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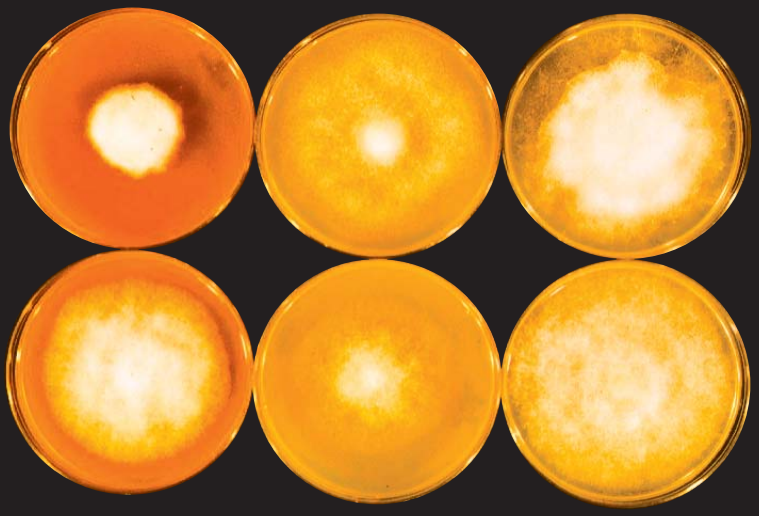
LABORATORY MANUAL FOR *P. INFESTANS* WORK AT CIP



MAY 2015

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Many of the methods presented here were developed in other laboratories, in particular Dr. Fry's laboratory in Cornell, U.S.A., Dr. Shaw's laboratory in Bangor, G.B., Dr. W. Flier in Wageningen and in Dr. D. Coocke in James Hutton Institute. Many thanks to these and other colleagues who have offered useful advice for our work on *Phytophthora infestans.*

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1 ISOLATION OF *P. infestans* FROM INFECTED TISSUE

There are different ways of isolating *P. infestans* from infected tissue but two common ways are 1) to transfer fungal hyphae and sporangia directly onto medium in a Petri dish, and 2) to place infected plant tissue on selective medium. The first of these generally involves growing the fungus on potato tubers and the second on tuber or leaf tissue. In our experience, potato genotypes are easier to isolate with method 1, and tomato isolates with method 2. In any case, it is always easier to isolate from a recent infection, which is just beginning to sporulate.

P. infestans is relatively easy to keep alive by repeated inoculations on living tissue. On the other hand, *P. infestans* is sometimes difficult to get into pure culture. Therefore, keep your isolate alive on living tissue until it is successfully purified.

1.1 Isolation *P. infestans* from infected potato leaves

1. Sporulating lesions on leaf tissue taken from the field are washed in fresh water and placed in a humid chamber (inverted Petri dish with water agar) with the leaf's abaxial side up.
2. Plates are incubated at 15-18°C for 1 day or until fresh sporulation appears.
3. Small pieces of infected tissue from the sporulating border of the lesion are cut and placed under potato slices in an empty Petri dish.
4. Dishes are incubated at 15-18°C for 1 week, until there is abundant sporulation on the upper side of the slice.
5. To re-inoculate leaves, pick sporangia from the top of the tuber and place them in a drop of water on a potato leaf or another tuber slice.

You can repeat steps 2-5 several times to keep your isolate alive (Figure 1).

1.2 Isolation of *P. infestans* from infected tomato leaves

1. Sporulating lesions on leaf tissue from the field are washed in fresh water and placed in a humid chamber with the leaf's abaxial side up.
2. Plates are incubated at 15-18°C with a 14hour light period for 1 day or until sporulation appears.
3. Small pieces of infected tissue from the sporulating border of the lesion are cut out and placed on top of a drop of water on the abaxial side of tomato leaves in a humid chamber (upturned Petri dish containing water agar).
4. Dishes are incubated at 15-18°C with a 14 hour light period for 1 week, or until there is abundant sporulation.
5. To re-inoculate leaves, pick sporangia from the top of the leaf and place them on a drop of water on a tomato leaf, or place small drop of water directly onto the sporulating lesion, wash the lesion several times with the same drop of water, then inoculate the abaxial surface of a new leaflet with the sporangial suspension obtained.
6. You can repeat steps 2-5 several times to keep your isolate alive.

This can be done as in Figure 1, but by substituting a tomato leaf for the tuber slice.

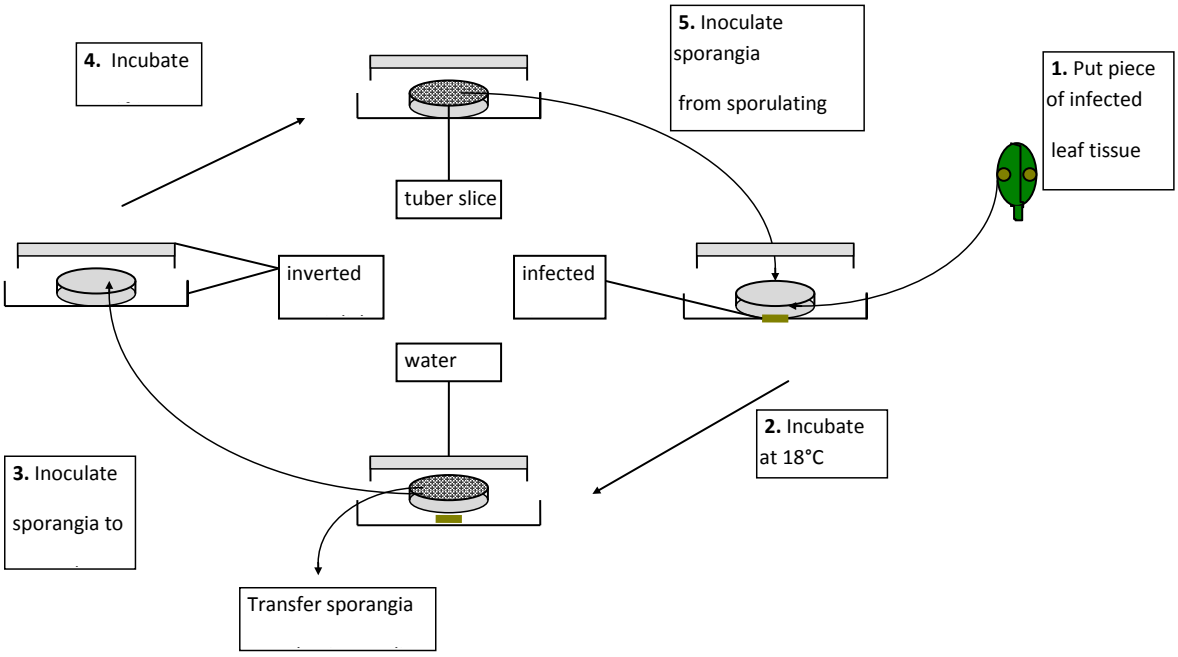
1.3 Isolation of *P. infestans* from infected tubers

If isolating from infected tubers, slice the tuber where infection has occurred and place in a moist chamber until sporulation occurs; then follow steps on next section.

1.4 Isolation in agar

1.4.1. After transfer from potato slices. When clean inoculum appears on the upper side of an infected tuber slice or leaf, the sporangia are harvested in a flow chamber, by picking them up with an inoculating needle and placing the sporangia on selective medium. Do not touch the potato slice or leaf with the needle. The use of a stereoscope is helpful.

1.4.2. Directly from infected leaf or stem tissue. It is possible to isolate the fungus directly from the infected leaf tissue, but it is advisable to produce fresh inoculum at least once. To isolate directly, a small piece of infected leaf from the sporulating border, including a little bit of green tissue, is cut out and passed through a 5% commercial bleach solution for 30 seconds, then rinsed in sterile distilled water twice, and dried off with sterile filter papers. The leaf pieces are then placed on top or inside a selective medium. The plates are incubated 18°C for 5-10 days, or until the fungus starts growing and feeding on the agar. Hyphal tips are then transferred to V8 or Rye B agar plates.



2 MULTIPLICATION OF *P. infestans*

We have two methods for multiplying *P. infestans*: 1) on potato slices or leaves, and 2) on pure culture on agar plates.

2.1. ON POTATO SLICES OR LEAVES

P. infestans is frequently multiplied on potato slices or leaves, primarily for inoculum production, but also for maintenance.

2.1.1. Inoculation of tubers

1. Select tubers from susceptible varieties, medium sized if available, without rots, severe damage or green coloration.
2. Wash the tubers thoroughly and let them dry.
3. Sterilize surfaces of tubers by dipping them in 70% alcohol for a few seconds, and burning off.
4. With a knife dipped in alcohol and burned off, cut the potato into about 1cm thick tuber slices. Each slice should have two cut surfaces. The outer most parts of the tubers are discarded.
5. Place the potato slices on laminated and sterilized wire mesh into plastic boxes with damp filter paper or a little amount of water at the bottom. The mesh should be at least 1cm above the bottom of the tray (a). Two drops of sporangia or zoospore suspension are placed on tuber slices.
6. Put another mesh over tuber slices (b), avoiding touching them and cover it with wet paper towel (c). The boxes are closed with the purpose to originate a moist chamber. Incubate at 15-18°C for 1 week under alternating light (12 h darkness and 12 h light).

A note on contamination! Be careful with contamination of tuber slices (and to some extent leaves) with Erwinia and other organisms that may attack potato. Always inoculate tuber slices with very clean (filtration as above) and very dilute amounts of inoculum. A 10-microliters drop with about 50 zoospores is plenty of inoculum for tuber slices and will guard against contamination. Also, you may just transfer pieces of hyphae and sporangia as shown in Figure 1.

2.1.2. Inoculation of leaves

1. Cut leaflets of fully expanded potato or tomato leaves, from greenhouse grown plants, which are not flowering, and place them in fresh water. Leaves must be healthy with no signs of disease or stress.
2. Rinse the leaves and pat them dry with paper towel.
3. Place leaves abaxial side up in the lids of inverted Petri dishes containing water agar, and inoculate with two drops of sporangial or zoospore suspension of P.infestans.
4. Incubate at 15-18°C for 1 day in the dark, then for 6 days with a 14-hour light cycle.

For maintenance of cultures of *P. infestans* either one of these two procedures may be repeated once a week. Only spores formed on the non-inoculated side of the potato slices of leaves are harvested. For inoculum production, spores from the inoculated side of the slices or leaves may also be harvested if no bacterial colonies are visible.

2.1.3. Harvesting of sporangia and zoospores

1. Sporangia are washed from the upper side of a sporulation lesion on a potato slice or tomato leaf with distilled water, and passed through a 30µm mesh filter to remove mycelium and other debris.
2. The filtrate is then passed through a 10-micron mesh filter, which traps the sporangia. These are washed several times with clean water, and then collected from the filter with a small amount of distilled water.
3. This sporangial suspension is incubated at 6°C for 2 hours to promote zoospore release.
4. To separate sporangia from zoospores, the suspension is passed again through the 10-micron mesh filter once the zoospores have been released, and the filtrate, containing only zoospores is collected.
5. The zoospore suspension is inoculated onto tuber slices or potato leaves (in the case of inoculum from potatoes) or tomato leaves (in the case of tomato inoculum) to keeps the fungus growing.

In CIP we use Millipore filters system (Complete Sterifil Aseptic System, 47 mm, Cat.XX11 047 00, Millipore Corporation, USA) and Nylon membranes of 10 and 30 µm (Spectra/Mesh Nylon filters cat. 146506, Cat. 146514).

2.2. ON AGAR PLATES

Basically all these recipes come from Caten and Jinks [Caten, 1968 #3268; Caten, 1970 #2098], and may have been slightly modified by Bill Fry's laboratory in Cornell.

2.2.1. V8-based media

Ingredients (for 1 liter)	10% Clarified	10% Unclarified	15% Unclarified	20% Unclarified
V8 juice	150ml	100ml	150ml	200ml
CaCO3	1.5g	1g	1.5g	2g
ß-sitosterol	0.05g	0.05g	0.05g	0.05g
Agar	15g	15g	15g	15g
Purpose	Mating type	Sporulation, Selective*	Sporulation	Sporulation

1. Combine V8 juice and enough distilled water up to bring up to 1 liter.
2. Add $CaCO_3$ and ß-sitosterol and mix well.
3. Then add agar and autoclave at 15 psi for 20 minutes.
4. Stir medium while dispensing to insure good mixing of $CaCO_3$.

For the clarified medium, first centrifuge the 150ml of V8 for 5 minutes at maximum speed, and then use 100ml of supernatant and follow the recipe above.

The selective media is prepared by adding antibiotics (see below)

2.2.2. Rye based media

Ingredients (for 1 liter)	Rye A	Rye B
Rye	60g	60g
Sucrose	20g	20g
ß-sitosterol	0	0.05g
Agar	15g	15g
Purpose	Maintenance	Sporulation, Selective*

The selective media is prepared by adding antibiotics (see below).

- Rye A

1. Soak rye grains in approx. 100ml distilled water for 36 hours. If less dH_2O is used, grains seem to germinate more quickly (24-30 hours).
2. Pour off and reserve liquid.
3. Blend the swollen grains for around 2 minutes (can add some dH_2O), and incubate for 3 hours at 50°C in distilled water. Don't modify time.
4. Filter through four thickness of gauze and discard the sediment.

5. Combine the original supernatant and the filtrate with agar and sucrose. Adjust volume to one liter.
6. Autoclave at 15 psi for 15 min., pour plates.

- Rye A slants

1. Soak rye grains in approx. 100ml distilled water for 36 hours. If less dH_2O is used, grains seem to germinate more quickly (24-30 hours).
2. Pour off and reserve liquid.
3. Blend the swollen grains for around 2 minutes (can add some dH_2O), and incubate for hours at 50°C in distilled water. Don't modify time.
4. Filter through four thickness of gauze and discard the sediment.
5. Combine the original supernatant and the filtrate with agar and sucrose. Adjust volume to one liter.
6. Heat agar on hot plate until agar begins to melt., and put 2-3 ml media/tube.
7. Cap tubes and autoclave at 15 psi for 15 min.
8. Slant the tubes and do not move until they have cooled.

- Rye B

1. Soak grains in distilled water for 36 hours.
2. Pour off and reserve liquid.
3. Boil the rye grains for 1 hour in enough distilled water to cover the grains.
4. Strain through 4 thickness of gauze, and combine filtrates.
5. Add sucrose, agar and ß-sitosterol, and then make up to 1 liter.
6. Autoclave at 15 psi for 15 minutes.

NOTE: Check water level often while boiling the rye, it boils off very fast! If all the water does boil off do not pour more water into dried out and very hot beaker (probably at this point containing burnt rye), it will crack.

2.2.3. Clean up/Selective media

We have two different selective media, one is used in Cornell, and the other developed by Hans Hohl (Hohl, 1991 #3726).

For the Cornell medium:

Antibiotics for Cornell media	
Vancomycin	100mg/L
Polymixin B	50mg/L
Ampicillin	200mg/L
Rifampicin	20mg/L
PCNB (75% WP)	67mg/L
Benlate (50% WP)	100mg/L

1. Prepare 10% unclarified V8, and after autoclaving let the media cool.
2. Add the antibiotic mix above, stir, and pour plates.

For the Hohl medium:

Antibiotics for Hohl media	
Griseofulvin	20mg/L
Nystatin	19mg/L
Benlate (50%WP)	10mg/L
Methoxypurine	5mg/L
Rifamycin	30mg/L
Nalidixic acid	5mg/L
8-azaguanine	40mg/L
Neomycin	30mg/L

1. Prepare Rye B agar and after autoclaving let media cool.
2. Mix the antibiotics in DMSO, and add them to the medium, mix thoroughly, and pour plates.

> You can also prepare antibiotic stocks in 10ml of DMSO and store 1ml aliquots in the freezer. Use 1ml of mix per 1 liter of media.

2.2.4. Other media

Pea Broth:

Ingredients	
Frozen or fresh peas	120g
Purpose	Harvest mycelium

1. Autoclave 120 g of peas in approximately 1 liter of distilled water for 15 minutes.
2. Filter using 6-8 gauze layers. Do not squeeze.
3. Complete the volume up to 1 liter and autoclave again for 15 minutes.

Unclarified V8/Lima bean Agar:

Ingredients (for 2 liters)	
Baby lima beans	160g
V8 juice	200ml
$CaCO_3$	2.8g
Agar	30g
Purpose	Sporulation

1. Autoclave baby lima beans in 400ml of distilled water for 10 min.
2. Strain through gauze and discard the lima beans.
3. Add $CaCO_3$ to V8 juice; bring the volume of the combined juices to 2 liters with distilled water.
4. Adjust pH to 6.0 with KOH or HCl.
5. Add agar and autoclave at 15 psi for 15 minutes and pour plates.

Cornmeal agar (CMA)

(Tuite 1969)

Commeal	60g
Agar	12g
Water	1L

Cornmeal agar (Dehydrated)

Difco Cornmeal agar	17g
V8 juice	1L

Lima bean – dextrose agar

(Thurston 1957. Phytopathoilogy. 47:137)

Fresh french bean	150g
Glucose	20g
Agar	20g
Vitamin B	0.5-1ml
Water	1L

Phytophthora selective medium (PAR)

(Kannwischer and Mitchell, 1978)

Difco cornmeal agar	17g
Deionized water	1L

Combine the ingredients and autoclave. Cool the medium to 45°C and add the following antibiotics as active ingredients:

Pimaricin	10 mg (50mg of 50% a.i.)
Rifampicin	10 mg (Rifampicin SV, 100% a.i.)
Ampicillin	250mg (100% a.i.)

Cheap culture media like V-8:

If you need to prepare V8- media and it is not possible to get a tin of V8 juice, you can use this recipe that works very well.

1. 250 g fresh tomato (organic tomatoes if possible)
2. Blending in 150 ml of distilled water and using only 150 ml of the solution, no filtering
3. Add 1.5 g $CaCO_3$ and ß-sitosterol, and then mix well
4. Add agar (15 g), bring up to 1 liter and autoclave.

3 STORAGE OF *P. infestans*

3.1. On Liquid Nitrogen

We store a dense suspension of sporangia freshly harvested from tubers or leaves.

1. The sporangia are washed with distilled water and concentrated in a filter (10 micron mesh).
2. The suspension is then mixed with 15% DMSO in the filter after the last wash. Wear gloves and work in a well ventilated area.
3. Once the suspension is in the vials they are cooled slowly in an alcohol bath with controlled cooling and constant stirring for 3-4 hours until they reach -50°C. The controlled cooling is done by an immersion cooler. We use a Neslab (cc 60 IIA) cooler and a Neslab Agitainer for holding and stirring the alcohol. Both are available via the big lab equipment vendors.
4. Quickly transfer the vials into the liquid nitrogen.

To thaw, put vials in tap water (20°C) for a few minutes, then the suspension can be put directly on potato slices.

3.1.1 Alternate method from Cornell

1. Put 1 ml 15% DMSO in 2 ml cryovial, autoclave for 15 min.
2. Add 5 plugs (cut with sterile instrument) taken at random 1cm from margin of 1-2 week old plate. A 40mm diameter colony will make 10 plugs if entire colony is used.
3. Snap vials onto cane, when canes are filled, put onto cardboard sleeve and into refrigerator in an open pipette can (4-5 canes/can); after the last cane is in the can, let them all sit for an additional 10 min.
4. Put top on pipette can and place into -80°C for 60 min, then place canes slowly into liquid nitrogen canister.

You can also put the cryotubes in a box and then in a Styrofoam container. Place this in the refrigerator for 30 min. then in the freezer for 25 min., afterwards in -80°C for one night. They can then be put in liquid nitrogen.

To thaw, put in tap water (same as CIP) and then the plugs are transferred to fresh medium.

For both the Cornell and the CIP procedure there is now in the market a container from Nalgene (Mr. Frosty) in which the vials are put in an isopropyl alcohol bath, put in an 'ultra freezer' (-80°C) for 4 hours and then the vials are put onto canes and in the liquid Nitrogen. The method claims its rate of cooling is 1°C/min. The vials can either have agar plugs or spore suspension. This information may be useful [Cunningham, 1973 #4843; Long, 1978. #4844].

3.2. On Agar slants

Isolates are maintained in Rye A agar slants (see media recipes) stored at 15°C. A small actively growing plug of mycelium is put at the bottom of the slant and tubes are sealed with parafilm. New transfers are made every 4-6 months.

3.3 On Screw tubes

1. Wash the rye and add water flooded rye, then place it at lower than 20°C for 24 h.
2. After pouring out the water, put the rye into a tube with screw cap. Every tube contains 3 g of rye and 10 ml of water.
3. The tubes are sterilized at 121°C for 30 min. Note that the tubes lids do not tighten before the sterilization completed, then, do it.
4. We cut the mycelia pathogen from the culture dish into small pieces of 0.25 cm^3, and then put five pieces into one tube. We suggest that each species is kept for at least five tubes. Then store the tubes in a dry and dark cabinet.

4 METALAXYL TESTS

Use 10% unclarified V8 media with the corresponding metalaxyl concentration. The fungicide is prepared in a stock of 100mg/ml. It is made by dissolving 1.1g of 90.6% technical grade metalaxyl in 10 ml of DMSO. Allow medium to cool to about 50°C before adding the metalaxyl.

Three different metalaxyl concentrations are tested and a control without fungicide:

Concentration	0µg/ml	5µg/ml	50ug/ml	100µg/ml
10% V8	1000 ml	1000 ml	1000 ml	1000 ml
DMSO	1.0 ml	0.95 ml	0.5 ml	0 ml
Metalaxyl stock	0 ml	0.05 ml	0.5 ml	1.0 ml

Set up duplicate plates of each of the three concentrations of metalaxyl.

1. Cut uniform size agar plugs from actively growing *P. infestans* cultures and put one plug per plate of metalaxyl. We use the end of sterile large volume Pasteur pipets for a 9 mm size plug.
2. After seven days measure the growth of the fungal colony at right angles, two diameters through the center of each plate. We usually include two known isolates in our tests as checks: one resistant and one sensitive.

Metalaxyl resistance is determined as follows:

Resistant: both 5 and 100µg/ml > 40 % growth of 0µg/ml

Intermediate: 5µg/ml > 40 % growth of 0µg/ml

Sensitive: both 5 and 100µg/ml < 40 % growth of 0µg/ml

5 DETACHED LEAF INOCULATION FOR VIRULENCE TESTS

Virulence is the ability of the pathogen to infect and reproduce on a plant with an identified gene for vertical resistance. This is usually tested with an inoculation on detached leaves. There are several ways of doing this but at this writing ours most resembles that of Cornell. The CIP approach is given below and then the Cornell procedure following.

5.1 CIP procedure for testing virulence

1. Cut leaflets of differentials in the morning. Leaflets should be taken from the upper third of 6-8 weeks old plants (before flowering) and completely healthy. Put the leaflets in plastic bags with water to transport to the lab.
2. Use 2 leaflets per differential per isolate and place them abaxial side up in Petri plates with water agar on the top. It is better to do no more than 10 isolates at a time.
3. A suspension of sporangia is prepared by washing one-week-old inoculated tuber slices or leaves.
4. Incubate suspension at 5°C to promote zoospore release, and once the zoospores are swimming pass the spore suspension through the 10 micron mesh filter, and collect filtrate containing only zoospores.
5. Calibrate zoospore concentration to 2000 per ml with a hemocytometer. Count two sets of five grids for a total of 20 zoospores. 10 grids x 1000 = zoospores / ml.
6. Place one 10µl drop of the calibrated inoculum on each side of the midrib of the leaflet.
7. Incubate the Petri plates at 15-18°C in the dark, then with a 14 hour light period starting the second day after the inoculation, for 6 days.
8. Assess virulence on the sixth day by determining a compatible or incompatible interaction.

5.2 Cornell procedure for testing virulence

The condition of the potato differentials is very important. Plants should be grown only in the green house. During these months the temperature and the photoperiod may be controlled (cooler temperatures and a 16 hour photo period are required to maintain plants for virulence testing).

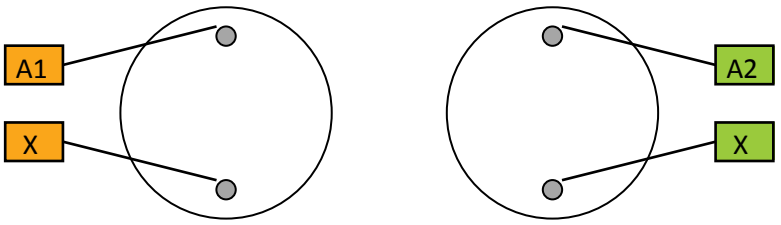
1. Inoculate plates of 10% V8. (If the isolate grows poorly on V8, rye B with ß -sitosterol or Pea agar with ß-sitosterol maybe used instead).
2. Between 1 1/2 and 3 weeks plates become ready for the virulence assay. (Using the binocular microscope, one must observe the plates in question to determine if they have produced enough sporangia for the assay. Plates may be used as soon as they have produced adequate numbers of sporangia. It is a good idea to check and mark the plates to be used about 2 days before you are planning to perform an assay. Plates older than three weeks should not be used. Unless you are only testing for a couple of virulence phenotypes, you will want to limit the number of isolates being tested to less than 10 at any particular time.).
3. Select leaves the morning of the assay. (Leaves selected for the assay should be dark green turgid leaves showing no signs of any disease. Leaves should never be taken from tuberizing or senescing plants. Selected leaves should be placed in bags with water in a Styrofoam box for transportation back to the lab. This protects the plants from the cold and maintains turgor.).

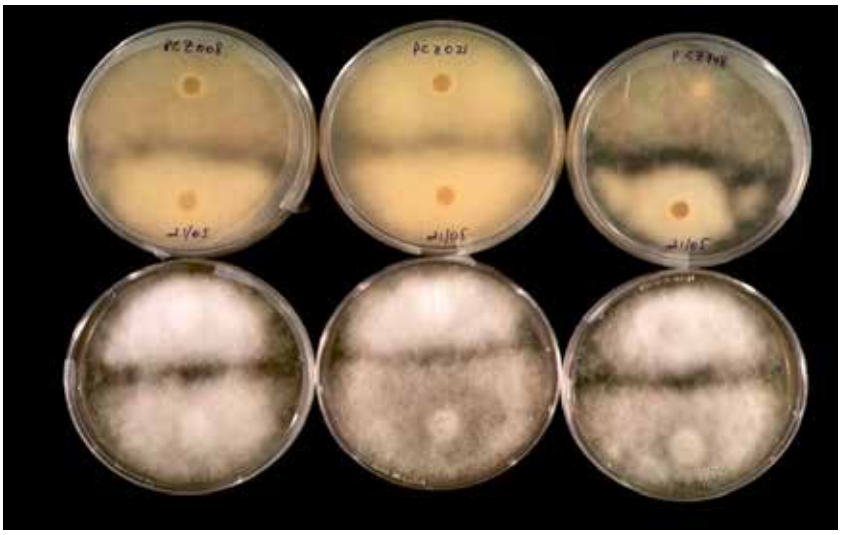
4. Use 4 leaflets per virulence phenotype. Place 2 leaflets into the lids of Petri dishes, randomizing leaflets so that plates to not contain two leaflets from the same leaf. Leaflets may place upside down in order to hold the drop of spore suspension better. (Remove any condensation that may have collected on the Petri plate lids beforehand.).
5. Harvest sporangia using 2-5 ml of sterile water. (The sporangia should detach from the mycelium on contact with water).
6. Determine spore concentration with a hemocytometer. Count two sets of five grids (10 grids x 1000 = sporangia / ml). Use 40.000 - 100.000 sporangia per ml for the virulence assay. (Spore concentration should be determined right after harvest because within an hour sporangia may start to germinate into zoospores complicating this task. After the spore concentration is determined, the spore suspension may be placed in 4°C until ready to be used (up to 3 hours).).
7. Inoculate each leaflet with one drop of the spore suspension using a Pasteur pipette. (Be careful that you don't knock the drop off of the leaflets while handling.).
8. Place plates in the 18°C chamber with a twelve hour photoperiod, 24 hours after inoculations, wrap the plates in parafilm.
9. Six days after inoculation, assess leaflets for virulence.
10. Leaves should be incubated in large Petri plates with water agar (plates should be no smaller than 140mm x 20mm).

NOTE: Each isolate should be tested at least twice. If two plates give enough sporangia for the first test the other two plates may be used to repeat the test.

6 MATING TYPE TEST

Use 10% clarified V8 medium. Place an agar plug of an actively growing isolate you want to test on one side of a Petri dish, and a plug of a known A1 isolate at the other side. In another Petri dish do the same with a known A2 isolate. Seal the dishes and incubate in the dark for 14-21 days, until the 2 colonies have come in contact with each other, and look for oospores using the microscope.





7 PRODUCTION, EXTRACTION AND VIABILITY OF OOSPORES FROM AGAR BASED MEDIA

7.1. *In vitro* production of oospores on solid agar media

1. Transfer isolates to be used as parents in crosses to Petri plates containing Rye A agar (RA) amended with 0.05 g l^{-1} ß-sitosterol and incubate for 10-14 d.
2. Take two or three small agar plugs (5 mm diam.) from the margin of the fast growing colony and place the plugs on one side of a 9 cm Petri plate containing RA amended with 0.10 g l^{-1} ß-sitosterol.
3. Place two or three agar plugs from the other parent on the other side of the plate, about 3 cm apart from the first parent.
4. Seal the plates with Parafilm and incubate in the dark at 15-20 ºC for 14 d (after 7d check for oospore formation at the junction were the two parental colonies meet).

NOTE: In literature, many agar media have been described that facilitate large numbers of oospores to be produced. We have encountered difficulties using V8 based media since a fairly large group of Mexican and western European isolates appears to grow slow on V8 Media. We have tested Pea Agar but found few oospores formed. The best advice is to try several media in your lab and select the one that performs best.

7.2 Extraction of oospores from agar based media

1. Mark the mating region (a distinct band visible by eye showing extensive stimulation of submerged hyphal growth at the interaction zone between the two parental strains) with a permanent marker on the bottom of the Petri dish.
2. Excise the mating region using a scalpel, transfer agar pieces into sterile 50 ml centrifuge ("blue cap") tubes containing 9 ml sterile double distilled water (ddH_2O).
3. Place a homogenizer (we are using a IKA T20 homogenizer with S20 probe) in a laminar flow cabinet and thoroughly sterilize the probe by 30 s full speed mixing using a beaker containing 96% Ethanol. Rinse twice with ddH_2O.
4. Blend the agar for 60 s at 20,000 rpm.
5. Quantify oospore concentration by counting oospores in three 50µl aliquots.

NOTE: A NovoZym treatment can be applied to lyses any mycelial fragments and sporangia in the oospore suspension.

6. Prepare a NovoZym 234 solution by adding 50 mg NovoZym 234 (Novo Biolabs) per ml of ddH_2O.
7. Sterilize the NovoZym solution using a 0.2 µm filter. Keep in refrigerator until use.
8. Add 1 ml of NovoZym solution to 9 ml of oospore suspension; incubate for 24 h at 20 ºC.
9. Wash oospores in three successive steps by adding 25 ml sterile ddH_2O, spinning down the oospores using a tabletop centrifuge, carefully remove supernatant using a pipette and re-suspended in 10 ml ddH_2O.

7.3. Methods for assessment of oospore viability

7.3.1. Oospore germination

1. Lyse mycelial fragments and sporangia in the oospore suspension using the NovoZym treatment.
2. Spread oospores (approx. 1 ml of oospore suspension) on a 9 cm Petri plate containing 10 ml sloppy water agar (5 g l^{-1}).
3. Tape the plates with Parafilm and incubated for 14 d at 20 º under cool blue fluorescent light.
4. Oospore germination can be assessed using a reversed microscope (at a magnification of 10 x 10).

7.3.2. Plasmolysis test

1. Extract oospores from agar plates as described before.
2. Add an equal volume of 4 M NaCl to the oospore suspension and mix by inversion.
3. Incubate the suspension for 3 h at 20 ºC.
4. Plasmolysis can be assessed by micoscopical observation. Viability is expressed as the percentage of oospores that were plasmolysed.

NOTE: In case of routine assessments of oospore viability, there is no need to include a NovoZym treatment prior to the plasmolysis or MTT staining technique, as the NovoZym treatment generally will reduce oospore viability.

7.3.3. MTT viability staining technique

1. Extract oospores from agar plates as described before.
2. Prepare a 0.1 M Phosphate buffer (pH 5.8) and add 0.1 % (0.1 g in 100 ml buffer) tetrazolium bromide (MTT), stir well.
3. Add an equal volume of MTT solution to an oospore suspension in a 50 ml disposable centrifuge tube, close the lid and mix well by inverting.
4. Incubate the oospore suspensions for 2 d at 35 ºC.
5. Transfer a small aliquot (approx. 50 µl) of the suspension to an object glass and examine the colour of individual oospores. Rose coloured oospores are considered to be dormant, blue-pink oospores are assumed to be activated (ready to germinate) and unstained or black oospores are considered to be non-viable.
6. Calculate the percentage viable oospores (dormant + activated) based on at least 250 examined oospores.

NOTE: Tetrazolium bromide is an extremely toxic compound; make sure to protect yourself from exposure to MTT and work according to your local safety regulations.

8 ISOZYME ANALYSIS

We are using 3 methods for this analysis: 1) potato starch gels, 2) cellulose acetate gels, and 3) polyacrylamide gels. We work with two enzymes: Glucose-phosphate dehydrogenase and Peptidase. The starch and cellulose acetate methods are basically Cornell's procedures.

8.1. STARCH GEL ELECTROPHORESIS

8.1.1. Grinding and protein extraction

1. Inoculate pea broth plates (2 plates/isolate; 2-3 plugs/plate) about 10 days to 2 weeks ahead.
2. Harvest mycelium (putting all plates of the same isolate together) and dry by vacuum filtration. Place into a 1.5ml Eppendorf tube, appropriately labelled, and put each tube on ice.
3. Add TC 7 gel "grinding buffer" to each tube. The amount of buffer may vary according to the amount of mycelium, from 100 to 200 µl.
4. Grind with a hand drill, which fit snugly inside the eppendorf tube.
5. Centrifuge tubes at high speed 1minute. They are ready to be used directly or kept in freezer.

Lyophilized mycelium can also be used. You would need only a small amount, just the tip of a spatula, about 1mg, add less grinding buffer, shake well and centrifuge; the samples are now ready to use.

Buffer	Chemical	Molarity	g/L
TC 7 (Grinding buffer)	Trisma base	0.135	16.4
	Citric acid	0.04	9.0
(Adjust pH to 7 with KOH or HCl)			

Measure 70 ml of solution and take up to 1000ml.

8.1.2. Making the Gels

1. Prepare all buffers the night before (gel and electrode) and store them in refrigerator. *Keep all buffers refrigerated.*

Buffer	Chemical	Molarity	g/L	g/4L
TC 6 (GPI)	Trisma base	0.135	16.4	65.6
(electrode)	Citric acid	0.04	9.0	36.0
(Adjust pH to 6 with KOH or HCl)				

Buffer	Chemical	Molarity	g/L	g/4L
TC 8.0 (PEP)	Trisma base	0.687	83.2	332.8
(electrode)	Citric acid	0.157	33	132
(Adjust pH to 8.0 with KOH or HCl)				

Buffer	Chemical	Molarity	g/L	g/4L
HIS 6 (GPI)	Histidine-HCl	0.01	2.1	8.4
(gel)				
(Adjust pH to 6 with KOH or HCl)				

Buffer	Chemical	Molarity	g/L	g/4L
TC 8.0 (PEP)	Trisma base	0.023	2.79	7.27
(gel)	Citric acid	0.005	1.05	3.15
(Adjust pH to 8.0 with KOH or HCl)				

2. Weigh 24g of sifted potato starch and put in 500ml vacuum flask.
3. Clamp plastic bars around edges of the glass plates, make sure edges of the bars
4. Touch each other at the corners, and put on top of plastic trays on a level table

Make gels:

a) Add 70ml of the gel buffer to the starch, and stir

b) Begin to heat 130ml of buffer in a 500ml volumetric flask

c) When buffer is boiling vigorously, pour into starch (swirling the starch mixture constantly)

d) Replace the mix onto the hot plate and allow to cook, with occasional swirling, until it is clear and thinner and bubbling actively

e) Remove bubbles by attaching flask to vacuum pump

f) Pour gel into plate with a circular motion and remove any bubbles or lumps with a Pasteur pipette

5. Cut wicks of thick blotting paper.
6. When gels have set, cover gel with plastic wrap.
7. Get samples and marker dye ready; put them in an ice water bath.
8. When gels have completely set, remove the two long (lengthwise) bars from tray.
9. Slice the gel lengthwise about 5cm from edge.
10. Pull slice back about 1cm, and insert wicks soaked in appropriate sample onto gel. Leave room at the end of the gel for the marker dye (add after other wicks).
11. When wicks are on, firmly push slice back up against the wicks leaving no air bubbles.
12. Fill buffer tanks with appropriate electrode buffer (about 1/2 full) and put in handy-wipes folded twice with folded side up.
13. Put handy-wipes onto both sides of the gel, making sure that they are straight and covering about 2 cm of the gel. Cover the gel with plastic film to prevent drying. Attach electrodes. Fill the tanks with more buffer. The level of buffer should be up to the electrode clip, but NOT touching. Turn on power and allow to run for about 15 minutes or until dye has traveled about 1 cm through gel.
14. Then turn off power. Remove wicks. Push the two parts of the gel firmly back together. Turn on power again and allow running for about 14 hours. For GPI set amperage at 75; for PEP set amperage at 30.

8.1.3. Staining

Prepare the following staining buffers and keep them refrigerated.

Buffer	Chemical		g/L
Tris-MgCl (GPI)	Trisma base	0.1M	12.1
(Staining)	MgCl2	0.1%	1.0

Buffer	Chemical	g/L
TBE 8.7 (PEP)	Trisma base	21.8
(Staining)	Boric acid	6.2
	Na EDTA	1.15

Measure 250ml of solution and take to 1000ml with distilled water

An agar overlay staining method is used. Mix all reagents in corresponding buffer and add dissolved agar last. Pour staining solution over gel, let set, incubate at 37°C preferably in the dark for a few minutes, and score.

Glucose phosphate isomerase (GPI)	Amount
Tris-MgCl pH 7	10ml
Fructose-6-phosphate	50mg
NAD	20mg
MTT*	10mg
PMS*	5mg
Glucose-6-Phosphate Dehydrogenase	50 units
Agar 1%	10ml

** Stocks can be made of NAD, MTT and PMS:*

NAD → 200mg + 10ml H2O → take 1ml

MTT → 100mg + 10ml H2O → take 1ml

PMS → 50mg + 10ml H2O → take 0.2ml

Peptidase (PEP)	Amount
TBE 8.7 gel	10 ml
Glycyl leucine	50mg
0-dianisidine	15mg
Peroxidase	4000 units
a-amino acid oxidase	1.5 units
Agar 1%	10ml

8.2. CELLULOSE ACETATE ELECTROPHORESIS

8.2.1. Preparation of Tissue Samples

Tissue can be from mycelia from broth cultures, scraped off plates or slants, or from washed sporangia from infected leaves or tubers. A very small amount is needed.

1. Put sample in 1.5 ml tube with 100 µl of distilled water; grind the tissue with a plastic or teflon pestle adapted to a hand drill.
2. Centrifuge for 1 minute to pellet cell debris. The extracts must be chilled before use to avoid enzyme degradation, or frozen for later use.

8.2.2. Gel and buffer preparation

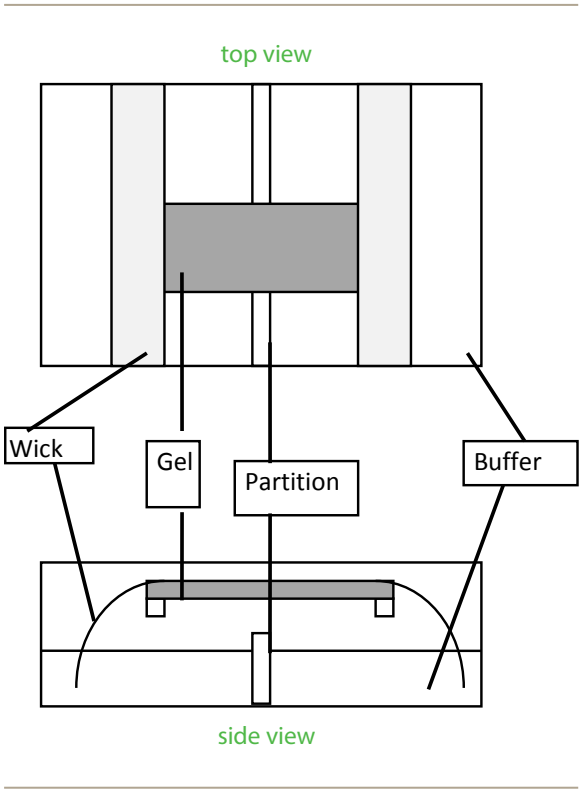
Buffer	Chemical	Molarity	g/L
TG x1	Trizma base	0.025	3g
pH8.5	Glycine, free base	0.192	14.4g

Place several cellulose acetate gels on rack and slowly fill the reservoir with electrode buffer. Care must be taken to avoid air bubbles and splashes. The gels must soak at least 20 minutes before use and can be kept refrigerated for several days.

8.2.3. Gel running

All equipment must be kept spotlessly clean.

1. Fill electrophoresis chambers with electrode buffer and soak in paper wicks. Position the wicks over the supporting rails.
2. Place 8 micro liters of sample on wells of sample plate.
3. Take one gel from buffer and blot dry between two blotting papers to remove excess buffer. Place gel on aligning base over a small drop of water to help the gel stay in place. Work quickly, or the membrane will dry out.
4. Pick up samples with applicator 2 - 4 times, and gently place the applicator in the guides and depress for a few seconds to transfer samples to the cellulose acetate gel. Place a small amount of marker dye (bromophenol blue) next to one extreme of samples.
5. Place gel over the moistened wicks cellulose side down with origin at cathode rail. Ensure a good contact between gel and wicks by placing 2 glass microscope slides on top of the gel.
6. Run the electrophoresis at 200 volts (and about 2mA) for 20 minutes for GPI and 15 minutes for PEP.



8.2.4. Staining

We use the overlay method. It saves staining reagents and produces less amount of toxic waste.

To stain, mix ingredients in buffer and add agar last. Pour solution over gel to cover, incubate until bands appear, then rinse with cold water. Score gel and then dry quickly with hair dryer to save as reference.

Quantities for 2 gels:

Glucose-6-phosphate isomerase (GPI)	Concentration	Amount
Tris-HCl, pH 8.0	0.05M	1.5 ml
Fructose-6-phosphate	20mg/ml	5 drops
NAD	3mg/ml	1 ml
MTT	10mg/ml	5 drops
PMS	2mg/ml	5 drops
Glucose-6-Phosphate Dehidrogenase	1U/µl	2 µl
Agar, @ 60°C	1.4%	2 ml

Peptidase (PEP)	Concentration	Amount
Tris-HCl, pH8	0.05 M	2 ml
Glycyl-leucine	15mg/ml	10 drops
Peroxidase	1000 U/ml	5 drops
o-Dianisidine	4mg/ml	8 drops
$MnCl_2$	20mg/ml	2 drops
a-amino acid oxidse	10 U/ml	5 drops
Agar, @ 60°C	1.4%	2 ml

8.3. PAGE GELS

8.3.1. Prepare the gel mold.

Clean carefully glass plates, wipe them with alcohol. Slightly grease the black rubber spacers, put them in place around the edges of the glass, making sure that the bottom joints are closed with vaseline. Carefully lay the second glass on top and clasp the two plates together. Check that the spacers have not moved. Put the comb in place. Stand the glass plates vertically and fill the mold with distilled water, wait for about 5 minutes to check that there are no leaks, then pour off the water and remove last drops with absorbent paper.

8.3.2. Prepare gel solutions.

The standard gel for isoenzymes contains 7.5% acrylamide.

1. Make a solution of 22.2% (w/v) acrylamide + 0.6% bis-acrylamide (dissolve the acrylamide before adding the bis).

N.B. Acrylamide is toxic when in powder or in solution.
Take care weighing it and wear gloves when dealing with the gel solution. Polymerized gels are not toxic.

22.2% acrylamide, 0.6% bis	35ml soln	35ml soln	30ml soln
	5% gel	7.5% gel	7.5%gel
	7.9ml	11.8ml	10.1
TG buffer (pH8.8)	7.5ml	17.5ml	15.0ml
Dist. water	9.5ml	5.5ml	4.7ml
Degas this solution for about 30secs.			
Add:			
10% Ammonium persulfate	200ul	200ul	171ul
TEMED	5ul	5ul	5ul

2. Mix gently, use a large syringe or pipette to slowly pour into gel mold. Be careful not to trap bubbles, as these are difficult to remove. Tilt the glass plates slightly to one side when reaching the comb and continue to fill the mold very slowly, as this helps prevent bubbles being trapped. Fill the mold completely; if the mold is not full the wells will not form properly. A few drops of distilled water may be gently added on top to prevent drying at the edges and to exclude air from the surface. Acrylamide does not polymerize if in contact with air.

Polymerization is accelerated in the light, so the gel can be put near a window or a lamp to accelerate polymerization.

8.3.3. Storing gels.

1. Gels may be stored for a day or two before use. The comb should be kept in place until use.
2. When the gel has polymerized, the clamps should be removed and the gel can either be completely wrapped in polythene film, or put into place in the electrophoresis apparatus, with the tank buffer in place to prevent desiccation.

8.3.4. Page with stacking gel

Results are usually superior (neater bands and a more horizontal migration) if a stacking gel is made above the main separating gel. The following method is taken from Davis B.J. 1964. Annals N.Y. Acad. Sci. 121: 404-427.

1. Put the mold together as described above, with the comb in place.

Make slightly less gel solution (30 - 32ml) and fill the mold up to about 1.0 -1.5cm below the comb.

In order to make a level surface, insert a syringe needle down towards the surface of the gel solution and very carefully add drops of distilled water to the surface until the water covers the entire width of the gel.

Make sure that the gel is standing on a level surface so that the top of the gel does not set at an angle.

Wait until the gel is polymerized (about 45 minutes) then tip or suck off the water.

2. Stock solutions for stacking gel

Buffer
Tris 5.98%
Adjust to pH6.7 with 1N HCl.
Add 0.2ml TEMED
Take up to 100ml with dist. water

Acrylamide
Acrylamide 20g
Bis acrylamide 5g

Take up to 100ml with water. This is slow to dissolve. Dissolve the acrylamide completely first. Keep in a dark bottle at room temp.

Catalist
Riboflavin 4mg
Take up to 100ml with water
Keep in a dark bottle, at 4°C

Sucrose 40g
Take up to 100ml with water
The gel may be made just prior to use.

3. For one gel in a new mold, mix

1 volume buffer
+ 1 volume acrylamide
+ 1 volume riboflavine
+ 4 volumes sucrose
+ 1 volume water.

1 volume =0.9ml

4. Pour into the mold as described above. Leave in the light to take place polymerization. Light is essential for the polymerization of riboflavin-catalyzed gels.

8.3.5. Setting up the apparatus

1. Remove clamps and the bottom spacer. Pour 2 liters of tank (electrode) buffer (Tris -Glycine pH8.8) into the bottom half of the apparatus, put the gel-holder in place and push the glass plates + gel into position. Melt a little old 2% agarose gel in the microwave, and use this to seal the joint around the glass and the holder. Check that there are no large bubbles trapped at the bottom of the gel, as this will reduce the flow of current through the gel. To remove these bubbles, lift the holder + gel out of the buffer, then slowly lower it in again, keeping it tilted so the air escapes to one side. If bubbles persist, it may be because there is too much Vaseline on the glass, in which case it may be necessary to wipe it off.
2. Carefully remove the comb. Add buffer to the upper part of the apparatus, making sure that the top of the gel is well covered and that there are no leaks. Rinse all the wells with tank buffer using a syringe + needle (this removes not polymerized gel).

8.3.6. Preparation of extracts

A spatula-tip of ground lyophilized mycelium (about 5mg) is put into a small Eppendorf tube, 100ml extraction buffer added, mixed with a vortex mixer, then spun down at 14000 rpm for 2 minutes. The supernatant can be used immediately or frozen for future use. Very little mycelium material is needed for GPI; more is needed for other enzyme systems.

Extraction buffer:

TC 7	800ul
40% sucrose	150ul
Blue juice	50ul

If the lyophilized mycelium was ground using (a spatula tip of) sodium metabisulphite, good results will be obtained with the buffer mix above. If the mycelium was ground without the addition of sodium metabisulphite, it is advisable to add beta mercaptoethanol to the extraction buffer, at 0.7% concentration.

8.3.7. Running the extracts

Rinse each well with tank buffer, using a syringe.

Slowly add extracts to wells.

	GPI	PEP
For the 15 well comb	12ul	20ul
For the 25 well comb	8ul	12ul

For the old equipment (thicker gels), run the gel at 10mA current for 30 minutes, then increase the current to 20mA for a further 30 minutes, and then increase it to 35mA for the rest of the run. Voltage should start at about 40 -55 V, gradually increasing during the run.

For the new equipment (1mm thick gels), run at 5mA for 30 minutes, and then increase to 10mA. If two gels are being run at the same time, double the amperage.

Gels should be run until the blue marker is at the bottom of the gel, or longer if desired.

Staining is carried out as for cellulose acetate gels. Pep activity is normally situated about halfway between the origin and the blue marker, whereas GPI migrates more slowly and is found in the upper part of the gel. Staining solution should be poured quickly and evenly over the appropriate areas.

9 DNA EXTRACTION OF *P. infestans*

1. Harvest mycelium from 8-10 day old pea broth cultures by vacuum filtration. Form the mycelium is a fairly flat shape, put it into 1.5ml microcentrifuge tubes, ensuring that air can circulate down to the bottom of the tube. This will speed up the lyophilization and prevent the drying mycelium from popping up out of the tube.
2. Freeze the mycelium rapidly, then lyophilize for at least 24 hours. Grind in liquid nitrogen with a little sand.
3. Weigh 30-35 mg of ground-lyophilized tissue, add 1ml of extraction buffer (microwave first 15-20 seconds) and incubate at 65°C for 1 hour. Once or twice during this time, gently mix the contents by inverting the tubes. Every 15 minutes, mix contents of the tubes by inverting them.
4. Add 333µl of 5M Potassium acetate, shake tube vigorously and put on ice for 20 minutes.
5. Spin tubes at maximum speed for 10 minutes, gently pour supernatant into a new sterile 2ml tube and add 800µl of cold isopropanol. Mix by inversion and put tubes on ice for 30 minutes.
6. Centrifuge for 5 minutes at maximum speed; gently pour off supernatant and dry pellets by inverting tubes on paper towels for about 10 minutes.
7. Resuspend pellet in 100µl of TE buffer.

DNA is of good enough quality now to use, but if you prefer you can continue with a second precipitation. We usually only do up to step 7.

For a 2nd precipitation resuspend pellet in 700µl of TE buffer.

8. Add 75µl of 3M Sodium acetate and 500µl of isopropanol. Mix well by inversion and spin down for 30 seconds.
9. Dump supernatant and wash pellet with 75% ethanol twice, let pellet dry and resuspend in 100µl of TE buffer.
10. DNA quality and concentration should be checked by electrophoresis on an agarose gel.
11. Dilute a part of the stock solution of DNA to 2 ng/µl with TE buffer before PCR.

Extraction Buffer

Buffer	Chemical	Final Concentration
Extraction	EDTA	0.05M
	Tris pH 8.0	0.1M
	NaCl	0.5M
	beta mercaptoethanol	0.7%
	SDS	0.25%

TE Buffer

Buffer	Chemical	Final Concentration
TE	Tris HCl pH8	10 mM
	EDTA	1 mM

9.1. DNA electrophoresis

Prepare the following TBE buffer:

Buffer	Chemical	Amount
TBE 5X STOCK	Tris base	54g
	Boric acid	27.5g
	EDTA (pH8)	20 ml of 0.5M stock

Dilute for 3 liters at a time (it's the minimum amount needed for the large gel and buffer tanks), to 0.5 X TBE buffer working solution (0.045 M Tris borate, 0.001M EDTA).

1. Weigh the corresponding amount of agarose in an Erlenmeyer. Add TBE buffer.

Get Size	Ingredients	Agarose Percentage	
		1%	2%
Small	Agarose 0.5X TBE	0.4g 40ml	0.8g 40ml
Big	Agarose 0.5X TBE	2g 200ml	4g 200ml

2. Heat in microwave until boiling, swirl, and heat again.
3. Cool for 5 minutes at 50°C in a water bath and add 1µl of 10mg/ml stock Ethidium bromide per 60ml of solution, swirl to mix. *Whenever dealing with ethidium bromide WEAR GLOVES*.
4. Carefully pour the agarose in a horizontal gel tray, avoiding bubbles, immediately insert the comb(s), and let gel set for 15 minutes.
5. Put the gel into the tank and pour TBE buffer over the gel until it is completely immersed. Remove the comb.
6. Prepare the samples and load (each mixed with 2µl of bromophenol blue on Para film or glass) slides.
7. Set voltage at 100 for the big gel and 80 for the minigel, and let run:

Purpose	Agarose concentration	Amount to Load on gel	Running time
DNA quality	1%	5 µl	1 hour
PCR products	1%	8 µl	1 hour
Restriction products	2%	29 µl	2.5 hours

DNA with Ethidium bromide can be visualized with UV light of 300nm on the transilluminator.
Wear safety glasses and gloves.

Dispose of Ethidium Bromide contaminated gel and buffer in the Ethidium Bromide Waste bucket.

10 mt DNA HAPLOTYPES

10.1 Polymerase chain reaction.

This method for detecting the different mitochondrial DNA types of P.infestans was developed by Gareth Griffiths and David Shaw in Bangor, University of Wales at 1998. (Polymorphism in Phytophthora infestans: Four Mitochondrial Haplotypes are detected after Amplification of DNA from Pure Cultures or from Host Lesions. Appl. Environ. Microbiol. 64(10): 4007-4014).

10.1.1 Preparation of samples

1. Prepare the following master mix in a stock, without the DNA. Make enough master mix to include a blank sample.
2. Vortex briefly and centrifuge for a few seconds.
3. Dispense 23 µl of master mix per sample and then add the DNA template to each tube.

Ingredients of Master Mix	Stock Concentration	Volume / Reaction	Final Concentration
$MgCl_2$	25mM	1.5 µl	1.5 mM
Thermo buffer	10x	2.5 µl	1x
dNTP's	1mM	2.5 µl	100 µM
Forward Primer	5 µM	1.6 µl	0.325 µM
Reverse Primer	5 µM	1.6 µl	0.325 µM
Taq DNA polymerase	5U/µl	0.3 µl	1.5 units
Water (distilled sterile)		13 µl	
DNA	2ng/µl	2 µl	4ng

4. Centrifuge briefly.
5. We use a 25µl final reaction volume.

10.1.2 Amplification

Primers sequences:

P1f 5'-GCAATGGGTAAATCGGCTCAA-3'
P1r 5'-AAACCATAAGGACCACACAT-3'

P2f 5'-TTCCCTTTGTCCTCTACC GAT-3'
P2r 5'-TTACGGCGGTTTAGCACATACA-3'

P3f 5'-ATGGTAGAGCGTGGGAATCAT-3'
P3r 5'-AATACCGCCTTTGGGTCCATT-3'

P4f 5'-TGGTCATCCAGAGGTTTATGTT-3'
P4r 5'-CCGATACCGATACCAGCACCAA-3'

Perform PCR using the following temperature profiles:

Purpose	Time	Temperature	Cycles
Denaturation	3 min	94°C	1 hour
Denaturation	30 sec	92°C	
Primer annealing	30 sec	55°C	35
Primer extension	1.10 min	72°C	
Final extension	5 min	72°C	1
Cooling	forever	4°C	

10.1.3 Restriction of PCR products

1. Pipet 10µl of PCR products in clean Eppendorf tubes.
2. Prepare a master-mix; for primer 1 use *Cfo*I, for primer 2 use *Msp*I, for primers 3 and 4 use *Eco*RI.

Ingredients	Stock Concentration	Volume / Reaction	Final Concentration
Restriction buffer	10x	3 µl	1x
Restriction enzyme	10U/µl	0.1 µl	1 unit
Distilled water		16.9 µl	

3. Vortex and centrifuge 2 seconds.
4. Label properly each microcentrifuge tube for each enzyme and isolate.
5. Add 20µl of the master mix to the DNA samples, vortex and centrifuge the samples 2 seconds at full speed, incubate for 1 hour at 37°C in a water bath.
6. Add 3µl of bromophenol blue to the samples and load the restriction products on a 2% agarose gel (see section 9.1.)
7. Load 5µl of 100 bp marker (0.1µg/µl) in one extreme of the gel.
8. Run at 60 V, for 2.5 hours.

10.1.4 Analysis of DNA bands

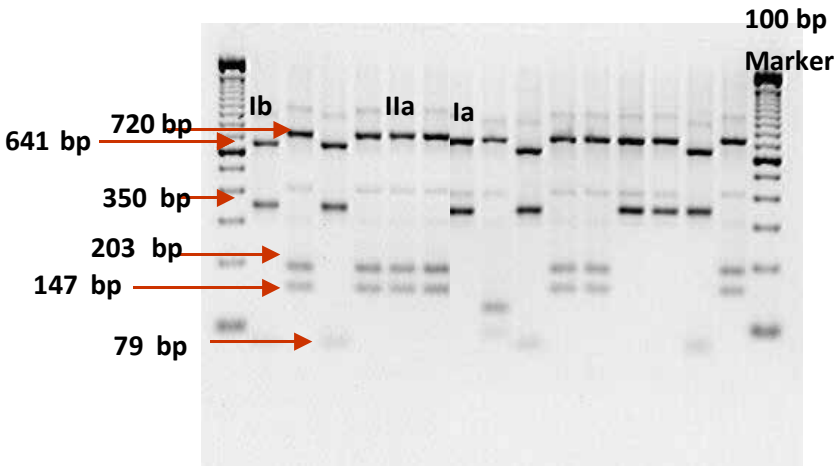
With CfoI it is possible to discriminate between IIa (1118 bp fragment) and Ia, Ib and IIb (907, 211 bp fragments).

With MspI it is possible to discriminate between Ib (641, 350, 79bp fragments),Ia, IIb (720, 350 bp fragments) and IIa (720,203,147 bp fragments).

With *Eco*RI and primer 3 it is possible to discriminate between Ia, Ib (1064, 228 bp fragments) and IIa, IIb (1292 bp fragment) isolates.

With *Eco*RI and primer 4 it is possible to discriminate between Ia, Ib (287,361,209 bp fragments) and IIa , IIb (596, 361 bp fragments).

mt DNA haplotypes detected in *P. infestans* Peruvian populations



MspI / primers P2f - P2rb.

11 RIBOSOMAL ITS REGION AMPLIFICATION

This is a PCR method developed by Paul Tooley et al 1997 (Appl. Env.Microbiol. 63,1467 -1475) for the early detection of P.infestans in tubers.

The primers used (ITS3: 5'-GCATCGATGAAGAACGCAGC-3' and PINF2: 5'CGATTCAAATGCCAAGCTAAAG -3' have been specifically designed to detect *P.infestans*, although they do also amplify *P.phaseoli* and *P.mirabilis*. PCR with these primers produces a single 456 bp product with these species. We have not yet used it for detection, but have used it successfully on purified DNA, as a check to see whether isolates from wild Solanaceae were *P.infestans.*

1. Extract DNA as indicated before (see section 9), dilute to 10ng per µl with TE.
2. Make up the following master mix. Multiply by 1.1X the number of samples + 1 control without DNA:

10X buffer		2 µl
25mM MgCl2	1.8mM	1.44 µl
2.5mM dNTP mix	100µM	0.8 µl
10µM primers ITS3	0.1µM	0.2 µl
PINF2	0.1µM	0.2 µl
taq		0.07 µl
H2O		14.29 µl
10ng\µl DNA		1µl
Total		20 µl

3. Dispense 14 µl into each tube and add 1µl of DNA.
4. Run the following amplification program:

94°	1 minute	1 cycle
94°	15 secs	
50°	1 minute	30 cycles
72°	45 secs	
72°	5 minute	1 cycle

These times are longer than specified in Tooley et al. because they are also suitable for several anchored microsatellite primers, which can be used for amplification at the same time.

5. Load 10µl on a 1.4% or 2% gel, alongside a DNA ladder, and view after 1.5 hours.

12 RFLP ANALYSIS (Restriction Fragment Length Polymorphism)

The detection of polymorphism through RFLP involves several steps: DNA extraction, DNA digestion using restriction enzymes, separation of restriction fragments by agarose gels electrophoresis, transfer to a solid support (membrane), hybridization with the labeled marker (DNA probes marked with radioactive isotopes or by non-radioactive methods), and signal detection by autoradiography or another method.

Probe RG57

The probe RG57 has been widely utilized to characterize *Phytophthora infestans* populations. The insert is 1 kb in length. RG57 hybridization patterns are moderately polymorphic, which makes them particularly useful for international studies of diversity and migration. Globally, over 25 bands hybridizing with RG57 have been described for the pathogen. Not all bands will be present in an individual isolate. Each combination of bands is called an RG57 genotype; a group of highly similar genotypes is considered to be a lineage (we infer that the group is related by descent).

We use the ECL Kit to label and detect probes. ECL- Direct nucleic acid labeling and detection systems provided by Amersham.

12.1. DNA restriction of *P. infestans*

1. Make up the mix for DNA restriction.

Always prepare an additional volume per every 10 reactions.

Restriction Buffer (10X)	2.5 µl
*Eco*RI (10 units/ µl)	1.5 µl
Spermidine (0.1 M)	0.6 µl
Water	5.4 µl
Total	10.0 µl

2. Add 10 µl of the restriction mix to 3 µg of DNA and incubate at 37°C overnight.

3. Prepare an agarose gel (0.7%) and run 1µl of each DNA sample in order to verify if DNA has been completely digested.

12.2 Agarose gel electrophoresis of digested DNA.

1. Prepare a 0.7% agarose gel with TAE in a gel bed of 20 x 25 cm (Tris acetate EDTA). (See Solutions and Buffers).

Use thin combs (2 mm) to form the wells, to ensure tight bands.

2. Place the gel in a clean tank, cover with fresh TAE buffer, and gently remove the comb.

3. Apply the samples of digested DNA with 20% of load buffer (See Solutions and Buffers).

> The load buffer makes the samples heavy, so that they sink to the bottom of the wells. In addition, the load buffer contains one or more dyes to allow you to see how far the gel has run.

4. Load run markers or DNA control to the extremes of the gel (1 kb ladder 200 pg).

> Run it slowly so that the bands are correctly separated (approximately 30 V overnight, preferably in cold).

5. Add ethidium bromide [10 mg/ml] only to the tank buffer, 1 µl per 100ml buffer.
6. When the gel has sufficiently run, approximately 18 cm (is desirable to obtain a good bands separation), remove it from the electrophoresis tank and take a photo.

> Take a photo using a transilluminator. This record helps you to compare with other gels.

12.3. Process the gel for DNA transfer

12.3.1. Depurination:

1. Place the gel in a container and cover it with depurination solution (250 mM HCl), for 15 minutes until the bromophenol blue becomes yellow in agitation.

> This process can also be carried out exposing the gel in light of a transilluminator (UV) only during 5 minutes up to 11 kb, the rest of the gel should be protected of the UV rays.

12.3.2. Denaturation:

1. Pour off the depurination solution and rinse the gel with distilled water.
2. Cover the gel with denaturation solution (see recipes), and agitate.
3. Wait for the bromophenol blue to return to its blue color, and then leave it in agitation for 25 minutes more.
4. Pour off the denaturation buffer and rinse the gel with distilled water.

12.3.3. Neutralization:

Cover the gel with neutralization solution (see recipes) and to leave in agitation for 30 minutes.

12.4. Southern Blot:

> "Southern blotting" is the transfer of denatured DNA fragments from a gel onto a membrane. The transfer can be done electrically or by capillary action with a high salt solution". (See figure below).

1. Fill a recipient (normally a glass or plastic dish somewhat larger and longer than the gel) with 20x SSC, to approximately 2 cm in depth.
2. Place a glass plate across the dish, to create a platform for the gel.
3. Place a piece of Whatman 3MM filter paper across the glass plate, so that the two ends of the filter paper reach into the buffer

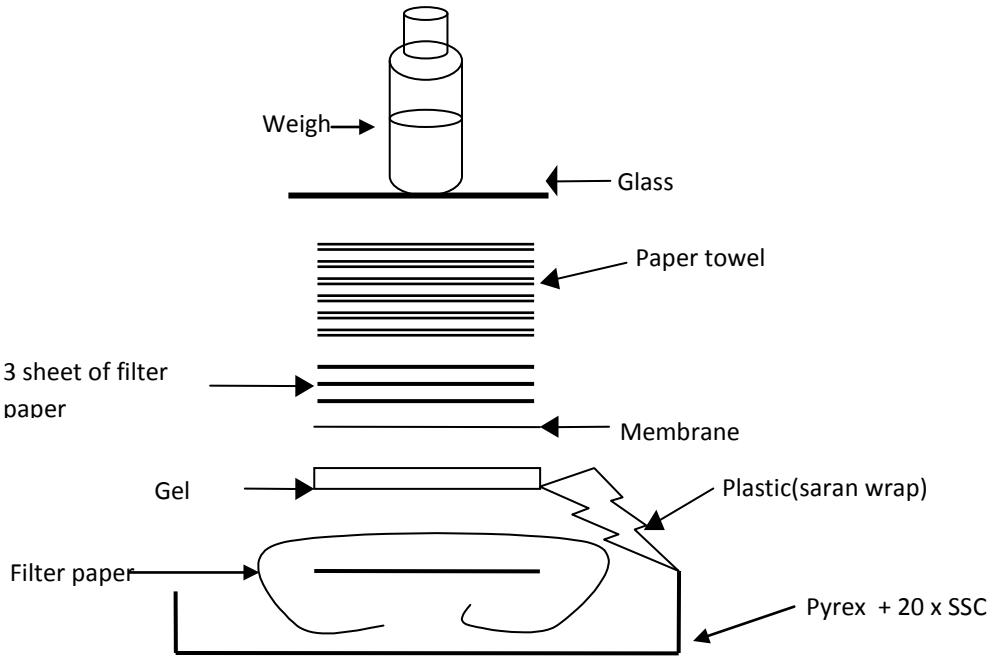
4. Place the gel (upside down) on the platform, on top of the filter paper. Put the gel down carefully, starting with one corner and laying it down gradually, to avoid the formation of bubbles.

> To ensure that the buffer goes through the gel and then through the membrane, it is recommended to cover the exposed surface of filter paper around the gel with plastic film (Saran Wrap). This avoids direct contact between the lower filter paper and membrane or the upper filter paper.

5. Put the membrane on top of the gel, again avoiding bubbles. Mark the location of the wells.

> The membrane can be made of nylon or nitrocellulose. CIP Mycology Laboratory uses membranes of nylon (Hybond -N^+, Amersham).

7. Add 3 layers of filter paper on top of the membrane, always avoiding bubble formation then put 7 cm of paper towel.
8. Finally, place another piece of glass and then a weight of approximately 400 g. *Do not crush the gel.*
9. Leave the gel in this configuration for several hours, preferably overnight.



10. The next day, gently remove the layers over the membrane. Remove the membrane from the now-thin gel, mark the position of the wells, and rinse it with 6X SSC for 1 minute.

> Always avoid touching the membrane with your fingers. Hold it by the edges with a pair of flat-tipped forceps. Gloves should be worn.

11. Let dry the membrane 2-3 minutes.
12. The DNA is permanently immobilized by ultraviolet irradiation (cross linker) or by baking it in an oven (80°C) for two hours.

> **Hybond-N+:** If not be use immediately, fixed blots can be stored dry wrapped in Saran Wrap at 2-8°C or, for up to several weeks, at room temperature under vacuum.

12.5. Solutions and buffers for RFLP

Depurination solution:

HCl	250 mM.

Denaturalization solution:

NaCl	1.5 M
NaOH	0.5 M.

Neutralization solution:

NaCl	1.5 M
Tris HCl	0.5 M

Adjust pH to 7.5

20x SSC:

Na3 citrate	0.3 M
NaCl	3 M

PH 7.0

Buffer of primary washing containing urea:

Urea 360g	6M
SDS 4g	0.4%
20xSSC 25 ml	0.5xSSC

Complete to a liter. Can keep at refrigerator until 3 months.

Secondary washing buffer:

20xSSC	100 ml

Complete to 1 liter. Can keep at refrigerator until 3 months.

TAE 50x:

Tris(base) 242g	2M
EDTA 18.6g	0.05M

Adjust to pH 8 with glacial acetic acid (~57 ml) and complete to 1 liter.

Loading buffer:

Azul of Bromophenol	0.05g
Xylene cyanol	0.05g
Glycerol	5.0 ml
EDTA	0.186g

Complete to 10 ml with TAE buffer.

1 kb ladder for Southern (200 pg/10µl):

Prepare 1000µl:

1 kb ladder (10ng//µl)	2µl
Loading	400µl
ddH2O sterile	598µl

1 kb ladder to label with the probe:

The 1kb ladder is 1µg/µl concentrated

Prepare 1000µl to a concentration of 10 ng//µl:

1 kb ladder	10µl
ddH2O sterile	990µl

Use 10µl to label DNA (Total:100ng)

Components that come in the ECL kit (Amersham):

1. Reagent of DNA labeling
2. Solution of glutaraldehyde
3. Water
4. Hybridization buffer (Gold)
5. Blocking agent
6. ECL detection Reagent 1
7. ECL detection Reagent 2.

Other materials needed:

1. Filter paper wathman 3MM
2. Extra thick blotting paper
3. Nylon Membrane (Hybond, Amersham)
4. Saran wrap
5. Agarose
6. Paper towel
7. Cassettes
8. X-omat film

13 AFLP ANALYSIS (Amplified Fragment Length Polymorphism)

I. Non-radioactive method:

The AFLP technique has been developed by the company Key Gene (Wageningen, The Netherlands), which has filed property rights on this technology (Zabeau and Vos 1993).

AFLP is based on the selective PCR amplification of restriction fragments from a total digested genomic DNA.

- The DNA is digested with two different restriction enzymes: a frequent cutter (the four-base restriction enzyme *Mse*I) and a rare cutter (the six-base restriction enzyme *Eco*RI). Specific synthetic adapters for each restriction site are then ligated to the digested DNA (we perform the restriction and ligation steps in a single reaction).
- The DNA ligated is then subjected to a preliminary PCR amplification using oligonucleotide primers that are specific to the adapter/restriction sites. An extra nucleotide is added, for example A, thereby only a subset of the fragments of the mixture is amplified (fragments in which the restriction site sequence is followed directly by an A).
- A second amplification is then carried out using similar oligonucleotide primers but with 2 extra bases (for example AC). Therefore, only a subset of the preliminary amplification reaction will undergo subsequent amplification during the second round of PCR (fragments in which the AC sequence follows the restriction site sequence).
- The subset of fragments is analyzed by denaturing polyacrylamide gel electrophoresis to generate a fingerprint and DNA bands are detected by silver stain.
- The AFLP technique detects polymorphism due to changes in the *Mse*I and *Eco*RI restriction sites. Different combinations of +2 primers allow the amplification of different subsets from the initial amplification products and increase the probability of finding useful polymorphisms.

Enclose you can find protocols for denaturing polyacrylamide gels and silver staining.

13.1. Mix for restriction-ligation reaction:

1. Make up the mix for restriction –ligation.

5X RL Buffer	10.0 µl
5 U *Eco*RI	0.5 µl
5 U *Mse*I	1.25 µl
Eco Adapter	1.0 µl
Mse Adapter	1.0 µl
Ligase [1U/µl]	1.0 µl
ATP (10 mM)	1.0 µl
DdH_2O	24.25 µl
	40.0 µl

2. In a microcentrifuge tube add the restriction-ligation mixture and 10µl of DNA (500 ng in 10 µl). *Total volume 50µl.*

> **NOTE:** Always prepare an additional volume per every 20 reactions.

3. Incubate at 37°C for 4 hours.
4. Make a dilution 1:4 (add 150 µl of TE 0.1 mM EDTA)
5. Store at -20°C.

13.2. Master mix for pre-amplification:

DdH_2O	10.80µl
Eco+A (50 ng/µl)	0.6
Mse+C (50 ng/µl)	0.6
DNTPs (5 mM)	0.8
PCR 10X	2.0
Taq DNA polymerase*	0.2
Total	15.0µl

1. Add to each PCR tube (0.2 ml): 5 µl of diluted DNA (digested and ligated) and 15 µl of pre-amplification mixture.

* Taq provided by the molecular marker laboratory at CIP, diluted 1:4

> Taq polymerases from Perkin Elmer (5U/µl) use 0.08 µl per reaction. Adjust the final volume with Milli-Q water

2. Amplify using the following cycle profile:

20 cycles at:

92°C for 60 sec

60°C for 30 sec

72°C for 60 sec

> For this and subsequent PCR we use a MJ Research thermocycler (PTC 200).

- Bring up to 200 µl the pre-amplified products with TE 0.1mM EDTA
- Store at 4°C (or -20°C for long term).

13.3. Master mix for selective amplification:

DdH_2O	10.9 µl
Eco (+2)	0.5
Mse (+2)	0.6
DNTPs (5 mM)	0.8
PCR 10X	2.0
Taq polymerase	0.2
Total	15 µl

1. Add to each PCR tube:

5µl of pre-amplification product and 15µl of amplification master mix

2. Conditions for amplification:

Cycle 1:

94°C 30 sec
65°C 30 sec
72°C 60 sec

Cycle 2-13:

94°C 30 sec
65 - 0.7°C every cycle 30 sec
72°C 60 sec

Cycle 14-36:

94°C 30 sec
56°C 30 sec
72°C 60 sec

13.4. Gel electrophoresis

1. Run in 6% polyacrylamide gel with 7.5 M of Urea.
2. Prepare the samples as follows:

   Mix 5µl of amplified DNA with 3 µl of loading buffer for PAGE.
3. Denature the samples for 5 min in thermocycler.

Load 10µ l of markers 1 kb and 50 bp in the wells extremes of the gel.

4. Place in the electrophoresis chamber:

   "Upper buffer" …… TBE 0.5X

   "Low buffer" …… TBE 1X

For gel preparation and silver staining follow corresponding protocols.

13. 5. AFLP initiators and adapters for *Eco*RI and *Mse*I

_Eco_RI Adapter:

Eco A1	5′-CTCGTAGACTGCGTACC
Eco A2	CTGACGCATGGTTAA-5′

Selective initiators:

Initiator + 0	E00	5′-GACTGCGTACCAATTC
Initiator + 1	E01	5′-GACTGCGTACCAATTCA
Initiators + 2	E11	5′-GACTGCGTACCAATTCAA
	E12	**5′-GACTGCGTACCAATTCAC**
	E13	5′-GACTGCGTACCAATTCAG
	E14	5′-GACTGCGTACCAATTCAT
	E15	5′-GACTGCGTACCAATTCCA
	E16	5′-GACTGCGTACCAATTCCC
	E17	5′-GACTGCGTACCAATTCCG
	E18	5′-GACTGCGTACCAATTCCT
	E19	5′-GACTGCGTACCAATTCGA
	E20	5′-GACTGCGTACCAATTCGC
	E21	5′-GACTGCGTACCAATTCGG
	E22	5′-GACTGCGTACCAATTCGT
	E23	5′-GACTGCGTACCAATTCTA
	E24	5′-GACTGCGTACCAATTCTC
	E25	5′-GACTGCGTACCAATTCTG

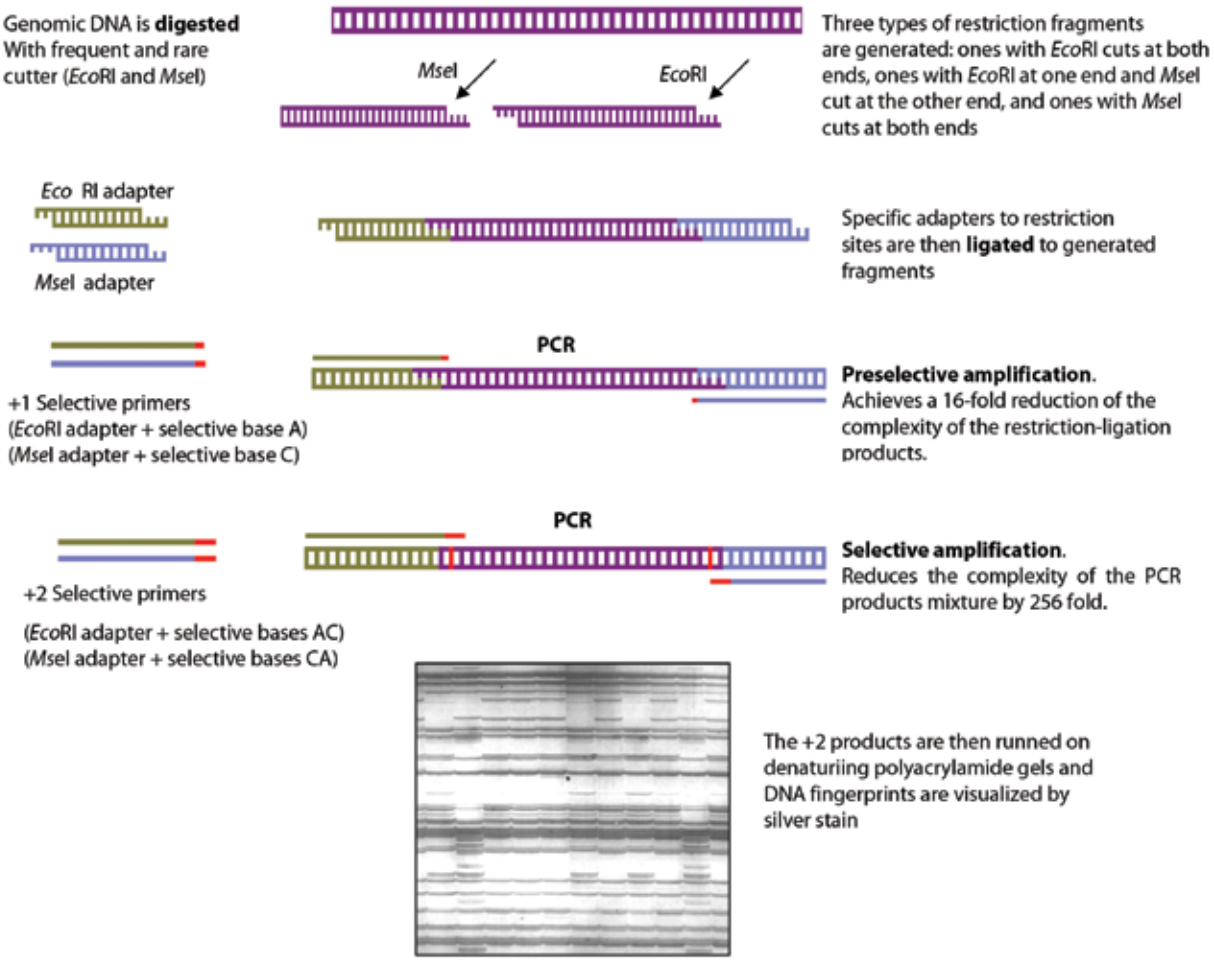
_Mse_I Adapter:

Mse A1	5′-GACGATGAGTCCTGAG
Mse A2	TACTCAGGACTCAT-5′

Selective initiators

Initiator +0	M00	5′-GATGAGTCCTGAGTAA
Initiators +1	M01	5′-GATGAGTCCTGAGTAAA
	M02	5′-GATGAGTCCTGAGTAAC
Initiators +2	M11	5′-GATGAGTCCTGAGTAAAA
	M12	5′-GATGAGTCCTGAGTAAAC
	M13	5′-GATGAGTCCTGAGTAAAG
	M14	5′-GATGAGTCCTGAGTAAAT
	M15	**5′-GATGAGTCCTGAGTAACA**
	M16	5′-GATGAGTCCTGAGTAACC
	M17	5′-GATGAGTCCTGAGTAACG
	M18	5′-GATGAGTCCTGAGTAACT
	M19	5′-GATGAGTCCTGAGTAAGA
	M20	5′-GATGAGTCCTGAGTAAGC
	M21	5′-GATGAGTCCTGAGTAAGG
	M22	5′-GATGAGTCCTGAGTAAGT
	M23	5′-GATGAGTCCTGAGTAATA
	M24	5′-GATGAGTCCTGAGTAATC
	M25	5′-GATGAGTCCTGAGTAATG

Schematic representation of the AFLP fingerprint



13.6. SOLUTIONS PREPARATION

▶ **Buffer PCR 10X:**

Reagents	Stock	(µl)
100mM Tris HCl pH 8.3	1M	1M
15 mM $MgCl_2$	1M	15
500 mM KCl	1M	500
Dd H_2O		385
Total		**1000**

Keep at--20°C

- **dNTPs 5mM:**

Add 200 µl of each of the nucleotides (dATP, dTTP, dCTP, dGTP) to 3,200 µl of Milli-Q water (or nucleases free water). Aliquot in micro centrifuge tubes and keep at - 20°C.

- **Restriction Ligation Buffer 5X (RL 5X):**

Reagents	Stock	(µl)
50 mM Tris HAc pH 7.5	1 M Tris HAc pH 7.5	
50 mM MgAC		
250 mM KAc		250
25 mM Dithiotreitol		
250 mg/µl BSA	10 µg//µl BSA	24
Dd H_2O		525
Total		**1000**

Keep at--20°C

- **1M Tris HAc pH 7.5:**

  Weigh 6.055 g of Tris bases for 50 ml. Take to pH 7.5 with acetic acid, complete with Milli-Q water. Keep at 4°C.

- **ATP 10 mM**
- **MgAc 1 M**
- **KAc 1 M**
- **DTT 250 mM**
- **BSA 10 µg/µl**
- **Tris HCl 1M pH 8.3:**

  Weigh 6.055 g of Tris bases for 50 ml; take to pH 8.3 with HCL, complete to 50 ml with Milli-Q water.

- **$MgCl_2$ 1 M**
- **KCl 1M**
- ***Eco* A and *Mse* A Adapters**

  Prepare adapters according to the stock concentration. For example:

Eco A (5µM):	Take	Stock	(µl)
Eco A1	17 µg	9,459 µg//µl	1.8
Eco A2	15 µg	13,268 µg//µl	1.1
Dd H_2O			597.1
Total			**600.0**

Mse A (50µM):	Take	Stock	(µl)
Mse A1	40 µg	9.437µg//µl	4.25
Mse A2	35 µg	10,721µg//µl	3.25
Dd H_2O			132.50
Total			**140.00**

- **Primers:**

  Prepare primers at 50 ng//µl concentrations.

- **TE 1.0 mM EDTA:**

Stock	[Final]	Take
Tris HCl 1 M pH 8	10 mM	10 ml
EDTA 0.5 M pH 8	1 mM	2 ml
Dd H_2O		988 ml

- **TE 0.1 mM EDTA:**

Stock	[Final]	Take
Tris HCl 1 M pH 7.6	10 mM	10 ml
EDTA 0.5 M pH 8	0.1 mM	0.2 ml
Dd H_2O		989.8 ml

NOTE: Use TE 0.1 mM of EDTA to dilute the DNA and primers.

- **TBE 10X for PAGE:**
  1M Tris
  1M Boric Acid
  20 mM EDTA
  Dd H_2O (up to 1 L).

- **Loading Buffer for PAGE:**

Xylene Cyanol	11.2 mg
Urea	8.4 g
Dd H2O	up to 14 ml.

- **1 Kb ladder for PAGE:**

1 kB (1µg//µl)	10µl
Loading buffer (PAGE)	130µl
1X TBE (PAGE)	60µl.

- **Marker of 50 bp for PAGE:**

50 bp (1µg//µl)	10µl
Loading buffer (PAGE)	130µl
1X TBE (PAGE)	60µl.

- **10% Ammonium persulfate**

- **Sodium thiosulfate (10 mg/ml)**

- **Bind (for short glass plates):**

Ethanol	3 ml
Glacial acetic acid	9µl
Bind silane	9µl

14 Polyacrylamide gels for DNA electrophoresis

NOTE: Denaturant polyacrylamide gels are utilized for sequencing of DNA and for the separation of AFLP products in electrophoresis equipment from BIORAD sequencing gels (Model Sequi Gen-GT 38x50 cm).

14.1. Preparation of sequencing plates:

The glass plates should be meticulously cleaned. Rinse the clean plates with deionized water to eliminate possible waste, finally wash with ethanol.

14.1.1. Short Glass Plate Preparation:

Treat the short glass plate with binding solution each time a gel is prepared.

1. Prepare fresh binding solution by adding 3µl of bind silane to 1ml of 95% ethanol and 3µl of acetic acid.
2. Wipe a scrupulously cleaned plate using a Kim Wipes® tissue saturated with 1 ml of freshly prepared binding solution. Make sure the plate is completely covered.

You may use a tissue paper of your preference that does not leave a trace.

3. After 4-5 min, apply approximately 2 ml of 95% ethanol to the plate and wipe with a tissue paper in one direction and then perpendicularly to the first direction pressing gently. (Rubbing hard will remove an excessive amount of the bind silane, and the gel may not adhere as well).
4. Repeat this operation three times, using a new tissue every time, to eliminate the excess of binding solution. This is important to prevent the contamination of the long glass plate, which could result in a torn gel.

14.1.2. Long Glass Plate Preparation:

1. Use new gloves before preparing the long plate to prevent cross-contamination with binding solution.
2. Wipe a scrupulously cleaned plate using a tissue saturated with Repel® solution.
3. After 5-10 min, remove the excess of Repel® solution by wiping the plate with a Kim Wipes® tissue. Excess Repel® may cause inhibition of staining.

In the event of contamination of either Bind silane or Repel on the respective glass, soak in 10% NaOH for 30-60 minutes.

14.2. Preparation of the sequencing gel:

1. Wait until the glass plates are completely dried (at least 30 min.).
2. Assemble the glass plates. There should be special care with filtration, uniform pressure of the clamps and combs and adequate spacers (use 0.4mm thick vinyl spacers and shark tooth comb).
3. Prepare the pre-mixture solution of 6% Polyacrylamide (20:1, acrylamide: bis acrylamide), 500 ml:

Urea (7.5 M)	225.22 g (Filter)
Acrylamide	28.5 g
Bis acrylamide	1.5 g
TBE 10X	50 ml
H2O Milli-Q sterile	Complete to 500 ml

Store at 4°C in a bottle covered with aluminum paper.

4. Prepare the gel as indicated below:

For one Gel:	
Pre-mixture of polyacrylamide	80 ml
Temed®	44 µl
Ammonium Persulfate 10%	440 µl

5. Pour the gel solution. Avoid bubble formation, in case there are place the plates vertically and strike smoothly. Place carefully the combs between the plates. Allow to polymerize horizontally at least for 2 hrs. Pre-run the gel to 1000 V by 15-30 min.
6. Load samples, run the gel at 500 V during the night or run a fast electrophoresis at 1600V for 5-6 hrs.
7. Stain with silver nitrate: Follow corresponding protocol.

15 Silver Stain for PAGE

Adapted from Promega protocols

NOTE: Silver stain is used for AFLP detection. Alternatively, the Promega stain kit (Catalogue No. Q4130) can be used.

The water quality influences a great deal on stain success.

15.1. Protocol:

15.1.1. Solutions:

1. **Fix/stop solution (10%glacial acetic acid):** add 200 ml of glacial acetic acid to 1,800 ml of ultra pure water (Milli-Q) or bidestilled water.
2. **Staining solution:** dissolve 2 g of silver nitrate ($AgNO_3$) in 2 L of ultra pure water and add 3 ml of 37% formaldehyde.
3. **Developing solution:** dissolve 60 g of sodium carbonate (Na_2CO_3) in 2 L of ultra pure water. Chill to 10°C in an ice bath. Immediately before use, add 3 ml of 37% formaldehyde and 400µl of sodium thiosulfate (10 mg/ml).

15.1.2. Separate the glass plates:

After electrophoresis, carefully separate the glass plates using a plastic wedge. The gel should be strongly adhered to the short plate.

15.1.3. Fix the gel:

Place the gel in a plastic tray, cover it with the fix/stop solution and agitate it for 20 min or until the tracking dyes is not visible (This step is critical for DNA precipitation and the elimination of the urea).

15.1.4. Wash the gel:

Rinse the gel twice using agitation in ultrapure water (2 min every time). Take out the plate from the tray and allow to drain for 10-20 seconds before placing it in the following washing.

15.1.5. Stain the gel:

Transfer the gel to the stain solution and agitate for 30 min. Complete preparation of the developing solution. Pour the pre-chilled developing solution into a tray.

Warning: The time of the next step (rinse) is very important. Prolonged rinsing time may cause a weak or lack signal.

15.1.6. Rinse the gel:

Submerge the gel briefly in the tray containing ultra pure water, drain and place the gel immediately into the tray of chilled developing solution. The time should not take more than 5-10 seconds to submerge the gel in water and transfer it to the developing solution.

15.1.7. Develop the gel:

Agitate the gel until the bands begin to appear. Continue to reveal until all bands are visible.

> The developed bands appear fairly light. Prolonged development times result in high background.

15.1.8. Fix the gel:

To finish the developing reaction, add stop solution (~ 500 ml) and leave in agitation for 2-3 min.

15.1.9. Rinse the gel twice:

In ultrapure water, 2 min every time.

15.1.10. Dry the gel:

Leaving it at room temperature or using a conventional dryer.

15.1.11. Permanent registries:

The APC films are the most desirable format to make permanent the registries of the silver dyed gels. The APC films produce a positive and opposite image of the original on a white background. The gel should be completely dry before exposing the APC film. Handle the plates with gloves to avoid fingerprints.

a) In dark room: place the dry gel adhered to the plate (gel side up) on a white light box with the safelight on.

b) Place the APC film, emulsion side down, over the gel to be copied.

c) Place a clean glass plate on the film to maintain contact between the gel and the film. Turn on the light box and expose the film for 1-2 min.

> The exposure time depends of the gel background or it may vary with different light. Optimize the exposure time exposing strips of the APC film for several time intervals. In general, exposure times of 1-2 minutes produce good results.

d) Processing the exposed APC film as follows:
Submerge in developing solution (Kodak® GBX Developer) for 1-5 min, wash during 1 min with deionized water, 3 min in fixer solution (Kodak® GBX Fixer) and 1 min in deionizer water.

16 AFLP ANALYSIS (Radioactive method)

Protocol used at Plant Research International, Wageningen, The Netherlands.

1. Digest high quality DNA (250 ng) with *Eco*R1 (10 U) and *Mse*1 (10 U) overnight at 37°C in Restriction Ligation (RL) buffer (10 mM Tris/HAc pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/ml BSA).

   We use 10 µl RL buffer, 1 µl *Eco*R1, 2 µl *Mse*1, and 37 µl MilliQ and DNA mix, adjusted to 250 ng DNA).

2. Check digestion on a 1.0% agarose gel using 10 µl of the digested product.
   Visualized by Ethidium Bromide and UV illumination.
3. Ligate AFLP adapters overnight to the restriction fragments at 10-12°C. 10 µl ligase/adaptor mix is added to 40 µl digested DNA fragments. Final concentrations are: 2.4 U T4 DNA ligase (Pharmacia), 0.1 µM *Eco*R1 adapter, 1.0 µM *Mse*1 adapter and 0.2 mM ATP.

   *Eco*RI adapter (5' CTCGTAGACTGCGTACC /CATCTGACGCATGGTTAA)

   *Mse*I adapter (5'GACGATGAGTCCTGAG / TACTCAGGACTCAT)

4. After ligation, dilute the products 1:9 with MilliQ water.
5. Perform non-selective fragment amplification with 0-primers using 5 µl diluted ligation product added to 20µl buffer PCR (10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2) with 60 mM dNTP, 5 ng/ml of primer E00 and M00, and 1 U Taq polymerase (Boehringer Mannheim).

   E00 5'GACTGCGTACCAATTC

   M00 5' GATGAGTCCTGAGTAA

6. Reactions are performed using a PTC200 thermocycler (MJ Research) under the following conditions: 2 min 94°C, 35 cycles of (30 sec at 94°C, 30 sec at 56°C, and 90 sec at 72°C), a final extension of 10 min at 72°C and cooling to 4°C.
7. Check the amplification of fragments on 1.0% agarose gels, visualized with Ethidium Bromide and UV illumination.
8. Perform the selective PCR amplification using 5 µl 20x diluted amplification product in 20 µl final reaction volume of the buffer described above, but using 200 mM dNTP, and 5 ng Cy5-labelled fluorescent E19 or E21 primer and 30 ng M16 primer.

   E19 5' GACTGCGTACCAATTCGA

   E21 5' GACTGCGTACCAATTCGG

   M16 5' GATGAGTCCTGAGTAACC

9. PCR conditions as performed using a PTC200 thermocycler: 2 min at 94°C, 13 cycles of (30 sec at 94°C, 30 sec at 65°C, and 60 sec at 72°C; annealing temperature was lowered 0.7°C each cycle), followed by 23 cycles of (30 sec at 94°C, 30 sec at 56°C, 60 sec at 72°C) and a final extension of 10 min at 72°C and cooling to 4°C.

10. Dilute PCR products with an equal volume of formamide dye (98% formamide, 10 mM EDTA and Blue Dextran), heat the diluted mixture for 5 min at 95°C to denature and immediately put on ice.
11. Load 5 µl of the denatured product on a 6% Sequagel (Biozym) polyacrylamide gels and run on an automatic sequencer ALFexpress (Pharmacia) at 1500 V, 60 mA, 35 W and 55°C. Running buffer used is 0.6 X TBE.
12. A fluorescent labeled 50 bp ladder (Pharmacia) (3 µl per lane) is loaded on the first and the last lane of the gel.

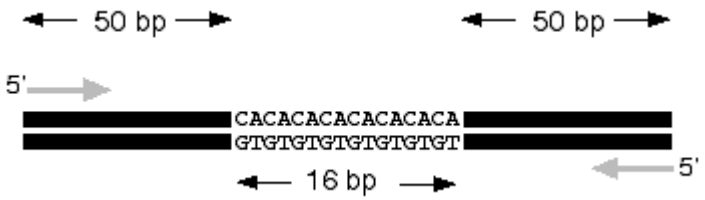
NOTE: This protocol is still being updated. More detailed information about the AFLP fingerprinting protocol and subsequent data analysis is available upon request. Please send us e-mail (*W.G.Flier@plant.wag-ur.nl; L.P.N.M.Kroon@plant.wag-ur.nl* or *G.B.M.Vandenbosch@plant.wag-ur.nl*) to receive the latest version of the protocols or more detailed information.

17 mt DNA HAPLOTYPES

Microsatellites are simple sequence repeats amplified by PCR. These are tandemly repeated motifs of 1-6 nucleotides that are densely and evenly distributed through out the genome and often exhibit substantial variation in the number of repeats. They are usually flanked by conserved sequences, which allow specific amplification of each microsatellite locus within species.

What makes microsatellites useful is the fact that at the same location within the genomic DNA the number of times the sequence (ex. AC) is repeated often varies between individuals, within populations, and/or between species. So, one population may commonly have 13 AC's repeated in a row while another population has 18 AC's repeated at the same location within the genomic DNA. Different regions of the DNA contain sequences that mutate at various rates. Some regions have a high rate of mutation while others have a low rate of change. In areas of the genome with high rates of mutation there is a wider range in the number of repeats found within individuals of a population (some individuals have 10 repeats others 11, 13, . . .). Each sequence with a specific number of repeated nucleotides is designated as an **allele**. So, a **locus** (a specific region within the genomic DNA) with 8 repeats is one allele and within another individual the same locus that contains 9 repeats is another allele.

The most common way to detect microsatellites is to design PCR primers that are unique to one locus in the genome and that base pair on either side of the repeated portion (figure 1). Therefore, a single pair of PCR primers will work for every individual in the species and produce different sized products for each of the different length microsatellites.



Microsatellites has proven to be a powerful tool for genetic studies of populations (Cheng y Crittenden, 1994), since they are very polymorphic, codominant (Both alleles at a locus are amplified and discriminated simultaneously), they present simple Mendelian heredity, they are easy to measure and analyze, and they are trustworthy, repetitive, and only tiny amount of DNA is needed.

Primers sequences used in CIP Quito and Perú. (*From Knapova and Allison Lees*)

Pi63 f:	ATGACGAAGATGAAAGTGAGG
Pi63 r:	ATTCATTATTGGCAATGTTGG
Pi66 f:	ACCGACAGCTTCTGAAACC
Pi66 r:	AAAATAAGAAGAGATTCGTGCC
Pi89 f:	GAGAACGCACAATGTAAGGC
Pi89 r:	ACATAAATACACGCTGAACGG
G11 f:	TGCTATTTATCAAGCGTGGG
G11 r:	TACAATCTGCAGCCGTAAGA
D13 f:	TGCCCCCTGCTCACTC
D13 r:	GCTCGAATTCATTTTACAGA
1F f:	CGAGAGTGAATGAGAGCGAG
1F r:	ACAATCTGCAGCCGTAAGAG
4B f:	AAAATAAAGCCTTTGGTTCA
4B r:	GCAAGCGAGGTTTGTAGATT
4G f:	CGCTGTGTGGATGACAAGTA
4G r:	TCGACCTGACATACGAGCTA
2D f:	AATTGAGTGAATGCGTCACC
2D r:	TTTCCTGCTATCCTCAGCAC

17.1 PRIMERS 4B and 2D

1. Master mix preparation when use this primers (10): Make a bulk mix for all reactions, add extra reactions to allow for pipetting errors.

Reagent	Stock	Final concentration	Volume for one reaction
Milli-Q water			1.50 µl.
Storage Buffer B (Promega)	10x	1x	1 µl.
$MgCl_2$	25 mM	3.50 mM	1.40 µl.
dNTP	2.5 mM	0.10 mM	0.40 µl.
Primer forward	10 µM	0.20 µM	0.20 µl.
Primer reverse	10 µM	0.20 µM	0.20 µl.
Taq Polymerase	5U/µl	1.5 U	0.30 µl.

2. Add in each PCR tube 5 µl of master mix and 5 µl of DNA (5ng/µl).

3. PCR profile:

Cycle	Temperature (°C)	Time
1	94	2 min
	94	40 seg.
35	57	40 seg.
	72	20 seg.
1	72	10 min.
1	4	Forever

* It is necessary to take attention on the annealing temperature for each primer pairs. In the case of **primers 2D and 4B the temperature is 57°C.**

17.2 PRIMER 1F

1. Master mix preparation when use this primers (10 µl): Make a bulk mix for all reactions, add extra reactions to allow for pipetting errors.

Reagent	Stock	Final concentration	Volume for one reaction
Milli-Q water			2 µl.
Storage Buffer B (Promega)	10x	1x	1 µl.
$MgCl_2$	25 mM	3.50 mM	1.40 µl.
dNTP	2.5 mM	0.05 mM	0.20 µl.
Primer forward	10 µM	0.10 µM	0.10 µl.
Primer reverse	10 µM	0.10 µM	0.10 µl.
Taq Polymerase	5U/µl	1.0 U	0.20 µl.

2. Add in each PCR tube 5 µl of master mix and 5 µl of DNA (5ng/µl).
3. PCR profile:

Cycle	Temperature (°C)	Time
1	94	2 min
	94	40 seg.
35	59	40 seg.
	72	20 seg.
1	72	10 min.
1	4	Forever

* Annealing temperature for **primer 1F is 59°C.**

17.3 PRIMER PI66

1. Master mix preparation when use this primers (10 µl): Make a bulk mix for all reactions, add extra reactions to allow for pipetting errors.

Reagent	Stock	Final concentration	Volume for one reaction
Milli-Q water			2.05 µl.
Storage Buffer B (Promega)	10x	1X	1 µl.
$MgCl_2$	25 mM	2.75 mM	1.10 µl.
dNTP	2.5 mM	0.05 mM	0.20 µl.
Primer forward	10 µM	0.20 µM	0.20 µl.
Primer reverse	10 µM	0.20 µM	0.20 µl.
Taq Polymerase	5U/µl	0.13U	0.25 µl.

2. Add in each PCR tube 5 µl of master mix and 5 µl of DNA (5ng/µl).
3. PCR profile:

Cycle	Temperature (°C)	Time
1	94	2 min
	94	30 sec.
35	58	30 sec.
	72	30 sec.
1	72	10 min.
1	4	Forever

* The annealing temperature for **primer Pi66 is 58°C.**

17.4 PRIMER D13

1. Master mix preparation when use this primers (10 µl): Make a bulk mix for all reactions, add extra reactions to allow for pipetting errors.

Reagent	Stock	Final concentration	Volume for one reaction
Milli-Q water			2 µl.
Storage Buffer B (Promega)	10x	1X	1 µl.
$MgCl_2$	25 mM	2.50 mM	1 µl.
dNTP	2.5 mM	0.10 mM	0.40 µl.
Primer forward	10 µM	0.20 µM	0.20 µl.
Primer reverse	10 µM	0.20 µM	0.20 µl.
Taq Polymerase	5U/µl	1.0 U	0.20 µl.

2. Add in each PCR tube 5 µl of master mix and 5 µl of DNA (5ng/µl).

3. PCR profile:

Cycle	Temperature (°C)	Time
1	95	2 min
	51	40 seg.
35	72	20 seg.
	94	40 seg.
1	72	20 min.
1	4	Forever

* The annealing temperature for **primer D13 is 51°C.**

17.5 PRIMER Pi 89

1. Master mix preparation when use this primers (10 µl): Make a bulk mix for all reactions, add extra reactions to allow for pipetting errors.

Reagent	Stock	Final concentration	Volume for one reaction
Milli-Q water			1.50 µl.
Storage Buffer B (Promega)	10x	1X	1 µl.
$MgCl_2$	25 mM	3.50 mM	1.40 µl.
dNTP	2.5 mM	0.10 mM	0.40 µl.
Primer forward	10 µM	0.20 µM	0.20 µl.
Primer reverse	10 µM	0.20 µM	0.20 µl.
Taq Polymerase	5U/µl	1.5 U	0.30 µl.

2. Add in each PCR tube 5 µl of master mix and 5 µl of DNA (5ng/µl).
3. PCR profile:

Cycle	Temperature (°C)	Time
1	94	2 min
	94	30 seg.
35	58	30 seg.
	72	30 seg.
1	72	10 min.
1	4	Forever

* The annealing temperature for **primer Pi 89 is 58°C.**

17.6 PRIMER G11

1. Master mix preparation when use this primers (10 µl): Make a bulk mix for all reactions, add extra reactions to allow for pipetting errors.

Reagent	Stock	Final concentration	Volume for one reaction
Milli-Q water			1.50 µl.
Storage Buffer B (Promega)	10x	1X	1 µl.
$MgCl_2$	25 mM	3.50 mM	1.40 µl.
dNTP	2.5 mM	0.10 mM	0.40 µl.
Primer forward	10 µM	0.20 µM	0.20 µl.
Primer reverse	10 µM	0.20 µM	0.20 µl.
Taq Polymerase	5U/µl	0.15 U	0.30 µl.

2. Add in each PCR tube 5 µl of master mix and 5 µl of DNA (5ng/µl).
3. PCR profile:

Cycle	Temperature (°C)	Time
1	94	2 min
	94	30 seg.
35	62	30 seg.
	72	30 seg.
1	72	10 min.
1	4	Forever

* The annealing temperature for **primer G11 is 62°C.**

17.7 PRIMER Pi 63

1. Master mix preparation when use this primers (10 µl): Make a bulk mix for all reactions, add extra reactions to allow for pipetting errors.

Reagent	Stock	Final concentration	Volume for one reaction
Milli-Q water			1.60 µl.
Storage Buffer B (Promega)	10x	1X	1 µl.
$MgCl_2$	25 mM	3.50 mM	1.40 µl.
dNTP	2.5 mM	0.10 mM	0.40 µl.
Primer forward	10 µM	0.20 µM	0.20 µl.
Primer reverse	10 µM	0.20 µM	0.20 µl.
Taq Polymerase	5U/µl	0.10U	0.20 µl.

2. Add in each PCR tube 5 µl of master mix and 5 µl of DNA (5ng/µl).

3. PCR profile:

Cycle	Temperature (°C)	Time
1	94	2 min
	94	30 seg.
35	62	30 seg.
	72	30 seg.
1	72	10 min.
1	4	Forever

* The annealing temperature for **primer Pi63 is 62°C.**

ELECTROPHORETIC RUN

Once amplified the samples, they should be first confirmed in 1% agarose gel. Run 1 µl of amplified samples. The bands observed would be in a range of 100 to 300 bp depending on the primers that were used.

POLYACRYLAMIDE GEL

1. Denature the samples by adding 2 µl of loading dye, mix and heat at 95°C for 5 min. Immediately place the denatured samples on ice to prevent annealing.
2. Remove clamps. Clean excess of polyacrylamide and urea from the top of plates with distilled water.
3. Place glass plates in the apparatus and add buffers. Pre run at 2000 V during 15 min, or 1500 V during 45 min.
4. Clean comb area with buffer before to place the comb. Insert the shark toothcomb between the glass plates with teeth facing downwards. Clean each well with 1X TBE buffer.
5. Load the amplified samples into each well (approx. 10µl).
6. Run the gel at 800V for Primer 4B and for the rest of primers at 600V over night. The next day you can rise the voltage without problem.
7. **Running time is variable depending on the size of PCR fragment.** To finish the run is necessary to let fall the two colors of dye. The first one that falls is the blue color, because its weight is 60 bp. The second color is light blue and it has a weight of 106 bp (this color is that you observe the next day).

   In the case of primer 4B, at 1500V wait 40 min after the light blue color falls. For Primer 2D, as soon as the light blue falls, immediately proceeds to stop the run.

   For primers 1F and G11 the light blue has to reach about 7 cm above the edge of glass plates.

   For primers Pi66, Pi63 y D13 stop the run when the light blue is about 4 cm above the edge of glass plates.

   In the case of primer Pi89 let the light blue falls completely and stop the run..
8. Turn off the power supply. Remove the plates from the tank. Separate the glass plates using a plastic wedge. And proceed with the silver stain.

SILVER STAIN PROTOCOL DESCRIBED BY Dr. Anna Karin Widmark			
	Total Volume to prepare **1000 ml**		
FIX 20′	Acetic Acid 10% Up to with water	100 ml 1000 ml	Keep this solution after use because can be reused or as stop solution.
3 WASHES WITH DISTILLED WATER **FOR 2 MINUTES**			
REDUCTION OF BACKGROUND 1-2′	Nitric Acid 1% Up to with water	10 ml 1000 ml	This solution can be used four times.
3 WASHES WITH DISTILLED WATER **FOR 2 MINUTES**			
STAIN 40′	$AgNO_3$ Formaldehyde 37% Up to with water	1 g 1.5 ml 1000 ml	This solution can be used three times.
1 WASH WITH DISTILLED WATER **FOR 5 TO 15 SECONDS**			
DEVELOPED To keep at 40C 2′- 25′	Na_2CO_3 Sodium Tiosulfate (10mg/ml) Formaldehyde 37% Up to with water	30 g 500 ul 1.5 ml 1000 ml	Add the Formaldehyde (37%) and Sodium Thiosulfate just 5′ before use.
STOP	Acetic Acid 10% Up to with water	100 ml 1000 ml	
WASH WITH DISTILLED WATER TWICE **FOR 10 MINUTES**			

18 POTATO DNA EXTRACTION

1. Weigh 500mg of fresh leaf tissue, grind with liquid Nitrogen, 250mg of beta-metabisulfite, and sand.
2. Add 2.5ml extraction buffer, keep on ice while adding to other samples.

Buffer	Ingredient	Concentration
Extraction pH 8.2	Sorbitol	350 mM
	Tris	100mM
	EDTA	5mM
Lysis	Tris	200mM
	NaCl	2M
	CTAB	2%
	EDTA	50mM

3. Add 2.5ml of lysis buffer (preheated to 60°C and 2%CTAB added just prior to use).
4. Add 100µl of 10% sarkosyl and then incubate tubes for 40 minutes at 65°C. Invert tubes from time to time.
5. Add 5ml of chloroform and mix gently, and centrifuge at 10000 rpm for 10 minutes.
6. Transfer aqueous supernatant to a new tube and add 1 ml of cold isopropanol. Mix by inversion and leave in fridge. Yield may also be increased by adding 100% ethanol.
7. Centrifuge at 10000 rpm for 5 minutes.
8. Remove isopropanol and rinse with 70% alcohol, let air dry and add 500µl of TE.
9. Treat with 1µl of 5mg/ml of RNAse for 30 minutes at 60°C.

NOTE: This method is also good for isolating *P.infestans* DNA, just use 1/5 of everything.

19 RNA EXTRACTION OF *P. INFESTANS* USING TRIZOL REAGENT

19.1 Homogenization

1. Dispense lyophilized mycelium 0.02g 1mL Trizol. Homogenize gently for 10 sec.
2. Mix by vortexing for 30 sec.
3. Incubate for 5 min (on ice).

19.2 Separation

1. Centrifuge at 10000 rpm for 15 min (4°C)
2. Transfer the supernatant to a new 2 ml tube.
3. Add 0.2mL of chloroform per 1 mL of Trizol.
4. Mix gently for 20 sec. And incubate for 5 min (on ice).
5. Centrifuge at 10000 rpm for 15 min (4° C)
6. Transfer supernatant to a new tube (1.5mL).

19.3 Precipitation and Washing

1. Add 0.5 mL of isopropanol per 1 mL of Trizol.
2. Mix gently for 15 seconds and incubate at room temperature for 10min.
3. Carry -20 or -70 C for 1 hour 30 min.
4. Centrifuge at 10000rpm for 20 min at 4 C.
5. Remove the supernatant, being careful not to lose the pellet.
6. Dry at room temperature for 5 min. (Inverted tube)
7. Wash the pellet with 1 mL of DEPC-70% ethanol or 75% 1mL Trizol. Wash gently.
8. Centrifuge at 10000 rpm for 8 min at 4° C.
9. Remove the supernatant, being careful not to lose the pellet.
10. Dry the pellet at room temperature for 10 min. "No dry completely"
11. Suspend the pellet in 50 -100uL with RNA storage solution or DEPC water.
    Mix gently to dissolve the pellet.

20 HELP FOR USING FTA CARDS TO SAMPLE POTATO LATE BLIGHT IN THE FIELD (SHOULD ALSO WORK FOR TOMATO)

Euroblight protocols www.euroblight.com.

20.1 Sample design

Below is a description of a study done in Kenya.

We have enclosed 14 FTA cards. This is enough to sample blight from 7 fields (8 samples/field) or 14 fields (4 samples/field) as they have 4 circles/card = 56 samples.

If sampling 7 fields: In each field identify 4 infected plants (not right next to each other) and take 2 lesions from each plant = 8 samples/field.

If sampling 14 fields: In each field identify 4 infected plants as above and take 1 lesion from each plant = 4 samples/field.

20.2 Picking lesions

Avoid dead leaves, old or drying lesions or those leaflets with many lesions. In the pictures below, the one on the left has a lesion that could work, but there are several more beside it. In the image on the right, the leaflet indicated by the arrow has only one good lesion. Choose those if possible. Here is what Alison Lees says, and she has experience, "…to be honest as long as it's a fairly decent spreading single lesion such as those in the photo's it will work (and even if it isn't it probably will still work). Wet, bacterial looking leaves where the disease is too far progressed and small restricted lesions will probably not work."

Follow the attached instructions for pressing the lesions onto the cards (Direct leaf Press method).

20.3 Using the FTA Cards

Direct leaf press:

1. Place leaf material directly onto the FTA card. Lay a piece of parafilm (you could also use a clean sheet of plastic or even a clean sandwich bag) over the leaf.
2. Apply moderate pounding/pressure to the leaf area with a blunt object such as a tack hammer or pestle.
3. When the extract is drawn through to the back of the FTA card the collection process is complete.
4. Samples applied to FTA cards are ready for immediate room temperature storage.

> **NOTE,** while the cards may still be wet it is important to not stack them in such a way that they might cross contaminate.

> **NOTE:** If samples are to be processed shortly after application on the FTA card, allow the sample to dry for one hour at room temperature prior to punching. Do not heat to shorten the drying period.

5. The sample is now ready for downstream processing or archiving.
6. Label the cards so they don't get mixed up. Here is an example used in a recent project:

Label the card in the following way:

Field 1, plant 1, lesion 1 (F1_P1_L1)

Field 2, plant 4, lesion 2 (F2_P4_L2)

Don't write in the circles.

Let the card air dry before closing.

For each field make a note (where possible) of:

1) The location

2) The variety

3) Date of sampling

4) How severe the blight is (scattered, large patches, extensive)

Send the cards to:

21 STORAGE OF PHYTOPHTHORA INFECTED LEAVES IN FIELDS

Materials

- RNA later (Ambion Cat#7023 20x 5ml)
- Scissors
- Paper like "Kim care, Kim wipe …"
- 70 % EtOH

Procedure

- Look for Phytophthora infected leaves. Choose leaves whose symptom is not only necrotophic and biotrophic.
- Wipe scissors by wet paper like "Kimcare" with 70% EtoH.
- Get 8 leaf discs (Diameter ~ 1cm) by Scissors and cut to tiny fragments, and put into RNA later (Fig.)
- Write location, date, sample ID to the bottle of RNA later.
- Store 4 °C when you arrive at the place where refrigerator is available. Until 1 week, samples can be stored at room temperature. When you arrive at your laboratory. The samples should be kept at -20 °C.

Notes

- You can use a cork borer instead of scissors. But scissors (3 min) is faster than cork borer (10 min.). If you use #4 cork borer, you should collect 8 leaf discs and cut a disc in half.
- RNA is stable at 4 °C within 1 month.

Storage at –20°C: Samples will not freeze at –20°C, but crystals may form in the storage buffer; this will not affect subsequent RNA isolation. Samples can subsequently be thawed at room temperature and refrozen without affecting the amount or the integrity of the recoverable RNA (Ambion RNA later protocol.)

22 RNA/DNA EXTRACTION FROM SAMPLES IN RNA LATER

You can use any RNA/DNA extraction kits for plant and fungi. The important thing is to remove RNA later solution from samples before putting into the extraction buffer of the kits.

Here, I explain protocols using QIAGEN RNeasy Plant Mini Kit and QIAGEN DNeasy Plant Mini Kit. We also use OmniPrep (BIOSCIENCE) for extracting DNA from infected materials.

RNA extraction from samples in RNAlater.

Materials

- Samples in RNAlater
- QIAGEN RNeasy Plant Mini Kit
- 2 ml Safe-lock tubes
- Disposable Homogenization Pestle or mortar& pestle

Procedure

- Prepare a 2ml tube
- Add 550 µl Buffer RLT with β-Mercaptoethanol to the tube.
- Remove RNA later solution from half of infected leaves on a paper towel.
- Add the leaves to the tube.
- Homogenize the leaves in the tube using disposable homogenization pestle or mortar& pestle.
- Vortex vigorously.
- A short 3 min incubation at 56°C
- Transfer the lysate to a QIAshredder spin column (lilac) placed in a 2 ml collection tube, and centrifuge for 2 min at full speed. Follow the manufacture protocol (from RNeasy Mini Hand book ,Plants and Fungi p54, 4).

DNA extraction from samples in RNAlater.

Materials

- Samples in RNAlater
- QIAGEN DNeasy Plant Mini Kit
- 2 ml Safe-lock tubes
- Disposable Homogenization Pestle

Procedure

- Prepare a 2ml tube
- Add 500 µl Buffer AP1 and 5µl RNase A stock solution (100 mg/ml) to the tube.
- Remove RNA later solution from infected leaves on a paper towel.
- Add the leaves to the tube.
- Homogenize the leaves in the tube using disposable homogenization pestle or mortar& pestle.
- Vortex vigorously.

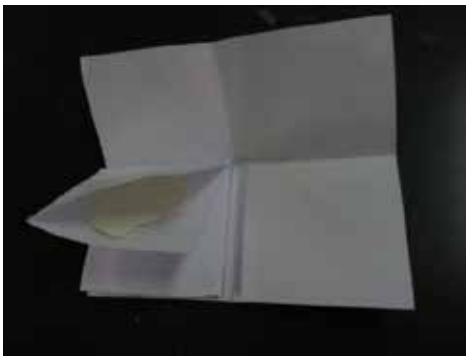
23 METHOD TO DRY *P. INFESTANS* MYCELIUM FOR STORAGE, TRANSPORT AND DNA EXTRACTION

The silica gel adsorb all of the water from the tissue, drying it rapidly, completely, and preserving the tissue very effectively. Here we present an easy and inexpensive method to prepare *P. infestans* mycelia for storage, shipment and subsequent DNA extraction that does not require sophisticated equipment, such as a lyophilizer and -70°C freezer. This mycelia is non-viable and can thus be to send internationally with no biological risk.

We recommend using orange silica gel since silica with blue indicator is very toxic.

1. Collect mycelium from pea broth using a Millipore filter system.

2. Place the mycelium in filter paper and wrap it using two filter papers.

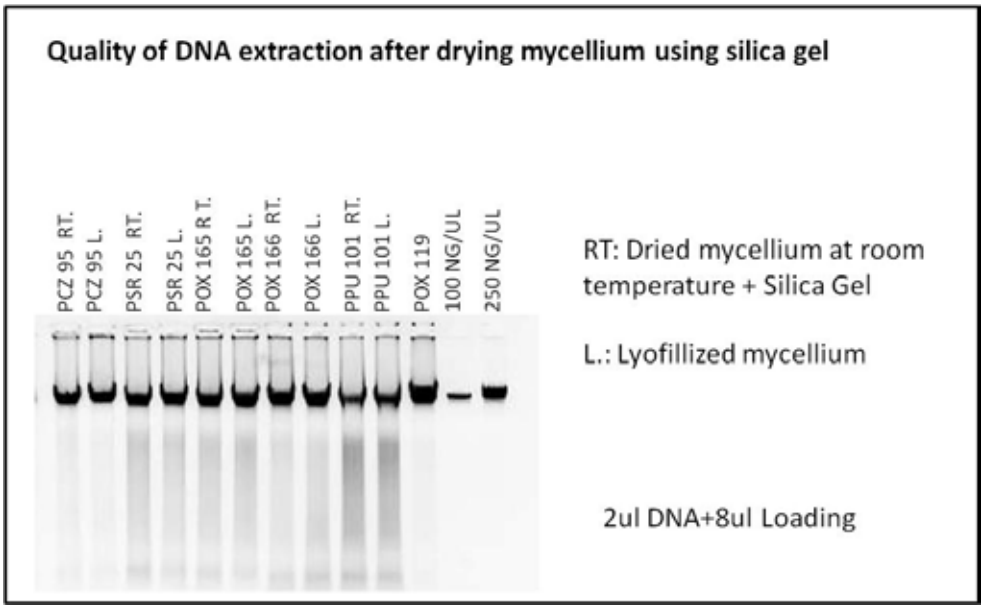


3. Coding each sample and placed in a bag containing silica gel.

4. Seal the bag and place it in a tightly closed container.

5. Store the container at room temperature for 7-10 days. If it is necessary, change the silica gel if it turned white.

6. Mycellium will be dried and ready for DNA extraction previous maceration with liquid nitrogen.



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