PCR Detection and DNA Isolation Methods
for Use in the *Phytophthora ramorum* National Program

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The validated protocols listed below are from Oregon State University and the University of California at Berkeley with limited modifications based on the work done in Beltsville by USDA APHIS PPQ CPHST. The following recommendations are provided based on our experience with DNA extraction and PCR protocols tested for *Phytophthora ramorum*.

I. SAMPLE PREPARATION:

IMPORTANT NOTE: Contamination of PCR samples can start prior to the PCR test during both sample selection and preparation, and DNA extraction. To minimize the potential for contamination during the sample selection make sure that the area used in the lab to select leaves is separated from the DNA extraction area. Make sure that disposable lab mats are used on the benches where samples are processed, and samples are cut in weigh boats changed between each sample. Gloves should be worn to select leaves and cut samples and changed between each sample to avoid contaminating the next sample. Mycelium and spores from *P. ramorum*-infected leaves can be transferred from infected leaf sets to healthy leaf sets and may result in a false positive PCR test. A good rule of thumb is to treat each sample as if it is infected and as if the next sample to be processed is healthy and take measures to minimize contamination of that next sample. Cutting implements should either be disposed of between samples, or dipped in ethanol and flamed between samples to avoid direct contamination.

- Leaf tissue for DNA extraction should be collected from the margin area between the necrotic lesion and healthy tissue of symptomatic leaves (see below). A No. 3 cork borer or a disposable blade can be used. Each plant disc cut out using a No. 3 cork borer weighs approximately 10mg. Leaf pieces cut with a disposable blade approximately 5 mm x 5mm square weighs approximately 5mg. Dispose of or flame sterilize the cutting implements between each sample. Each leaf showing necrotic symptoms should be sampled at least once. If only a few leaves are present in the sample they can be sampled two or more times from the same leaf. We have found that taking samples from non-symptomatic areas do not yield target PCR products. In addition, while taking the sample requires including necrotic areas of the leaf try to make sure that the necrotic margin is in the middle of the sample area. Our experience has been that too much green area in a sample may result in a PCR false negative because of a low amount of pathogen, as will

too much necrotic area in a sample because of PCR inhibition from the necrotic tissue. The maximum amount of tissue used in the DNeasy Plant Mini kit is 100mg (9-10 leaf discs using a No. 3 cork borer, or 15-16 of the 5mm x 5mm cut leaf pieces).

A. Sampled areas from *P. ramorum*-infected Rhododendrum

B. Sampled areas from *P. ramorum*-infected Camelia

II. MOLECULAR METHODS: DNA EXTRACTION

DNA must be extracted using the CPHST validated protocols using either the Qiagen DNeasy Plant Mini kit or the OSU DNA extraction protocol that consists of a CTAB-based extraction that finishes with the Qiagen DNeasy Tissue kit (#69504). The PCR amplification levels were comparable using both protocols. We, however, recommend use of the Qiagen kit over the OSU protocol because it is quicker, user friendly, and all reagents and kit components are standardized and quality assured. We also note that the Qiagen kit produces higher yields of DNA, which appears to be of better quality based on gel analysis. We make this recommendation for culture and leaf material only. We also recommend the Qiagen DNeasy kit so that labs performing the DNA extraction are providing a similar quality of DNA to the PCR testing labs that will provide consistency in the results obtained.

IMPORTANT NOTES: In order to avoid cross contamination please designate separate rooms or lab areas for each segment of the work and use separate designated sets of pipettes. Use aerosol-resistant barrier pipet tips. Centrifuge any DNA-containing tubes before opening so that any liquid near the rim of the tube is removed; centrifuge rotors designed for aerosol containment are recommended. Use microfuge tube openers to avoid contact with the top rim of the microfuge tubes. It is a good practice to store plant samples or extracts in a separate freezer or freezer compartment from PCR reaction components. If samples are contaminated with soil rinse them in sterile water and pat-dry them with a hand towel. Wear gloves and change them often, particularly between different segments of the DNA extraction procedures. Use disposable lab mats to cover bench areas and change them between each set of extractions. Never autoclave any *P. ramorum*–contaminated plant material, culture plates, or soil in an autoclave used to sterilize buffers, glassware, or plastic ware used in the SOD DNA extraction because of potential contamination from aerosols within the autoclave. All racks, tube openers, and other plastic materials used in the procedure should be decontaminated between each set of extractions by soaking in a 10% bleach solution (a 1:10 dilution of commercial bleach in water) for 30 minutes followed by 2 rinses with water to remove the bleach solution. Bench areas, pipettes, centrifuge rotors, lab chairs, drawer handles, and other knobs, etc. in the environment of the bench used to do DNA extractions should be wiped down every couple of days with a DNA elimination solution such as DNA Away.

A. PLANT DNA EXTRACTION using QIAGEN DNeasy Plant Mini Kit (Cat # 69104)

This protocol is based on the Qiagen's protocol for total DNA extraction from plant tissue using the mini columns. A booklet is included with each kit and the protocol is on page 16. Please, read carefully all information provided in the booklet before starting work. We have made a few changes in the protocol. Here are some helpful tips:

- Consider extracting as many samples at once as your centrifuge rotor can hold. We always process 12 or less. Too many samples processed by one person can result in some columns beginning to dry before the next step in the procedure.
- Prepare and label all necessary tubes and columns in advance (columns should be labeled on the top of the lid, not on the side of the collection tubes). Place them on a rack in rows:
  - First row – the QIAshredder columns (lilac colored columns supplied)
  - Second row – 1.5 ml tubes
  - Third row – DNeasy columns (white columns supplied)
  - Forth row – 1.5ml tubes with lids cut off (if necessary).
  - Fifth row – 1.5 ml tubes for the DNA extracts clearly labeled with the sample ID and date of extraction.
- Set up a water bath or thermostat at 65ºC.

- First time you use the kit add the appropriate volume of 95-100% of ethanol to AW and AP3/E buffers. Make a note that EtOH added and on what date on the bottle cap and bottle. (If a date does not exist on an opened bottle and doubt EtOH has been added, smell the bottle for the smell of ethanol.)
- Always check buffers AP1 and AP3/E for precipitates that can form upon storage. To dissolve the precipitate, warm the buffers up to 65ºC.
- Wear gloves during extraction. Change them often and if you have any reason to believe they have been contaminated with sample extracts.
- Open tubes and columns carefully to avoid aerosol formation and spills. Use a tube opener. When you transfer samples try not to touch the edge of the tubes with the pipetor tip.
- When handling columns, please hold them for the top of the collection tube, not for the column. Column only sits on the top of the collection tube. If you hold the column only, the collection tube may disconnect and drop (potential contamination or losing your sample). When transfer columns from one collection tube to another, please do not touch the bottom of the column.
- Once you start the extraction, please do not stop and leave your samples for any amount of time longer than prescribed by the protocol.
- Pre-warm portion of the AE buffer to 65ºC in water-bath or thermostat.
- Centrifugation steps are carried out at room temperature.
- Most centrifugation steps are to be preformed at 20,000 x g or 14,000 rpm. If you do not have a centrifuge capable of that speed double the time.

PROTOCOL

1. Use cork borer No 3 (0.6 cm in diameter) to cut out leaf tissue from suspect *P. ramorum* infected samples. Sterilize cork borer after each separate sample by dipping in EtOH and flaming. You may use disposable blades instead of the cork borer and cut out a square piece of plant tissue with sides of 0.5 cm long. Max weight of tissue to be used for DNA extraction with the kit is 100 mg. Change gloves and cutting surface (weight boat or paper) between samples.
2. Place a glass bead (5mm diameter) in a 2 ml screw-cap tube then add pieces of tissue then another glass bead. Tightly screw tube cap and drop the tube in liquid nitrogen for about 30 sec. Take tube out and place it immediately on a Bead-Beater. Homogenize for 30 sec at 2500 rpm (settings 3 for time and 25 for speed). Remove the tube and place it in liquid N_2 again for 30 sec. Repeat freezing and beating 2 more times. Place tube in ice while homogenizing next sample. Alternatively you can use mortar and pestle to grind samples.
3. Add 600µl of buffer AP1 and 6µl of RNase A to each tube, vortex vigorously and incubate at 65ºC for 10min (15 min if you use a thermostat not a water bath). Mix tubes time to time by inverting. Spin tubes very briefly after incubation and proceed to step 4.
    a. Do not mix the AP1 and RNase A stock before use.
    b. The volume of the AP1 buffer was increased because we have noticed that plant tissue from some of the *P. ramorum* hosts (rhododendron) absorbed

most of the buffer and that may affect extraction efficiency. The volumes of the RNase A and AP2 buffer were increased accordingly.

4. Add 195µl of buffer AP2 to each sample, mix well by inverting and incubate on ice for 5 min.
5. Centrifuge samples on micro centrifuge for 5 min at 20,000x g (14,000 rpm).
6. Transfer supernatant to a labeled QIAshredder spin column (lilac) placed in a 2ml collection tube and centrifuge for 2 min at 20,000x g (14,000 rpm). Discard the column, keep the flow-through.
7. Transfer the flow-through fraction from step 6 to a new 1.5 ml tube. Try not to disturb pellet formed on the bottom of the tube. Record the volume for each sample. Calculate and add 1.5 volumes of AP3/E buffer to each sample. Immediately mix well by pipetting up and down.
8. Transfer 650µl of the mixture from the step 7 to the DNeasy Mini Spin Column (white) sitting in a 2ml collection tube. Spin for 1 min at 6,000 x g (8,000 rpm). Keep the column discard the flow-through. Place the column in the same collection tube and proceed to step 9.
    a. The flow-through contains AP3/E buffer with guanidine hydrochloride that is hazardous. Please, discard this fraction according to your lab hazardous waste regulations.
9. Repeat step 8 with the remaining of the sample. Discard the flow-through as in step 8, keep the column.
    a. The DNaesy column volume is 650µl and the volume of the sample in step 7 is usually more than that. That's why it is necessary to apply the sample in 2 consequent loads (steps 8 and 9) so that all extracted DNA binds to the column. Please be careful not to mix up samples at this point.
10. Place the DNeasy Mini Column in a new 2ml collection tube (supplied). Add 500µl of buffer AW and centrifuge for 1 min at 6,000 x g (8,000 rpm). Discard the flow-through, keep the column.
11. Add second portion of 500µl of buffer AW and centrifuge for **2 min** at 20,000 x g (14,000 rpm). Discard the flow-through, keep the column.
12. Place the column in a new 1.5ml tube without a lid. Pipet 100µl of preheated (65ºC) buffer AE directly to the membrane. Incubate for 5 min at room temperature. Spin for 1 min at 6,000 x g (8,000 rpm). Discard the column keep the flow-through.
    a. We do not recommend doing a second elution.
13. Transfer the flow-through (total plant DNA extract) to a new, properly labeled 1.5ml tube and store it at -20ºC until used for PCR.

Specific Equipment and Reagent Information for DNeasy Plant Mini Kit (Qiagen Cat # 69104)

Reagents and Equipment to Be Supplied by User

- Equipment for disrupting plant tissue. Such as a bead beater
- Ethanol (96–100%)
- Liquid nitrogen

For the DNeasy Plant Mini Kit:

- Microcentrifuge tubes (1.5 ml)
- Microcentrifuge with rotor for 2 ml tubes
- Screw-cap plastic tubes – 2 ml volume (general lab vendors)

- Glass beads 5mm diameter (Fisher Scientific cat. # 11-312C).
- Mini Bead-beater (Cole Parmer, Cat# A-36270-02)

B. Modified OSU DNA Extraction Protocol

We tested this protocol only with mountain laurel infected and healthy leaf tissue. It's efficacy for tissue samples from other sources remains to be determined. All stock solutions should be prepared in advance. When processing many samples, aliquot the necessary volume of the stock solution for the number of the samples plus 2-3 more in a new tube to prepare a working solution. The extraction should be conducted in a room or an area separate from the location used to set up master mix and PCR reactions.

PROTOCOL

1. Make 2% CTAB (cetiltrimethylammonium bromide SIGMA) buffer, 100 mM Tris, pH 8.0; 20 mM Na_2EDTA pH 8.0; 1.4 M NaCl; 1% polyvinylpirolidone, 0.1% 2-mercaptoetanol [ME]). (We prepare the buffer without 2-ME. Before using it, take the necessary volume for the number of samples (600 µl per sample) and add 2-mercaptoethnol to 0.1%, in a fume hood.)
2. Pre-warm CTAB buffer to 65 ^{o} C.
3. Use a No. 3 cork borer (0.6 cm in diameter) to cut out leaf tissue from suspect SOD-infected samples. Sterilize cork borer after each separate sample by dipping in EtOH and flaming. After flame-sterilizing the cork borer it will not be sufficiently sharp, so you may want to use disposable blades and cut out a square of plant tissue with sides of 0.5 cm long.
4. Place the plant disc into a 2 ml screw-cap plastic tube, add two glass beads, screw the cap tightly and drop the tube into liquid nitrogen for around 30 sec.
5. Take the tube out of the liquid nitrogen and immediately place it onto Mini Beat-Beater. Beat for 30 sec at 2,500 rpm (setting 3 for time and 25 for speed). If you composite several (up to 10) samples, you will need to repeat freezing and grinding 2 more times.
6. Add 500 µl of pre-warmed CTAB buffer. Vortex.
7. Incubate for 1 hour at 65 ^{o} C, mixing time to time.
8. Add 500 µl phenol: chloroform: isoamyl alcohol (25:24:1) (we do not take glass beads out at this point). Vortex for 1 min.
9. Spin on a microcent ifuge for 10 min at 13,000 rpm.
10. Carefully remove the supernatant (approx. 450 µl) and transfer it into a new microcentrifuge tube. Add 450 µl chloroform: isoamyl alcohol (24:1) and vortex for 1 min.
11. Spin again as at step 9.
12. Carefully remove the supernatant and transfer it into a new microcentrifuge tube containing 200 µl of 100% EtOH (200 proof)). Mix well by inverting.
13. Pour the mix into DNeasy column (DNeasy Tissue Kit from Qiagen Cat. #69504).
14. Spin for 1 min at 8,000 rpm on microcentrifuge at room temperature (RT). Pour out the flow-through, keep the column.
15. Wash the column with 500 µl AW1 buffer (from the kit). Spin 1 min / 8,000 rpm at

RT. The AW1 and AW2 buffers are provided with the kit and have to be diluted with 100% EtOH prior to use (see manufacturers instructions).

16. Transfer the column into a new collection tube and wash with 500 µl AW2 buffer. Spin for 3 min at 8,000 rpm at RT until the buffer passes through the column leaving the column dry.
17. Transfer the column into 1.5 ml microcentrifuge tube (you may need to cut off the lid to fit into the microcentrifuge).
18. Add 50 to 100 µl of the Elution Buffer exactly on the top of the filter column, not the walls. Incubate at RT for 1 min.
19. Spin for 1 min at 8,000 rpm at RT.
20. If you have cut off tube lids transfer resulting DNA in a new tube, label and keep DNA at -20ºC.

Specific Equipment and Reagent Information for OSU DNA Extraction Protocol

- Reagents for CTAB buffer (2% CTAB (cetyltrimethylammonium bromide), 100mM Tris, pH 8.0; 20mM Na2EDTA pH 8.0; 1.4M NaCl; 1% polyvinylpirolidone, 0.1% 2-mercaptoetanol).
- Phenol: chloroform: isoamyl alcohol (25:24:1) from Invitrogen Cat # 15593-031.
- 200 Proof (100%) Ethanol.
- DNeasy Tissue Kit from Qiagen Cat. # 69504.
- Screw-cap plastic tubes – 2 ml volume.
- Glass beads 5mm diameter from Fisher Scientific cat. # 11-312C.
- Mini Bead-beater (Cole Parmer, Cat# A-36270-02, ~$800.00, 1800-323-4340)

III. Molecular Methods: PCR

Amplification Modified UCB DNA

Amplification (PCR) Procedure:

All reagents should be kept at -20 ^{o} C. Before PCR take them out to thaw, vortex briefly and spin down shortly to collect the content on the bottom. The Taq polymerase is stored in glycerol and does not require thawing. It should remain in the freezer or on ice at all times. While preparing the master mix, all reagents should be kept on ice. The master mix should be prepared in a laminar flow hood, or in a room where PCR products are not generated or analyzed. Use a separate set of pipettes for the master mix. Change tips and gloves often.

The PCR mix for the first round of amplification (using specific PCR primers Phyto 1 and Phyto 4) consists of:

10X PCR Buffer	2.50 µl	
10mM dNTPs	0.50 µl	
50mM $MgCl_2$	1.00 µl	
5 µM Phyto 1/4primer mix	2.50 µl	(0.5 µM final concentration)
Platinum Taq polymerase (5u/ml)	0.25 µl	
dH_2O	12.00 µl	
Total mix volume	18.75 µl	
Add DNA	6.25 µl	
Total reaction volume	25.00 µl	

Prepare **master mix** for the number of the samples tested, plus 1-2 extra and keep on ice. Don't forget to include positive and negative (water used to make the master mix and 0.1X TE buffer used for DNA dilutions) controls. Aliquot 18.75µl of the master mix to each PCR tube. Take tubes to the PCR station and add 6.25 µl of each DNA sample with a pipette used only for this purpose. No mineral oil needed if you run PCR on thermocycler with heated lid.

First round PCR program:

Denaturation:	1 cycle	94 ^{o} C / 1min 25 sec
Amplification:	34 cycles	93 ^{o} C / 35 sec 62 ^{o} C / 55 sec 72 ^{o} C/ 50 sec
Extension:	1 cycle	72 ^{o} C/ 10 min
Hold:		4 ^{o} C
Ramp rate :	3.3 ^{o} C/ sec heating and 2.0 ^{o} C/ sec cooling (if possible).	

Take special care while preparing diluted template samples for the second round of PCR in order to avoid cross contamination. In a laminar hood or PCR set up area prepare Eppendorf tubes with 500 µl sterile distilled water for the number of the samples you are running. Take those tubes to the PCR station/area and add 1 µl of corresponding first round PCR reactions to make 1:500 dilutions. **Close tubes tightly, vortex and spin down briefly**. Change gloves between opening the PCR tubes and tubes with diluted templates –first round products. Be careful to avoid any aerosol formation. In the laminar hood make up the PCR mix for the **second round** of the nested PCR.

The PCR mix for the second round of amplification (using Universal PCR primers Phyto 2 and Phyto 3) consists of:

10X PCR Buffer	2.50 µl	
10mM dNTPs	0.50 µl	
50mM $MgCl_2$	1.00 µl	
5 µM Phyto 2/3 primer mix	2.50 µl	(0.5 µM final concentration)
Platinum Taq polymerase (5u/ml)	0.25 µl	
dH_2O	12.00 µl	
Total mix volume	18.75 µl	
Add DNA	6.25 µl	
Total reaction volume	25.00 µl	

Dispense 18.75 µl of the master mix to each tube and take tubes to the PCR station. Add 6.25 µl of each diluted first-round sample, mix and place on the block. Include new controls: sterile water used for dilution, water used for master mix and positive control (diluted first round product).

Second round PCR program:

Denaturation:	1 cycle	94 ^{o} C / 1min 25 sec
Amplification:	34 cycles	93 ^{o} C / 35 sec 62 ^{o} C / 55 sec 72 ^{o} C/ 50 sec
Extension:	1 cycle	72 ^{o} C/ 10 min
Hold:		4 ^{o} C
Ramp rate :	3.3 ^{o} C/ sec heating and 2.0 ^{o} C/ sec cooling (if possible).	

To visualize PCR results run 10 µl of each PCR sample, from the second round only on 1.5% agarose gel in 1X TAE buffer at 100V for 1 hour. Stain gels with the EtBr according to you lab protocol.

IV. Equipment and Reagents Necessary for SOD Detection:

Protocol 1 PCR Primers and Reagents

A. PCR machine -we use T-gradient from Biometra (others could be used).
B. Primers:
   a. Specific *P. ramorum* primer pair:
      i. Phyto 1: 5'-CAT GGC GAG CGC TTG A-3'
      ii. Phyto 4: 5'-GAA GCC GCC AAC ACA AG-3'
   b. *Phytophthora* universal primer pair:
      i. Phyto2: 5'-AAA GCC AAG CCC TGC AC-3'
      ii. Phyto3: 5'-GGT GGA TGG GGA CGT G- 3'
   c. We ordered primers desalted from Invitrogen (you can use your usual provider) and prepare primer mixes the following way:
      i. Tubes with lyophilized primers were spun briefly before opening the tubes. Primers were rehydrated to 100 µM concentration in autoclaved
      ii. 0.1 X TE buffer pH 8.0 and stored at - 20 ^{o} C.
      iii. 50 µM stocks of primer mixes of the specific pair (Phyto 1 and 4) and the control pair (Phyto 2 and 3) were made.
      iv. 5 µM working mixes were prepared by diluting an aliquot of the 50 µM stocks 1:10 in 0.1 X TE buffer pH 8.0.
C. Reagents
   a. Enzyme– Platinum *Taq* Polymerase from Invitrogen (cat. No 10966-034). Supplied as a kit with 10X PCR Buffer and 50mM $MgCl_2$.

b. 10mM **dNTP Mix** from Sigma (D-7295).
c. **Thin-wall 0.2ml PCR** tubes from Cole-Parmer (No. 67103-90).
d. Molecular grade d H_2O from Geno Technology Inc. (1-800-628-7730).
e. Sterile 0.1X TE buffer pH 8.0
f. Sterile distilled water.

Vendors:

- **Cole Parmer (800-323-4340) www.coleparmer.com (search for bead beater, then select the Mini Bead Beater $847.00)**
- **Qiagen (800-426-8157) www1.qiagen.com/products (select Genomic DNA Stabilization and Purification, then select DNA purification from Animal and Plant Samples, be sure to select the MINI kit)**
- **Invitrogen (800-955-6288)**
- **Geno Technology Inc. (800-628-7730)**
- **Sigma (800-325-3010)**
- **Biospec Products, Inc (800-617-3363)**
- **Fisher Scientific (800-766-7000) www.fishersci.com**
- **LabRepco (800-521-0754)**