience in the biological sciences (e.g. plant pathology, microbiology, biotechnology or other related field) and one year previous experience in bacterial ring rot testing. Trainees will have appropriate education or work experience and undergo a course of training with a qualified trainer.

The training of each person who performs bacterial ring rot testing procedures is documented on the TRAINING RECORD sheet. The trainee first observes the trainer performing the procedure, and later performs the procedure under the supervision of the trainer. On both these occasions, both the trainer and trainee date and initial the TRAINING RECORD sheet. Also documented is the date of completion of proficiency testing (see section 4 below).

Each procedure is broken down into steps and the training for each step is documented separately. Thus, a person could be trained to perform the rinsing of ELISA plates, for example, without needing to know all the preceding and subsequent steps.

4. Proficiency testing and on-site inspection

In order to ensure the accuracy of bacterial ring rot testing and maintain the quality system, the testing laboratory has a program of proficiency testing and on-site laboratory inspections.

Proficiency testing will consist of testing a panel of samples provided by USDA-APHIS. All persons in the laboratory qualified to perform the testing must successfully complete the periodic proficiency panels in order to continue to perform tests. If the results of an individual's proficiency test are not 100% correct, another proficiency panel must be completed, until a perfect score is attained. On the second page of the TRAINING RECORD sheet there is a space to document completion of the panel. The results of the proficiency panels will be kept in a secure location.

The MPDTL will undergo occasional on-site inspections (audit). The inspector will be appointed by APHIS. The parameters of the inspection will be determined by the inspector and the laboratory to be inspected prior to the first inspection date. This external audit will be conducted at the convenience of the APHIS inspector.

In addition to the audit conducted by APHIS, an annual INTERNAL AUDIT will be conducted by the lab technicians and manager. A check list is in place that is used to record each step in the audit. Any abnormalities are listed on a separate form and corrections are also made and documented at the time of the audit. The forms for the audit are included with this protocol.

We have chosen to have an INTERNAL AUDIT at the beginning of the testing season. The month of August will be targeted for that audit.

The Seed Testing Laboratory will conform to all State, Local, and fire safety ordinances that govern its operation.

5. Equipment location

Staff is responsible for laboratory equipment. All testing equipment is located in a containment facility that is in a separate area from where samples are received. This area is locked and entrance is only available to lab-authorized personnel. The containment facility has been APHIS-approved for pathogen testing and equipment and lab bench surfaces are regularly surface-sterilized in order to avoid sample cross-contamination. Lab Staff will have access to Lab Equipment Manuals which will be kept in a designated location.

6. Equipment maintenance and calibration record

Equipment used for bacterial ring rot testing should be reliable, i.e. it should perform in a uniform and stable manner. To assure the required performance, equipment is maintained on a regular schedule. Controlled temperature devices are monitored when in use for bacterial ring rot testing. Calibration is also scheduled for those pieces of equipment requiring it. Laboratory equipment is serviced by certified Quality Control companies when necessary.

EQUIPMENT MAINTENANCE AND REPAIR LOG. Recorded on this form are: any damage done to an item, repair work or other service done, performance anomalies and subsequent corrective action taken, calibration performed and regularly scheduled maintenance. Reports of calibration and service can be attached to this form or available by electronic means. If a temperature controlled item (e.g. an incubator) has not been used for bacterial ring rot testing and is not being monitored, or it has undergone a repair, temperature stability and uniformity must be established. This is also noted on the log. All entries are dated and initialed.

Controlled temperature equipment is monitored with maximum/minimum thermometers (if the equipment does not have a built in temperature monitoring unit) to ensure the correct temperature range when they are in use for bacterial ring rot testing. The max/min temperatures are recorded on the TEMPERATURE LOG. Readings are taken periodically by lab technicians, or by way of remote data logging. If readings occur outside the allowable range, corrective action must be taken.

Regular maintenance and calibration programs are summarized in the table below. Maintenance of each piece of equipment should always follow the manufacturer’s instructions, if available. The significance of performance irregularities is evaluated when they occur and corrective action is taken, when required.

Instrument	Maintenance	Calibration
Refrigerator: 4°±3°C	Clean annually in spring Monitor temp when in use for BRR testing	Establish stable temp initially and after repair
Freezer: -20°±10°C	Clean annually in spring Monitor temp when in use for BRR testing	Establish stable temp initially and after repair
Autoclave	Clean after each use Check temperature weekly	none
Incubator: 37°±2°C	Clean as needed Monitor temp when in use for BRR testing	Establish stable temp initially and after repair
Max/Min thermometer	NIST guaranteed for one year, purchase annually	None required
Balance	Clean each use	Check against traceable calibration weights annually
pH meter	Store electrode according to manufacturer’s instructions	Calibration check with 2 buffers monthly
Microscope for IFAS	Clean & service annually	Calibrate stage micrometer initially
Pipettors	Clean as needed	Calibrate annually
Plate reader	Remove dust	Calibration plate test annually
Centrifuge	Balance all loads symmetrically Clean up after spills	None required
Shaker	Clean up after spills	None required
PCR	Clean as needed	Monitor outputs/discrepancies

7. Changing protocols and quality assurance procedures:

Periodically, this manual will be reviewed and changed if needed. Changes can only be made on approval of: the laboratory manager, the Director of the Department of Agriculture, Conservation and Forestry, and the person(s) overseeing the Quality Assurance. All responsible parties must sign and date the signature page included in this document.

SAMPLE CHECK-IN

Introduction

The Maine Potato Disease Testing Laboratory will be provided with a sample of 400 tubers, labeled with a certification number provided by the Division of Animal and Plant Health Seed inspectors. Sample collection will be performed under the supervision of the Seed Inspectors who will verify the sample lot, origin, variety and generation. Upon receipt at the Maine Seed Potato Testing Laboratory, the tuber sample will be inspected and entered into a logbook. The inspection process ensures that the sample meets certain criteria of tuber number, size, and condition that will not compromise the accuracy and sensitivity of subsequent tests. If a sample does not meet these criteria, a new sample can be requested before processing begins. Assigning a number to the sample and entering it in a logbook are required for sample tracking and chain of custody documentation. The sample will be stored at room temperature in a locked room. Samples will not be stored more than 7 days prior to processing. Proper storage between the time of sample arrival and processing is needed to ensure that sample integrity is not compromised prior to testing.

Guidelines for Sample Submission

- The submission information enclosed with the sample should include the following information (MDACF Seed Potato Certification Sample Submission Form):
 - submitter's name;
 - submitter's mailing address;
 - submitter's telephone number;
 - submitter's email address (optional);
 - submitter's fax number (optional);
 - submitters preferred contact information
 - date of collection;
- The box or container should be well sealed to protect sample integrity.

Guidelines for Sample Check-In at the Testing Lab

- Upon arrival, information on each sample will be entered into a log book or computer database. This information will include:
 - a unique identifying number that will be used to label all bags, forms, tubes, slides and other materials generated from that sample;
 - tuber variety and generation;
 - the date of receipt of the sample;
 - whether the sample is adequate for testing (see below for criteria);
 - the date of core processing (if applicable);
 - the date ELISA performed (if applicable);
 - the date PCR performed (if applicable);
 - the date test results are reported to the Maine Department of Agriculture, Conservation and Forestry (if applicable).
- A standard intake form will also be completed for each sample (see attached form). A copy of this form is provided to the submitter.

- Any additional documentation supplied by the submitter will be attached to the intake form.
- Samples will be inspected to make sure they meet basic criteria that will not jeopardize test results. Samples will be rejected and a replacement sample requested if:
 - the shipping box or container has been crushed or broken;
 - the bag containing the tubers has ripped and is leaking;
 - the number of tubers is less than 390
- If the sample does not meet the basic criteria outlined above, any deficiency will be noted on the intake form.
- If a sample is rejected for any reason, the client will be contacted promptly to explain the cause of rejection and to request a new sample. Any such contact will be documented on the intake form.
- Rejected samples will be promptly autoclaved and discarded.
- Samples that are not rejected will be kept in locked room for up to 7 days prior to processing.

PROCESSING POTATO CORES FOR EXTRACTION OF BACTERIA

Introduction

After the sample of 400 tubers has been inspected and logged in (see SAMPLE CHECK-IN) it must next be processed to extract the bacteria from the tuber tissue for the ELISA and PCR tests. Preferred method for this lab is the Shaker Method.

Shaker Method

1. If cores are frozen, thaw them at room temperature 30-60 minutes before processing. Never thaw by heating.
2. Cores are divided into two 200-core samples and labeled, A and B respectively.
3. Weigh each subsample and record weight on Core Processing Worksheet.
4. Place each subsample into a separate sterile 1 liter flask and add 1ml sterile distilled water per gram of tissue. Water should cover cores. If there is not enough water to cover the cores, increase the water to not more than 1.5 ml per gram of tissue.
5. Cover each flask and place on a shaker at room temperature for overnight at 160rpm.
6. Using sterile/filtered pipet tips remove a 1ml aliquot of extract from each sub-sample and place into a clean, sterile microcentrifuge tube. The tube should be labeled with the sample info and the subsample number.
7. Make a 1:10 and 1:100 serial dilution from each tube. You should have three tubes representing

each of the subsamples. To make serial dilutions, place 900 µl of sterile water in each of two tubes. Take a 100 µl aliquot of sample and put it in the first tube (1:10.) Mix. Take a 100 µl aliquot from the 1:10 tube and put it in the next tube (1:100.) Mix.

8. Remove 1mL of soakate and put it in a labeled 1.5mL tube. Spin at 10,000rpm for 10 minutes. Discard supernatant, resuspend pellet in 1mL of 20% glycerol and freeze for long term storage.
9. Store Aliquots at -20 C for ELISA and PCR testing.
10. All waste materials are autoclaved. Re-usable items are sterilized/decontaminated by flame, bleach, autoclave, alcohol or commercial disinfectants that contain phenol.

Lab benches and sinks are decontaminated using commercial disinfectants containing alcohol.

References

1. Protocols for diagnosis of Bacterial Ring Rot. Jan. 6, 2003. Wisconsin Seed Potato Certification Program.
2. Dinesen, I. G. 1984. The extraction and diagnosis of *Corynebacterium sepedonicum* from diseased potato tubers. Bulletin OEPP/EPPO Bulletin 14, 147-152.
3. Phytosanitary Procedures: *Clavibacter michiganensis* subsp. *Sepedonicus*: Inspection and Test Methods. PM 3/25(1) English.
4. NDSU Cms Detection Export Protocol. Version 6.0

Instructions for coring tubers

Line seed rack with brown craft paper. Paper is changed between each sample (not necessary to change the paper between subsamples).

Attach a clean plastic bag to the bag holder on the seed rack.

Place the 400 tuber sample on the seed rack.

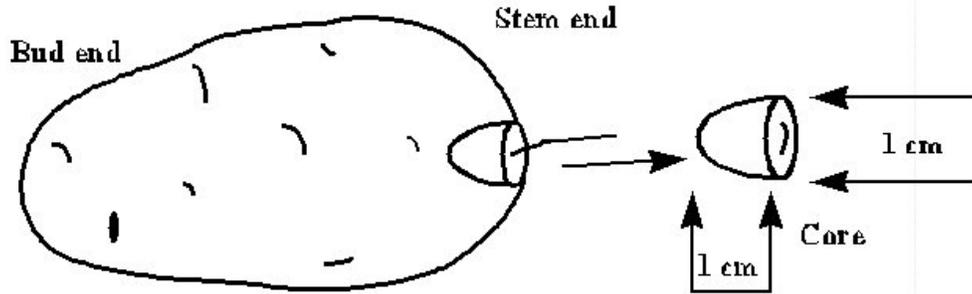
Place each tuber in the plastic bag as the tubers are cored.

When there are 200 tubers cored, take the cores to the lab to wash them.

Place the 200 cores on a paper towel to remove excess moisture.

Weigh the cores. They should weigh approximately 100.00 grams.

- Disinfect coring tools between subsamples
- Cores must be taken at the stolon attachment site and must be **conical** or **semi-spherical** in shape, approximately 1 cm in diameter at the top and 1 cm deep (see diagram below). Each core should weigh between 0.5-1.0 g, and include as much of the vascular ring radiating from the stolon attachment as possible.
- All cores from samples must be kept until testing is complete. If testing is negative, cores may be thrown out. If positive, or undetermined, do not discard until further testing is completed.



Note: The core in this diagram is drawn to scale.

- Place all cores of a subsample in a sandwich bag while processing the sample. Label the sandwich bag with the unique sample ID number and indicate whether it is subsample A or subsample B.

POLYMERASE CHAIN REACTION FOR *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS*

Realtime polymerase chain reaction (PCR), also known as quantitative PCR, is similar to conventional PCR in that a target sequence is amplified. The difference is that the amplification can be viewed in ‘real time’. We have adopted two different approaches to viewing amplicons in “real time”. One approach, sometimes called Taqman® assays, relies on tagging of a probe with a fluorescent dye that is released each time the ‘probe’ is disassembled by the polymerase enzyme. The dyes are tagged to the probes on one end, and on the other end of the probe is a quencher, which prevents the dye from fluorescing when it is associated with the probe. Once the dye and the quencher are separated by the polymerase during the reaction, the dye can fluoresce. With each cycle of realtime PCR, fluorescence is released and measured if the target sequence is present. The maximum number of realtime PCR cycles is somewhat arbitrary, but generally, a maximum between 30 and 40 cycles of realtime PCR is common. In our assay, we run the assays for 40 cycles. A second approach, known as SYBR® green assays, is to use an intercalating dye, such as SYBR green, which intercalates with the target amplicon as it is being amplified with each cycle. This second approach does not require use of a probe, and for detection of the BRR pathogen, we have found it to be more sensitive in optimization tests we have conducted. However, if SYBR green assays are used, an additional follow up step, known as a melting temperature analysis, should be implemented since SYBR green assays can be less specific in some instances. Melting temperature analysis allows comparison of the melting temperatures of the amplicon and the positive control; the temperatures should be the same to be considered positive.

Two concerns with pathogen detection are false positives and false negatives. False negatives might arise from sampling error, inhibitors present in the sample, poorly designed

primers/probes, or bad reagents. To reduce the potential for false negatives due to inhibitors, samples can be diluted. In our lab, reactions are carried out on 10^0 , 10^{-1} , and 10^{-2} dilutions of soakate (the 10^0 dilution is the raw soakate removed from the cups), in duplicate, from each subsample. Another way to minimize the chance of false negatives, particularly those due to inhibition, is to include an internal control; however, for our *Cms* assays, a satisfactory internal control has not yet been developed. Additional steps we take to reduce false negatives is to perform basic optimization steps to optimize for annealing temperature and primer/probe concentration whenever we change machines or master mixes. Efficiency of primers and probes is also determined. If low efficiency is due to poor probe design, the probe can be omitted in favor of SYBR green assay. Specificity should be established by published research; the specificity of the primers we use have been demonstrated (Gudmestad et al, 2009). Thus, altering primers themselves to improve efficiency should be avoided, unless it can be shown that any changes to the primers does not compromise their published specificity.

False positives can result from non-specific detection or contamination. In realtime PCR, amplification of non-target sequences or primer/probe dimers can lead to false positives. To minimize the potential for false positives, realtime PCR can be used to validate positive results obtained from other tests such as ELISA or IFA. If real time PCR is used as the sole test (in absence of ELISA), different sets of primer/probe combinations that target different parts of the genome can be used to minimize false positives, and all reactions must yield an amplicon for the sample to be considered positive. Alternatively, a melting temperature analysis can be performed, or the amplicon can be directly sequenced. Risk of contamination is minimized by proper handling of samples and reagents. To further reduce the chances of false positives, only primers that have been shown to be specific (for example, based on published results) should be used; if specificity results are not in the public domain, test results indicating an appropriate approach for testing for specificity should be made available on request.

The use of commercially available pre-made master mixes offers at least two advantages: it minimizes potential for contamination and improves consistency from one assay to another. Acceptable realtime master mixes for use with primers and probes developed for *Cms* are available, and choosing which master mix to use depends in large part on the realtime PCR machine used, the type of sample being tested, and whether a Taqman assay or SYBR green assay is adopted.

Procedure

A. Primers and Probes used: For all qPCR reactions, we use the primer/probe sequences as published (Gudmestad et al, 2009). We may use the primer/probe sets known as CelA or Cms50 and Cms72a for validation of ELISA results, or for initial screening. For all qPCR reactions, we have modified the parameters in the following ways: 1. The working concentration of primers and probes is 10 μ mole, with final concentrations varying depending on optimization steps for the realtime qPCR machine and master mixes used; 2. Probes for CelA, Cms50, and Cms72a primers are tagged with the fluorescence dye known as FAM or other that is compatible with the realtime qPCR machine used; and 3. Previously, the Cms50 and Cms72a sets have been optimized to be run in duplex; however, due to improved efficiency, as of 18 May 2015, the Mills primers are run in simplex. Primer and probe concentrations and fluorescence dyes used may change depending on master mixes and machines used. The CelA primer/probe set yields one amplicon of 149 base pairs (bp). The set of primers designated as Cms50/Cms72a, first devised by Mills et al (Mills et al Phytopathol. 87:853-861) and adapted for realtime PCR by Gudmestad et al (Gudmestad et

al Plant Dis. 93:649-659) yields a 192-bp amplicon from the Cms50 set and a 213-bp amplicon for the Cms72a set. Both primer/probe sets Cms50 and Cms72a must yield a positive result for the reaction to be considered positive. If only one amplicon occurs in the Cms50/Cms72a reactions, the sample is negative.

Use of qPCR to validate ELISA: If ELISA is used for initial screening, either CelA or Cms50/Cms72a or other acceptable published primer/probe set can be used for validation of positive results.

B. IMPORTANT NOTE Regarding Dyes and Quenchers used for the Probes and working concentrations of Primers and Probes: Dyes and quenchers as published in Gudmestad et al Plant Dis. 93:649-659 are not necessarily optimal for other qPCR machines. Be sure to use dyes and quenchers that are appropriate for the qPCR machine used. Each machine may have different optimal dye/quencher combinations. The available light channels in a machine and whether duplexing or multiplexing is desired play important roles in choosing the dye/quencher combinations. Primer and probe concentrations can also be adjusted for different labs. Stock solutions of our primers and probes are re-suspended at a concentration of 100µmole and working concentrations for primers and probes are 10µmole.

Primer set	Mills Cms50	Mills Cms72a*	CelA
Forward Primer Sequence	GAGCGCGATAGAAGAGGAACTC	CTACTTTCGCGGTAAGCAGTT	TCTCTCAGTCATTGTAAGATGAT
Reverse Primer Sequence	CCTGAGCAACGACAAGAAAAATATG	GCAAGAATTCGCTGCTATCC	ATTCGACCGCTCTCAAA
Probe Sequence	TGAAGATGCGACATGGCTCCTCGGT	GATCGTGAATCCGAGACACGGTGACC	TTCGGGCTTCAGGAGTGCGTGT
Cycling Parameters	<u>Step One:</u> 95 C for 600 sec <u>Step Two, 40 cycles:</u> 94 C, 10 sec 60 C, 45 sec 72 C, 10 sec		<u>Step One:</u> 95 C for 600 sec <u>Step Two, 40 cycles:</u> 95 C, 30 sec 59 C, 45 sec 72 C, 30 sec
Expected amplicon size	192 bp	213 bp	149 bp
Expected melting temperature			~87.4C

*Modified to facilitate binding of probe in realtime PCR (Gudmestad et al Plant Dis. 93:649-659)

C. Controls: Positive controls are used for each assay: either from extracted DNA; from soakate from Cms-infested tuber cores (preferred) (Provided by AGDIA); or cells from pure culture suspended in sterile, distilled water (about 10⁸/ml). The negative control is soakate from tubers known to be free of the pathogen (Provided by AGDIA). A water blank or NTC may also be included as an additional negative control. Internal controls (e.g. primers/probes that target plant DNA or general bacterial DNA) have not yet been developed for the assays, as of 18 November 2014.

D. Realtime PCR reaction mixes:

CelA Primer/Probe Mix	Volume per Rxn (µl)	Mills Primer/Probe Mix – SIMPLEX ONLY	Volume per Rxn (µl)
10 µmole CelA F	1.0	12.5 µmole Mills50-F OR Mills72aF	1.0
10 µmole CelA R	1.0	12.5 µmole Mills50-R OR Mills72aR	1.0
10 µmole CelA probe (SYBR;melt temp)	0.5(0.0)	Probe-50 OR Probe-72a (SYBR; melt temp)	0.5(0.0)
Water (SYBR; melt temp)	8.0(8.5)	Water (SYBR; melt temp)	8.0 (8.5)
BioRad SsoFast Probes Super Mix (2X; cat. no. 172-5230 or 172-5231) or equivalent; Melt temp-(BioRad SsoFast SYBR green Mix)	12.5	BioRad SsoFast Probes Super Mix (2X; cat. no. 172-5230 or 172-5231) or equivalent Melt temp-(BioRad SsoFast SYBR green Mix)	12.5
DNA	2.0	DNA	2.0
Total rxn volume	25.0	Total rxn volume	25.0

Interpretation of qPCR results:

A positive reaction with qPCR is indicated by fluorescence that is greater than background fluorescence, in a sigmoidal curve. The lowest number of qPCR cycles (among dilutions) at which the amount of fluorescence exceeds the background fluorescence is reported, and this value is referred here as the "crossing threshold", Ct. Background fluorescence is adjusted automatically on the BioRad machine.

- **NEGATIVE RESULT:** Where no Ct value is reported (for example, 0 or NA, depending on the machine used), the amount of fluorescence of a sample did not exceed the fluorescence threshold, and the pathogen was not detected or Ct >39.
- **POSITIVE RESULT:** For samples with positive ELISA, Ct ≤ 30.0 with CelA primer /probe set *or* both Cms50 and Cms72a primer/probe sets means the pathogen was detected, and the result is reported as POSITIVE without any additional re-testing required AS LONG AS ELISA was also positive. HOWEVER: If ELISA was NOT used at all, then any positives where Ct ≤ 39.0, obtained with one method (e.g. CelA or Cms50/Cms72a), must be validated using another method, e.g. use of other primer set(s) CelA or Cms50/Cms72a or other acceptable published primer/probe set; SYBR melting temperature analysis; or sequencing of amplicon(s); or SYBR green assays using validated primers. NOTE: Whenever Cms50 and Cms72a primers/probes are used, BOTH Cms50 AND Cms72a must be positive for the result to be considered positive.
- **RE-TEST REQUIRED:** If 30<Ct≤39, results should be validated using another method, such as qPCR with a second primer/probe set (for example, if CelA is used for the first test and the Ct value is 30<Ct≤39, Cms50 and Cms72a primers/probes should be used for the re-test), melting temperature analysis, sequencing (may require re-amplification),

even if ELISA were completed.

- **NEGATIVE RESULT:** Negative results occur when one PCR test detects the pathogen within 39 cycles but the re-test (using a different set of primer/probe) does not.
- **POSITIVE RESULT:** All three primer/probe combinations (CclA, Cms50, Cms72a) produce a Ct value ≤ 39 .
- **Additional tests that can be used to validate suspected positives:**
 - **MELT TEMPERATURE ANALYSIS - optional:**
 - **NEGATIVE RESULT:** If MELTING TEMPERATURE ANALYSIS shows that melting temperature is not consistent with that of the positive controls
 - **POSITIVE RESULT:** If MELTING TEMPERATURE ANALYSIS shows that melting temperature is consistent with the melting temperature of the positive controls
 - **SEQUENCING – optional:** Further evidence of a true positive can be obtained by direct sequencing of amplicons. Many commercial options are available for reasonable cost. Sequences can then be compared (e.g. via BLAST) with those submitted to a database (e.g. the NCBI database or other acceptable database). NOTE: Sometimes the concentration of the resulting amplicon is too low for quality sequencing. To resolve this problem the amplicon can be re-amplified to obtain a sufficient concentration. One caution of this approach is that a higher risk of contamination can exist, which can lead to a false positive.

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.
3. NDSU Cms Detection Export Protocol. Version 6.0

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS*

Introduction

Extract obtained from processing of tuber cores (see CORE PROCESSING) may be tested for *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) using an Enzyme Linked Immunosorbent Assay (ELISA). This test may be used as confirmatory step, but will not supersede results obtained by PCR protocols. The analytical sensitivity of this test is approximately $10^5 - 10^6$ cells per gram of tissue, but diagnostically it is recognized that Cms ELISA fails to detect non-mucoid strains. We use the double antibody sandwich (DAS) ELISA test provided by Agdia® in their Cms PathoScreen Kit. This product uses the 1H3 monoclonal antibody and an alkaline phosphatase enzyme. Products from other companies could also be used if approved by APHIS.

Procedure

Follow the manufacturer instructions for Agdia®'s Cms PathoScreen Kit. They can be found at <http://www.agdia.com> Catalog number SRA 70002.

The manufacturer instructions are used, with the following exceptions:

1. The sample is prepared according to the method for processing potato cores described in this document.
2. 4 positive and 4 negative controls are run in one column. Controls are provided by AGDIA, INC.
3. For each A and B subsample, a 1:1 (raw soakate) and 1:10 dilution (soakate:MEB) is tested.

A copy of the manufacturer instructions can be found at [www. Agdia.com](http://www.agdia.com) and a copy of those instructions is maintained with the SOPs.

The ELISA RECORD sheet is completed and initialed as each part of the procedure is performed. A copy of the ELISA Record is included in this SOP.

Additional Precautions

- Prior to use, check plates for dust, scratches, and irregularities that may interfere with the test. Do not use wells with abnormalities. Cover wells when not they are being filled or washed. Check for contaminants (dust, fuzz, hair, etc.) in the reagents as well.
- Wash plates carefully to avoid cross-contamination of wells. Discard contents of wells directly into the sink so that well contents do not contaminate surrounding wells.
- Before inserting plates into the plate reader, inspect them visually for dust or any contaminants that might interfere with plate reading.

Interpretation of results

If $P/N \geq 10$, use the Alternative Method for Positive Threshold calculation.

- For a plate to be valid, by default the positive control should reach a minimum absorbance of 0.400. Or, determine ELISA threshold using procedure outlined below in the Alternative Method.
- An ELISA well is positive if the absorbance value is 0.200 or greater (Or, based on the Alternative Method described below).
- This lab prefers to utilize the Alterative Method described below.

Alternative Method:

- Record the absorbance value for the negative tuber control. (N)
- Calculate the average absorbance value for the positive control wells. (P)
- Divide P by N to get the signal-to-noise ratio. If the P/N value is greater than 10 use the formula for positive threshold value below. If ratio is less than 10, use 2N or greater absorbance as the positive threshold value.

- Calculate the threshold value using the formula:

$$\text{Threshold} = 0.06 (P-N) + N.$$
- If a sample dilution absorbance value is less than the threshold value, it is negative. If a sample dilution absorbance value is greater than or equal to the threshold value, it is positive.
- If sample absorbance value is $\geq 2N$, the sample will be retested.
 - o If both values of any sample dilution are positive, then the sample requires a confirmatory test if not already completed (PCR)
 - o If one dilution is positive, redo the ELISA test.
 - If retest of ELISA on positive dilution is again positive, proceed to confirmatory tests with PCR.
 - o If both dilutions are negative, the ELISA test is negative. No confirmatory test is required.
 - o Reporting of results is discussed in the REPORTING SOP.

References

1. Protocols for diagnosis of Bacterial Ring Rot. Jan. 6, 2003. Wisconsin Seed Potato Certification Program.
2. Agdia Product Documentation for *Cms*PathoScreen Kit: DAS ELISA, alkaline phosphatase label. User's manual can be found at www.agdia.com.
3. Bacterial Ring Rot of Potato: Protocol for Indexing and Confirmation of Diagnosis. December 5, 2002. National Potato Council Seed Potato Certification Subcommittee. Protocols for diagnosis of Bacterial Ring Rot. January 6, 2003. Wisconsin Seed Potato Certification Program.
4. De Boer, S.H. and T. L. Haan. 1996. Validation of thresholds for serological tests that detect *Clavibacter michiganensis* subsp. *sepedonicus* in potato tuber tissue. Bulletin OEPP/EPPO Bulletin 26:391-398.
5. De Boer, S.H. and T. L. Haan. Version 2.0, May 4, 2001. Protocol for the detection of *Clavibacter michiganensis* subsp. *sepedonicus*, the bacterial ring rot pathogen in potato. Centre of Expertise for Potato Diseases, Canadian Food Inspection Agency. Charlottetown, Prince Edward Island, Canada

Adoption of New Testing Platforms or Methods:

MPDPTL staff are required to record and show documentation of the following in order to adopt new testing platforms or methodology for the detection of *Cms*.

- 1.) Published literature on the specific platform or methodology to include its statistics on Sensitivity (Analytical (Pathogen per volume) and/or Diagnostic (True positive rates), and Specificity (False positive rates).
- 2.) Professional opinions of recognized leaders in the potato pathology field (University Faculty/ APHIS lab personnel). Gather information from others regarding history or experience with such platforms or methodologies.
- 3.) On-site beta testing/research of new platforms or methodologies. Compare testing results of proposed new testing with current testing platforms being utilized at MPDPTL. Compare and document sensitivity and specificity statistics, as well as time and financial efficiencies of the testing methods.
- 4.) MPDPTL must communicate to USDA-APHIS proposed methodology changes. USDA-APHIS officials must approve proposed changes and their suitability to trade agreements with other countries (e.g. CFIA).

Reporting Results:

Reporting results for CMS testing:

For export samples, test results are reported to the grower of the sample and to the Division of Animal and Plant Health. The form for reporting results is included in this document. Voluntary testing requested by growers will only be reported to the requesting grower unless a sample is confirmed positive for CMS, at which it will be reported to the Division of Animal and Plant Health.

Concerns or Disputes of Results:

Growers may call or email the lab with concerns, comments, or questions in regards to results (aplant@mainepotatoes.com). Formal protest or appeal of results needs to be brought to the MDACF-Seed Potato Certification Supervisor (Chap. 252 Rules Governing Certification of Seed Potatoes in the State of Maine). A formal request or query maybe applied for in writing to:

Eric Hitchcock, Eric.Hitchcock@maine.gov

Seed Certification Program Manager

744 Main Street, Suite 9

Presque Isle, ME 04769

Original samples maybe re-tested by the MPDTL, or sent to another USDA-APHIS approved BRR laboratory for testing.

Signature Page:

Signatures below indicate that those so identified have read, understood, and approved this document:

Andrew Plant, Laboratory Manager

A handwritten signature in cursive script, appearing to read "Andrew B. Plant".

Ann Gibbs, Director Division of Animal and Plant Health

A handwritten signature in cursive script, appearing to read "E. Ann Gibbs".

Dr. Larry Feinstein, Assistant Professor of Biology Univ. of Maine, Presque Isle

A handwritten signature in cursive script, appearing to read "Larry Feinstein".

TRAINING RECORD

Trainee:

Page 1 of 2

Procedure or Step	Observed Trainer			Performed Supervised		
	Initials		Date	Initials		Date
	Trainer	Trainee		Trainer	Trainee	
Sample Check-in						
Criteria for rejection						
Digital tracking						
Core Processing						
Shaker Method						
ELISA						
Preparing buffers						
Pipetting						
Dilute samples, randomize & load						
Prepare enzyme conjugate						
Washing						
Prepare PNP						
Plate reader operation						
Calculate threshold						
Complete ELISA form						
PCR						
Complete PCR Form and Plate map						
Prepare primers and probes						
Dilute, load samples						
Dilute & load primers and probes						
Load qPCR						
Program qPCR						
Analyze qPCR output						
Melt Temp analysis						

with Sybr Green						
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Training completed: Date:
 Trainer:
 Trainee:

Proficiency panel:	Date completed	
ELISA		
PCR		

Maine Department of Agriculture, Conservation & Forestry
Division of Animal & Plant Health
744 Main Street, Suite 9
Presque Isle, Maine 04769
Phone: (207)764-2036 Fax: (207)764-2035

Official Seed Potato Sample Data Sheet

Grower Name: _____

Address: _____
_____, Maine _____

Telephone: _____ Fax: _____

Variety: _____

Certification #: _____

Seed Class/Generation #: _____

Acreage: _____

Date Official Sample Collected: _____

State/Federal Officer: _____

Grower Signature: _____ Date: _____

COATING PLATES WITH THE CAPTURE ANTIBODY FOR CMS

Date:

Coating Buffer prep:

Carbonate Coating buffer lot#:

Volume of buffer needed:

Volume of buffer:

Volume of distilled water:

Capture Antibody lot #:

Volume of Capture Antibody added to the buffer:

Number of wells coated:

Incubate for 4 hours at room temperature or overnight in the refrigerator

Signature _____

PLATE MAP/LOADING DIAGRAM

	Manufacturer/Product							Lot				Plate #	
	1	2	3	4	5	6	7	8	9	10	11	12	
A													
B													
C													
D													
E													
F													
G													
H													

Filename of Run:		
Date:	Time:	Operator:
Thermocycling protocol:		

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
10 µM Forward Primer	1.0			
10 µM Reverse Primer	1.0			
10 µM Probe	0.5			
Water	8.0			
BioRad SsoFast Probes Super Mix (2X; cat. no. 172-5230 or 172-5231)	12.5			
DNA	2.0			
Total rxn volume	25.0			

Note- If conducting Melting Temperature Analysis, replace Probe with water (0.5), and replace Probe Supermix with SYBR Green Supermix.

STATE OF MAINE
DEPARTMENT OF AGRICULTURE, CONSERVATION AND FORESTRY
POTATO DISEASE TESTING LABORATORY
744 MAIN STREET
PRESQUE ISLE, MAINE 04769



JANET MILLS
GOVERNOR



AMANDA BEAL
COMMISSIONER

To:
Date:
Re:

Testing has been completed for the following samples. ELISA and/or Realtime-PCR (Cela, Mills Cms50, and/or Mills Cms72a primers) were used to detect and/or confirm the presence of *Clavibacter michiganensis* subsp. *sepedonicus* (Cms), which can cause Bacterial Ring Rot in potatoes.

Variety Cert # Generation #Tubers Cms Result

Note: Results reported by the Potato Disease Testing Laboratory are based only on the samples submitted for testing by the customer. The Potato Disease Testing Laboratory makes no representations, guarantees or warranties, expressed or implied as to the test results.

If you have any questions, please call the lab at 769-5061.

Thank you,

A handwritten signature in black ink, reading "Andrew B. Plant".

Andrew Plant, Lab Manager

