Evaluation of the PCR Detection and DNA Isolation Methods for Use in the *Phytophthora ramorum* National Pilot Survey.

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Rationale: Evaluate the two existing PCR methods utilized by the Oregon State University and the University of California, Berkeley for the detection of *P. ramorum* the causal agent of Sudden Oak Death (SOD).

Approach: Evaluate the methods according to the laboratory procedures for PCR amplification and DNA extraction, evaluate each PCR method using DNAs extracted by either DNA extraction protocol, evaluate the ease of operation of the two PCR methods and associated DNA extraction methods, evaluate the sensitivity of the two PCR methods, and include any changes to the extraction or PCR protocols that did not effect the overall performance of the methods.

I. BACKGROUND:

Methods tested:
We tested two PCR protocols from UCB and OSU. We used a **Biometra T-gradient thermocycler (LabRepco).**

A. **OSU (Hansen) protocol** is designed to detect *P. lateralis* and uses 2 pairs of primers: PLAT primer pair is specific for *P. lateralis* and generates product of 738bp, and the universal control pair (NS1 and NS2) based on the ITS region of ribosomal DNA and generates product of 550bp. Those two pairs were tested separately and in multiplex format as well.

B. **UCB (Garbelotto) protocol** is a nested PCR using *P. ramorum* specific primers Phyto 1 and 4 (687 bp product) for the first round and *Phytophthora* specific primers Phyto 2 and 3 (291 bp product) for the second round.

DNA/ plant, culture materials utilized/provided:

DNA extracts provided by Everett Hanson's lab at Oregon State University were *P. ramorum* 07 from California*, P. lateralis,* and 3 DNA extracts (778, 779, and 785) from field samples (host names not provided).

DNA extracts provided by Matteo Garbelotto's lab at UC Berkeley were *P. boehemeriae* 325, *P. parasitica* 331, *P. megasperma* 336, *P. erythroseptica* 355, *P. gonapodyides* 393, *P. palmivora* 427, *P. cryptogea* 438, *P. cinamomi* mating

type A1 P 3679, *P. cinamomi* mating type A2 P 2444, *P. cambivora* P 198513, *P. lateralis* E 16, *P. ramorum* 0-63, *P. syringae* 115773A.

Plant material, mountain and bay laurel leaves only, infected with *P. ramorum* isolates 0-217 and PR-G2, and healthy mountain and bay laure,l was provided by Paul Tooley, USDA-ARS. Ft. Detrick. Fungal cultures of *P. ramorum, P. cinamomi, P. cambivora, P. ilicis, P. infestans, P. lateralis, P. syringae* were obtained from the same lab.

II. EVALUATION OF THE PCR TESTS USING DNAS PROVIDED BY THE OSU AND UCB LABS, BOTH DNA SOURCES USED IN EACH TEST:

A. OSU (Hansen's) PCR PROTOCOL:

The OSU PCR protocol was used on DNAs provided by OSU and UCB (Fig.1). The specific primer pair PCR gave positive results with *P. lateralis, P. ramorum* and field samples 779 and 785 (provided by OSU). The specific band generated using *P. ramorum* DNA was very faint at the 1:100 dilution (as seen in Fig. 1) and almost completely disappeared at 1:1000 dilutions (data not shown). The NS primer pair reacted with all 5 DNAs provided by OSU including field sample 778 (which had been previously reported to be negative for PLAT.)

The multiplex PCR worked well for both PCR products, but it worked best at 1:100 dilutions and decreased with dilutions of 1:1000 and higher. DNA provided by the UCB lab worked well in the multiplex PCR when tested with OSU primers. Positive PCR results using the PLAT specific pair were obtained only with *P. lateralis* and *P. ramorum*. NS1/2 universal primer pair PCR gave positive results for all 13 DNA samples. The efficiency of the multiplex PCR was not always good most likely due to the nature of the multiplex reaction, specifically template competition. No attempts have been made to optimize the reaction.

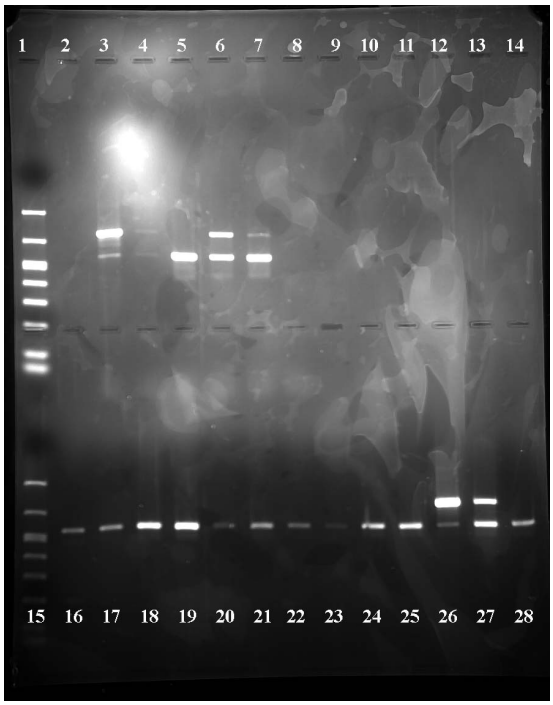


Figure 1. Multiplex PCR – OSU Protocol using pure culture DNAs provided by OSU and UCB.

1. Marker	15. Marker
2. Empty	16. *P. boehemeriae*
3. *P. lateralis*	17. *P. parasitica*
4. *P. ramorum*	18. *P. megasperma*
5. Field sample 778 (reported negative for *P. ramorum*)	19. *P. erythroseptica*
6. Field sample 779	20. *P. gonapodyides*
7. Field sample 785	21. *P. palmivora*
8. Water control	22. *P. cryptogea*
9. Empty	23. *P. cinamomi* mating type A1
10. Empty	24. *P. cinamomi* mating type A2
11. Empty	25. *P. cambivora*
12. Empty	26. *P. lateralis*
13. Empty	27. *P. ramorum*
14. Empty	28. *P.syringae*

DNAs number 3-5 were from OSU and used in 1:100 dilutions in 0.1X TE buffer
DNAs number 16-27 were from UCB and used in 1:500 dilutions in 0.1X TE buffer

Overall conclusion of the OSU (Hanson) PCR: Multiplex PCR works well with pure culture DNAs provided by OSU and UCB.

B. UCB (Garbelotto's) PCR PROTOCOL:
DNA provided by OSU lab worked very well in the Garbelotto's PCR giving expected results. *P. lateralis* did not react, altogether, with field sample 778 (reported to be negative by the providing lab). *P. ramorum* DNA and field samples 779 and 785 did amplified with very good efficiency (Fig. 2).

DNA of 13 *Phytophthora* species provided by the UCB lab was extensively tested in the OSU PCR protocol for cross-reactivity of the primers. DNA was initially used in the recommended dilutions of 1:5000. At this dilution positive results were seen for *P. lateralis* (a very faint band not visible in the picture below) and *P. ramorum* (Fig. 2). *Phytophthora cambivora* gave bands time to time usually associated with higher DNA concentrations. When PCR products were run on silver stained 5% polyacryl amide gels, more non-specific reactions could be seen. Silver stained PAGE analysis is more sensitive than agarose electrophoresis.

Overall conclusion of the UCB (Garbelotto) PCR: The nested PCR works well with pure culture DNAs provided by OSU and UCB.

End-point dilutions and cross reactivity of primers. Ten-fold serial dilutions were made to find out the end-point dilution at which the pathogen DNA can be detected. Dilutions were made from the pure DNA extracts provided from the UCB lab. An accurate DNA concentration could not be determined with this DNA. Using *P. ramorum* purified DNA a dilution of 1:50,000,000 was the endpoint (Fig. 3). Using *P. lateralis* purified DNA a dilution of 1: 5,000 was the endpoint. A very faint band could be seen *for P. cambivora* at 1:500 dilution. These are results are consistent with statements made by the UCB laboratory that at high DNA dilutions their PCR assay is very specific and no cross-reactivity occurs.

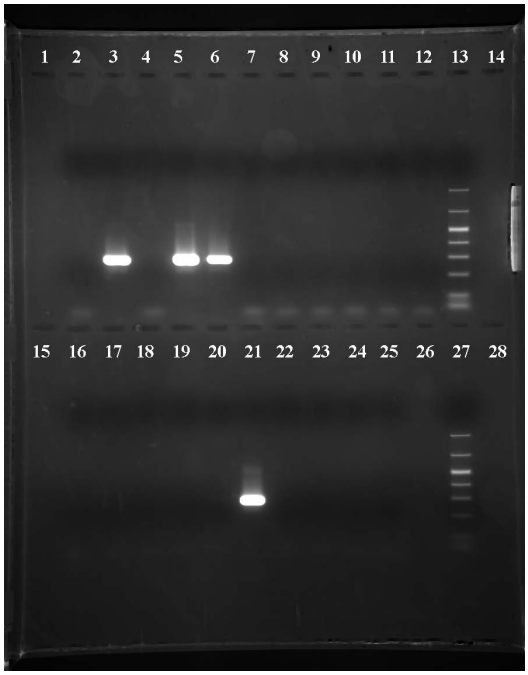


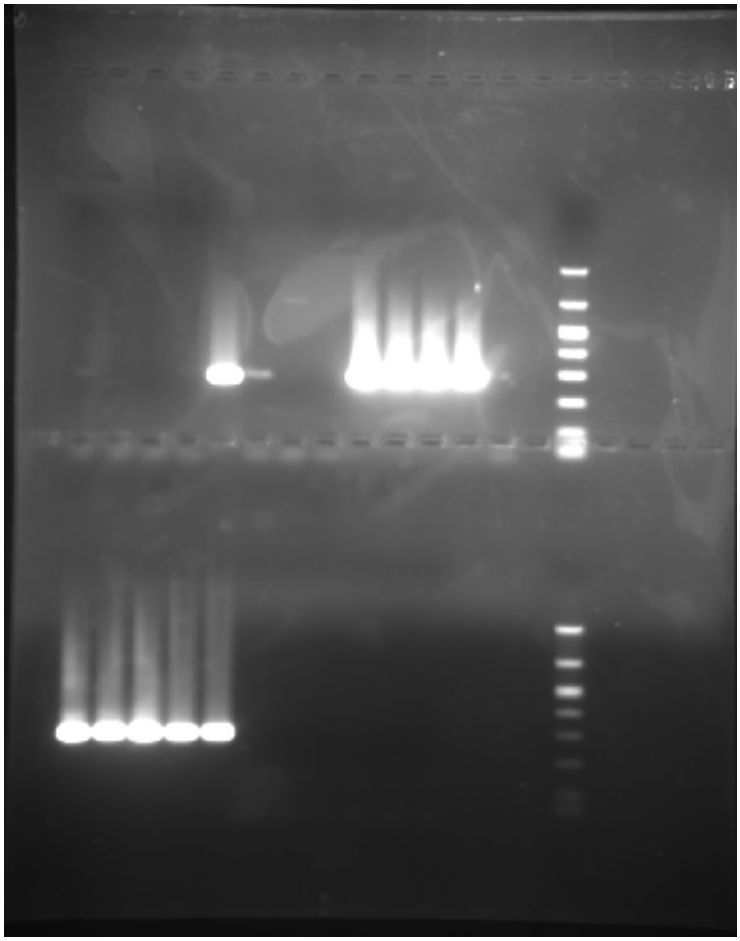
Figure 2. Nested PCR – UCB Protocol using pure culture DNAs provided by OSU and UCB. Second round of nested PCR shown.

1. Empty	15. Empty
2. *P. lateralis*	16. *P. cryptogea*
3. *P. ramorum*	17. *P. cinamomi* mating type A1
4. Field sample 778 (reported negative for *P. ramorum*	18. *P. cinamomi* mating type A2
5. Field sample 779	19. *P. cambivora*
6. Field sample 785	20. *P. lateralis*
7. *P. boehemeriae*	21. *P. ramorum*
8. *P. parasitica*	22. *P.syringae*
9. *P. megasperma*	23. water control first round
10. *P. erythroseptica*	24. 0.1X TE buffer
11. *P. gonapodyides*	25. water control second round
12. *P. palmivora*	26. Empty
13. Marker	27. Marker
14. Empty	28. Empty

DNAs number 2-6 were from OSU and used in 1:100 dilutions in 0.1X TE buffer and DNAs number 7 - 22 were from UCB and used in 1:500 dilutions in 0.1X TE buffer.

Fig. 3 Nested (UCB) PCR – second round PCR products of *P. ramorum*, *P. lateralis* and *P. cambivora* DNAs in dilution series – cross reactivity of the primers at lower dilutions.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

1.	*P. cambivora* 1:500	16.	*P. ramorum* 1:5,000
2.	*P. cambivora* 1:5,000	17.	*P. ramorum* 1:50,000
3.	*P. cambivora* 1:50,000	18.	*P. ramorum* 1:500,000
4.	*P. cambivora* 1:500,000	19.	*P. ramorum* 1:5,000,000
5.	*P. lateralis* 1:500	20.	*P. ramorum* 1:50,000,000
6.	*P. lateralis* 1:5,000	21.	*P. ramorum* 1:500,000,000
7.	*P. lateralis* 1:50,000	22.	*P. ramorum* 1:5,000,000,000
8.	*P. lateralis* 1:500,000	23.	*P. ramorum* 1:50,000,000,000
9.	DNA infected tissue-sample 1 1:100	24.	*P. ramorum* 1:500,000,000,000
10.	DNA infected tissue-sample 1 1:500	25.	0.1X TE
11.	DNA infected tissue-sample 2 1:100	26.	Water control second round
12.	DNA infected tissue-sample 2 1:500	27.	Empty
13.	Water control	28.	Empty
14.	Empty	29.	Empty
15.	Marker	30.	Marker

III. EVALUATION OF THE DNA EXTRACTION PROCEDURES IN COMBINATION WITH THE PCR TESTS DESCRIBED ABOVE:

We tested the OSU and UCB protocols for DNA extraction using *P. ramorum* infected and healthy mountain laurel leaf tissue from P. Tooley. The extraction protocols were tested in at least 2 experiments.

A. The OSU protocol is based on CTAB extraction with further use of DNeasy tissue kit Cat # 69504 from Qiagen. Plant tissue is ground in a mini bead beater mill using glass beads (5mm glass beads from Fisher). We froze the closed tubes containing plant tissue (discs) and 5mm glass beads in liquid nitrogen and then set them up for grinding at the recommended settings of 4200 rpm for 30 sec. At those settings the **tubes broke**! We determined that a speed set lower at 2500 rpm worked safely and the tissue appeared to have the same degree of homogenization.

The resulting DNAs were tested in both PCR protocols and they worked very well. Using the nested PCR approach of the UCB, *P. ramorum* was detected at 1:10,000 dilution and higher (Figs. 5 and 6). Using the multiplex PCR approach of OSU, DNA was detected at 1:1,000 and higher (Fig. 4).

B. The UCB protocol is more complicated. It calls for freeze-drying of plant material before homogenization. It's most likely a way to preserve the tissue for long storage and might be important for wood samples. Leaf tissue could also be store at -80°C. The tissue was ground in a bead-beater using chrome-steel ball bearings (3.2 mm from Biospec Products Inc, cat # 11079132c, 1-800-617-3363). The grinding was performed as described. The freeze-dried tissue was pulverized so well that it was actually embedded in the entire tube surface. CTAB buffer was used for the extraction followed by a lengthy procedure of freezing and thawing. GENECLEAN Turbo Nucleic Acid Purification Kit Cat # 1102-600 from Q-BioGene was used for further purification.

DNA purified using this method worked in both the OSU and UCB PCR protocols, but DNA could not be dilute out very far. Using the UCB nested PCR, DNA from this extraction had a dilution end-point of 1:5,000 (Fig. 6). Using the multiplex OSU PCR, the detection limits were even lower – 1:100 (Fig. 4). Interestingly, undiluted DNA did not always work most likely due to inhibition of the PCR by high DNA concentrations. Healthy tissue and the empty tube control did not generate any PCR bands (Fig. 5).

C. We used the DNeasy tissue kit Cat # 69504 from Qiagen alone for DNA extraction from infected leaves. The kit worked very well although it is not

specifically designed for plant DNA extraction. In the UCB nested PCR *P. ramorum* DNA could be detected at dilutions of 1:10,000 and beyond (Fig. 6).

D. We tested the Qiagen DNeasy Plant Mini Kit Cat # 69104 on *P. ramorum* infected bay laurel, healthy bay laurel, and healthy material of rhododendron, pieris and viburnum. DNAs extracted using this kit worked well using both PCR protocols (Fig. 7). DNA extracts of healthy rhododendron, pieris and viburnum did not inhibit PCR reaction when added to *P. ramorum* DNA. The DNeasy Plant Mini Kit is very user friendly and produces DNA of quality good without a phenol: chloroform extraction used in both the OSU and UCB extraction protocols.

E. We evaluated tissue batching using the OSU DNA extraction protocol on *P. ramorum* infected tissue (mountain laurel) mixed with healthy tissue. We were able to detect *P. ramorum* in 1:9 ratio of infected/healthy tissue using the UCB nested PCR.

We also evaluated tissue batching using the Qiagen DNeasy Plant Mini Kit DNA extraction protocol on *P. ramorum* infected bay laurel leaves with a mix of healthy tissue (bay laurel, rhododendron, viburnum and pieris). DNA extracts were then diluted 10-fold and UCB PCR run. Positive results were observed when samples were batched infected to healthy at ratios of **1:9**, **0.5:9.5**, and **0.5:4.5** (Fig. 7). We feel that batching up to 10 samples in one extraction tube won't compromise sensitivity of the PCR detection but without additional experimentation we would not recommend batching more than 10 samples. One of the problems with batching is efficiency of tissue grinding (maximum up to 100 mg of tissue should be used for Qiagen DNeasy Plant Mini Kit) and consequently extraction efficiency. Some tissue is harder, rhododendron for example. One critical step is repeating the freezing/ grinding procedure 3 times (see protocol provided) or using mortar and pestle. This additional grinding and freezing produces tissue of similar grind-size to non-batched samples.

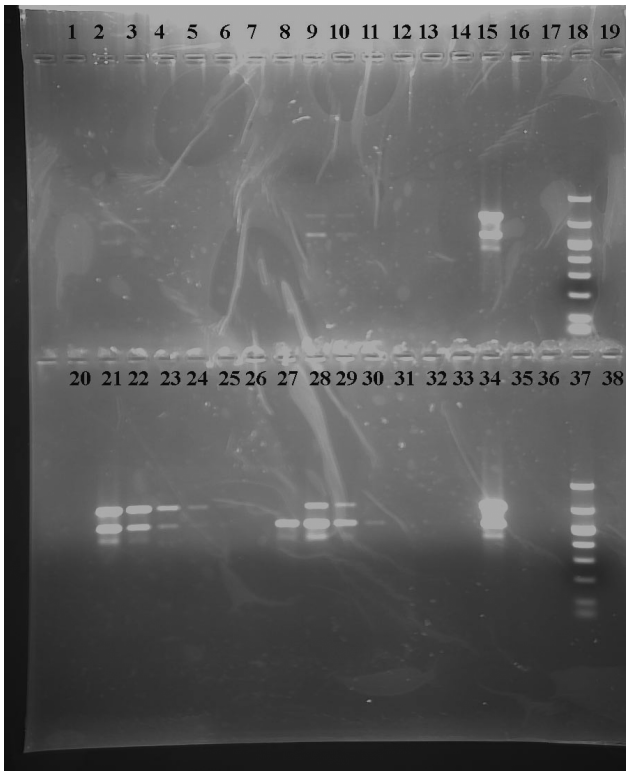
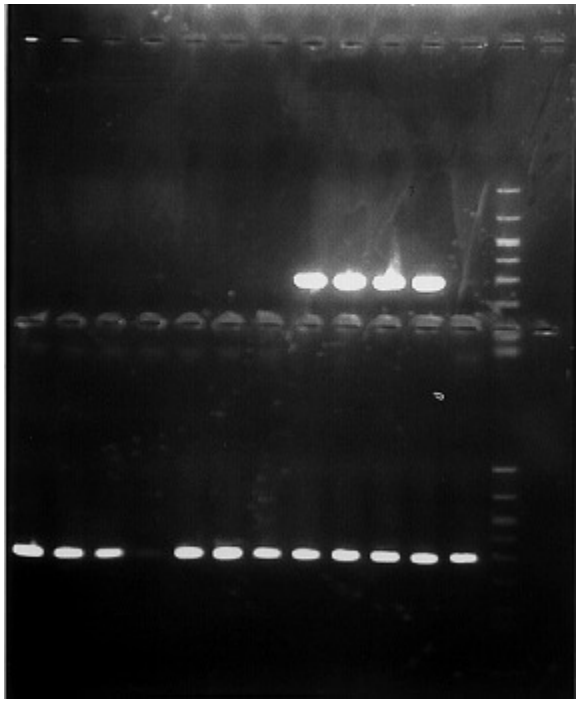


Figure 4. Multiplex PCR (OSU) protocol following DNA extraction of *P. ramorum* isolate MD 0-217 on mountain laurel using either the OSU or UCB extraction protocol (DNA diluted in 0.1X TE).

UPPER	**GEL**
UCB DNA extraction, homogenized 10s, 4200 rpm	UCB DNA extraction, homogenized 30s, 2400 rpm
1. Undiluted	8. Undiluted
2. 1:10	9. 1:10
3. 1:100	10. 1:100
4. 1:1,000	11. 1:1,000
5. 1:5,000	12. 1:5,000
6. 1:10,000	13. 1:10,000
7, empty well	14. Empty
	15. positive control
	16. 1:10 TE buffer
	17. empty
	18, Marker
	19. Empty
LOWER	**GEL**
OSU DNA Extraction Homogenized 30s, 2400 rpm Samples frozen -80°C	OSU DNA Extraction Homogenized 30s, 2400 rpm Samples frozen in LN_2
20. Undiluted	27. Undiluted
21. 1:10	28. 1:10
22. 1:100	29. 1:100
23. 1:1,000	30. 1:1,000
24. 1:5,000	31. 1:5,000
25. 1:10,000	32. 1:10,000
26. empty well	33. Empty
	34. positive control
	35. 1:10 TE buffer
	36. empty
	37. Marker

Figure 5. UCB nested PCR of serial dilutions of DNA from healthy and *P. ramorum*-infected mountain laurel using OSU and P. Tooley (lines 23 to 26) extraction protocols. Resulting DNA were diluted in 0.1X TE.

1 2 3 4 5 6 7 8 9 10 11 12 13 14

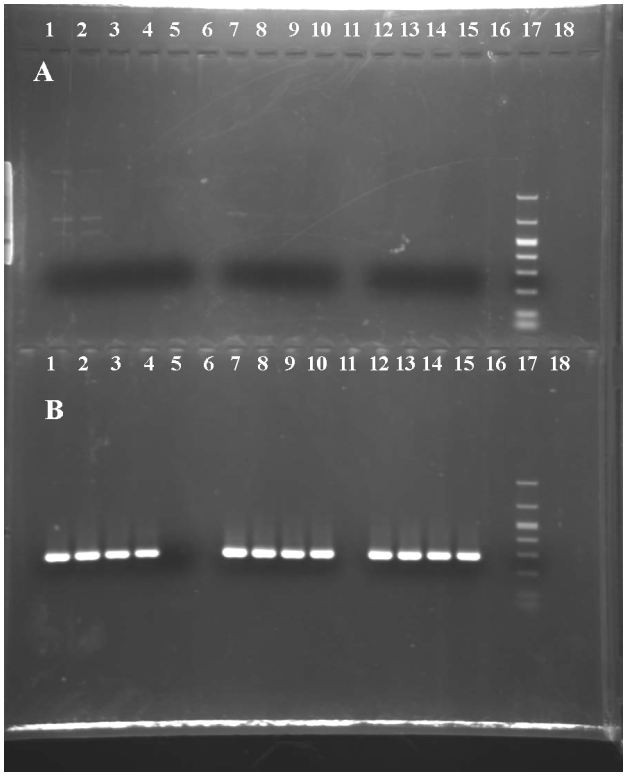


15 16 17 18 19 20 21 22 23 24 25 26 27 28

This gel is included to show the specificity of the assay for *P. ramorum* by a lack of amplification of DNA from healthy plant material.

1.	Water	15	0-217 infected 1:10 (second sample)
2	Empty tube 1:10 (OSU)	16	0-217 infected 1:100
3	Empty tube 1:100	17	0-217 infected 1:500
4	Healthy 1:10	18	0-217 infected 1:1000
5	Healthy 1:100	19	PR G2 infected 1:10
6	Healthy 1:500	20	PR G2 infected 1:100
7	Healthy 1:1000	21	PR G2 infected 1:500
8	0-217 infected 1:10	22	PR G2 infected 1:1000
9	0-217 infected 1:100	23	0-217 infected 1:10 (P. Tooley)
10	0-217 infected 1:500	24	0-217 infected 1:100
11	0-217 infected 1:1000	25	0-217 infected 1:500
12	0.1X TE	26	0-217 infected 1:1000
13	Marker	27	Marker
14	Empty well	28	Empty well

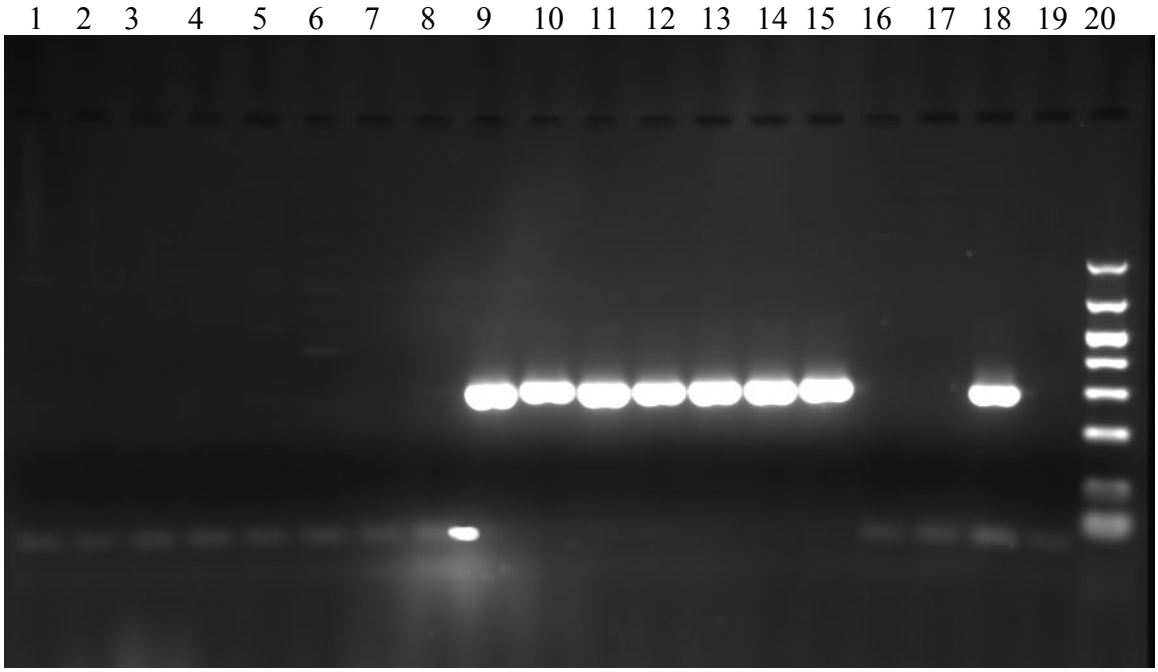
Figure 6. Nested (UCB) PCR protocol using a dilution series of DNA extracted using the UCB, OSU, and Qiagen DNeasy tissue extraction kit. DNA extracted from *P. ramorum* infected mountain laurel.



Bands in the first round (A) are faint, yet present. Bands are strong in the second round (B) as demonstrated in the lower gel. The OSU and Qiagen extraction procedures yield greater amounts of DNA as demonstrated by the level of dilution of the DNA. The OSU and Qiagen procedures have strong bands at a dilution of 1:50,000, whereas the UCB extraction was only diluted out to 1:5,000 having no detectable product at a dilution of 1:10,000 (lane 5).

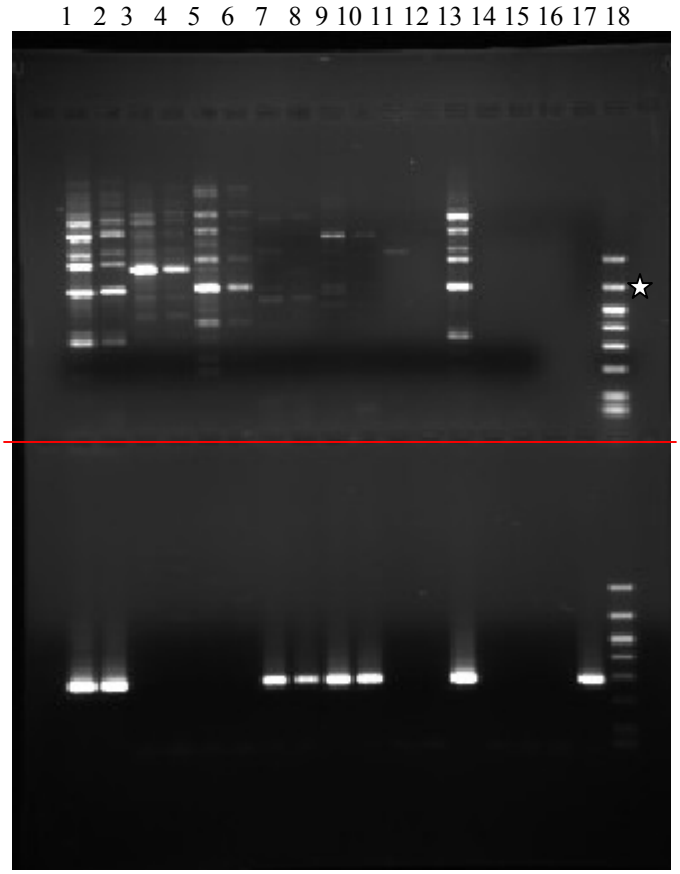
A. First round products of nested PCR	B. Second round products of nested PCR
1. UCB protocol-DNA 1:10 very faint	1. UCB protocol-DNA 1:10
2. UCB protocol-DNA 1:100 very faint	2. UCB protocol-DNA 1:100
3. UCB protocol-DNA 1:1,000	3. UCB protocol-DNA 1:1,000
4. UCB protocol-DNA 1:5,000	4. UCB protocol-DNA 1:5,000
5. UCB protocol-DNA 1:10,000	5. UCB protocol-DNA 1:10,000
6. Empty	6. Empty
7. OSU protocol-DNA 1:1,000 very faint	7. OSU protocol-DNA 1:1,000
8. OSU protocol-DNA 1:5,000 very faint	8. OSU protocol-DNA 1:5,000
9. OSU protocol-DNA 1:10,000	9. OSU protocol-DNA 1:10,000
10. OSU protocol-DNA 1:50,000	10. OSU protocol-DNA 1:50,000
11. Empty	11. Empty
12. Qiagen protocol-DNA 1:1,000	12. Qiagen protocol-DNA 1:1,000
13. Qiagen protocol-DNA 1:5,000	13. Qiagen protocol-DNA 1:5,000
14. Qiagen protocol-DNA 1:10,000	14. Qiagen protocol-DNA 1:10,000
15. Qiagen protocol-DNA 1:50,000	15. Qiagen protocol-DNA 1:50,000
16. Empty	16. Empty
17. Marker	17. Marker

Fig. 7. Electrophoresis in 1.5% agarose gel of second round nested PCR products (UCB protocol). DNA extracted from batched tissue using Qiagen DNeasy Plant Mini Kit.



1 to 8	DNA extracted from healthy Rhododendron leaves.
9.	DNA from 1 disc infected / 9 healthy (1:10 ratio), undiluted
10.	DNA from 1 disc infected / 9 healthy (1:10 ratio), diluted additionally 1:10 in 0.1X TE prior to PCR
11.	DNA from ½ disc infected / 9 ½ healthy (1:20 ratio), undiluted
12.	DNA from ½ disc infected / 9 ½ healthy (1:20 ratio), diluted additionally 1:10 in 0.1X TE prior to PCR
13.	DNA from ½ disc infected / 4 ½ healthy (1:10 ratio), undiluted
14.	DNA from ½ disc infected / 4 ½ healthy (1:10 ratio), diluted additionally 1:10 in 0.1X TE prior to PCR
15.	*P. ramorum* DNA positive control
16.	0.1X TE buffer used for DNA dilutions
17.	PCR water control
18.	*P. ramorum* DNA positive control for the second round
19.	PCR water control (water used for 1:500 dilutions of the first round PCR product)
20.	Bio Venture Low Marker

Fig. 8. Electrophoresis of first and second round PCR products of the UCB nested PCR protocol in 1.5% agarose in TAE buffer.



PCR products after first (upper gel) and second (lower gel) round of nested PCR protocol (UCB). Pure culture DNAs and total plant DNAs extracted with OSU protocol were used. The bright band in row 13 in the upper gel corresponds to the 700 bp marker band in lane 18. This band is generated with *P. ramorum* specific primer pair (Phyto 1 and 4). The universal primer pair (Phyto 2 and 3) generate a single band of 291 bp seen in the lower gel. (10ul of each product was loaded on the gel)

1.	P. ramorum DNA 1:5,000	10.	P. ramorum 217 infected plant, 1:500
2.	P. ramorum DNA 1:50,000	11.	Healthy mountain laurel 1:100
3.	P. lateralis DNA 1:5,000	12.	Healthy mountain laurel 1:500
4.	P. lateralis DNA 1:50,000	13.	P. ramorum DNA PCR control
5.	P. cambivora DNA 1:5,000	14.	0.1X TE buffer
6.	P. cambivora DNA 1:50,000	15.	PCR water control
7.	P. ramorum PR-G2 infected plant, 1:100	16.	PCR water control, second round
8.	P. ramorum PR-G2 infected plant, 1:500	17.	PCR positive control, second round
9.	P. ramorum 217 infected plant, 1:100	18.	Marker

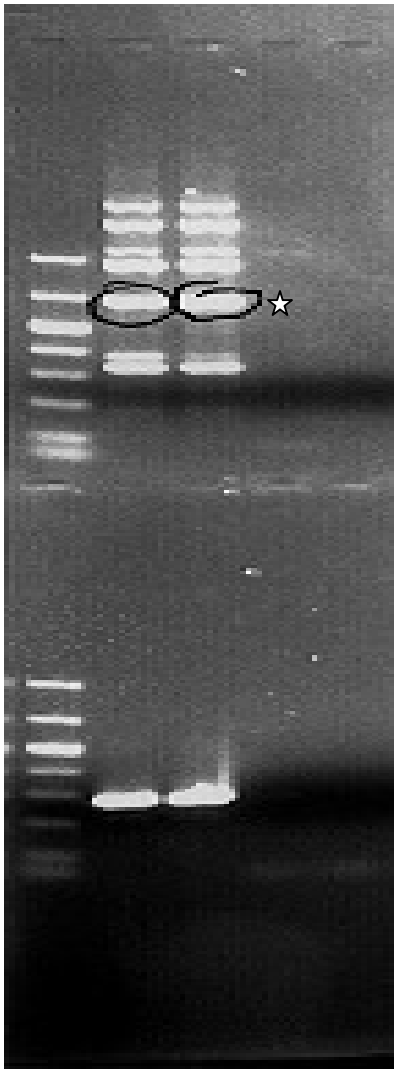


Figure 9. First round nested PCR bands are clearly visible among the additional bands when DNA is extracted from pure cultures of *P. ramorum*. Several visible bands are produced in the first round, however, the second round produces a single, strong, and clear single band for diagnostics.

Figures 8 and 9 demonstrate that there could be a problem utilizing solely the first round of the nested PCR as a single diagnostic. Bands are visible clearly using DNAs generated from mycelium from cultures. In Fig. 8, lanes 7 to 10, a few faint bands are observed with DNA from *P. ramorum* infected mountain laurel after the first round of PCR (upper gel). Additional faint bands of different sizes can be seen in healthy tissue as well which could make correct diagnosis difficult.

IV. SURVEY RECOMMENDATIONS:

Based on the work done in Beltsville by USDA APHIS PPQ CPHST the following recommendations are being given based on our experience with DNA extraction and PCR protocols tested.

- DNA can be extracted either using the OSU DNA extraction protocol or the Qiagen DNeasy Plant Mini kit. PCR amplification was comparable using both protocols. One reason we would recommend the kit over the OSU protocol is because it is easier to use and all reagents and kit components are standardized and quality assured. We also note that the kit produces higher yields of DNA and it appears to be of better quality. We make this recommendation for culture and leaf material only.
- The PCR for use in the survey should be the nested PCR test developed at UC Berkeley. This test in combination with the DNeasy kit or the OSU, CTAB-based extraction gave excellent sensitivity demonstrated in figure 6 where samples could be diluted out 1:50,000. We have modified the OSU protocol slightly and the modified protocol is included in this document.
- The nested PCR test should be run. This utilizes both reaction 1 (the specific PCR) and reaction 2 (the universal PCR). We found that some infected mountain laurel gave weak bands in the first round of PCR which could potentially lead to false negatives in some samples if only the first round is used in the survey. Figures 8 and 9 demonstrate the difference in the PCR profiles for reaction one and reaction two when using DNA extracted from fungal culture versus extraction from infected plant material.
- From limited experiments we have determined that 10 samples can be batched for DNA extraction and subsequent PCR.
- Any sample reacting positive in the PCR test will have to be confirmed by USDA APHIS PPQ NIS and the CPHST lab in Beltsville, MD. Any such sample is considered a suspect positive until confirmation. All suspect positives must be reported to Jonathan Jones of APHIS PPQ ISPM in Riverdale, MD, and the USDA APHIS PPQ State Plant Health Director in your state. USDA APHIS will arrange for the transport of suspect positives to the lab in Beltsville, MD. Jonathan Jones can be contacted at 301-734-5038.

V. METHODS AND MATERIALS NEEDED:

NOTE: In order to avoid cross contamination please designate separate rooms or lab areas for each segment of the work and use separate sets of pipettes. Use aerosol-resistant tips. Centrifuge any DNA-containing tubes before opening so that any liquid near the rim of the tube is removed. It's 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 hand towel. Wear gloves and change them regularly and between different procedures. Use disposable lab mats to cover bench areas and change them often. Never autoclave any *P. ramorum* –contaminated plant material, culture plates, or soil in an autoclave used to sterilize buffers, glassware, or plasticware used in the SOD PCR because of potential contamination from aerosols within the autoclave.

MODIFIED OSU DNA EXTRACTION PROTOCOL:

We tested this protocol only with mountain laurel infected and healthy leaf tissue. All stock solutions should be prepared in advance. When process many samples aliquot the necessary volume of the solution for the number of the samples plus 2-3 more in a new tube. Don't take from the stock solution to prevent contamination. The extraction should be conducted in a room or an area separate from location used to set up mastermix and PCR reactions.

1. Make 2% CTAB (cetiltrimethylammonium bromide) buffer, 100mM Tris, pH 8.0; 20mM Na2EDTA pH 8.0; 1.4M NaCl; 1% polyvinylpirolidone, 0.1% 2-mercaptoetanol [ME]). We make the buffer without 2-ME. Before using it, take the necessary volume for the number of samples (600µl per sample) and add 2-mercaptoethnol to 0.1%.
2. Pre-warm CTAB buffer to 65°C.
3. Use cork borer No 3 (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 several flamings cork borer won't be sufficiently sharp anymore 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 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 30sec at 2,500rpm (setting 3 for time and 25 for speed). If you batch several (up to 10) samples you 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°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 microcentifuge for 10min at 13,000rpm.
10. Carefully take the supernatant (approx. 450 µl) and transfer it into a new Eppendorf tube. Add 450 µl chloroform: isoamyl alcohol (24:1) and vortex for 1 min.
11. Spin again as at step 9.
12. Carefully take the supernatant and transfer it into a new Eppendorf 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 1min 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 1min / 8,000rpm 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 3min 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 Eppendorf 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.

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

(modified for batched samples)

1. Pre-warm AE buffer to 65ºC in water-bath or thermostat.
2. Use cork borer No 3 (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 several rounds of flaming cork borer won't be sufficiently sharp anymore so you may want to use disposable blades and cut out a square of plant tissue with sides of 0.5 cm long. Max weight of tissue to be used for DNA extraction with the kit is 100mg.
3. Place a glass bead (5mm diameter) in a 2 ml screw-cap tube then add 10 pieces of tissue (could be 10 different samples) 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.
4. Add 600µl of buffer AP1 and 6µl of RNase A to each tube, mix well by inverting and incubate at 65ºC for 15 min. Mix tubes time to time by inverting.
5. Add 195µl of buffer AP2 to each sample, mix well by inverting and incubate on ice for 5 min.
6. Centrifuge samples on micro centrifuge for 5 min at full speed (usually 10,000 rpm).

7. Transfer supernatants to QIAshredder spin columns (lilac colored tubes) placed in a 2ml collection tubes and centrifuge for 2 min at max speed.
8. Transfer 450µl of the flow-through fraction (try not to disturb pellets formed on the bottom of the tubes) to a new 1.5 ml tube and add 1.5 volumes of AP3/E buffer (675µl) and mix well by pipetting. If you recover less or more than 450µl calculate how much AP3/E buffer you need to add.
9. Follow protocol provided with the kit.
10. To elute DNA use 100µl of pre-warmed AE buffer. Don't do second elution.
11. Label tubes and keep extracted DNA at -20ºC.
12. For PCR dilute DNA 1:10 in sterile 0.1X TE buffer pH 8.0.

MODIFIED UCB DNA AMPLIFICATION (PCR) PROCEDURE:

All reagents should be kept at -20°C. Before PCR take them out to thaw, vortex briefly and spin down shortly to collect the content on the bottom. 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	
5µM Phyto 1/4primer mix	2.50	µl	(0.5µM final concentration)
Platinum Taq polymerase (5u/µl)	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:	94°C / 1min 25 sec
1 cylce	
Amplification:	93°C / 35 sec
34 cycles	62°C / 55 sec
	72°C/ 50 sec
Extension:	72°C/ 10 min
1 cycle	
Hold:	4°C

Ramp rate: 3.3°C/ sec heating and 2.0°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 H2O 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	
5µM Phyto 2/3 primer mix	2.50 µl	(0.5µM final concentration)
Platinum Taq polymerase (5u/µl)	0.25 µl	
dH_2O	12.00 µl	
Total 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:	94°C / 1min 25 sec
1 cylce	
Amplification:	93°C / 35 sec
34 cycles	62°C / 55 sec
	72°C/ 50 sec
Extension:	72°C/ 10 min
1 cycle	
Hold:	4°C

Ramp rate: 3.3°C/ sec heating and 2.0°C/ sec cooling (if possible).

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

VI. EQUIPMENT AND REAGENTS NECESSARY FOR SOD DETECTION:

UBC (M. Garbelotto's) nested PCR protocol primers and reagents

A. PCR machine - we use T-gradient from Biometra (others could be used).

B. Primers:

Specific *P. ramorum* primer pair:

- Phyto 1: 5'-CAT GGC GAG CGC TTG A-3'
- Phyto 4: 5'-GAA GCC GCC AAC ACA AG-3'

Phythopthora universal primer pair:

- Phyto2: 5'-AAA GCC AAG CCC TGC AC-3'
- Phyto3: 5'-GGT GGA TGG GGA CGT G- 3'

We ordered primers desalted from Invitrogen and prepare primer mixes the following way:

- Tubes with lyophilized primers were spun briefly before opening the tubes. Primers were rehydrated to 100 µM concentration in autoclaved 0.1 X TE buffer pH 8.0 and stored at - 20°C.
- 50 µM stocks of primer mixes of the specific pair (Phyto 1 and 4) and the control pair (Phyto 2 and 3) were made.
- 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

1. Enzyme– Platinum Taq Polymerase from Invitrogen (cat. No 10966-034). Supplied as a kit with 10X PCR Buffer and 50mM $MgCl_2$.
2. 10mM **dNTP Mix** from Sigma (D-7295).
3. **Thin-wall 0.2ml PCR** tubes from Cole-Parmer (No. 67103-90).
4. Molecular grade d H_2O from Geno Technology Inc. (1-800-628-7730).
5. Sterile 0.1X TE buffer pH 8.0
6. Sterile distilled water.

OSU DNA extraction protocol

1. 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).

2. Phenol: chloroform: isoamyl alcohol (25:24:1) from Invitrogen Cat # 15593-031.

3. 200 Proof (100%) Ethanol.

4. DNeasy Tissue Kit from Qiagen Cat. # 69504.

5. Screw-cap plastic tubes – 2 ml volume.

6. Glass beads 5mm diameter from Fisher Scientific cat. # 11-312C.

7. Mini Bead-beater (Cole Parmer, Cat# A-36270-02, ~$800.00, 1800-323-4340)

Qiagen DNeasy Plant extracaction protocol

1. DNeasy Plant Mini Kit from Qiagen Cat # 69104.

2. Glass beads 5mm diameter from Fisher Scientific cat. # 11-312C.

VENDORS:
Cole Parmer (800-323-4340)
Qiagen (800-426-8157)
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)
LabRepco (800-521-0754)