



# **Rapid Diagnostic Tools for *Phytophthora* on Horticultural Crops**



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eastern and central africa

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# Rapid Diagnostic Tools for *Phytophthora* on Horticultural Crops

May 9 – 13, 2016

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# Rapid Diagnostic Tools for *Phytophthora* on Horticultural Crops

## Workshop Agenda

### May 9 - 13, 2016

#### Monday – May 9<sup>th</sup>

<b>3.30PM – 4.00PM</b>	<b>Theme Lecture</b>	The history and importance of <i>Phytophthora</i>
<b>4.00PM – 5.00PM</b>	<b>Lecture 1</b>	Isolation and morphological identification

#### Tuesday – May 10<sup>th</sup>

<b>9.00AM – 9.30AM</b>	<b>Lecture 2</b>	Lucid keys for identification of unknowns
<b>9.30AM – 10.30AM</b>	<b>Lab 1</b>	Identifying unknown species by morphology
<b>10.30AM – 10.45AM</b>	<b>Tea/coffee break</b>	
<b>10.45AM – 11.45PM</b>	<b>Lab 2</b>	DNA extractions using NaOH – Fresh and dried material ( <b>p. 27</b> ) ; simple quick PCR ( <b>p. 28 – 29</b> )
<b>11.45AM – 1.00PM</b>	<b>Computer Lab</b>	Using a Lucid key to identify unknowns
<b>4:30PM – 5.00PM</b>	<b>Review/quiz</b>	

#### Wednesday – May 11<sup>th</sup>

<b>11.45AM – 1.00PM</b>	<b>Lab 3</b>	Gel electrophoresis of quick PCR (Lab 2) ( <b>p. 28 – 29</b> )
<b>1.00PM – 2.00PM</b>	<b>Lunch</b>	
<b>2.00PM – 3.00PM</b>	<b>Lecture 4</b>	Molecular identification – PCR, sequence based methods, rapid diagnostics
<b>3.00PM – 3.30PM</b>	<b>Lab 4</b>	Set up PCR-RFLP ( <b>p. 33 – 36</b> )
<b>3.30PM – 4.30PM</b>	<b>Lab 5</b>	Set up LAMP reaction ( <b>p. 45</b> )
<b>4.30PM – 5.00PM</b>	<b>Review/quiz</b>	

#### Thursday – May 12<sup>th</sup>

<b>9.00AM – 10.30AM</b>	<b>Lab 6</b>	Set up digest of PCR-RFLP product (Lab 4) ( <b>p. 33 – 36</b> ); Gel electrophoresis of LAMP reaction (Lab 5) ( <b>p. 45</b> )
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<b>2.00PM – 4.30PM</b>	<b>Lab 7</b>	Gel electrophoresis of restriction digest (Lab 6) <b>(p. 33 – 36)</b>
<b>4.30PM – 5.00PM</b>	<b>Review/quiz</b>	
<b>Friday – May 13<sup>th</sup></b>		
<b>9.30AM – 10.00AM</b>	<b>Lab 8</b>	LAMP repeat
<b>12.00PM – 1.00PM</b>	<b>Lab 9</b>	LAMP analysis and electrophoresis
<b>1.00PM – 2.00PM</b>	<b>Lunch</b>	
<b>2.00PM – 4.30PM</b>	<b>Lecture 5</b>	Data analysis and interpretation
<b>4.30PM – 5.00PM</b>	<b>Review/quiz</b>	

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Protocol derived from this reference:

National Plant Diagnostic Network (NPDN) News 4: Vol. 2 Issue 3. Gail Ruhl, Plant and Pest Diagnostic Laboratory, Purdue University.

## **Float incubation technique for *Phytophthora* and *Pythium* diagnostics**

This float incubation technique is an excellent way to induce the production of sporangia as well as mycelial growth from herbaceous tissue for diagnostic purposes. A 1% unsterilized soil extract solution works well for stimulating sporangia production from *Phytophthora*- infected tissue. This float technique may also be used to stimulate sporangia production from mycelium growing on agar plugs.

1. Prepare a 1% unsterilized soil extract by swirling 10 g soil and 1 L distilled water together in a 2 L flask.
2. Slowly pour extract solution through filter paper lined funnel into media storage bottle and store in refrigerator until needed. Wash funnel and replace filter paper for each bottle.
3. Pour enough refrigerated soil extract solution into Petri dish containing herbaceous roots, stems, and/or leaves to just cover the plant material.
4. Cover Petri dish and incubate on benchtop for 24 hrs.
5. Examine herbaceous material with a compound microscope while it is still floating in the soil extract solution. Look for sexual (oogonia with antheridia or double walled oospores) or asexual (sporangia; *Pythium* globose and *Phytophthora* lemon shaped) reproductive structures.

Note: Do not confuse protozoans which thrive on herbaceous material floating in unsterilized soil extract solution with sporangia or zoospores.

Protocol from the lab of Jason H. Brock and Glenn H Beard, University of Georgia, 2002. Reference: Miscellaneous Publication 104, The Cooperative Extension Service, University of Georgia College of Agricultural and Environmental Science.

## **A Simplified Technique for recovering *Pythium* and *Phytophthora* from infected plant tissue**

### **Materials needed:**

Petri Dish  
Scalpel or knife  
Sterile water  
Acid Fuschin stain (preferred over water)  
Compound microscope

### **Procedure:**

1. Wash the plant tissue under a gentle stream of tap water.
2. Slice multiple sections of tissue from the plant. Select tissue from the border of diseased and healthy areas. To prevent contamination, use a blade that has been flamed over a burner.
3. Place the sectioned tissue in the Petri dish containing sterile water and cover.
4. Leave the dish undisturbed for 24 hr. *Pythium* and *Phytophthora* spp. should begin producing sporangia between 24 and 48 hr after being placed in water.
5. Remove one section, including mycelial growth, from the dish. Use the scalpel or blade to macerate the tissue in the acid fuschin stain.
6. Use the compound microscope to find sporangia, oogonia or oospores. If none are preset, allow the tissue samples to continue floating in water.

Note: Lack of mycelial growth or reproductive structures does not always rule out the presence of *Pythium* and *Phytophthora*. Growth of pathogens can be influenced by temperature and light. Further evaluation may be needed.

### **Identification of *Pythium* and *Phytophthora*:**

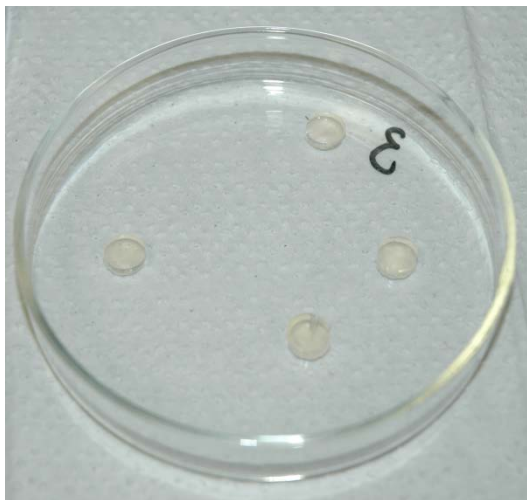
A shared morphological characteristic of *Pythium* and *Phytophthora* spp. is coenocytic hyphae (lacking cross walls). Most true fungi produce hyphae with septa, although some true fungi also lack septa. The presence of coenocytic hyphae provides evidence for *Pythium* and *Phytophthora* but, in itself, is not conclusive. In order to make a conclusive identification, reproductive structures must be observed. *Pythium* and *Phytophthora* have similar sexual reproductive structures. Asexual reproductive structures are sporangia, and allow for an easier differentiation between *Pythium* and *Phytophthora*. Sporangia of *Pythium* are globose to oval or may have an irregular shape, while sporangia of *Phytophthora* are typically lemon-shaped. These are general characteristics that can be used to identify *Pythium* and *Phytophthora*; however, diversity within each genus can make identification much more complicated.



## **Stimulating sporangia formation of *Phytophthora* in vitro**

1. Using a #6 or large cork borer (or larger), transfer multiple plugs of the suspected *Phytophthora* isolate into an empty Petri dish. It is best to use cultures less than 7 days old; isolates growing on corn meal agar work best. (Cultures on PARP have worked okay sometimes, but many *Phytophthora* species do not sporulate well on media containing antibiotics).
2. Flood the Petri dish with 2% soil extract solution or dilute V8, until the solution covers the surface of the agar plugs.
3. Label Petri dish and incubate at room temperature.
4. Using a dissecting scope, continually check agar discs 48 – 96 hours after flooding to look for mycelial growth and sporangia production.
5. Once sporangia are detected, the agar plugs can be magnified using an inverted scope, or removed from the flooded Petri dish and smashed on a slide with lacto-phenol blue to observe sporangial morphology at higher magnification.

Sporangia of *Pythium* species tend to be globose to oval or have an irregular shape, while sporangia of *Phytophthora* species are typically lemon-shaped.



## **Purification of *Phytophthora* cultures contaminated with bacteria**

This protocol can be used to purify *Phytophthora* cultures that are contaminated with bacteria.

1. Choose a plate of media containing antibiotics that was poured thinly (< 1 cm thick). PARP(H) is a good choice, but anything with antibiotics should work.
2. Cut the plate into quarters with a sterile scalpel and set aside.
3. Using a sterile scalpel, remove a thin layer of media from the growing hyphal edge of the contaminated culture. The circumference of the sliver should be about that of a pencil eraser.
4. Place this sliver in the center of an empty, sterile Petri dish.
5. Using a sterile spatula, place a quarter-piece of the antibiotic media on top of the sliver (Fig. 3).
6. Allow 2-4 days for the *Phytophthora* mycelia to grow up through the media. The bacteria should not be able to move vertically through the media.
7. Using a sterile scalpel, remove a thin layer of media containing uncontaminated hyphae, being careful not to penetrate too deeply through the media.
8. Transfer this wedge onto nutrient agar to make sure that the decontamination was successful.

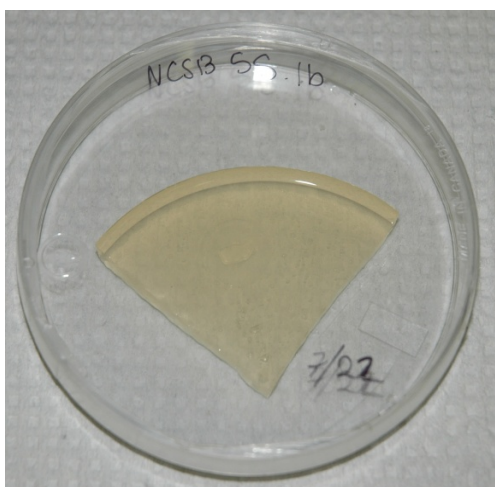


Fig. 1. A quarter-piece of PARP is placed on top of a piece of contaminated *Phytophthora* culture.

## **Isolation and detection of *Phytophthora* using *Rhododendron* leaf baits**

This protocol is used to detect and/or isolate *Phytophthora* from infested soil or tissue samples.

### **PART 1: Leaf Bait Preparation:**

1. Collect unblemished leaves from native *Rhododendron*.
2. Rinse in 10% Clorox for 1 min.
3. Triple rinse with distilled water.
4. Blot dry and place in plastic bag.
5. Store at 4 °C (refrigerator) overnight.

### **PART 2: Leaf Baiting and Isolation:**

1. Set incubator temperature at 12 °C both day and night. Turn lights OFF.
2. Place soil or plant tissue to be baited in large gallon ZIPLOC ® bag.
3. Float two rhododendron leaves per soil sample, one bottom side up and the other bottom side down.
4. Incubate baited leaves at 12 °C in total darkness for 4 days.
5. Remove leaves from sample, blot and gently wipe soil from leaf surfaces with paper towel. Infected leaves will have water soaked tissue (Fig. 4), however the water soaking can dry out if culturing is delayed and prime isolation sites can disappear.
6. Plate water-soaked sections of each rhododendron leaf onto PARP or PARPH by completely submerging leaf sections in agar.
7. Check plates for hyphal growth starting at 3-4 days, up until 14 days.



Fig. 2. Water-soaked veins of *Rhododendron* leaf.

# Methods of isolation of *Phytophthora* species

## A. Introduction to methods of culture.

Some species of *Phytophthora* such as *P. capsici* are readily cultured via simple surface disinfestation and plating on a selective media. Other species, such as *P. infestans* from tomato, are more recalcitrant and require passage through a living clean tomato leaf before isolation of sporangia onto antibiotic amended agar. All species can be simply stored on lima bean or corn meal agar disks in sterile water with sterile hemp seed for long term storage. More elaborate and expensive cryostorage in liquid nitrogen can also be done (Tooley, 1988).

The following methods and media are provided to assist in the isolation of a *Phytophthora* species in culture and are not meant to be an inclusive list. Further methods can be found in Erwin and Ribeiro (1996), Gallegly and Hong (2008) and Ribeiro (1978). Contamination by bacteria is common in cultures and should be eliminated before production of asexual and sexual structures for identification. Plating isolates on antibiotic amended media is useful and then transfer to nonamended V-8 or lima bean agar should be done to confirm that bacteria have been eliminated from cultures. Once a clean isolate is obtained, production of asexual sporangia, chlamydospores and sexual structures are needed in order to proceed with morphological identification. See methodologies recommended in Erwin and Ribeiro (1996), Gallegly and Hong (2008) and described briefly below for production of structures for morphological identification.

## B. Isolation.

Most *Phytophthora* species can be isolated from small pieces of infected plant tissue after surface disinfestation in 0.05% sodium hypochlorite for 3-5 minutes, followed by rinsing in sterile distilled water and blotting on sterile paper towels. *Phytophthora* species can also be baited from soil or water with rhododendron leaf disks (Erwin and Ribiero, 1996; Larkin et al, 1995). Small pieces of surface disinfested plant tissue are plated on antibiotic amended media such as TPT, PAR(PH) or P<sub>10</sub>VP media. *Phytophthora* species are Oomycetes and have coenocytic hyphae. First, mycelium growing from tissue pieces should be observed for coenocytic hyphae. Then proceed to observe morphological characters of asexual and asexual structures.

### 1. Isolation Media.

#### a. Triple P media (TPT)

Add amendments to one liter of corn meal agar (17g/L) after autoclaved and cool.

	<u>PPM</u>	<u>Stock</u>	<u>Use</u>
Penicillin G - sodium salt*	50 mg/L	50 mg/ml	1.0 ml
Polymyxin B Sulfate*	50 mg/L	50 mg/ml	1.0 ml
Pimaricin (50ai)*(store in dark)	30 mg/L	20 mg/ml	3.0 ml

\*Add amendments separately in a laminar flow hood while stirring to media after sterile and cooled to 50 °C. Fungicide and antibiotic stock solutions should be made in sterile bottles with sterile distilled water and stored at 5 °C or frozen in sterile plastic vials in aliquots for 1 liter. Pimaricin is sold under the trade name Delvocid (50ai) from Nelson-Jameson, Inc, Marshfield, WI.

### **b. PAR(PH) Media for *Phytophthora***

Add to one liter of corn meal agar (17g/L) or V-8 Agar (see below).

	<u>PPM</u>	<u>Stock</u>	<u>Use</u>
Pimaricin (50ai)*	10 mg/L	20 mg/ml	1.0 ml
Ampicillin*	250 mg/L	25 mg/ml	10.0 ml
Rifampicin*	10 mg/L	10 mg/ml	1.0 ml

#### Optional

PCNB (96ai)*	100 mg/L	5.2 mg/ml	20 ml
Hymexazol (99.5ai)**	50 mg/L	25 mg/ml	2.0 ml

\*Add amendments separately in a laminar flow hood while stirring to media after sterile and cooled to 50 °C. Dissolve ampicillin and rifampicin in 70% ethanol and PCNB in 95% ethanol. All fungicides and antibiotic stock solutions should be prepared aseptically and stored at 5 °C or frozen in sterile plastic vials in aliquots for 1 liter. \*\*Hymexazol is added to suppress *Pythium* species. PCNB is used to suppress soil fungi and is useful for soil dilution plating. Can use Terraclor (PCNB 75%WP) as an alternative to the active ingredient.

### **c. Modified P<sub>10</sub>VP Corn Meal Agar**

Add to one liter of corn meal agar (17g/L)

	<u>PPM</u>	<u>Stock</u>	<u>Use</u>
Pimaricin*	10 mg/L	20 mg/ml	1.0 ml
Vancomycin*	200 mg/L	50 mg/ml	4.0 ml
PCNB(96ai)*	100 mg/L	5.2 mg/ml	20 ml

#### Optional

Hymexazol (99.5ai)**	25 mg/L	25.1 mg/ml	1.0 ml
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\*Add amendments separately in a laminar flow hood while stirring to media after sterile and cooled to 50 °C. Dissolve PCNB in 95% ethanol. All fungicides and antibiotics should be prepared aseptically and stored at 5 °C or frozen in sterile plastic vials in aliquots for 1 liter. \*\*Hymexazol is added to suppress *Pythium* species.

### **d. Goodwins Media (for isolation of *Phytophthora infestans* from plant tissue)**



After preparing 10% V-8 Juice Agar add

<u>Antibiotics:</u>	<u>PPM</u>	<u>Stock</u>	<u>Use</u>
Rifamycin	20 mg/L	10 mg/ml	2.0 ml
Polymixin B Sulphate	50 mg/L	50 mg/ml	1.0 ml
Ampicillin*	200 mg/L	25 mg/ml	8.0 ml

<u>Fungicides:</u>			
PCNB 75 WP	50 mg/L	67 mg/ml	1.0 ml
Benomyl 50 WP	100 mg/L	10 mg/ml	2.0 ml

\*Dissolve ampicillin in 70% ethanol. Add amendments separately in a laminar flow hood while stirring to media after sterile and cooled to 50 °C. All fungicides and antibiotics should be prepared aseptically and stored at 5 °C or frozen in sterile plastic vials in aliquots for 1 liter. Surface sterilize tissue in 70% Ethanol (15s) then 10% Clorox (2-5 min). Rinse in distilled water. Plate sporangia from lesions on media and incubate plates at 18 °C in the light.

## 2. Soil Dilution Plating – for isolation of soilborne species

Soil dilution plating can be performed to isolate some species with a soilborne phase (ie, *P. capsici*, *P. nicotianae*, *P. cinnamomi*, *P. ramorum*). Forty grams of soil is added to 160 ml 0.25% sterile water agar, stirred for 5 min, and 1 ml aliquots are plated onto each of 5 plates of Masago's selective medium. At higher inoculum levels, additional 1:5 serial dilutions are needed. Plates are incubated in the dark at 24 °C for 72 hr, rinsed under running water to remove soil residue, and colonies are counted. Gravimetric soil water content (g water /g dry soil) of the soil samples is determined at the time of soil dilution and used to calculate inoculum density per g dry soil.

**Masago's** (*Phytophthora* isolation from soil) (Masago et al, 1977).

Potato Dextrose Agar 39g/L

	<u>PPM</u>	<u>Stock</u>	<u>Use</u>
PCNB (96ai)*	25 mg/L	5.2 mg/ml	5.0 ml
Benomyl (50 wp)	10 mg/L	10 mg/ml	2.0 ml
Ampicillin (100ai)*	500 mg/L	25 mg/ml	20.0 ml
Rifampicin (100ai)*	10 mg/L	10 mg/ml	1.0 ml
Nystatin (100ai)	25 mg/L	25 mg/ml	1.0 ml
Hymexazol (99.5ai)	25 mg/L	25.1 mg/ml	1.0 ml

\*Dissolve ampicillin and rifampicin in 70% ethanol and PCNB in 95% ethanol. Add separately to media after sterile and cooled to 50 °C in a laminar flow hood while stirring. All fungicides and antibiotics should be prepared aseptically and

stored at 5 °C or frozen in sterile plastic vials in aliquots for 1 liter. Hymexazol will inhibit most *Pythium* species.

**3. Growth media.** Most species of *Phytophthora* will grow on lima bean agar or V-8 juice agar.

**a. Fresh Lima Bean Agar.** A good general media for most species of *Phytophthora*.

Boil 200g lima beans for 30 minutes in 1 liter of water. Strain through cheesecloth and bring up to 1 liter. Add 1g glucose and agar (17g/L). Autoclave.

Optional:

2 ppm beta- sitosterol (0.8ml of .25% ETOH) for oospore production. Add before autoclaving.

**b. V-8 CaCO<sub>3</sub> Agar.** A good growth medium for most species and useful for sporangia formation but difficult to see through media for observation of sexual oospores.

V-8 juice (200ml), 800ml distilled water, CaCO<sub>3</sub> (2g), and agar (17g/L).

All ingredients are mixed and autoclaved for 30 minutes.

**c. V-8 Broth for Vermiculite Culture.** Used to prepare inoculum for soil infestation.

Prepare V-8 juice broth; V-8 juice (200ml), distilled water (800ml), CaCO<sub>3</sub> (2g). Add 250ml V-8 broth to 500cm<sup>3</sup> of vermiculite in 1 qt mason jars and autoclave for 1 hour with a vented lid (plugged with foam stopper) in water-filled pan on two successive days. Alternatively standard laboratory Erlenmeyer flasks can also be used. Cool and cover lid with a plastic bag. Seed vermiculite with inoculum plug of *Phytophthora* sp. Shake after 2 or 3 weeks. This vermiculite media is useful for inoculation of soil with soilborne species to conduct Koch's postulates.

**d. Clarified V-8 Juice Agar.** A good medium for observing oospores.

Clarify V-8 juice by centrifuging at 4340 g for 10 minutes. Mix 200 ml V8 supernatant, CaCO<sub>3</sub> (2g), distilled water (800 ml), and filter through Whatman #1 filter paper. Then add 17g agar and autoclave.

Optional:

2 ppm beta- sitosterol (0.8ml of .25% ETOH) for oospore production. Add before autoclaving.

**e. V-8 Rye Agar.** A medium for growth of *P. infestans*.

Soak 50g rye seed (nonfungicide treated) in 1100ml distilled water at 24 °C for 24 - 36 hours followed by autoclaving for 30 minutes. Filter supernatant through 4 layers of cheesecloth, adjust final volume to 1000 ml with distilled water. Add: 5% V-8, 0.02% CaCO<sub>3</sub>, 2% Bacto agar. Autoclave.

**f. Pea Broth.** Autoclave 120 g of frozen peas in 1 L of water for five minutes. Filter the supernatant through cheesecloth, pour into bottles and autoclave. This is a general broth for growing mycelia cultures of *Phytophthora* species.

### C. Production of structures for morphological identification

1. **Growth.** Isolates can be tested for growth on lima bean agar at a range of temperatures of 20, 25, 30 and 35 °C.
2. **Sporangia** – Sporangia are the cells or vessels in which zoospores are formed. Agar disks containing mycelium from cultures removed from either lima bean or V-8 juice agar are placed in sterile petri dishes and covered with a thin layer of sterile distilled water or sterile or non-sterile soil extract. Non-sterile soil extract is prepared by adding 1000 ml distilled water to 15 g air-dried field soil. Soil is stirred vigorously for at least 4 hr and allowed to settle overnight. The supernatant is filtered through two layers of cheesecloth, centrifuged at 1935 *g* for 15 min, filtered through coarse filter paper and can be either autoclaved or used non-sterile. Non-sterile soil extract is more effective than sterile soil extract for sporangia production. Store in the refrigerator at 4 °C. (Jeffers and Aldwinkle, 1987).

A thin layer of sterile distilled water or soil extract is added to petri plates containing disks removed from cultures. Do not submerge the disks. Plates are incubated under cool white fluorescent light for 1-3 days and observed daily under the dissecting scope for sporangia. Slides can be made and sporangia type (papillate, semipapillate, or nonpapillate), number of apices, shape and size can be observed and measured. Length and breadth (width) of 10 sporangia are measured with an ocular micrometer and length /breadth ratio's are calculated.

3. **Sporangiophore.** Sporangiospheres are the hyphal strands on which sporangia are borne. Morphology of sporangia can be observed using a binocular microscope from the agar disks described above (2). Sporangiospheres can be branched or unbranched to form compound or simple sympodia. The sporangiophore emerges from the base of previous sporangium in either a lax or close manner in a simple sympodium. Sporangia can form in umbels (an inverted umbrella-like cluster of sporangia) on the sporangiophore, or very long irregular branches. Sporangia may proliferate internally through previously formed sporangia on the sporangiophore. Sporangia can be borne in tight or botryose clumps on the sporangiophore.

4. **Cauducity.** Sporangial disks produced as described above (2) are placed on a microscope slide and agitated to dislodge sporangia in water. Cauducous sporangia will break or fall away from the sporangiophore readily when agitated and can be observed. Pedicel (sporangial stalk) length is measured. The pedicel is the hyphal strand left attached to the sporangium in cauducous species. Pedicel lengths can be grouped into small (< 5  $\mu$ m), medium (5-10  $\mu$ m) and long (> 10  $\mu$ m) categories.
5. **Oospores** - Oospores of heterothallic isolates are produced by placing an agar disk containing mycelium of an unknown isolate 2-3 cm apart from a tester isolate on clarified V8 (CV8) agar or lima bean agar. Tester isolates of known opposite mating type (A1 or A2) are needed for pairing with each unknown isolate and serve as controls. Homothallic isolates do not require pairing and should produce oospores in single culture. Cultures are incubated in the dark at 20-22 C for approximately 1 mo. Oogonia and antheridia should form within 7 days but oospore formation may take longer. Oospores formed in a distinct band between opposite mating types will confirm heterothallic species.
6. **Oogonia and oospore diameter** can be measured. Oospore diameter is measured using the outer wall of the oospore. Oogonial diameter is measured using the outer oogonial wall contained within the oospore. Measurements in two directions are usually done and at least 10 oospores or oogonia should be measured.
7. **Antheridial characters** should be observed. The antheridium is the male gametangium, and is a multinucleate, swollen hyphal tip that can be affixed to the basal side of the oogonium (paragynous) or the oogonial stalk can grow through the antheridium so that the antheridium surrounds the oogonial stalk (amphigynous). Antheridia may be 1 or 2 celled.
8. **Hyphal characteristics** including hypha swellings and presence of chlamydospores can be observed in thin plates of lima bean agar.

**E. Stock cultures.** Stock cultures of most species can be maintained on cornmeal agar or lima bean agar slants covered with sterilized mineral oil at 20C. Agar disks of most species can also be stored in sterilized water containing autoclaved hemp seed in 1 ml vials at room temperature.

## References

- Erwin, D. C. and Ribeiro, O. K. 1996. *Phytophthora* diseases worldwide. Amer. Phytopathol. Soc. Press, St. Paul, MN. 562 pp.
- Gallegly, M. and Hong, C. 2008. *Phytophthora*: Identifying species by morphology and DNA fingerprints. American Phytopathological Society Press, St. Paul, Mn. 158pp.
- Jeffers, S.N., and Aldwinkle, H. S. 1987. Enhancing detection of *Phytophthora cactorum* in naturally infested soil. *Phytopathology* 77:1475-1482.
- Larkin, R.P., Ristaino, Jean B., and Campbell, C. L. 1995. Detection and quantification of *Phytophthora capsici* in soil. *Phytopathology* 85:1057-1063.
- Masago, H., Yoshikawa, M., Fukada, M., and Nakanishi, N. 1977. Selective inhibition of *Pythium spp.* on a medium for direct isolation of *Phytophthora spp.* from soils and plants. *Phytopathology* 67: 425-428.
- Ribeiro, O. K. 1978. A source book of the genus *Phytophthora*. , J. Cramer, Vaduz Liechtenstein. 417 pp.
- Tooley, P. W. 1988. Use of uncontrolled freezing for liquid nitrogen storage of *Phytophthora* species. *Plant Dis.* 72:680-682.



## PCR Protocols used in Lucid Key

This PCR protocol can be used to amplify ITS sequences and the 5' end of the mitochondrial *cox 1* gene (BOL, Barcode of Life Region) to identify species of *Phytophthora*. The primers ITS6 and ITS4 amplify both spacer regions and the 5.8S rDNA (White et al., 1990). Restriction digestion of the amplified ITS region with restriction enzymes can be done instead of sequencing for identification of some species (Ristaino et al., 1998). See further methods for use of restriction analysis fingerprints at <http://phytophthora-id.org/files/Phytophthora-ID%20sequencing%20protocols.pdf>. See ITS and BOL maps of gene regions amplified with primers below.

### Master mix for each 50µl reaction

		<u>Final concentration</u>
ddH <sub>2</sub> O	35.25 µl	
10X PCR buffer	5 µl	20 mM Tris-HCl; 50 mM KCl
dNTPs (2mM each)	2.5 µl	0.1 mM of each dNTP
Primer-F (10µM)	2 µl	0.4 µM
Primer-R (10µM)	2 µl	0.4 µM
MgCl <sub>2</sub> (50mM)	1.8 µl	1.8 mM
BSA (20mg/ml)	0.25 µl	0.1mg/ml
Taq (5U/µl)	0.2 µl	1 U

Add 49 µl of master mix to 1µl template DNA (5-10 ng)

### Cycling parameters:

Initial denaturation:	96 °C	2 min
35 cycles:	96 °C	1 min
	56 °C	1 min
	72 °C	2 min
Final extension:	72 °C	10 min

### Reagents:

**10X PCR buffer** (200 mM Tris-HCl, pH 8.4; 500 mM KCl) & **50 mM MgCl<sub>2</sub>** come with **Taq DNA polymerase** (Invitrogen)

**2 mM dNTPs:** (300 ml)

Add 24 µl of dNTPs mix (100 mM) (Bioline) to 276 µl ddH<sub>2</sub>O

### **Primer stock soln (100 µM)**

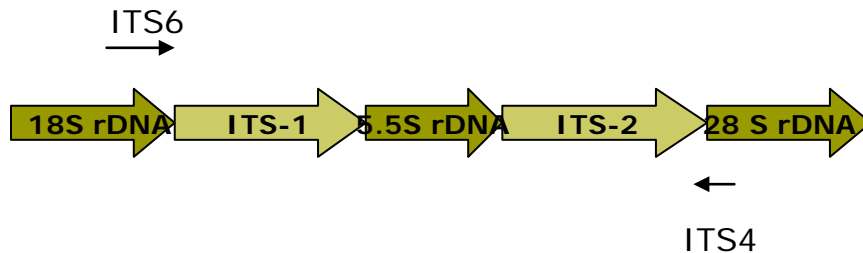
Base on the value of nmole on the tube, we add 10 times of that value of ddH<sub>2</sub>O to make 100µM stock soln.

**BSA (20mg/ml)** – Roche

**Primers:**

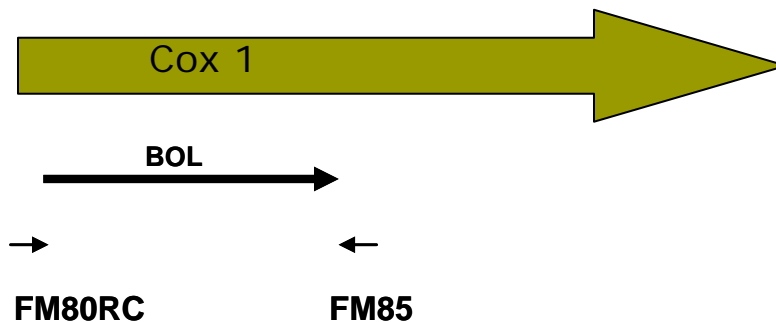
ITS region –

- ITS6 – GAAGGTGAAGTCGTAACAAGG
- ITS4 – TCCTCCGCTTATTGATATGC



BOL region –

- FM80RC – TTTCAACAAATCATAAAGATATT
- FM85 – AACTTGACTAATAATACCAA

**VI. REFERENCES**

Blair, J.E., Coffey, M.D., Park, S-Y., Geiser, D.M., and Kang, S. 2008. A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences. *Fungal Genetics and Biology* 45: 266–277.

Bonants, P.J.M., Hagenaar-de Weerd, M., van Gent-Pelzer, M., Lacourt, I., Cooke, D.E., and Duncan, J.M. 1997. Detection and identification of *Phytophthora fragariae* Hickman by the polymerase chain reaction. *Eur. J. Plant Pathol.* 103: 345-355.

Bonants, P.J.M., van Gent-Pelzer, M.P.E., Hooftman, R., Cooke, D.E.L., Guy, D.C., and Duncan, J.M. 2004. A combination of baiting and different PCR formats, including

measurement of real time quantitative fluorescence, for the detection of *Phytophthora fragariae* in strawberry plants. European Journal of Plant Pathology 110: 689-702.

Cooke, D. E. L., Drenth, A., Duncan, J. M., Wagels, G. and Brasier, C. M. 2000. A molecular phylogeny of *Phytophthora* and related Oomycetes. Fungal Gen. Biol. 30:17-32.

Forster, H., M. P. Cummings, and Coffey, M. D. 2000. Phylogenetic relationships of *Phytophthora* species based on ribosomal ITS I DNA sequence analysis with emphasis on Waterhouse groups V and VI. Mycol. Res. 104:1055-1061.

Gallegly, M. and Hong, C. 2008. *Phytophthora*: Identifying species by morphology and DNA fingerprints. American Phytopathological Society Press, St. Paul, Mn.158pp.

Kang, S., Blair, J.E., Geiser, D.M., Khang, C., Park, S., Gahegan, M., O Donnell, K., Luster, D.G., Kim, S.H., Ivors, K.L., Lee, Y., Lee, Y., Grunwald, N.J., Martin, F.N., Coffey, M.D., Veeraraghavan, N., Makalowska, I. 2006. Plant pathogen culture collections: It takes a village to preserve these resources vital to the advancement of agricultural security and plant pathology. Phytopathology. 96:920-925.

Kong, P., Hong, C., Richardson, P.A. and Gallegly M. E. 2003. Single-strand-conformation polymorphism of the ribosomal DNA for rapid species differentiation in the genus *Phytophthora*. Fung Gen Biol 39:238-249.

Kroon, L.P.N.M., Bakker, F.T., van den Bosch, G.B.M., Bonants, P.J.M., and Flier, W.G. 2004. Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. Fungal Genet. Biol. 41: 766-82.

Martin, F. N. and Tooley, P. W. 2003. Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. Mycologia 95:269-284.

Mills, S.D., Forster, H. and Coffey, MD. 1991. Taxonomic structure of *Phytophthora cryptogea* and *P. dreschleri* based on isozyme and mitochondrial DNA analysis. Mycol Res 95:31-48.

Park, J., Park, B., Veeraraghavan, N., Jung, K., Lee, Y., Blair, J., Geiser, D., Isard, S., Mansfield, M., Nikolaeva, E., Park, S., Russo, J., Kim, S., Greene, M., Ivors, K., Balci, Y., Peiman, M., Erwin, D. C., Coffey, M. B., Rossman, A., Farr, D., Cline, E., Grünwald, N. J., Luster, D. G., Schrandt, J., Martin, F., Ribeiro, O., Makalowska, I., and Kang, S. 2008. *Phytophthora* Database: A Forensic Database Supporting the Identification and Monitoring of *Phytophthora*. Plant Dis. 92: 966-972.

Ristaino, J. B., Madritch, M., Trout, C. L. and Parra, G. 1998. PCR amplification of ribosomal DNA for species identification in the plant pathogen genus *Phytophthora*. Appl. Environ. Microbiol. 68:948-954.

Ristaino, J. B. 2012. A Lucid Key to the common species of *Phytophthora*. Plant Disease 96:897-903.

Schena, L., Duncan, J.M., and Cooke, D.E.L. 2008. Development and application of a PCR-based 'molecular tool box' for the identification of *Phytophthora* species damaging forests and natural ecosystems. Plant Pathology 57: 64–75.

Tooley, P.W., Bunyard, B.A., Carras, M.M., and Hatziloukas, E. 1997. Development of PCR primers from Internal Transcribed Spacer region 2 for detection of *Phytophthora* species infecting potatoes. Appl. Environ. Microbiol. 63: 1467-1475.

Trout, C.L, Ristaino, J.B., Madritch, M, and Wangsomboondee, T. 1997. Rapid Detection of *Phytophthora infestans* in late blight infected tissue of potato and tomato using PCR. Plant Disease 81: 1042-1048.

Wang, H., Qi, M., and Cutler, A. J. 1993. A simple method of preparing plant samples for PCR. Nuc. Acids. Res. 21:4153-4154.

White, T.J., Burns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White T.J. (eds). PCR Protocols: A guide to Methods and Applications. Academic Press, San Diego, CA.

### **Other useful detection method papers**

Choi, Y-J., Beakes, G., Glockling, S., Kruse, J., Nam, B., Nigrelli, L., Ploch, S., Shin, H-D., Shivas, R.G., Telle, S., Voglmayr, H., Thines, M. 2015. Towards a universal barcode of oomycetes – a comparison of the *cox1* and *cox2* loci. Mol. Ecol. Res. DOI: 10.1111/1755-0998.12398.

Martin, F.N., Abad, Z. G., Balci, Y., Ivors, K. 2012. Identification and detection of *Phytophthora*: Reviewing our progress, identifying our needs. Plant Disease 96: 1080–1103.

Miles, T. D., Martin, F. N., Coffey, M. D. 2015. Development of rapid isothermal amplification assays for detection of *Phytophthora spp.* in plant tissue. Phytopathology 105: 265-278.

Robideau, G. P., de Cock, A. W. A. M., Coffey, M. D., Voglmayr, H., Bonants, P. J. M., Ristaino, J. B., Chitty, D., Rintoul, T., Désaulniers, N., Eggertson, Q., Bala, K., Gachon, C. M. M., Smith, M. L., Lévesque, A. 2011 DNA barcoding of oomycetes with cytochrome c oxidase subunit I (COI). Mol. Ecol. Res. 11: 1002-1011.

Sikora, K., Verstappen, E. Mendes, O., Schoen, C., Ristaino, J. and Bonants, P. 2012. A Universal Micro-array Detection Method for identification of Multiple *Phytophthora* Species using Padlock Probes. Phytopathology 102:635-645.

Van Doorn, R., Sławiak, M., Szemes, M., Dulleman, A.M., Bonants, P., Kowalchuk, G.A., Schoen, C.D. 2009. Robust detection and identification of multiple oomycetes and fungi in environmental samples by using a novel cleavable padlock probe-based ligation detection assay. *Applied and Environmental Microbiology*, 75 (12) pp. 4185-4193.



## **Molecular identification of *Phytophthora* isolates using a DNA Sequence Based Approach**

### **I. Introduction**

Many *Phytophthora* species can be difficult to identify based on morphology due to a lack of physically distinguishing characteristics and variability of morphological characteristics under different culture conditions. Additionally, it may take several days or weeks for an isolate to mature enough to develop identifying characteristics. Because time is often of the essence in identifying and assessing the potential risk of a newly isolated pathogen, DNA sequence-based identification is frequently used to augment and complement morphological data.

To serve as a baseline for identification, classification, and risk assessment of new *Phytophthora* isolates, PD cataloged genotypic and phenotypic information on isolates of previously described species in a web-accessible and searchable format. To support the identification of new *Phytophthora* isolates via comparison of their sequences at one or more loci with the corresponding sequences derived from the isolates archived in PD, sequence data from up to nine loci have been generated from more than 2,000 isolates from known and novel species (94 in total) and deposited the data in PD so that these loci can be used for species identification (Blair et al., 2007; Park et al., 2008). The characterized loci include the following: (i) two loci in the nuclear ribosomal RNA (rRNA) encoding genes: the internal transcribed spacer (ITS) regions and the 5' portion of the large subunit rRNA gene, (ii) nuclear genes encoding 60S ribosomal protein L10, beta-tubulin, enolase, heat shock protein 90, TgaA fusion protein, and translation elongation factor 1 alpha, and (iii) a mitochondrially-encoded *coxII* gene and spacer region between *coxI* and *coxII*. PCR reaction conditions for amplifying these marker loci, including the sequences and positions of primers used, can be found in the Genetic Markers section of PD and are hyperlinked from marker names throughout the PD user interface.

A comprehensive phylogenetic analysis was performed (using *Pythium vexans* as an outgroup) to establish evolutionary relationships among the characterized species (Blair et al., 2007), in which sequences of seven loci (all the markers described above except the ITS and *cox* regions) derived from 228 isolates from 83 species were utilized. The result is shown in the form of a genus-wide phylogenetic tree via individual species pages in PD and will be updated periodically. Sequences employed in this analysis and sequence alignments are available for downloading. Data search and analysis tools in PD (Fig. 7) include BLAST, Phyloviewer (a program for building phylogenetic trees using sequences of selected isolates), and Virtual Gel (a program for generating expected restriction patterns for given sequences). The PD also provides a customized means of storing and sharing data via the web.

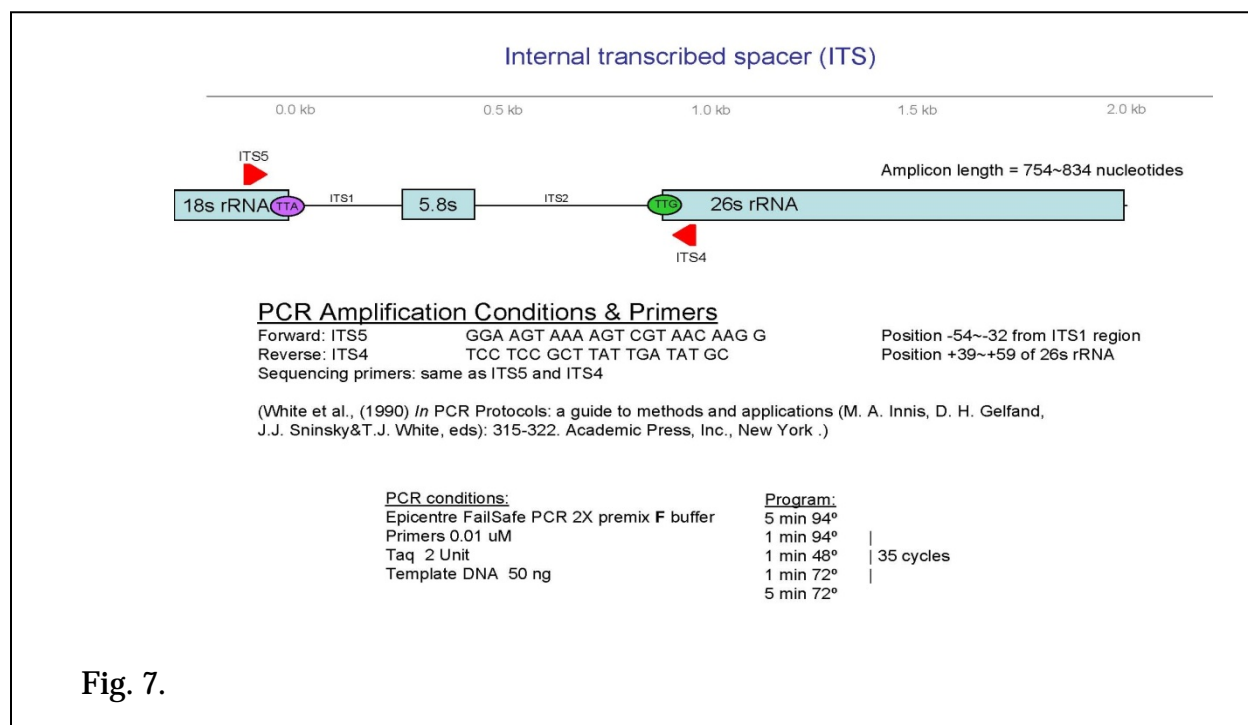
## II. Sequence-Based Strain Identification

The following flowchart protocol will provide a descriptive and pictorial explanation of how to use DNA sequence data along with the *Phytophthora* Database (PD; <http://www.phytophthoradb.org>) and GenBank search functions in order to identify new isolates. Detailed descriptions of how to use search functions in PD and of how data can be moved from one analysis tool to another can be found in the user manual at the PD web site. The BLAST tool allows for the identification of an unknown isolate by querying the sequence database in PD (and GenBank) using one or more of the marker sequences described above. Given the comprehensive set of ITS sequences available for the archived isolates, we suggest users to begin the identification process using this locus, which should establish its identity at or near the species level.

1. Grow single-spore isolate and extract genomic DNA



2. PCR using Internal Transcribed Spacer (ITS) primers and conditions described below



3. Check for positive PCR amplification on an electrophoresis gel, purify PCR products and sequence



4. Manually edit sequence data using appropriate program



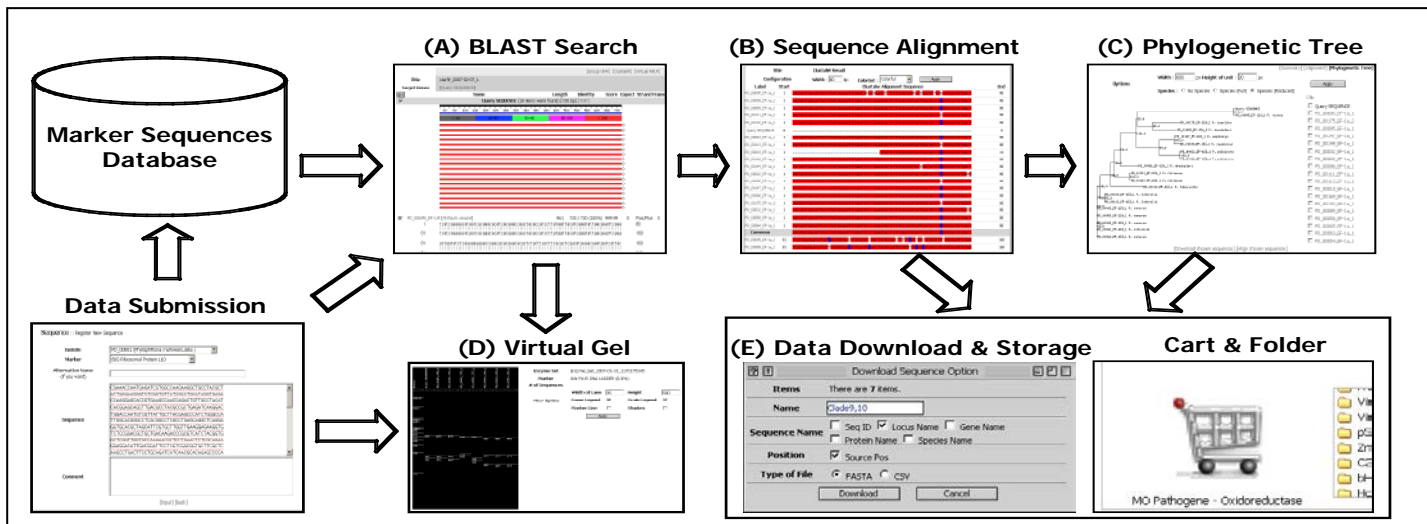
5. Compare target isolate sequence to other ITS sequences using the BLAST tool in PD ([www.phytophthoradb.org](http://www.phytophthoradb.org)) or GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>)



6. Evaluate the search results

Once you have submitted your target sequence, the BLAST tool will compare your sequence to sequences contained in your database of choice. The output you will receive will be a list of the CLOSEST but not necessarily EXACT matches (for example if your sequence represents a new species you may not have an exact match). In PD, you will also be provided with links to individual species names and descriptions in the order of the closest matches to your submitted sequence. It is important to look at the output you receive as a COLLECTIVE body of information that suggests what your isolate is most closely related to, rather than a single answer consisting of the top closest match. There are several reasons why this is important: (i) the top closest match may be a misidentified isolate, therefore, using this match as a single identifier for your isolate may be perpetuating a mistake; (ii) even if your isolate has 99% sequence identity to an already described species, several small or singular sequence differences between your isolate and the closest match may suggest that you have a novel species. Given the intensive curation performed on the data archived in PD, the first problem is unlikely (but not impossible). The second situation illustrates why it is often necessary to take the search process further by generating an alignment with the top closest matches and performing a base-by-base visual sequence comparison to determine if there are true differences.

If the closest match exhibits substantial sequence differences from your sequence, it is possible that the unknown isolate may belong to a new species. In the latter case, one may sequence all or some of the seven loci used for the genus-wide phylogenetic analysis (Blair et al., 2007) to investigate this possibility further. Characterization of morphological and biological traits (e.g., growth characteristics, pathogenicity on plants) will also be needed to formally describe a new species.



**Fig. 3. Overview of the functionality and data flow in PD.** This diagram illustrates (A) BLAST, (B) Clustal W: a tool that will align and illustrate a base-by-base comparison between your isolate and the closest matches, (C) Phyloviewer: a tool to visualize the evolutionary relationship between your isolate and related described species, and (D) the Virtual Gel which displays predicted RFLP patterns from selected sequences (your own and the closest sequence matches). Another helpful feature of PD is that your sequence information, along with the closest matches, alignments, phylogenetic trees, and virtual gels, can be stored in a personal folder (E). You can also restrict or share this data with personalized options.

### III. Literatures Cited

Blair JE, Coffey MD, Park S-Y, Geiser DM, Kang S (2007) A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete pathogen genomes. *Fungal Genet. Biol.* 45:266-277.

Park J, Park B, Veeraraghavan N, Blair JE, Geiser DM, Isard S, Mansfield MA, Nikolaeva E, Park S-Y, Russo J, Kim SH, Greene M, Ivors KL, Balci Y, Peiman M, Erwin DC, Coffey MD, Jung K, Lee Y-H, Rossman A, Farr D, Cline E, Grünwald NJ, Luster DG, Schrandt J, Martin F, Ribeiro OK, Makalowska I, Kang S (2008) *Phytophthora* Database: A cyberinfrastructure supporting the identification and monitoring of *Phytophthora*. *Plant Dis.* 92: 966-972.

## Quick NaOH DNA Extraction from Dried Leaf Samples

1. Using forceps or your fingers, remove a piece of leaf tissue from the healthy leaf that is approximately 2 mm in diameter and place it in a clean 1.5 ml tube.
2. Add 90  $\mu$ l of 0.5 N NaOH.
3. Grind tissue using a clean konte pestle until the sample is liquified (approximately 1-2 minutes). You will still see fine particles of leaf in the liquid.
4. Immediately transfer 3  $\mu$ l of the solution with the ground leaf tissue to a new tube containing 300  $\mu$ l, 100 mM Tris buffer, pH 8.0 (use 1:10 dilution of 1M Tris-HCl (pH 8.0) stock from above).
5. Vortex briefly to mix or shake vigorously until tube contents are well mixed.
6. Place tube on ice.
7. Repeat steps 1-6 for infected leaf tissue. Sample tissue from the visible lesion on the leaf. Make sure you use a clean pellet pestle to grind the sample and also a clean pipette tip each time you work with a new sample.

0.5 N NaOH :	$\frac{100 \text{ ml}}{2\text{g}}$	$\frac{\text{per L}}{20\text{g}}$	$\frac{\text{Formula FW}}{\text{NaOH (40)}}$
0.5 N NaOH			
Add to 100 ml H <sub>2</sub> O.			

### Reference:

Wang, H., Qi, M., and Cutler, A. J. 1993. A simple method of preparing plant samples for PCR. Nuc. Acids. Res. 21:4153-4154.





## PCR with Ready-To-Go™ PCR beads

1. Obtain 4 tubes containing a Ready-To-Go™ PCR bead. The bead contains:
  - a. Taq polymerase -- the enzyme that catalyzes the reaction
  - b. nucleotides -- the building blocks of DNA
  - c.  $MgCl_2$  -- it brings more ions to the reaction solution
  - d. buffer -- it buffers the reaction
2. Add 13  $\mu$ l sterile water and 2  $\mu$ l of each primers to each tube.
3. Label 4 tubes and add reagents as follows:
  - a. Add 8  $\mu$ l of sterile water to tube 1
  - b. Add 8  $\mu$ l of diluted DNA from the healthy potato leaf to tube 2
  - c. Add 8  $\mu$ l of diluted DNA from the infected leaf tissue to tube 3
  - d. Add 8  $\mu$ l of the positive control DNA from the positive control to tube 4
4. Gently mix the reagents in each tube by pipeting the mixture up and down several times.
5. Place on ice.
6. When everyone's reactions are set up and the machine is programmed and ready to go, place the tubes in the thermal cycler and run the following PCR program:

### ***Cycling Parameters:***

Initial denaturation	94°C	2 min.
35 Cycles	94°C	15 sec
	55°C	15 sec
	72°C	15 sec
	72°C	5 min.
Final extension	72°C	5 min.
Hold	4°C	

Primers: (Primers can be ordered from Invitrogen at <http://www.invitrogen.com/>)

Forward primer -- **ITS5**: GGAAGTAAAAGTCGTAACAAGG

Reverse primer -- **PINF2**: CTCGCTACAATAGCAGCGTC

### **Preparing Primer stock soln (100 $\mu$ M):**

Base on the value of nmole on the tube, we add 10 times of that value of ddH<sub>2</sub>O to make 100 $\mu$ M stock soln.

### **Preparing 5 $\mu$ M Primer soln from primer stock soln:**

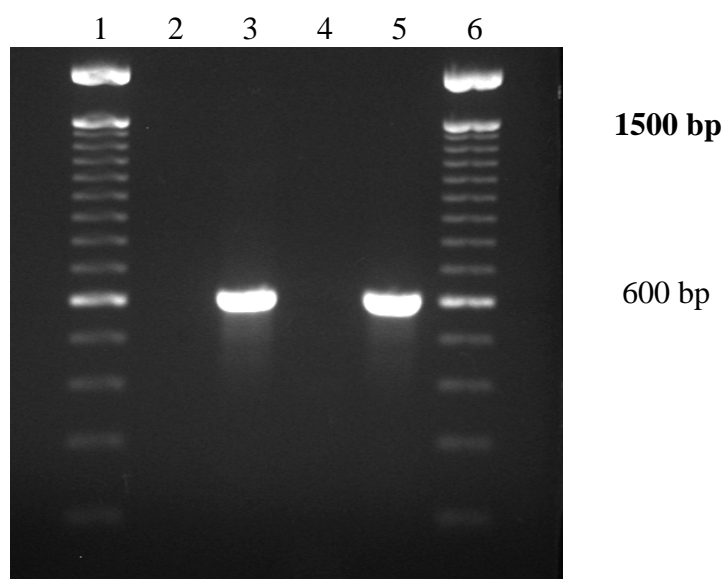
Add 10  $\mu$ l of 100  $\mu$ M primer stock soln into 190  $\mu$ l of sterilized distilled water.

\*Ready-To-Go™ PCR beads -- can be ordered from GE healthcare with cat# 27-9559-01 at <http://www.gelifesciences.com/webapp/wcs/stores/servlet/productById/en/GELifeScience-us/27955901>

### Gel Preparation

1. Seal the ends of the gel tray with tape and inset the comb.
2. Make a 1.5% molten agarose at approximately 55-65°C and add 5 ul of gel red
3. Pour the molten agarose into the tray to form a gel approximately one quarter inch thick. Allow the gel to solidify completely. The gel should be cloudy when it is completely solidified. This takes at least 20 minutes.
4. Carefully remove the combs from the gel and place the gel into the gel rig and cover it with 1X TBE buffer.

### Expected Results



**Figure 4.** Amplified PCR products from *Phytophthora infestans* DNA from mycelium and potato leaf tissue. Lanes: 1 and 6 are 100 bp ladder ; 2, negative no template control; 3, *P. infestans* DNA (positive control); 4, dried, healthy potato leaf; 5, dried potato leaf infected with *P. infestans*.

## CTAB Extraction of Fungal DNA

1. Grow mycelia in pea broth culture 7-10 days or until sufficient mycelia.
2. Harvest mycelia by vacuum filtration and freeze at  $-20^{\circ}\text{C}$ .
3. Add 150  $\mu\text{l}$  Extraction Buffer, vortex. Grind mycelia with sterile Konte pestle.
4. Add 150  $\mu\text{l}$  Nuclei Lysis Buffer and 60  $\mu\text{l}$  5% Sarkosyl, vortex to mix.
5. Incubate at  $65^{\circ}\text{C}$  for 15-30 min.
6. Add 1 volume ( $\sim 300\ \mu\text{l}$ ) Chloroform ( $\text{CHCl}_3$ ): Isoamyl Alcohol (24:1), invert to mix.
7. Centrifuge 15 min, 12K rpm, room temperature.
8. Transfer aqueous phase to a new microfuge tube. Repeat chloroform extraction. Centrifuge 15 min, 12K rpm, room temperature.
9. Transfer aqueous phase to a new tube. To aqueous phase add 0.1 volumes 3M Sodium Acetate ( $\text{NaOAc}$ ), pH 8.0 and 2 volumes of cold 100% Ethanol.
10. Allow DNA to precipitate overnight at  $-20^{\circ}\text{C}$ .
11. Centrifuge to pellet DNA, 10 min, 12K rpm, room temperature. Pour off supernatant.
12. Wash pellet twice with 70% Ethanol.
13. Dry pellet in speed vacuum.
14. Resuspend pellet in Te buffer, pH 8.0.

Extraction Buffer:	<u>250 ml</u>	<u>per L</u>	<u>Formula (FW)</u>
0.35 M Sorbitol	15.94 g	63.77 g	$\text{C}_6\text{H}_{14}\text{O}_6$ (182.2)
0.1 M Tris	3.03 g	12.11 g	$\text{C}_4\text{H}_{11}\text{NO}_3$ (121.1)
0.005 M EDTA(pH 7.5)	0.47 g	1.86 g	$\text{C}_{10}\text{H}_{14}\text{N}_2\text{O}_8\text{Na}_2 \cdot 2\text{H}_2\text{O}$ (372)
0.02 M Sodium Bisulfite	0.95 g	3.8 g	
Adjust pH to 7.5 with HCl. Do not autoclave and store at $4^{\circ}\text{C}$ .			

CTAB (Nuclei Lysis Buffer):	<u>250 ml</u>	<u>per L</u>	<u>Formula (FW)</u>
0.2 M Tris	6.05 g	24.2 g	$\text{C}_4\text{H}_{11}\text{NO}_3$ (121.1)
0.05 M EDTA pH 7.5	4.65 g	18.6 g	$\text{C}_{10}\text{H}_{14}\text{N}_2\text{O}_8\text{Na}_2 \cdot 2\text{H}_2\text{O}$ (372.2)
2.0 M NaCl	29.2 g	116.88 g	$\text{NaCl}$ (58.44)
2% CTAB (cetyltrimethylammonium bromide)	5 g	20 g	$\text{C}_{19}\text{H}_{42}\text{NBr}$ (364.5)

5% Sarkosyl:  
5 g N-lauryl sarcosine per 100 ml  $\text{H}_2\text{O}$ . Autoclave.

3M Sodium Acetate:	<u>250 ml</u>	<u>per L</u>	<u>Formula (FW)</u>
3M Sodium Acetate	61.52 g	246.09 g	$\text{C}_2\text{H}_3\text{O}_2\text{Na}$ (82.03)
Adjust pH to 8.0 with HCl and adjust volume to 1 liter. Dispense and autoclave. Store at room temperature.			

## **Buffers**

1M Tris HCl (pH 8.0): 100 ml per L                      Formula (FW)  
1M Tris HCl                      15.76 g                      157.6 g  $C_4H_{11}NO_3 \cdot HCl$  (157.6)  
Add Tris into 80 ml  $H_2O$ . Adjust pH with HCl. Bring to final 100ml volume. Sterilize by autoclaving

0.5 M EDTA (pH 8.0):                      100 ml                      per L                      Formula (FW)  
0.5 M EDTA                      18.6 g                      186g                       $C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$  (372)  
Add EDTA to 70 ml water and stir vigorously. Adjust pH to 8.0 with NaOH. Adjust to 100 ml volume and autoclave.

Te Buffer (pH 8.0):                      50ml  
10 mM Tris-HCl                      0.5 ml of 1 M Tris-HCl (pH 8.0)  
0.1 mM EDTA                      0.01 ml of 0.5M EDTA  
 $H_2O$                       49.49 ml

TE Buffer (pH 8.0):                      50ml  
10 mM Tris-HCl                      0.5 ml of 1 M Tris-HCl (pH 8.0)  
0.1 mM EDTA                      0.1 ml of 0.5M EDTA  
 $H_2O$                       49.4 ml

70% Ethanol: 73 ml 95% ETOH + 27 ml  $H_2O$ .

## **DNeasy Plant Mini Kit Extraction**

Before starting, preheat a water bath or heating block to 65°C. Make sure 96 - 100% ethanol has been added to the AW1 and AW2 buffers. Buffer AP1 and AW1 (pre-ethanol) may form precipitates during storage, which can be rectified by warming to 65°C.

1. Grow mycelia in pea broth culture 7-10 days or until sufficient mycelia.
2. Place approximately 100mg wet weight mycelia into a microcentrifuge tube.
3. Add 400µL Buffer AP1 and 4µL RNase A solution. Grind mycelia with sterile Konte pestle. Vortex vigorously.
4. Incubate at 65°C for 10 minutes. Invert the tube two or three times during incubation to mix.
5. Add 130µL Buffer P3, mix, and place on ice for 5 minutes.
6. Centrifuge the lysate for five minutes at 14,000 rpm.
7. Pipet the supernatant into a purple QIAshredder mini spin column (in a 2mL collection tube). Centrifuge for 2 minutes at 14,000 rpm.
8. Transfer the flow through into a new tube without disturbing the cell debris pellet.
9. Add 1.5 volumes of Buffer AW1 to flow through and mix by pipetting (e.g. if there is 450µL of flow through, add 675µL Buffer AW1).
10. Pipet 650µL of the mixture to a clear DNeasy mini spin column (in a 2mL collection tube). Centrifuge for 1 minute at 8000 rpm or greater. Discard flow through and reuse tube and column in next step.
11. Repeat step 10 with the remainder of the mixture. Discard the flow through and collection tube (save the column!).
12. Place the column in a clean 2mL collection tube and add 500µL Buffer AW2. Centrifuge for 1 minute at 8000 rpm or greater. Discard flow through and reuse tube.
13. Add 500µL Buffer AW2 and centrifuge for 2 minutes at 14,000 rpm. This will dry the membrane.
14. Transfer the column to a clean 1.5-2mL microcentrifuge tube.
15. Pipet 100µL Buffer AE directly onto the DNeasy membrane. Incubate for five minutes at room temperature. Centrifuge for 1 minute at 8000 rpm or greater to elute.
16. Repeat step 15 once.

## ***Phytophthora* spp identification using PCR-RFLP technique**

PCR allowed the amplification of DNA fragments. DNA fragments are used to identify species using restriction enzymes. Restriction enzymes cut specific sites inside the amplified fragments and generate a band pattern that allowed the identification of specific organisms according to the band length.

On this laboratory we will identify *Phytophthora* spp. using the PCR-RFLP technique, using restriction enzymes.

For the PCR we will use two specific primers A2 forward and I2 reverse from Drenth *et al.* (2006) (table 1).

Drenth, A., Wagels, G., Smith, B., Sendall, B., O'Dwyer, C., Irvine, G., Irwin, J.A.G. 2006. Development of a DNA-based method for detection and identification of *Phytophthora* species. *Australasian Plant Pathology* 35: 147 – 159.

Table 1. Specific primers used for the genera *Phytophthora* identification.

Primer	Sequence (5' - 3')
A2 forward	ACTTTCACGTGAACCGTTTCAA
I2 reverse	GATATCAGGTCCAATTGAGATGC

Later we will use the PCR product to make a digestion using the *Msp I* enzyme.

### **PCR**

1) Prepare 2 DNA samples and a negative control (water), each reaction will have a final volume of 25 µl. Prepare the master mix according to the next table (make calculations for 3 reactions:

	<b>1x = 25µl</b>	<b>3 X</b>
dSH <sub>2</sub> O	12.6 µl	
Buffer (10X)	2.5 µl	
dNTPs (2 mM each)	2 µl	
primer A2 (10 µM)	2 µl	
primer I2 (10 µM)	2 µl	
MgCl <sub>2</sub> (50 mM)	1.7 µl	
Taq polymerase (5 U/µl)	0.4 µl	
<b>Subtotal</b>	<b>23.2 µl</b>	<b>x</b>
DNA ( <i>add at the end</i> )	2 µl	Add to each tube
<b>Total reaction</b>	<b>25 µl</b>	<b>x</b>

2) Close the tubes and put them in the thermocycler using the following program:

Initial denaturation 94°C for 5 min



35 Cycles:    Denaturation 94°C for 30 s  
                   Annealing 65°C for 45 s  
                   Extension 72°C for 2 min  
 Final Extension 72°C for 10 min

3) Once the amplification is over, prepare a 1.6% agarose gel (make your own calculations for the agarose to prepare 50 ml TBE 0.5X gel). Dissolve the agarose in the gel and heat in the microwave (be careful it is not boiling over). Add 5 µl of Gel Red and mix. When it is getting warmer (close to 60 °C), pour inside the chamber and put the comb in.

4) Add buffer TBE 0.5X inside the electrophoresis chamber.

5) Use 8 µl from each sample and add 2 µl of loading dye (blue) inside each well. At the end add 6 µl of ladder (molecular marker of 100 bp) on the first one and annotate the order of your samples on the next table:

Well	Line	Sample	Well	Line	Sample
1	top		1	bottom	
2	top		2	bottom	
3	top		3	bottom	
4	top		4	bottom	
5	top		5	bottom	
6	top		6	bottom	
7	top		7	bottom	
8	top		8	bottom	
9	top		9	bottom	
10	top		10	bottom	
11	top		11	bottom	
12	top		12	bottom	

6) Connect the cables correctly. Turn on the power and program the electric field to 90V.

7) Once the blue bands get close to the inferior border (close to 1cm) stop the power and look at the gel under UV Light. Take a picture.

**RFLP**

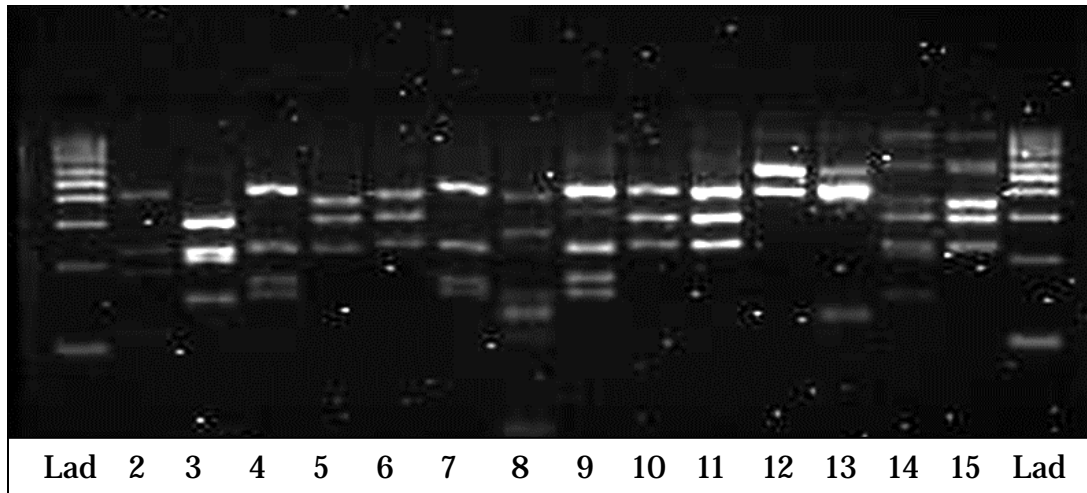
1) Prepare the enzymatic mixture as follows:

	<b>For 15 reactions</b>
dSH <sub>2</sub> O	18 µl
Tango Buffer	6 µl
Msp I	6 µl
<b>Total</b>	<b>30 µl</b>

- 2) Take 2 µl from the enzymatic mixture and add 8 µl from the PCR product.
- 3) Incubate at 37 °C for 1 h
- 4) Repeat steps 3 – 7 from last section.

## RFLP Band Patterns for 16 Species of *Phytophthora*

Note: Gel was run using a 100bp ladder



2	<i>P. cactorum</i>
3	<i>P. capsici</i>
4	<i>P. cinnamomi</i>
5	<i>P. citricola</i>
6	<i>P. citrophthora</i>
7	<i>P. drechsleri</i>
8	<i>P. erythroseptica</i>
9	<i>P. fragariae</i>
10	<i>P. infestans</i>
11	<i>P. infestans</i>
12	<i>P. nicotianae</i>
13	<i>P. palmivora</i>
14	<i>P. syringae</i>
15	<i>P. tropicalis</i>

### **All *Phytophthora* TaqMan PCR**

The generic All *Phytophthora* TaqMan PCR will be performed with 2 µl DNA extract as published by Kox et al (2007) and described below. Real-time (TaqMan) PCR will be performed on the ABI7500 instrument in 96-well plates. In all cases 2 µl of the DNA-extract will be used in 30 µl master mix. Negative controls with Milli-Q water will be run as negative controls in each run. Real-time (TaqMan) PCR will be performed with TaKaRa Premix, Lonza (Verviers, Belgium).

Positive or negative results will be based upon the *cycle threshold* (Ct) value (number of cycles after which a positive real-time PCR-signal has been obtained). The C<sub>T</sub> value will be calculated by the software of the real-time PCR machine (AB7500).

Kox, Linda; Heurneman, Ilse; Vossenbergh van den, Bart; Beld van den, Ineke; Bonants, Peter and Gruyter de, Hans (2007). Diagnostic values and utility of immunological, morphological and molecular methods for in planta detection of *Phytophthora ramorum*. *Phytopathology* 97: 1119-1129.

	Final conc.	Stock	x1
Premix TaKaRa	1x	2x	15 µl
ROX Dye II	1x	50x	0.6 µl
F ITS-15Ph-Phos	250 nM	10 µM	0.75 µl
RITS-279Ph-Phos	250 nM	10 µM	0.75 µl
All_phy probe	83 nM	5 µM	0.5 µl
MQ	-	-	X µl
gDNA	100 ng – 1pg		2µl

Forward Primer: F ITS-15Ph-Phos 5'- Phosphate-TGC GGA AAG GAT CAT TAC CAC ACC

Reverse Primer: RITS-279Ph-Phos 5'- Phosphate-GCGAGCCTAGACATCCACTG

Probe: All-phy probe 5'-FAM-TTGCTATCTAGTTAAAAGCA-MGBNFQ-3'

#### PCR program :

2 min 50°C

10 min 95°C

40 cycles: 95°C 15 sec

60 sec 60°C

#### **Samples to run in the 96 well plates - do 3 reps**

1. Use a no-template control - buffer and no pathogen DNA
2. Unknown *Phytophthora* DNA from each of 12 groups
3. Positive control *P. infestans* DNA

One group will set up a dilution series of *P. infestans* DNA at known concentrations

## All *Phytophthora* TaqMan PCR with Internal Amplification Control

The generic All *Phytophthora* TaqMan PCR will be performed with 2 µl DNA extract as published by Kox et al (2007) and described below. Real-time (TaqMan) PCR will be performed on the ABI7500 instrument in 96-well-plates. In all cases 2 µl of the DNA-extract will be used in 30 µl master mix. Negative controls with Milli-Q water will be run as negative controls in each run. Real-time (TaqMan) PCR will be performed with TaKaRa Premix, Lonza (Verviers, Belgium).

Internal amplification control primers and probe will be included as described by Klerks et al. (2006).

Kox, Linda; Heurneman, Ilse; Vossenbergh van den, Bart; Beld van den, Ineke; Bonants, Peter and Gruyter de, Hans. 2007. Diagnostic values and utility of immunological, morphological and molecular methods for in planta detection of *Phytophthora ramorum*. *Phytopathology* 97: 1119-1129.

Klerks, M.M., van Bruggen, A.H.C., Zijlstra, C., Donnikov, M. 2006. Comparison of methods of extracting *Salmonella enterica* serovar *enteritidis* DNA from environmental substrates and quantification of organisms by using a general internal procedural control. *Applied and Environmental Microbiology* 72 (6), pp 3879-3886.

Positive or negative results will be based upon the *cycle threshold* (Ct) value (number of cycles after which a positive real-time PCR-signal has been obtained). The C<sub>T</sub> value will be calculated by the software of the real-time PCR machine (AB7500).

	Final conc.	Stock	x1
Premix TaKaRa	1x	2x	15 µl
ROX Dye II	1x	50x	0.6 µl
F ITS-15Ph-Phos	250 nM	10 µM	0.75 µl
RITS-279Ph-Phos	250 nM	10 µM	0.75 µl
All_phy probe	83 nM	5 µM	0.5 µl
FPgfp	75 nM	5 µM	0.45 µl
RPgfp	75 nM	5 µM	0.45 µl
PYYgfp	50 nM	5 µM	0.3 µl
IAC DNA	-	1.7 pg/µl	1 µl
MQ	-	-	X µl
gDNA	100 ng – 1pg		2µl

Forward Primer: F ITS-15Ph-Phos 5'- Phosphate-TGC GGA AAG GAT CAT TAC CAC ACC

Reverse Primer: RITS-279Ph-Phos 5'- Phosphate-GCGAGCCTAGACATCCACTG

Probe: All-phy probe 5'-FAM-TTGCTATCTAGTTAAAAGCA-MGBNFQ-3'

Forward Primer: FPgfp 5'-TGGCCCTGTCCTTTTACCAG-3'

Reverse Primer: RPgfp 5'-TTTTCGTTGGGATCTTTTCGAA-3'

Probe: PYYgfp 5'-YY-AACCATTACCTGTCCACACAATCTGCCC-3'

IAC DNA is a mix of genomic DNA from the E.coli host and the gfp containing plasmid DNA

PCR program :

2 min	50°C	
10 min	95°C	
40 cycles:	95°C	15 sec
60 sec	60°C	



## **12-plex Microsatellite (SSR) Genotyping of *Phytophthora infestans***

Microsatellites can be used for genotyping lineages of *P. infestans*. Li and Cooke (2013) have developed a protocol that multiplexes 12 diagnostic SSR primer sets in a single tube for more rapid analysis and genotyping. The protocol uses fluorescently labeled primers, which can then be read by a capillary analyzer for analysis. This protocol is optimized for use with an ABI 3730xl DNA analyzer with a 5 dye set (6-FAM, VIC, NED, PET, and LIZ size standard). The following protocol is from Li and Cooke, with modifications implemented by the lab of Bill Fry at Cornell University.

Li, Y.; Cooke, D.E.L.; Jacobsen, E.; van der Lee, T. 2013. Efficient multiplex simple sequence repeat genotyping of the oomycete plant pathogen *Phytophthora infestans*. *Journal of Microbiological Methods* 92: 316-322.

### **Primers (5' – 3')**

Locus	Dye	Product size range (bp)	Primer sequence
PiG11	NED	130-206	FwdNED-TGCTATTTATCAAGCGTGGG Rev-GTTTCAATCTGCAGCCGTAAGA
Pi02	NED	255-275	FwdNED-ACTTGCAGAACTACCGCCC Rev-GTTTGACCACTTTCCTCGGTTT
PinfSSR11	NED	325-360	FwdNED-TTAAGCCACGACATGAGCTG Rev-GTTTAGACAATTGTTTTGTGGTCGC
D13	FAM	100-210	FwdFAM-TGCCCCCTGCTCACTC Rev-GCTCGAATTCATTTTACAGACTTG
PinfSSR8	FAM	250-275	FwdFAM-AATCTGATCGCAACTGAGGG Rev-GTTTACAAGATACACACGTCGCTCC
PinfSSR4	FAM	280-305	FwdFAM-TCTTGTTTCGAGTATGCGACG Rev-GTTTCACTTCGGGAGAAAGGCTTC
Pi04	VIC	160-175	FwdVIC –AGCGGCTTACCGATGG Rev-GTTTCAGCGGCTGTTTCGAC
Pi70	VIC	185-205	FwdVIC – ATGAAAATACGTCAATGCTCG Rev-CGTTGGATATTTCTATTTCTTCG
PinfSSR6	VIC	230-250	FwdVIC-GTTTTGGTGGGGCTGAAGTTTT Rev - TCGCCACAAGATTTATTCCG
Pi63	VIC	265-280	FwdVIC – ATGACGAAGATGAAAGTGAGG Rev-CGTATTTTCCTGTTTATCTAACACC
PinfSSR2	PET	165-180	FwdPET-CGACTTCTACATCAACCGGC Rev-GTTTGCTTGGACTGCGTCTTTAGC
Pi4B	PET	200-295	FwdPET – AAAATAAAGCCTTTGGTTCA Rev-GCAAGCGAGGTTTGTAGATT

Instead of individually pipetting each primer into the master mix, a 10X multiplex primer mix is made that includes all primers. The primer mix is made as follows (makes 400µl):

Primer	Volume of 100µM primer stock (µl)
PiG11F	2
PiG11R	2
Pi02F	2
Pi02R	2
PinfSSR11F	2
PinfSSR11R	2
PinfSSR4F	2
PinfSSR4R	2
Pi04F	2
Pi04R	2
Pi70F	2
Pi70R	2
PinfSSR6F	2
PinfSSR6R	2
Pi63F	2
Pi63R	2
PinfSSR2F	2
PinfSSR2R	2
D13F	6.4
D13R	6.4
PinfSSR8F	12
PinfSSR8R	12
Pi4BF	12
Pi4BR	12

Combine with 303.2µL of 10 mM Tris buffer (pH=8.0) to make 400µl of primer mix.

The master mix can be made using either the Qiagen multiplex PCR kit (Qiagen, cat. No 206145) or the Qiagen Type-it Microsatellite PCR kit (Qiagen, cat. No. 206243). For the purposes of this protocol we use the Type-it microsatellite PCR kit.

<b>Reagent</b>	<b>Volume per reaction (µl)</b>
2X Type-it master mix	6.25
10X multiplex primer mix	1.25
PCR grade water	4

Total reaction mix volume per sample (µl)	11.5
---	------

1µl of template DNA is added to bring the total volume per sample to 12.5 µl.

Thermocycling program:

1 cycle	95C	5 min.
30 cycles	95C	30 sec.
	58C	90 sec.
	72C	20 sec.
1 cycle	60C	30 min.

Before loading on a DNA analyzer, samples must be prepared with the LIZ size standard (Applied Biosystems LIZ500, cat. No. 4322682) and suspended in an appropriate loading solution. For use on an ABI 3730xl DNA analyzer we use highly deionized formamide (hi di formamide, Applied Biosystems, cat. No. 4311320). Check with your local source for fragment analysis for preparation and submission protocols specific to their facilities.

Master mix for analysis preparation:

<b>Reagent</b>	<b>Volume per reaction (µl)</b>
Hi-di formamide	10
LIZ500 size standard	0.3

Total volume per sample (µl)	10.3
------------------------------	------

Add 0.5 µl of template DNA to bring the total volume per sample to 10.8 µl.

## Loop-mediated isothermal amplification (LAMP)

LAMP is an isothermal PCR method that has been utilized in the development of detection assays for multiple plant pathogens. Instead of *Taq* polymerase, it utilizes *Bst* polymerase (New England Biolabs, cat. No. M0275S), which will amplify at 65°C without the need for thermal cycling. As a result, LAMP can be performed using a heat block, a thermocycler, or a water bath.

LAMP utilizes three sets of primers (6 in total): F3, B3, FIP, BIP, LoopF, and LoopB. LoopF and LoopB are not required for successful amplification, but are included to reduce amplification time and increase specificity. While all primers can be used as standard desalted primers, HPLC purification of the FIP and BIP primers has been noted to greatly increase reliability of the reaction.

The reaction can be modified for detection on a lateral flow device (LFD). By labeling the 5' ends of the LoopF and LoopB primer with digoxigenin (or fluorescein, i.e. FAM) and biotin, respectively, the resulting product can be detected on a LFD designed for detection of labeled PCR products (e.g. PCRD nucleic acid detector, <https://www.altabioscience.com/shop/nucleicaciddetection/nucleic-acid-detector/>).

### Examples of LAMP primer design software:

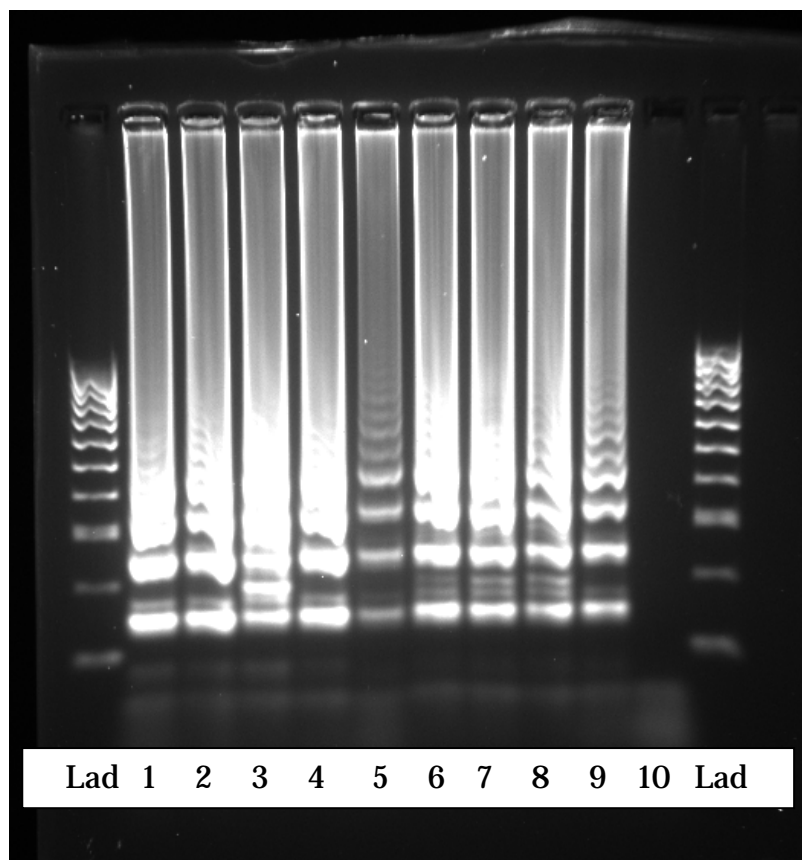
PrimerExplorer (free, requires Java): <https://primerexplorer.jp/e/>

LAMP Designer (7 day trial): <http://www.optigene.co.uk/lamp-designer/>

### An example of LAMP primers for *Phytophthora ramorum* from Tomlinson et al. (2007) (5' – 3')

<i>Pram</i> F3	CTAAAAACTTTCCACGTGAAC
<i>Pram</i> B3	CTTCATCGATGTGCGAGC
<i>Pram</i> FIP	TCAAGCGCTCGCCATGATAGAGTCAAACCCTTAGTTGGGGGCT
<i>Pram</i> BIP	ACTTTTTTAAACCCATTCTAAATACTGAACATCCACTGCTGAAAGTTGC
<i>Pram</i> F-Loop	CGAAGCCAGCCGAACAGA
<i>Pram</i> B-Loop	GTGGGGACGAAAGTCTCTG

Samples can be visualized on a gel or through the use of a visual dye such as SYBR green, which turns from orange to green in the presence of dsDNA.



An example of positive LAMP reactions run on a gel. All samples except lane 10 contained DNA.

### References

Ravindran, A., Levy, J., Pierson, E., Gross, D. 2012. Development of a loop-mediated isothermal amplification procedure as a sensitive and rapid method for detection of '*Candidatus* Liberibacter solanacearum' in potatoes and psyllids. *Phytopathology* 102: 899 – 907.

Tomlinson, J.A.; Barker, I.; Boonham, N. 2007. Faster, simpler, more-specific methods for improved molecular detection of *Phytophthora ramorum* in the field. *Applied and Environmental Microbiology* 73: 4040 – 4047.

## Detection of *Phytophthora infestans* using LAMP technique

LAMP reactions are being developed for the detection of multiple species. In this laboratory, we will use primers developed to detect *P. infestans* to run a LAMP reaction

1.) Prepare 2 samples of *P. infestans* DNA and a negative control (water). It is important to maintain a low temperature while preparing the reaction, so keep all tubes on ice if not actively working with them. Prepare the master mix according to the following table (make calculations for 4 reactions):

Reagent	Volume per Reaction (µl) (1X)	4X
Isothermal buffer (10X)	2.5	
MgSO <sub>4</sub> (50mM)	3	
dNTP mix (10mM each)	3.5	
F3 (10 µM)	0.5	
B3 (10 µM)	0.5	
FIP_HPLC (10 µM)	0.5	
BIP_HPLC (10 µM)	0.5	
LoopF (100 µM)	0.25	
LoopB (100 µM)	0.25	
Betaine (5M)	4.5	
WarmStart <i>Bst</i> polymerase (8U/µl)	1	
ddH <sub>2</sub> O	7	

2.) In each PCR tube, combine 24 µl of master mix and 1 µl of DNA. Incubate in the thermocycler at 65 °C for 1 hour followed by 5 minutes at 80 °C.

3.) To visualize, prepare a 1.6% agarose gel with 5 µl of Gel Red (see page 34 for instructions). Prepare samples for loading by mixing 2 µl of loading dye with 5 µl of product and load onto the gel. At each end of the gel add 1µl of 100bp ladder mixed with 2µl of loading dye. Connect the cables correctly and turn on the power. Set the power supply to 90V. Allow the gel to run for at least 5cm before removing and visualizing with a UV light.

4.) Alternate visualization: Add 1µl of 1000X SYBR green directly to the product and gently mix. Positive reactions will result in a change in color from orange to green.

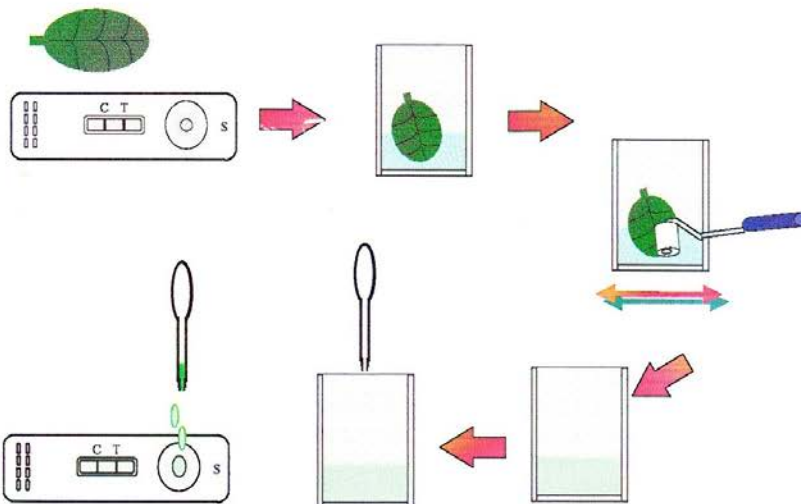




A diagram of a single cell. At the top is a large circle labeled 'S' (stroma). Below it is a smaller circle labeled 'C' (nucleus). Inside the nucleus is a smaller circle labeled 'T' (nucleolus). To the right of the nucleus is a rectangular structure labeled 'CT' (crista). Below the cell are four small circles representing other cells.



2



Invalid

## INSTRUCTIONS FOR USE

1. Remove a sample (3cm-4cm square, part of a lesion and neighbouring tissue) from the infected root/leaf and add to the extraction bag on one side of the mesh (B).  
*Smaller pieces of material from several parts of the plant may give better results.*
2. Add the contents of the extraction bottle (A) to the extraction bag (B) containing the plant sample. Use the extraction device (C) to macerate the sample. *Do not add too much material to the extraction bag.*
3. Remove the lateral flow device (D) from the foil pack.
4. Draw into the transfer pipette (E), about half of the liquid from the extraction bottle from the side of the mesh opposite to the beet sample.
5. Add 2 drops to the sample well on the lateral flow device. *Maintain the device in a horizontal position. The drops should be absorbed in about 30 seconds and a blue dye should appear in the viewing window. If this does not happen, add one more drop to the well. Do not add any more.*
6. Wait until the blue control line (C) appears and read the test (2-3 minutes). If the control line does not appear, repeat the test with a new device.
7. If the control line does not appear and the sample material is high in tannins and other contaminants then repeat the test with a diluted sample. This is achieved by adding approximately 1ml from the initial extract to a fresh extraction solution bottle, using the graduated transfer pipette provided.

**Positive result** - intensity of the blue colour on the sample line (T) will vary depending on amount of pathogen in the sample extract. If the control line is a strong blue colour, then a faint sample line should be interpreted as positive.

**Negative result** - the target pathogen was not present at detectable levels in the sample tested. May also mean that the sample tested is not representative of the infection, or that the area of sampling from the plant was only recently infected.

**Invalid result** - conduct a further test with a fresh device.

If in doubt conduct another test. If still in doubt submit a sample to a laboratory.

## IMPORTANT INFORMATION

**Preservatives:** The extraction buffer contains sodium azide (0.05%). This is a toxic substance and should be handled accordingly. Avoid ingestion and contact with skin. Materials Safety Data Sheet available on request.

A small sachet of silica gel is enclosed in each foil pouch. Avoid contact with skin and keep away from children.

## STORAGE AND HANDLING OF DEVICES

- Sealed foil pouches can be stored at room temperature for up to 12 months.
- Once opened, use the devices as soon as possible to avoid deterioration.
- Each device should be used once only.
- Take care when handling devices not to touch the test window.

## TERMS AND CONDITIONS OF SUPPLY

The tests are supplied subject to Neogen Europe Ltd terms and conditions of supply. Copy available on request. Particular attention is drawn to the following.

The tests supplied are for the detection of the pathogen stated on the foil pack. The tests should be used to provide the basis for a presumptive diagnosis. A negative result cannot be taken as conclusive evidence of freedom from the specific pathogen under test. If in doubt, repeat the test or submit to a diagnostic laboratory for confirmation.

These products are for diagnostic use only. They are supplied and service, information and advice rendered, on the understanding that the customer is solely responsible for determining the suitability for the intended purpose. Neogen Europe Ltd shall not be liable for any indirect, special or consequential damages of any kind resulting from their use. The sole and exclusive remedy of the customer is limited to the replacement of goods shown to be substandard.



## **Additional Sampling Instructions – Choosing and Collection**

### **Choosing plants to test:**

1. Select a plant or plants showing symptoms which you consider typical of the problem. Avoid using dead plants if possible.
2. If several plants are affected take material from each and bulk together for testing.
3. When there is a history of disease outbreaks, sample as soon as there are any indications of poor plant health.
4. Plants showing any type of symptom e.g. yellowing, wilting, stunting, dieback, leaf or stem lesions, root rotting may be tested.

### **Sample collection:**

1. Leaf lesions - Collect several small areas of affected tissue. Avoid leaf tissue that has dried out or that is soft and wet.

Note: Leaf symptoms such as yellowing may indicate a problem at the stem base or roots and not a direct attack on its leaves. Check the base of the plant and test tissue from it if root rot or stem lesions are found.

2. Stem lesions - If dark sunken areas are present on the stem, scrape away bark tissue and look for discoloration taking several slivers of affected tissue. If crown tissue is affected include pieces of it.
3. Root rots - Wash the roots thoroughly under running water to remove soil or compost and blot them dry. Collect several pieces of affected root and ensure some pieces from near the crown area are included. If crown tissue affected use knife to include pieces of it.
4. Stembase lesions and crown rots - Concentrate particularly on slivers from the stem base/crown area plus several pieces of affected root. Scrape away the bark tissue and look for discoloration. Remove any discoloured tissue for testing.

For larger plants take a 5cm length of stem or crown including discoloured tissue and cut it transversely to expose the pith and cortex. Place in bag and macerate as best as possible.

Contact Neogen Europe Ltd for further information if required  
[info@neogeneurope.com](mailto:info@neogeneurope.com)



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Web: [www.agdia.com](http://www.agdia.com)  
Email: [info@agdia.com](mailto:info@agdia.com)

## USER GUIDE: *Phytophthora* ImmunoStrip® (Product Number 92601)

### KIT INFORMATION

#### Intended Use

The *Phytophthora* ImmunoStrip is designed for on-site testing of plant tissues suspected of infection with *Phytophthora* species. The test is recommended for use as a preliminary screening tool in survey programs for *Phytophthora* species such as *P. ramorum* (Sudden Oak Death) and *P. kernoviae*. The test is also suitable for detection of *Phytophthora* species that affect other important crops such as *P. fragariae* in strawberry or *P. infestans* in potato. A complete list of detectable *Phytophthora* species can be found on Page 2 of this document.

#### Storage of Kit

ImmunoStrips should be stored refrigerated (4°C) between uses and tightly sealed in the desiccated container at all times.

Test strips and SEB1 sample extraction bags (if applicable) should be warmed to room temperature prior to use.

#### ImmunoStrip Kit (ISK) Includes

- ImmunoStrips
- SEB1 sample extraction bags
- User guide

ImmunoStrips (STX) purchased separately do not include buffer filled mesh bags.

#### What's required to perform the assay?

- Scissors, knife or razor blade.
- SEB1 sample extraction buffer
- Sample extraction device (Agdia sample extraction bags are recommended)
- Letter holder or other device to hold sample extraction bags.

### PERFORMING THE ASSAY (\*Special Attention Required)

#### Prepare Sample



Collect a sample section that is approximately \*1 inch square in size.

Samples should be taken from areas of the plant that exhibit symptoms of disease. It is best to select tissue from areas where symptomatic tissue comes into contact with areas that appear healthy.



Cut open the sample extraction bag along the top of the label.

\*SEB1 Buffer is required to perform this assay.



Insert the sample between the mesh linings near the bottom of the sample extraction bag.



Extract the sample by rubbing it gently between the mesh linings with a blunt object such as a pen or permanent marker.

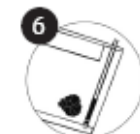
Depending on the sample type, the color of the solution will turn a light brown or green color once the sample is adequately extracted.

#### Perform Assay



Insert the ImmunoStrip into the channel portion (no mesh) of the buffer filled bag.

\*Be sure to insert the "sample" end of the strip no more than 1/4" or to the white line on the ImmunoStrip label.



Allow the ImmunoStrip test to remain in the sample extract for 30 minutes. Positive results may be visible in as little as 5 minutes. Lower titer samples may take up to 30 minutes.

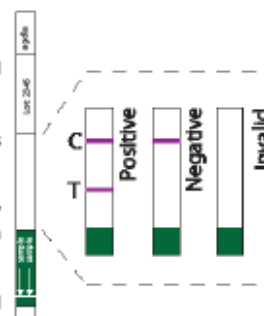
#### 7 Interpret Results

Remove test strip from extract and interpret results (see illustration).

If only the control line (C) is visible, this indicates a negative result.

If the test line (T) is also present at any intensity of pink / purple, this indicates a positive\*\* result.

If no lines are present, the test is invalid (see troubleshooting).





## FTA® Technology

[Home](#) > [DNA Extraction & Quantitation](#) > [Other Extraction Techniques](#) > [Other Techniques](#) > FTA® Technology



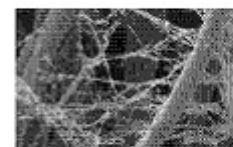
FTA® is an acronym for fast technology for analysis of nucleic acids. It was originally developed by Burgoyne and Fowler at Flinders University in Australia in the 1980s as a means of protecting nucleic acid samples from degradation by nucleases and other processes. The concept was to apply a weak base, chelating agent, anionic surfactant or detergent, and uric acid (or a urate salt) to a cellulose based matrix (filter paper). A sample containing DNA could then be applied to the treated filter paper for preservation and long term storage.

Whatman® licenses the FTA® technology from Flinders University. They offer a line of products using this technology, most notably filter paper cards.



**Click here to read more about filter paper cards on Whatman's website.**

Biological samples, such as blood and saliva, adhere to the paper through the mechanism of entanglement, while the mixture of chemicals




lyses cells and denatures proteins. Because nucleases are inactivated, the DNA is essentially stable when the sample is properly dried and stored. Nucleic acid damage from nucleases, oxidation, ultraviolet light (UV) damage, microbes, and fungus is reduced when samples are stored on the FTA card.<sup>09, 10</sup>

A marketable advantage of the FTA® technology is that samples spotted on treated cards may be stored at room temperature. The chemicals on the FTA cards enhance the preservation of the DNA and inactivate many dangerous pathogens that may be found in liquid blood samples or dried biological stains. Because the cards are small in size (approximately 3.5" x 5"), they are easily packaged, shipped, and stored for databasing.

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Source: [http://www.nfstc.org/pdi/Subject03/pdi\\_s03\\_m04\\_02\\_d.htm](http://www.nfstc.org/pdi/Subject03/pdi_s03_m04_02_d.htm)

Product Overview	
<b>Test Label:</b> NA <b>Test Format:</b> ImmunoStrip <b>Capture Reagent:</b> Polyclonal <b>Detection Reagent:</b> Monoclonal	
<h3>Intended Use:</h3> <p>The <i>Phytophthora</i> ImmunoStrip is designed for on-site testing of plant tissues suspected of infection with <i>Phytophthora</i> species. The test is recommended for use as a preliminary screening tool in survey programs for <i>Phytophthora</i> species such as <i>P. ramorum</i> (Sudden Oak Death) and <i>P. kernoviae</i>. The test is also suitable for detection of <i>Phytophthora</i> species that affect other important crops such as <i>P. fragariae</i> in strawberry or <i>P. infestans</i> in potato.</p>	
Technical Information	
Related Products	

Source:

[https://orders.agdia.com/InventoryD.asp?loc=IN&collection=ISK%2092601&attribute\\_Size=25](https://orders.agdia.com/InventoryD.asp?loc=IN&collection=ISK%2092601&attribute_Size=25)

## Phytophthora spp. ALERT-LF™


Categories » *Phytophthora* spp. » *Phytophthora* spp. ALERT-LF™

ALERT-LF™ offers a range of devices for the detection of key fungal pathogens in symptomatic plant material.

These kits provide real-time, meaningful results promoting rapid disease identification and decision making.

No other equipment required, all materials included.

No training or laboratory skills required.



Source:

[http://plant.neogeneurope.com/product.asp?strParents=&CAT\\_ID=161&P\\_ID=614&numCurrencyID=2](http://plant.neogeneurope.com/product.asp?strParents=&CAT_ID=161&P_ID=614&numCurrencyID=2)



## PuReTaq Ready-To-Go PCR Beads

### Description

illustra™ PuReTaq™ Ready-To-Go™ PCR Beads are premixed, predispensed, single-dose reactions optimized for performing standard PCR amplifications. The use of recombinant PuReTaq DNA Polymerase and other high-purity reagents ensures reliable and robust performance in both end point and real-time fluorescence-based PCR amplification, and ensures the lowest possible levels of contaminating prokaryotic and eukaryotic nucleic acid. PuReTaq Ready-To-Go PCR Beads are preformulated to ensure greater reproducibility between reactions, minimize pipetting steps, and reduce the potential for pipetting errors and contamination. The only additional reagents required are water, primers, and template DNA. The beads are provided predispensed into either 0.2-ml or 0.5-ml PCR tubes. The 0.2-ml tubes are also supplied in a 96-well (8 × 12) plate format that allows individual strips of eight tubes to be easily removed. This flexibility allows use of either the entire 96-well plate, strips of eight, or individual 0.2-ml tubes.

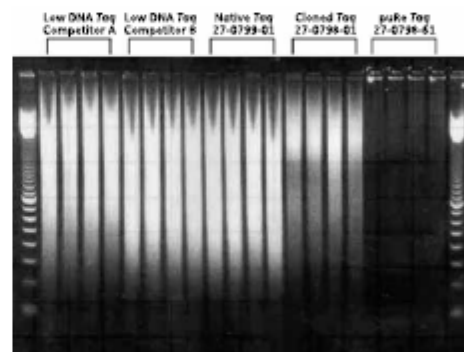


Fig 1. PCR amplifications were performed with two different commercial sources of "low DNA" Taq DNA polymerase, native and cloned Taq DNA polymerases, and with PuReTaq DNA polymerase. Random primers were used without addition of template DNA. All reactions were amplified via standard PCR protocol with 25- $\mu$ l reactions: 95 °C for 5 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. Reactions were analyzed by gel electrophoresis with a 10  $\mu$ l loading volume.

### Source:

[https://www.gelifesciences.com/gehcls\\_images/GELS/Related%20Content/Files/1314750913712/litdoc11002607AB\\_20110831023628.pdf](https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314750913712/litdoc11002607AB_20110831023628.pdf)

# GelRed™ & GelGreen™



**G**elRed and GelGreen are a new generation of fluorescent nucleic acid gel stains designed to replace the highly toxic ethidium bromide (EtBr). Developed by scientists at Biotium, GelRed and GelGreen are superior to EtBr and other EtBr alternatives by having a combination of low toxicity, high sensitivity and exceptional stability.

EtBr has been the predominant dye used for nucleic acid gel staining for decades because of its low price and generally sufficient sensitivity. However, EtBr is a highly mutagenic material. The safety hazard and costs associated with decontamination and waste disposal can ultimately make the dye expensive to use. For this reason, alternative gel stains, such as SYBR® dyes, have become commercially available in recent years. Although these alternative dyes have reduced mutagenicity, they often have to sacrifice other aspects of the dyes. For example, SYBR® Safe has very limited sensitivity while SYBR® Green and SYBR® Gold are much less stable than EtBr. SYBR® dyes also enter cells rapidly to stain mitochondria and nuclear DNA, making it more likely for the dyes to be toxic at high enough concentrations. Indeed, SYBR® Green I is known to strongly potentiate mutation caused by UV light or another mutagen (Ohta, et al. *Mutat. Res.* 492, 91(2001)).

To make GelRed and GelGreen safe, scientists at Biotium used a novel yet very simple concept: reducing genotoxicity by preventing the dyes from entering living cells. We believe that a DNA-binding dye can be made non-mutagenic or substantially so by denying its chance to be in contact with genomic DNA in living cells. Thus, we engineered the chemical structures of GelRed and GelGreen such that the dyes are incapable of crossing cell membranes. The Ames test confirmed that GelRed and GelGreen are nonmutagenic at concentrations well above their working concentrations used for gel staining. Furthermore, environmental safety tests showed that GelRed and GelGreen are nonhazardous and nontoxic to aquatic life. As a result, GelRed and GelGreen can be disposed of down the drain or in regular trash. For more information, please download the GelRed/GelGreen Safety Report on Biotium website.

GelRed and GelGreen are highly sensitive either as precast gel stains or post gel stains. Designed primarily for use with a 312/302 nm UV transilluminator, GelRed is much more sensitive than EtBr, and at least as sensitive as or brighter than SYBR® Gold in post gel staining. Unlike SYBR® Gold, GelRed can also be used as a highly sensitive precast gel stain. GelGreen is developed to meet the needs of researchers who use a 488 nm laser-based gel scanner or a Dark Reader that uses a visible blue light for excitation. GelGreen is spectrally similar to SYBR® Safe, but is far more sensitive than the latter.

Another major advantage of GelRed and GelGreen is their remarkable stability. You can handle the two dyes the same way you do with EtBr. This means that the dyes are perfectly stable in water at room temperature for long-term storage, and they can be microwaved for making precast gels. Both dyes are also very photostable, permitting their use under normal room light without exercising special precaution.

A complete list of GelRed and GelGreen products is shown in Table 1.

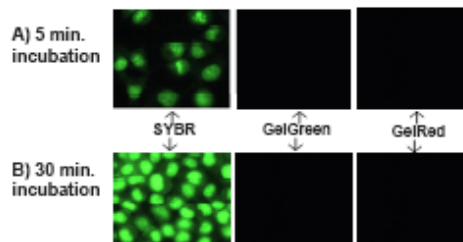


Figure 4. HeLa cells were incubated at 37 °C with 1X of SYBR® Green I, SYBR® Safe, GelGreen™ and GelRed™, respectively. Images were taken following incubation for 5 min (panel A) and 30 min (panel B), respectively. SYBR® Green I and SYBR® Safe entered into cells rapidly as evident from the bright green nuclear staining (images from SYBR® Safe not shown). However, GelRed™ and GelGreen™ were unable to cross cell membranes as shown by the lack of any fluorescence staining.

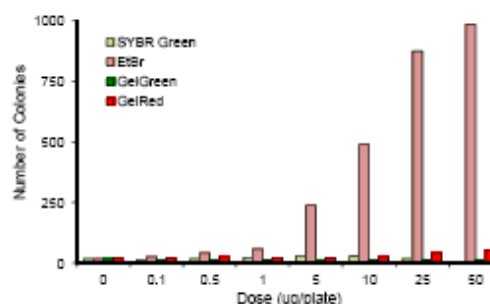


Figure 5. GelRed™ and GelGreen™ are non-mutagenic. Comparison of mutagenicity among GelGreen™, GelRed™, SYBR® Green I and EtBr in +1 frameshift Salmonella indicator strain TA98 in the presence of S9 fraction. SYBR® Green I became cytotoxic at 50 ug/plate. For more information, download the GelRed™ and GelGreen™ Safety Report at [www.biotium.com](http://www.biotium.com)

Table 1. Gel Stain Product List

Cat.#	Product Name	Unit Size
41003	GelRed™, 10,000X in H <sub>2</sub> O	0.5 mL
41003-1	GelRed™, 10,000X in H <sub>2</sub> O	10 mL
41001	GelRed™, 3X in H <sub>2</sub> O	4 L
41002	GelRed™, 10,000X in DMSO	0.5 mL
41005	GelGreen™, 10,000X in H <sub>2</sub> O	0.5 mL
41005-1	GelGreen™, 10,000X in H <sub>2</sub> O	10 mL
41004	GelGreen™, 10,000X in DMSO	0.5 mL

\* GelGreen™ and GelRed™ and their uses are covered by US patent numbers 7950496, 7802943, and 8232050.

\*\* SYBR is a trademark of Molecular Probes, Inc.

Source:

[http://www.biotium.com/product/product\\_info/flyer/GelRed%20&%20GelGreen%20Flyer.pdf](http://www.biotium.com/product/product_info/flyer/GelRed%20&%20GelGreen%20Flyer.pdf)

## DNeasy Plant Mini Kit

Print Share

Add to wishlist

For isolation of up to 30 µg total cellular DNA from plant cells and tissues, or fungi



- Pure DNA, free from contaminants and enzyme inhibitors
- Rapid isolation of ready-to-use DNA
- No organic extraction, no ethanol precipitation

The DNeasy Plant Mini Kit provides fast and easy silica-based DNA purification in convenient spin column format. Typical yields are 3–30 µg of high-quality DNA, depending on the samples used. Purification of DNA using the DNeasy Plant Mini Kit can be automated on the QIAcube.

Source: <http://www.qiagen.com/Products/Catalog/Sample-Technologies/DNA-Sample-Technologies/Genomic-DNA/DNeasy-Plant-Mini-Kit>

## Gentra Puregene Yeast/Bact. Kit

Print Share

Add to wishlist

For purification of archive-quality DNA from yeast and Gram-positive or Gram-negative bacteria



- Archive-quality DNA for long-term storage
- Reproducible DNA purification
- A complete solution for sample-to-storage purification
- Convenient, scalable purification procedure

The Gentra Puregene Yeast/Bact. Kit enables purification of high-molecular-weight (100–200 kb) DNA suitable for archiving. The scalable purification procedure gently removes contaminants and inhibitors and allows samples to be purified for

use as long-term references.

Source: <http://www.qiagen.com/Products/Catalog/Sample-Technologies/DNA-Sample-Technologies/Genomic-DNA/Gentra-Puregene-YeastBact-Kit>

## Phytophthora Species ID Worksheet

<b>Name:</b>				
<b>Morphological Identification</b>				
<b>Asexual Structures</b>		<b>Sexual Structures</b>		<b>Chlamydospores, hyphae, culture characteristics</b>
<b>Sporangia</b>		<b>Reproductive behavior</b>		
Papillate		Homothallic		Present
Semipapillate		Heterothallic		Absent
Nonpapillate				
		<b>Antheridia</b>		<b>Hyphal Swellings</b>
<b>Number apices</b>		Amphigynous		Present
one		Paragynous		Absent
more than one				
		<b>Oogonia Size</b>		<b>Culture growth habit</b>
<b>Caducous</b>		<30 um		rosette
yes		>30-40 um		not rosette
no		>40-50 um		
		>50		<b>Culture growth rate</b>
<b>Pedicle Length</b>				slow
short - up to 5 um		<b>Oogonium features</b>		not slow
medium - up to 10 um		ornamented		
long - up to		tapered base		<b>Temperature Optimum</b>
				low < 22 C
<b>Sporangium shape</b>		<b>Oospore size</b>		moderate 22-28 C
spherical		<20 um		high > 28 C
ellipsoid		20-30 uim		
ovoid		>30 um		
obpyriform		>40 um		
obturinate				
obovoid		<b>Oospores</b>		
distorted		plerotic		
		aplerotic		
<b>Sporangium base</b>				
tapered				
not tapered				
<b>Sporangium length/breadth ratio</b>				
<1.6				
>1.6				

### ***Phytophthora* Species ID Worksheet ctd.**

<b>Asexual Structures Ctd.</b>				
<b>Sporangium length</b>				
less than 45 um				
45-75 um				
>75 um				
<b>Sporangiophore features</b>				
sympodia compound				
sympodia simple				
long irregular				
umbellate				
internal proliferation				
basal swelling				
intercallary swelling				
clumps				

Species	Sex	Temp (°C)	Sporangia	Oospores	Antheridia	Oogonia	Chlamydospore	Hypheal Swelling
<i>P. boehmeriae</i>	homothallic	25-32	cauducous, papillate, short pedicels	plerotic	amphigynous	spherical	some	absent
<i>P. cactorum</i>	homothallic	25	caducous, papillate, short pedicels	plerotic	paragynous	spherical or tapering toward the base	present	absent
<i>P. capsici</i>	heterothallic	28	caducous, papillate, very long pedicels, variable in shape	plerotic	amphigynous	spherical	rare	rare
<i>P. cinnamomi</i>	heterothallic	24-28	noncaducous, non- papillate	plerotic	amphigynous	funnel-shaped base	grape-like clusters	coralloid hyphae
<i>P. citricola</i>	homothallic	25-28	noncaducous, semipapillate	mostly aplerotic	paragynous	spherical; funnel- shaped base	rare	absent
<i>P. citrophthora</i>	heterothallic	24-28	noncaducous, papillate	plerotic	amphigynous	N/A	some	absent
<i>P. drechsleri</i>	heterothallic	28-31	noncaducous, non- papillate	plerotic	amphigynous	slightly tapered	some	absent
<i>P. erythroseptica</i>	homothallic	27.5	noncaducous, non- papillate	aplerotic	amphigynous	spherical	absent	absent
<i>P. fragariae</i>	homothallic	18-22	noncaducous, non- papillate	aplerotic	paragynous	tapered bases	absent	small, in clumps
<i>P. nicotianae</i>	heterothallic	27-32	noncaducous, papillate	aplerotic	amphigynous	round and smooth	terminal and intercalary	present
<i>P. meadii</i>	homothallic/ heterothallic	25-30	caducous, papillate, medium pedicels	aplerotic	amphigynous	spherical to pyriform	rare	absent
<i>P. palmivora</i>	heterothallic	27.5-30	caducous, papillate	aplerotic	amphigynous	spherical	terminal and intercalary	absent
<i>P. syringae</i>	homothallic	15-20	noncaducous, semi- papillate	plerotic	paragynous	round	absent	absent
<i>P. tropicalis</i>	heterothallic	24-28	caducous, papillate	plerotic	amphigynous	smooth-walled; spherical; brown pigment	present	absent
<i>P. infestans</i>	heterothallic	20	caducous, semipapillate	aplerotic	amphigynous	globose	absent	absent