

Rapid Diagnostic Tools for *Phytophthora* on Horticultural Crops

Implementación de herramientas de diagnóstico rápido para
Phytophthora en cultivos agrícolas en Centro América

Universidad de Costa Rica
[June 28 – July 2, 2010]



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June 28 – July 2, 2010

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Rapid Diagnostic Tools for *Phytophthora* on Horticultural Crops
Workshop Agenda
June 28 – July 2, 2010

Sunday – June 27th

Registration –

1:30 – 3:00 pm Hotel check-in at D' Galah hotel San Pedro de Montes de Oca, San José, Costa Rica (506) 2280-8092.

6:00 pm Social and Dinner

Monday – June 28th

8:30 – 9:30 am Welcome speech & Introduction of instructors
Speaker: Dr. Felipe Arauz

9:30 – 10:00 am Snack

10:00 – 11:00 am History and importance of the genus *Phytophthora*
Speaker: Dr. Jean Ristaino

11:00 – 12:00 am Isolation and morphological description of the genera
Speaker: Dr. Kelly Ivors

12:00 – 1:30 pm Lunch

1:30 – 3:00 pm **LAB 1:** Field collection of *Phytophthora*
Organizer: Kelly Ivors and Monica Blanco

3:00 – 3:30 pm Snack

3:30 – 5:00 pm **LAB2:** Isolation and morphological observations of unknown species
Organizer: Jean Ristaino, Kelly Ivors and Monica Blanco

6:00 pm Dinner

Tuesday – June 29th

8:30 – 9:30 am *Phytophthora* identification with dichotomous and Lucid keys
Speaker: Dr. Jean Ristaino

9:30 – 10:00 am Snack

10:00 – 11:00 am *Phytophthora* identification with “The *Phytophthora* Database”
Speaker: Dr. Kelly Ivors

LAB4: Use US and NL Phytophthora database and NCBI sequence to identify unknown species

Organizer: Kelly Ivors

11:00 – 12:00 am Digital distance diagnosis, NPDN and the NCSU PDIC
Speakers: Jean Ristaino and Kelly Ivors

12:00 – 1:30 pm Lunch

1:30 – 3:00 pm **LAB3:** Use Lucid Key to identify unknown species
Organizer: Jean Ristaino

3:00 – 3:30 pm Snack

3:30 – 5:00 pm **LAB6-1:** DNA extraction methods: simple to complex and kits
Organizer: Monica Blanco, Luis Gomez, and Peter Bonants

6:00 pm Dinner

Wednesday – June 30th

8:30 – 9:30 am Introduction to molecular techniques
Speaker: Luis Gomez and Monica Blanco

9:30 – 10:00 am Snack

10:00 – 11:00 am **LAB5:** DNA extraction – Direct PCR from fresh/dried tissue (PINF/ITS)
Organizer: Jean Ristaino, Monica Blanco, Luis Gomez, and Peter Bonants

11:00 – 12:00 am **LAB6-2:** DNA extraction methods: simple to complex and kits
Organizer: Monica Blanco, Luis Gomez, and Peter Bonants

12:00 – 1:30 pm Lunch

1:30 – 3:00 pm **LAB7:** PCR-RFLP of ITS amplicons
Organizer: Monica Blanco, Luis Gomez, Jean Ristaino, and Peter Bonants

3:00 – 3:30 pm Snack

3:30 – 5:00 pm **LAB8:** Digest PCR-RFLP of ITS amplicons; Run gel for the ITS PCR products from Lab5
Organizer: Monica Blanco, Luis Gomez, Jean Ristaino, Peter Bonants

6:00 pm Dinner

Thursday – June 30th

8:30 – 9:30 am *Phytophthora* identification: PCR, TaqMan, SSCP, SSR, AFLP, PLP
Speaker: Dr. Peter Bonants

9:30 – 10:00 am Snack

10:00 – 11:00 am **LAB9:** *Phytophthora* identification with TaqMan real time PCR
Organizer: Peter Bonants, Jean Ristaino, and Kelly Ivors

11:00 – 12:00 am **LAB10:** Run gels from PCR-RFLP of ITS amplicons from Lab8
Organizer: Monica Blanco, Luis Gomez, Jean Ristaino, and Peter Bonants

12:00 – 1:30 pm Lunch

1:30 – 3:00 pm **LAB11:** Pack Lock Probe (PLP): Ligation & Exonuclease
Organizer: Peter Bonants, Jean Ristaino, and Kelly Ivors

3:00 – 3:30 pm Snack

3:30 – 5:00 pm **LAB12:** PLP: SYBRGreen PCR of ligated samples; PCR-RFLP analysis for Lab10
Organizer: Peter Bonants, Monica Blanco, and Kelly Ivors

6:00 pm Dinner

Friday – July 1st

8:30 – 9:30 am **LAB13:** PLP: Hybridizations
Organizer: Peter Bonants, Jean Ristaino, and Kelly Ivors

9:30 – 10:00 am Snack

10:00 – 11:00 am **LAB14:** Novel detection methods
Organizer: Dr. Kelly Ivors

11:00 – 12:00 am *Phytophthora* Genome sequences: Uses: AFLP, SSR's, avirulence genes
Speaker: Dr. Peter Bonants

12:00 – 1:30 pm Lunch

1:30 – 3:00 pm **LAB15:** PLP: Stain microarray and interpretation

Organizer: Peter Bonants

“Unknown species revealed” to students

3:00 – 3:30 pm

Snack

3:30 – 5:00 pm

Wrap up: Evaluation, suggestions, questions, discussion
Workshop evaluation forms- Jean Ristaino

6:00 pm

Closing dinner – certificates and travel reimbursements

Table of content

Day 1 –	Lab 1: Field collection of <i>Phytophthora</i>	p. 8 – p. 12
species	Lab 2: Isolation and morphological observations of unknown	p. 13 – p. 19
Day 2 –	Lab 3: Use Lucid Key to identify unknown species	p. 20 – p. 23
sequence to identify unknown species	Lab 4: Use US and NL <i>Phytophthora</i> database and NCBI	p. 24 – p. 27
Day 3 –	Lab 5: DNA extraction – Direct PCR from fresh/dried tissue	p. 28 – p. 31
(PINF/ITS)	Lab 6: DNA extraction methods: simple to complex and kits	p. 32 – p. 33
	Lab 7: PCR-RFLP of ITS amplicons	p. 34 – p. 35
	Lab 8: Digest PCR-RFLP of ITS amplicons; Run gel for the ITS	p. 35 – p. 36 & p. 31
PCR products from Lab5		
Day 4 –	Lab 9: Phytophthora identification with TaqMan real time PCR	p. 37 – p. 40
	Lab 10: Run gels for PCR-RFLP of ITS amplicons from Lab8	p. 35 – p. 36
	Lab 11: Pack Lock Probe (PLP): Ligation & Exonuclease	p.41 – p. 42
	Lab 12: PLP: SYBRGreen PCR of ligated samples; PCR-RFLP	p. 42 & p. 35 – p. 36
analysis for Lab10		
Day 5 –	Lab 13: PLP: Hybridizations	p. 42 – p. 43
	Lab14: Novel detection methods	p. 45 – p. 47
	Lab 15: PLP: Stain microarray and Interpretation; “Unknown	p. 43 – p. 44
species revealed” to students		

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 present, 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*:

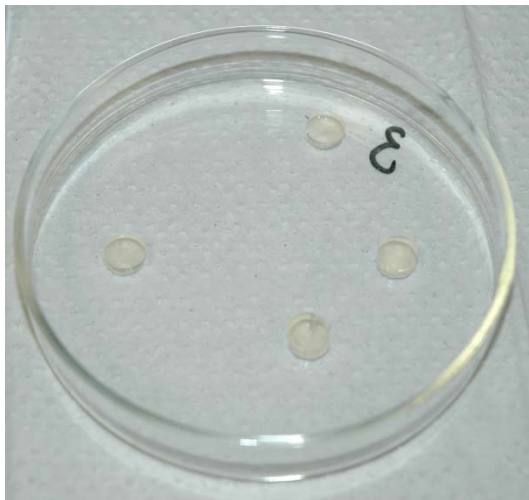
A shared morphological characteristic of *Pythium* and *Phytophthora* spp. is coenocytic hyphae (lacking cross walls). Most true fungi produce hyphae with septa, although some true fungi also lack septa. The presence of coenocytic hyphae provides evidence for *Pythium* and *Phytophthora* but, in itself, 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.

General lab protocol

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.



Original protocol published in *Inoculum*, 56 (6), 2005 by Susan Kaminskyj.

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.

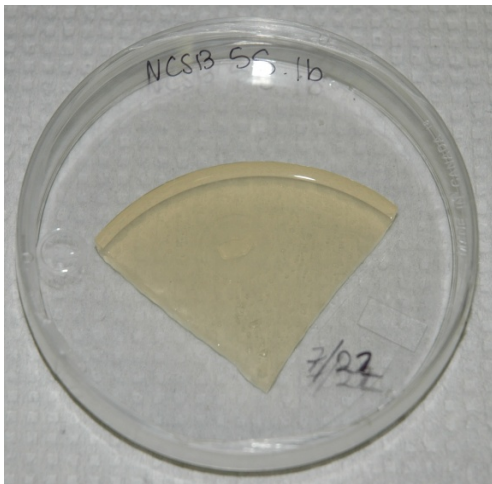


Fig. 3. A quarter-piece of PARP is placed on top of a piece of contaminated *Phytophthora* culture.

Protocol from the lab of Kelly Ivors, North Carolina State University, 2004.

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. 4. 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 disinfection 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, chlamydo spores 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 disinfection 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 P₁₀VP 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
<u>Optional</u>			
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 P₁₀VP 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
<u>Optional</u>			
Hymexazol (99.5ai)**	25 mg/L	25.1 mg/ml	1.0 ml

*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>	<u>PPM</u>	<u>Stock</u>	<u>Use</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 CaCO₃ 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, CaCO₃ (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), CaCO₃ (2g). Add 250ml V-8 broth to 500cm³ 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, CaCO₃ (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% CaCO₃, 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 nonsterile 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 μm), medium (5-10 μm) and long (> 10 μm) 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.

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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 www.Phytid.org/methods. See ITS and BOL maps of gene regions amplified with primers below.

Master mix for each 50µl reaction

		<u>Final concentration</u>
ddH ₂ 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 ₂ (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 MgCl₂** come with **Taq DNA polymerase** (Invitrogen)

2 mM dNTPs: (300 ml)

Add 24 µl of dNTPs mix (100 mM) (Bioline) to 276 µl ddH₂O

Primer stock soln (100 µM)

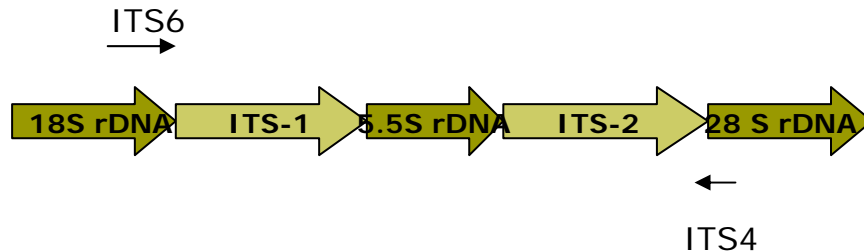
Base on the value of nmole on the tube, we add 10 times of that value of ddH₂O to make 100µM stock soln.

BSA (20mg/ml) – Roche

Primers:

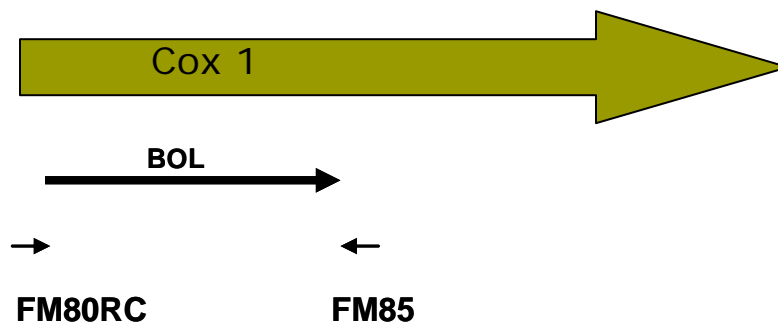
ITS region –

- ITS6 – GAAGGTGAAGTCGTAACAAGG
- ITS4 – TCCTCCGCTTATTGATATGC



BOL region –

- FM80RC – TTTCAACAAATCATAAAGATATT
- FM85 – AACTTGACTAATAATACCAA



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Protocol from the lab of Seogchan Kang (Michele Mansfield and Seogchan Kang), Penn State University, 2008.

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 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.

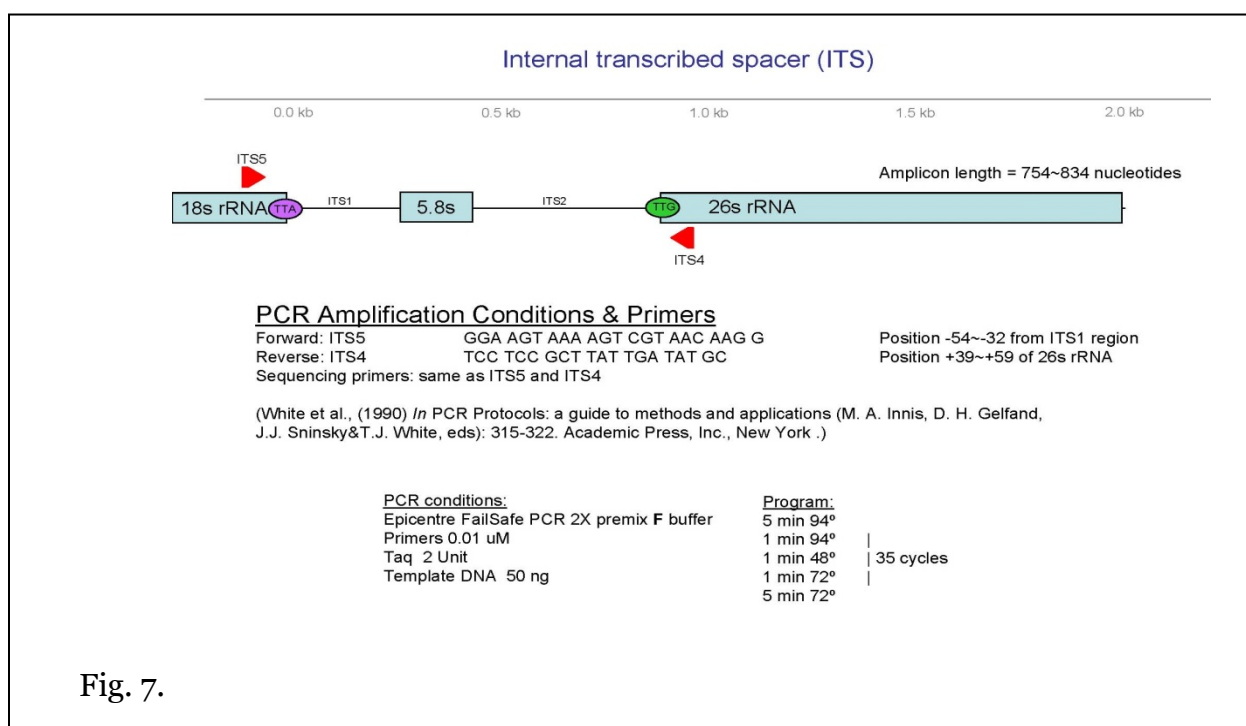
II. Sequence-Based Strain Identification

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) 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.

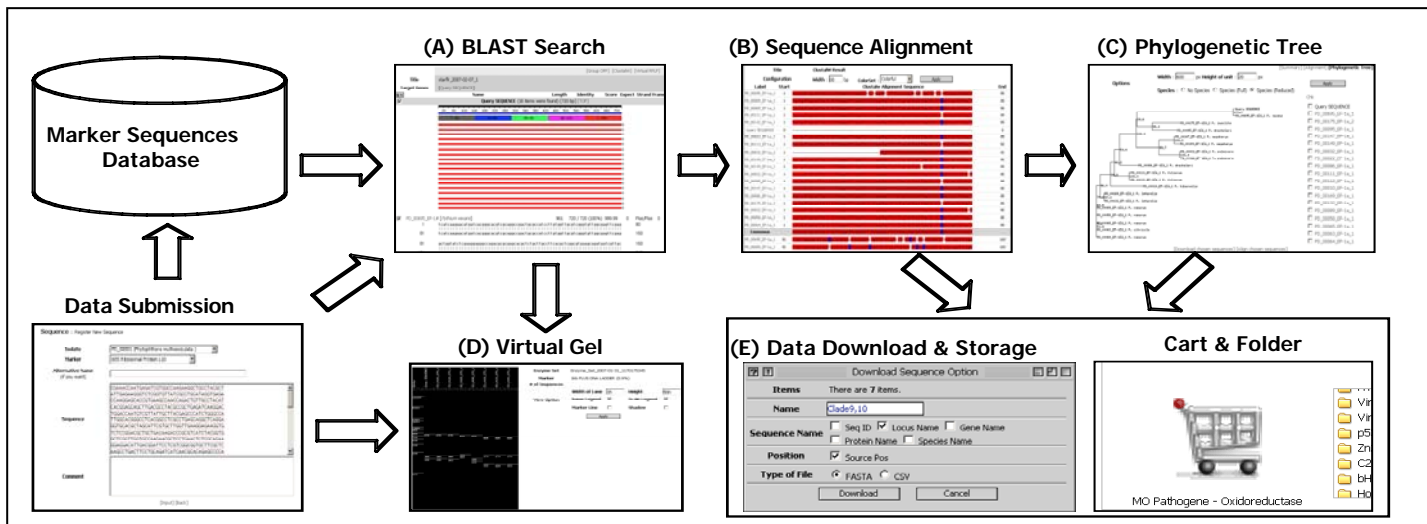


Fig. 8. Overview of the functionality and data flow in PD. This diagram illustrates (A) BLAST, (B) Clustal W: a tool that will align and illustrate a base-by-base comparison between your isolate and the closest matches, (C) Phyloviewer: a tool to visualize the evolutionary relationship between your isolate 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

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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 μ 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 μ l of the solution with the ground leaf tissue to a new tube containing 300 μ 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 :	<u>100 ml</u>	<u>per L</u>	<u>Formula FW</u>
0.5 N NaOH	2g	20g	NaOH (40)
Add to 100 ml H ₂ O.			

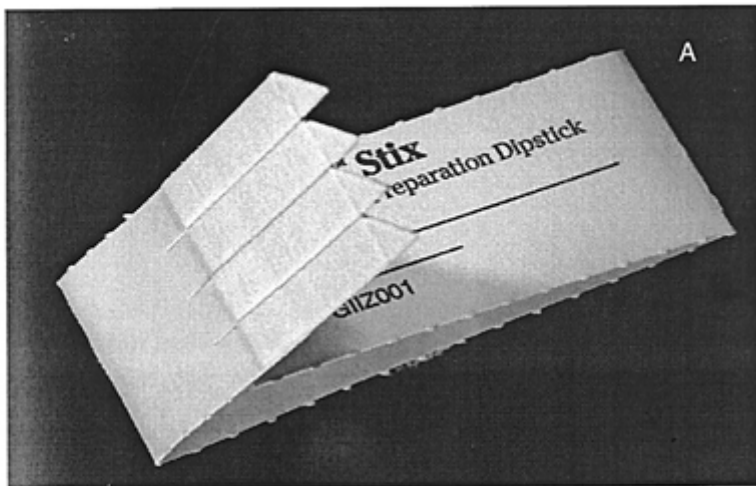
Reference:

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



Quick DNA Extraction with IsoCode™ Stix

1. Remove a small piece of tissue (1 cm²) containing the late blight pathogen from an infected leaf or tuber lesion containing *P. infestans*.
2. Grind the sample in a few drops of water with a clean konte pestle.
3. Place the tissue onto the end of one of the four triangular sample areas of the IsoCode Stix (Fig. 1A).
4. Add a pure culture of the pathogen as a positive control.
5. Dry samples at 80°C for 5 to 10 min in a hot-air oven.
6. Then place each sample triangle over a sterile microcentrifuge tube, and close the lid while pulling the end of the stick to detach the sample triangle directly into the tube.
7. Add 50 µl of sterile distilled water into each tube and wash the sample three times by pulse-vortexing for at least 5 seconds each.
8. Remove the water with a sterile pipette after washing and then centrifuge for 5 seconds to remove residual droplets of water.
9. Resuspend the sample with 50 µl of sterile distilled water.
10. Incubate the tube at 95°C for 30 min and gently mix after incubation to release the DNA from the membrane.
11. Remove the water containing the template DNA to a new tube and use 5 to 10 µl of DNA for each PCR reaction.



*IsoCode Stix can be ordered from Krackeler Scientific, Inc. (www.krackeler.com) with cat.# 32-10495015 (PK of 20) or 32-10495020 (PK of 50)

Fig.1A IsoCode Stix.

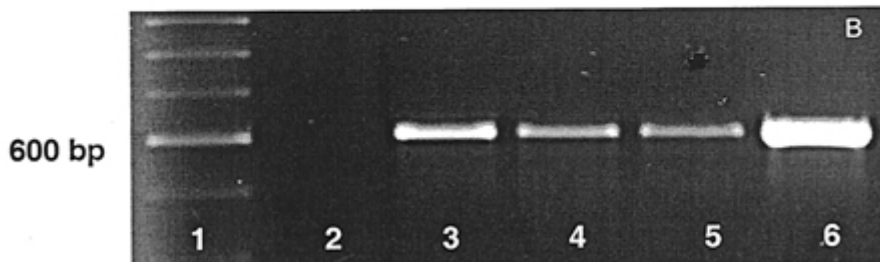


Fig.1B PCR amplification of *P. infestans* DNA by IsoCode Stix. Lane1, 100-bp DNA ladder; lane2, no template control; lane 3-5 contain amplified DNA products from IsoCode Stix extracted from mycelium of *P. infestans*, tubers and leaf lesions, respectively; lane 6, positive control.

PCR with Ready-To-Go™ PCR beads

1. Obtain 5 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. MgCl₂ -- it brings more ions to the reaction solution
 - d. buffer -- it buffers the reaction
2. Add 13 µl sterile water and 2 µl of each primers to each tube.
3. Label 4 tubes and add reagents as follows:
 - a. Add 8 µl of sterile water to tube 1
 - b. Add 8 µl of diluted DNA from the healthy potato leaf to tube 2
 - c. Add 8 µl of diluted DNA from the infected leaf tissue to tube 3
 - d. Add 8 µl 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
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 µM):

Base on the value of nmole on the tube, we add 10 times of that value of ddH₂O to make 100µM stock soln.

Preparing 5 µM Primer soln from primer stock soln:

Add 10 µl of 100 µM primer stock soln into 190 µl 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/aptrix/upp01077.nsf/Content/Products?OpenDocument&moduleid=164859>

Gel Preparation

1. Seal the ends of the gel tray with tape and inset the comb. Pour 1.5% molten agarose at approximately 55-65°C 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.
2. Carefully remove the combs from the gel and place the gel into the gel rig and cover it with 1X TBE buffer.

Expected Results

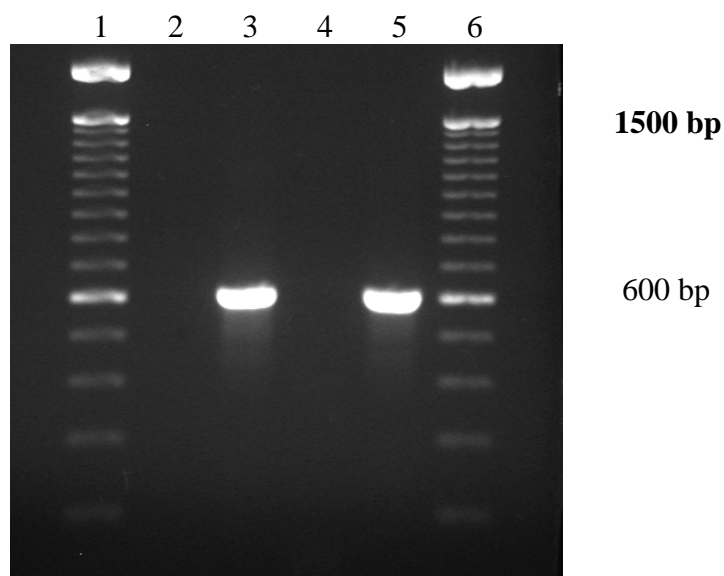


Figure. Amplified PCR products from *Phytophthora infestans* DNA from mycelium and potato leaf tissue. Lanes: 1 and 6 are 100 bp ladder ; 2, negative no template control; 3, *P. infestans* DNA (positive control); 4, dried, healthy potato leaf; 5, dried potato leaf infected with *P. infestans*.

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°C .
3. Add 150 μl Extraction Buffer, vortex. Grind mycelia with sterile Konte pestle.
4. Add 150 μl Nuclei Lysis Buffer and 60 μl 5% Sarkosyl, vortex to mix.
5. Incubate at 65°C for 15-30 min.
6. Add 1 volume ($\sim 300\ \mu\text{l}$) Chloroform (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 (NaOAc), pH 8.0 and 2 volumes of cold 100% Ethanol.
10. Allow DNA to precipitate overnight at -20°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°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	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 H_2O . 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 .

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

Neiva Sánchez-Chiang
Luis Gómez Alpizar

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).

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

Primer	Secuence (5' - 3')
A2 forward	ACTTTCCACGTGAACCGTTTCAA
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 μ l. Prepare the master mix according to the next table (make calculations for 3 reactions):

	1x = 25μl	3 X
dSH ₂ O	12.6 μ l	
Buffer	2.5 μ l	
dNTPs	2 μ l	
primer A2	2 μ l	
primer I2	2 μ l	
MgCl ₂	1.7 μ l	
Taq polymerase	0.4 μ l	
Subtotal	23.2 μl	x
ADN (<i>add at the end</i>)	2 μ l	Add to each tube
Total reaction	25 μl	x

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

Initial denaturation 94°C for 5 min
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 and getting out from the tube). Add 5 µl of Gel Red and mix. When is getting warmer (close to 60 °C), put inside the chamber and put the comb.

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	botton	
2	top		2	botton	
3	top		3	botton	
4	top		4	botton	
5	top		5	botton	
6	top		6	botton	
7	top		7	botton	
8	top		8	botton	
9	top		9	botton	
10	top		10	botton	
11	top		11	botton	
12	top		12	botton	

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

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

RFLP

1) Prepare the enzymatic mixture as follow:

	15 x de 8 µl c/u
dSH ₂ O	18 µl
Tango Buffer	6 µl
Msp I	6 µl
Total	30 µl

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

Protocol 1. Generic ITS-PCR fungi

Generic ITS-PCRs will be performed with DNA Engine (PTC-200) instrument (Veenendaal, Nederland) in 96-wells-plates as described below. In all cases 1 µl of the DNA extract will be used in 24 µl master mix, and all samples will be tested in duplo. Negative controls with Milli-Q water will be used in each run. ITS-PCR will be performed with Roche mastermix (Almere, Nederland).

Samples will be analyzed on agarose gel and stained with GelRed.

The positive or negative result will be based on presence/absence of the amplicon of correct size.

PCR Reaction (25 µl): Mastermix per reaction	Mastermix 24 µl and 1 µl target	Final conc
Roche buffer (10x)	2.5 µl	1x
dNTP (600 µM)	2.5 µl	60 µM
ITS1 (5 µM)	1.0 µl	200 nM
ITS4 (5 µM)	1.0 µl	200 nM
Taq polymerase (5 U/µl)	0.125 µl	0.625 U
Water	16.875 µl	

Forward Primer ITS1	5'- TCCGTAGGTGAACCTGCGG-3'
Reverse Primer ITS4	5'- TCCTCCGCTTATTGATATGC-3'

PCR program:

2 min	94°C
35 cycles:	30 sec 94°C
	30 sec 56°C
	60 sec 72°C
5 min	72°C

Protocol 2a. 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-wells-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_T 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-TCCGTAGGTGAACCTGCGG-3'
 Reverse Primer: RITS-279Ph-Phos 5'- Phosphate-TCCTCCGCTTATTGATATGC-3'
 Probe: All-phy probe 5'-FAM-TTGCTATCTAGTTAAAAGCA-MGBNFQ-3'

PCR program :

2 min 50°C
 10 min 95°C
 40 cycles: 15 sec 56°C
 60 sec 60°C

Protocol 2b. 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-wells-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_T 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-TCCGTAGGTGAACCTGCGG-3'
 Reverse Primer: RITS-279Ph-Phos 5'- Phosphate-TCCTCCGCTTATTGATATGC-3'
 Probe: All-phy probe 5'-FAM-TTGCTATCTAGTTAAAAGCA-MGBNFQ-3'

Forward Primer: FPgfp 5'-TGGCCCTGTCCTTTTACCAG-3'
 Reverse Primer: RPgfp 5'-TTTTCGTTGGGATCTTTCGAA-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:	15 sec	95°C
	1 min	60°C

Protocol 3. *Phytophthora* Identification with a padlock probes mixture and a micro-array system**Ligation:**

If the All *Phytophthora* TaqMan signal (Protocol 1) is positive, 1 µl of the amplicon (10x diluted) will be added to the ligation mixture according to scheme below.

	Final conc.	x1
MQ	-	5.5 µl
<i>Pfu</i> ligase Buffer 10X	1X	1 µl
ss DNA	20 ng	1 µl
PLP mix	100 pM	1 µl
<i>Pfu</i> ligase 4 U/µl	1 U	0.5 µl
Amplicon	1 µl of 10 x dilution	1 µl

Pfu ligase, Stratagene (Bio-Connect, Huissen, Netherlands)

Ligation will be performed in a DNA Engine (PTC-200) instrument (Veenendaal, Nederland) in 96-wells-plates according to protocol described below:

Ligation program :

5 min 95°C
 10 cycles: 30 sec 95°C
 5 min 65°C
 95°C 15 min
 4°C for ever

Perform the exonuclease treatment directly after the ligation.

Exonuclease treatment:

Exonuclease treatment will be performed on ligation mixtures according to protocol described below:

	Final conc.	x1
MQ	-	8.8 µl
Buffer 10x	1X	1 µl
Lambda exo 5 U/µl	1 U	0.2 µl

Add 10 µl of Lambda exo mixture to each ligation tube.

Lambda Exo, New England Biolabs (Westburg, Leusden, Netherlands).

Exonuclease treatment will be performed in a DNA Engine (PTC-200) instrument (Veenendaal, Nederland) in 96-wells-plates according to protocol described below:

Exonuclease Program:

37°C 10 min

75°C 10 min

SYBR Green PCR:

After exonuclease treatment SYBR Green PCR will be performed on the ABI7500 instrument in 96-wells-plates according to protocol below. In all cases 3 µl of the exo mixture will be used in 25 µl master mix, and all samples will be tested in duplo. Negative controls with Milli-Q water will be run as negative controls in each run. Real-time SYBR Green PCR will be performed with SYBR Green premix (ABI, Nieuwerkerk aan de IJssel, Nederland).

	Final conc.	Stock	x1
MQ	-	-	8 µl
SYBR premix (ABI)	1x	2x	12.5 µl
P1-f19-Biot	300 nM	10 µM	0.75 µl
P2-r20-Phos	300 nM	10 µM	0.75 µl
Template	3 µl of ligation/exo mix		3 µl

SYBR Green PCR program:

2 min 50°C

10 min 95°C

20 cycles: 15 sec 95°C

1 min 60°C

Determine the dissociation curve.

Hybridization:

After SYBR Green PCR, samples will be hybridized to the micro-array as described below.

Chip conditioning and hybridization:

- Wash rubbers in 96% EtOH, dry them completely at 65C
- Put rubbers on the slide, and press to the kimwipe tissue
- Add 100 µl of MQ water to each chamber.
- During incubation with water, prepare 5 x amplicon dilution with 1,5 x TMAC (4.5 M TMAC, 0.15% N-lauroylsarcosine, 75 mM Tris-HCl pH 8.0, 6 mM EDTA). To prepare

55 µl of hybridization mix you need: 11 µl of SYBR Green amplicon, 36.7 µl of 1.5x TMAC, 5.9 µl MQ, 1.4 µl of 10 µM corner spot.

- Denature the mixture 95°C/5 min/ice
- During the denaturation remove water from slide and add 100 µl 1 x TMAC (prewarmed) to each chamber. Incubate 2 min/50°C in humidity chamber.
- Go back to the samples: after denaturation place them on ice, than go to incubator for slide. Remove buffer with pipette, do not allow chambers to dry out. Apply 50 µl of each sample to the microarray, don't make any air bubbles on the surface nor leave liquid droplets on the rubbers. Seal the slide with tape.
- Place the slide in the humidity chamber. Incubate 2h/50°C on a shaker. At the same time place the first washing buffer in the incubator.

Washing:

- 1x with 100 µl of 2 x SSC/0.1%SDS (prewarmed) 50°C/2 min
- 1x with 100 µl of 2 x SSC RT/2 min
- 1x with 100 µl of 0.2 x SSC RT/2 min
- Apply and remove every buffer with multichannel pipette

Blocking:

- Add 100 µl of 6xSSPE, 2% milk, 0.05% Triton X-100 with multichannel pipette. Incubate RT/15 min in humidity chamber.

Conjugation:

- Add 50 µl 1:5000 dilution (in 6xSSPE, 0.01% Triton X-100) of streptavidine-conjugated horseradish peroxidase to each chamber (with multichannel pipette, add fast)
- Incubate RT/1 h in humidity chamber, cover with aluminium foil (light sensitive)
- Washing 2 x 1 min with 1 x PBS
- Washing 1 x 30 sec with citric buffer (4.2 g citric acid in 1 l MQ, adjust pH to 4.3 with NaOH)

Staining:

Add 50 µl of o-dianisidine solution to each chamber with a multi channel. Add just before staining enough solution to 8 PCR tubes for pipetting 3x 50 µl with multi channel

- Incubate RT/1-10 min
- Carefully remove o-dianisidine with a pipette, measurement on dry surface
- Optional: Add 2 x 100 µl of PBS, remove carefully with pipette

Visualisation and signature identification:

Signal of the micro-array will be scanned by the Array Reader developed by MicroDiscovery and data analysis will be performed in Excel.
Signatures can also be scored visually by a microscope and a digital camera.

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.



ALERT LF™ *Phytophthora* spp

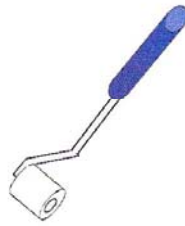
1



(1)



(2)



(3)

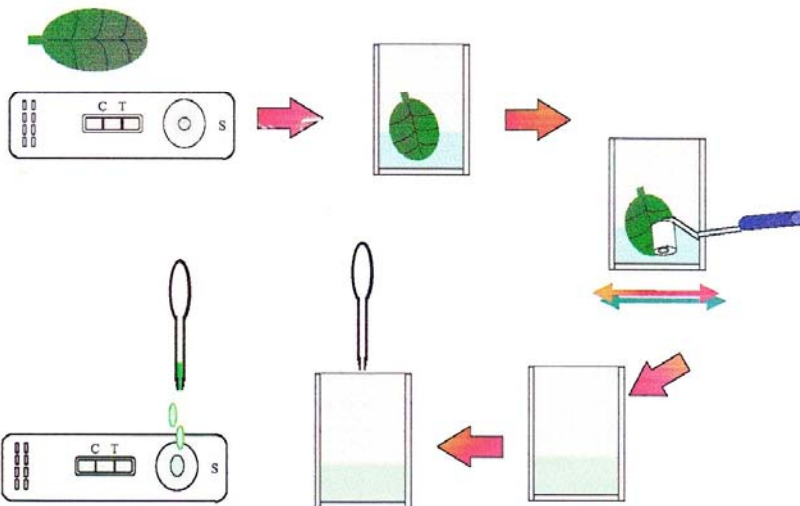


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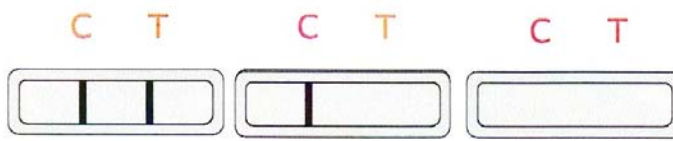


(5)

2



3



Positive

Negative

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 (2).
Smaller pieces of material from several parts of the plant may give better results.
2. Add the contents of the extraction bottle (1) to the extraction bag (2) containing the plant sample. Use the extraction device (3) to macerate the sample. Do not add too much material to the extraction bag.
3. Remove the lateral flow device (4) from the foil pack.
4. Draw into the transfer pipette (5), 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