Full Dickeya Protocol as used by the Maine Department of Agriculture, Conservation and Forestry, Seed Certification Lab

Generalized protocol scheme: cutting samples, then extracting bacteria, then extracting DNA, then using conventional PCR and gel electrophoresis to test for positives, then using CVP plating to grow and double-check positives.


Lab Basics

This protocol requires (chemicals/reagentsbuffers not listed):

- Sterile rubber gloves
- 2 lab coats- one ‘dirty’ for sampling and sample cutting, and one ‘clean’ for processing, DNA extraction, cPCR work, gel electrophoresis, and plating
- An autoclave with the capacity to maintain 240°F and 15lb/in^2 for 15 minutes.
- A centrifuge with the capacity to spin 50mL tubes at 10,000 rpm for 10 minutes.
- A centrifuge with the capacity to spin 1.5mL tubes at 16,000 x g for 3 minutes
- A conventional PCR unit and accompanying tubes and lids
- A gel electrophoresis power unit, buffer tank, gel mold, and comb
- Prosan- a quaternary ammonia based cleaner effective against plant pathogens
- Swiss army peeler/coreing tools
- 90-100% ethanol for diluting
- Large containers for holding tuber samples
- A refrigerator
- A freezer
- A vortex
- A mallet
- Plastic bags that have a seal with the capacity to hold liquids without leaking
- Autoclave bags
- A Sigma GenElute™ Bacterial Genomic DNA Kit, or other suitable bacterial DNA extraction kit
- 50mL screw-top centrifuge tubes and accompanying racks
- 250mL screw-top plastic Nalgene jars
- 1.5mL microcentrifuge tubes and accompanying racks
- Sterile, disposable plastic knives
- Sterile, disposable plastic weigh boats or other similar disposable trays
- Fine point sharpies
- Pipettes and tips with the capacity to pipette from 1uL to 1mL. (Recommended: 0.1uL-2uL pipette, 2-20uL pipette, 20-200uL pipette, 200-1000uL pipette)
- Dry hot bath with the capacity to maintain 55°C for 30 minutes
- Plastic disposable 90mm petri dishes
- Plastic disposable hockey-stick culture spreaders, or a glass hockey-stick culture spreader and the capacity to use flaming-ethanol sterilization techniques
- Plastic disposable loop culture streaking tools, or a metal loop culture streaking tool and the capacity to use flaming sterilization techniques
- Glass 1L flasks
- Glass beakers with the capacity to pour 12mL and 15mL amounts
A relatively clean lab coat is worn at all times, along with clean gloves. If at any time, moisture is noticed on gloves, gloves are discarded into an autoclave bag and new are acquired. The only exception to this rule is coreing, where a designated ‘dirty’ lab coat is worn, and gloves are only changed between samples.

Surfaces are regularly sterilized with prosan or alcohol. Any spills are cleaned up immediately and the area is sterilized with prosan or alcohol.

When pipetting, tips are never reused and are discarded with each use into an autoclave bag. Pipettes are wiped regularly with alcohol. Appropriate tips are used with pipettes. Pipetting is done free of bubbles and without sucking liquid into the pipette itself. If bubbles occur, draw liquid up slower. If there is danger of sucking the liquid into the pipette, a filter tip should be used. Equipment such as centrifuges, hot baths, autoclaves, and hoods are used according to manufacture instructions and with proper attention to safety.

All laboratory equipment that comes into direct contact with samples must either be disposable (and is autoclaved before disposal) or must be scrubbed and autoclaved before reusing. If an item such as a pipette tip, rubber glove, or tube is dropped onto the floor, that item is now considered non-sterile and must either not be used, or sterilized before being used again.

All tubes, jars, and bags are labeled with the sample name.

Step 1: SAMPLING

- All samples require a Dickeya sample submission form- available at the lab and may be filled out during delivery of samples. Sample names must be written on the form exactly as they appear on the sample’s container.
- Leaking or saturated bags of samples should not be brought to the lab, as this can spread disease, and may be rejected.
- Dormant tubers: Tubers must be taken from a variety of locations in the field or in the potato house- do not grab all tubers from a single area, as this does not give an accurate representation of the whole lot. Up to 200 tubers can be tested as a single sample. Tubers don’t need to be washed, but should be free of mud and excessive dirt. BURLAP SACKS ARE NOT ACCEPTED. Paper bags only. If bag is seeping or saturated, it may be rejected.
- Symptomatic tubers: The tuber displaying symptoms should be wrapped in paper (newspaper or a brown paper bag will suffice) to prevent liquefying, and sealed in a plastic bag to prevent spread of disease. Any seeping bags or containers may be rejected. (Minitubers follow this same sampling protocol.)
- Water test: A sample of the water will be taken in a plastic, sterile, screw-top jar provided by the lab. The jar may have water poured in or may be dipped into a body of water- if jar is dipped, please either sterilize the outside of the jar after sealing, or seal jar in a plastic bag. Water samples should be delivered to the lab no more than 24 hours after being taken.
- Plant material: An entire plant should not be brought into the lab and may be rejected. When a symptomatic or suspicious plant is found, the grower should cut the stem at the base, as close to the dirt as possible, and then remove leaves and offshoots, leaving just the stem displaying blackleg symptoms. The stem should be wrapped in paper (newspaper or a brown paper bag will suffice) to prevent liquefying, and sealed in a plastic bag to prevent spread of disease. Any seeping bags or containers may be rejected.

Step 2: SAMPLE PROCESSING/BACTERIAL EXTRACTION

- Dormant tubers
  - The bag of tubers is loaded onto a covered coreing rack. Gloves may be worn for the entire sample, and then are changed before moving on to the next sample.
Using a coreing tool (non-rotating vegetable peeler with sharpened triangular tip), the stolon end is peeled down to the flesh. The tip of the tool is inserted into the stolon and is rotated to remove a conical section of tissue - the core.

- Discard peel, keep core.
- Repeat with up to 200 tubers.
- Gloves, coreing tool, coreing rack cover, and core tray are all changed before beginning the next sample. Peels, dirt, and other debris is cleaned away.
- Cores are put in a 250mL Nalgene screw-top jar.
- Extraction buffer (see appendix for instructions) is poured into jar over the cores. Enough extraction buffer is added to barely cover the cores.
- Jars are tightly sealed and put on the orbital shaker. Jars are shaken overnight at 100-200 rpm.
- The liquid, now containing bacteria extracted from the cores, is decanted into a 50mL tube.
- 50mL tubes are put into the large volume centrifuge and spun at 10,000 rpm for 10 minutes.
- Supernatant from spun tubes is discarded into an autoclave bag, leaving the pellet in the tube.
- Add 2mL of 0.25% Ringer’s solution to the 50mL tube and vortex to resuspend pellet.
- Liquid is pipetted into two 1.5mL tubes - one for storage and plating, if need be, and one for PCR.

**Symptomatic Tubers**

- Using a sterile plastic knife and a weigh boat as a cutting tray, take a slice of tuber at the border of health and infection. The slice should be about the size of a dice.
- Put the tuber tissue slice into a 50mL tube and add enough 0.25% Ringer’s solution to cover the slice.
- Let sit for at least 10 minutes, vortexing or shaking occasionally.
- Liquid can be pipetted into two 1.5mL tubes - one for storage and plating, if need be, and one for PCR - or may remain in the 50mL tube with the tissue slice as a sample stock.

**Water Samples**

- If sample has debris or appears cloudy, pour 40mL into a 50mL tube and centrifuge at 180 rpm for 2 minutes to clarify. Supernatant is kept, while the dirt pellet is discarded. If the water appears clear and free of particulate matter, skip this step.
- Pour 25mL of sample and 25mL of pectate enrichment broth (see appendix for instructions) into a 50mL tube. Invert a few times to mix, then incubate at 37°C for 48 hours.
- Vortex, then centrifuge 50mL tube of PEB and sample at 10,000 rpm for 10 minutes.
- Pour off supernatant carefully. Pellet may be fragile or may be large and gelatinous.
- Add 2mL of 0.25% Ringer’s solution to 50mL tube. Vortex to resuspend pellet.
- Liquid is pipetted into two 1.5mL tubes - one for storage and plating, if need be, and one for PCR.

**Minitubers**

- Put minituber in a sterile plastic bag and seal or fold end to keep well contained. Center tuber in the bag to prevent leaking.
- Smash tuber in the bag with a mallet. If processing multiple minitubers in the same bag, each minituber must at least be split to expose flesh.
- If bag tears, put tuber bits in a new bag or into a 50mL tube.
- Pour enough 0.25% Ringer’s solution to cover tuber bits.
- Let sit for at least ten minutes, shaking or vortexing occasionally.
- Liquid can then be pipetted into two 1.5mL tubes- one for storage and plating, if need be, and one for PCR- or into a 50mL tube as a sample stock.

**Other Symptomatic Material (stems, roots, etc)**
- Using a sterile plastic knife and a weigh boat as a cutting tray, take a slice of tissue at the border of health and infection. Put tissue slice into a 50mL tube.
- Pour enough 0.25% Ringer's solution into the tube to cover the sample slice.
- Let sit for at least ten minutes, vortexing or shaking occasionally.
- Liquid can then be pipetted into two 1.5mL tubes- one for storage and plating, if need be, and one for PCR- or left in the 50mL tube as a sample stock.

### Step 3: DNA EXTRACTION

- This extraction is performed using a Sigma GenElute™ Bacterial Genomic DNA Kit. This can be ordered through Sigma Aldrich, cat# NA2120-1KT. The protocol for this kit can be found at [http://www.sigmaaldrich.com/technical-documents/protocols/biology/genelute-bacterial-genomic-dna-kit.html](http://www.sigmaaldrich.com/technical-documents/protocols/biology/genelute-bacterial-genomic-dna-kit.html). This kit is recommended for extractions on plant tissue. For tuber samples or others likely to gum up, it may be advisable to use the MP FastDNA Spin Kit for Soil.

### Step 4: PCR AND GELS

In each protocol, include at least one positive control and one no-template control.

*Primer sequences can be found in Humphris et al, Table 1. Cycling conditions, ratios, dilutions, and primer sets are directly from Humphris et al. The primers pelADE1 and pelADE2 (here simply referred to as ADE1 and ADE2) are currently recognized as the best diagnostic method for detection of dickeya species.*

- **Conventional PCR Protocols:**
  - **Dickeya spp**
    - **Mix:**
      - **H2O** - - - - 9.5 uL
      - **Jumpstart RedTaq ReadyMix** - 12.5 uL
      - **ADE 1 (10pmol/uL)** - - 1 uL
      - **ADE 2 (10pmol/uL)** - - 1 uL
      - **DNA template** - - - 1 uL
    - **Cycling:**
      - 94 C - 5:00
      - 94 C - 1:00
      - 72 C - 2:00
      - GOTO step 2 x25
      - 72 C - 7:00
  - **Blackleg (Dickeya spp and Pectobacterium spp)**
    - **Mix:**
      - **H2O** - - - - 9.5 uL
      - **Jumpstart RedTaq ReadyMix** - 12.5 uL
      - **SR3f (7.5pmol/uL)** - - 1 uL
      - **SR1cR (7.5pmol/uL)** - - 1 uL
      - **DNA template** - - - 1 uL
    - **Cycling:**
      - 95 C - 0:30
      - 60 C - 0:45
      - 72 C - 0:45
      - GOTO step 1 x40
      - 72 C - 8:00
• Gel Setup:
  o Mixing the gel (50 lane tank):
    Agarose - 3.0 g
    50x TAE buffer - 4 mL
    H2O - 196 mL
    GelRed - 10 uL

    Microwave for 2 minutes. Pour into tank with comb and allow to cool for approx. 45 minutes.
    (Recommended to pour the gel immediately following loading the PCR and allowing to cool the duration of the protocol.)
  o Load 5uL of sample from the PCR into each lane, including a ladder.

Step 6: PLATING

Samples that receive a positive result in the PCR are then plated to confirm results, and to discern whether bacteria is live or dead. Culture may be scraped from plates to return to step 3, DNA extraction, and step 4, PCR.

• Double-Layer Crystal Violet Pectate media recipe (also known as CVP, or CVM, or CPM.)
  This recipe requires two layers to be made separately: basal layer and overlayer. It is recommended that the basal layer is made first and put in the autoclave. The overlayer may be made while the basal layer autoclaves, and then may be autoclaved while pouring the basal layer. Plates should be given at least 10 minutes to dry well before being stacked upside-down in a sterile bag, sealed, and stored in the refrigerator. This recipe makes approximately 30 complete plates.
  o Basal layer:
    ▪ Mix-
      • 4.0g peptone from casein
      • 1.2g yeast extract
      • 2.0g NaCl
      • 0.1g SDS
      • 1.0g L-Asparagine
      • 3.0g tryptone
      • 6.0g agar
      • 500mL water
    ▪ Before autoclaving, mix 0.05g of 2,3,5-triphenyltetrazolium chloride with 5mL of water in a small lidded jar.
    ▪ Autoclave both mix and small jar for 15 minutes at 250 F.
    ▪ Add 0.5mL of the autoclaved triphenyltetrazolium chloride to the mix.
    ▪ Add 400uL (32ug/mL) polymxin B sulfate.
    ▪ Mix gently, careful not to form bubbles.
    ▪ Dispense 12mL of mix into each petri dish.
  o Overlayer:
    ▪ Mix-
      • 1.0mL 0.075% w/v crystal violet solution
      • 6.5mL 10% CaCl2
      • 1.0g NaNO3
      • 2.5g tri-Na citrate dehydrate
      • 2.0g agar
      • 500mL water
- Add 7.5g pectin after everything else has been well mixed.
- Autoclave for 15 minutes at 250 F.
- Add 300uL (1.2ug/mL) polymixin B sulfate.
- Mix gently, careful not to form bubbles.
- Dispense 15mL of mix into each petri dish, directly on top of basal layer.

- Plating Samples
  - Dilution/Lawn method (recommended for first isolation attempt, liquid to culture)
    - Dilute sample into six tubes. Concentrations are $10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$.
    - Dispense 100uL of each dilution onto the center of a CVP plate.
    - Use a sterile disposable hockey stick cell spreader to spread out the sample across the surface of the plate, being cautious not to damage the media.
    - Incubate at 37 C for 48 hours.
  - To replate semi-defined colonies that indicate the presence of dickeya bacteria (red colonies that sink into the media, and sink the area around it, creating a wide divot or 'pit'), use the t-streak method on a fresh CVP plate. (Recommended for second isolation attempt, culture to culture)
  - To replate defined colonies that are confident to be dickeya, use the star streak method on a fresh nutrient agar plate. (Recommended for third isolation attempt, culture to isolate)

**APPENDIX**

1) Pectate enrichment broth (PEB)
   - 0.64g Epsom salt
   - 2.16g Ammonium sulfate
   - 2.16g Potassium phosphate
   - 3.4g Pectin
   - 1000mL Water
   - Mix well, autoclave before using, refrigerate for storage.

2) Extraction buffer
   - 4.26g Sodium phosphate dibasic
   - 2.72g Potassium phosphate monobasic
   - 1000mL Water
   - Mix well, autoclave before using, refrigerate for storage.