The Biotechnology Centre (UG) & The Institute for Genomic Diversity (Cornell University)
Training Course on the Use of Molecular Markers for Efficient Crop Improvement
30 June – 11 July 2008

Laboratory Manual

Laboratory Practices & Safety:

All enzymes used in these procedures are very **expensive**! Always keep the enzyme on ice and remove the enzyme from the freezer just before starting the required step of your experiment! Hold enzyme stocks at the top of the tube. Enzymes are proteins that are degraded by heat so do not put your "hot little hands" on portions of the tube containing enzyme.

Laboratory equipment is also very **expensive** and, sometimes (e.g. in the case of centrifuges) potentially **dangerous**.

Please do not use any equipment you are unfamiliar with without asking for help!

Ultraviolet light is dangerous to the eyes. Goggles should be worn unless other shielding is available.

Of course, hands should be washed after any laboratory practices and especially before any food is eaten.

All of the liquid chemicals listed below are highly toxic and hazardous compounds. Take extreme care when handling these compounds and **always wear a labcoat, gloves and safety glasses**. On contact with skin/eyes wash immediately with water.

- **Acrylamide : Bisacrylamide** (19:1) 40% solution is bought ready made. Acrylamide is a neurotoxin and possible carcinogen. It is most dangerous when dry (inhalation hazard) so wipe any spilled liquid acrylamide off the bench before it has a chance to dry.
- **Chloroform** a carcinogenic and toxic solvent, readily absorbed through the skin, degreases and deproteinates the skin; dispense in the fumehood. Chloroform is a hazardous solvent and readily melts some plastics such as polystyrene: ***Take great care when handling.*** Avoid chloroform on outside of tubes as it erases labels.
- **Alcohol** highly flammable
- **Ethidium bromide** may cause heritable genetic damage.
- **Liquid nitrogen** risk of cryogenic burns, always handle in a well aerated and open area.
- **Silver nitrate** a possible carcinogen, causes burns, absorbed through the skin and stains the skin.
- **Sodium thiosulphate** irritant, dispense stock in the fumehood.
- **TEMED** highly flammable, harmful by inhalation; causes burns.
- **All other chemicals in powder or crystal** form that you are likely to encounter on this course will generally be irritants in one way or another, possibly causing irreversible effects on contact with the skin or on inhalation, always wear gloves when weighing, dispensing and handling solutions.

Genomic DNA isolation

Fulton TM, Chunwongse J, and Tanksley SD. (1995) Microprep Protocol for Extraction of DNA from Tomato and other Herbaceous Plants. Plant Molecular Biology Reporter 13 (3): 207-209.

1. Collect 50-100 mg (approximately 4-8 new leaflets, up to 1.5 cm long) from a 1-3 week old tomato seedling[1] and nestle loosely in the bottom of a 1.5 mL Eppendorf tube.[2]
2. Prepare fresh microprep buffer (see recipe below), keep at room temperature.
3. Add 200 µL of buffer and grind tissue with power drill and plastic bit (rinsing pestle with water between samples); add another 550 µL of buffer and either vortex lightly or shake entire rack by hand.
4. Incubate in 65C waterbath for 30-120 minutes.
5. Fill the tube with chloroform/isoamyl (24:1). Mix well. (This can be done by vortexing each tube or sandwiching tubes between two racks and vigorously inverting or shaking up and down 50-100 times).
6. Centrifuge tubes at 10,000 rpm for 5 minutes.
7. Pipet off aqueous phase into new microfuge tubes. Add 2/3-1 times the volume of cold isopropanol to each tube. Invert tubes until DNA precipitates.
8. Immediately spin at 10,000 rpm for 5 minutes (no more), pour off isopropanol and wash pellet with 70% ethanol.[3]
9. Dry pellet by leaving tubes upside down on paper towels for approximately 1 hr or placing on sides in seed dryer for 15 minutes (longer if necessary).
10. Resuspend DNA in 50 µl of TE at 65ºC for 15 min.
11. Spin 10 min at 10,000 rpm, store at 4ºC for up to 1 week, -20ºC for longer storage.
12. For RFLP use, digest 15-25ul for one Southern blot (can expect 5-10ug DNA, use 15-20 units of enzyme). For PCR, use 1 µL.

Optional:
Remove any contaminating RNA by addition of 1 of a 10 µg/mL stock solution of RNase and incubate at 37ºC for 30 min.

Recover DNA by the addition of 1/10 volume of 3M sodium acetate (pH 6.8) and 2 volumes of 96% ethanol to the DNA containing solution.

Incubate on ice for 10 min

Centrifuge at maximum speed for 5 min at room temperature, to pellet the DNA.

Wash and dry DNA as above and finally dissolve in 50 sdH2O.

[1]If only PCR is needed, can use as little as 1 cotyledon, resuspend in 50 µL in Step 10 but use 5 µL to PCR
[2]Tissue can be harvested and kept at room temperature for up to 3 hours or stored at 4C for up to 3 days
[3]Can stop here, storing pellet in 70% EtOH at -20C indefinitely

General Notes:
Yield should be approximately 10-20 µg of DNA, enough for 2-4 Southerns or 50-100 PCR reactions. The number of samples can be maximized by using 2 drills with drill stands concurrently and a foot pedal on/off switch. One experienced person can do up to several hundred samples in one day. This protocol is known to work on tomato, pepper, apple, tobacco, strawberry, and artichoke.

Materials and Solutions required

Drill: Heavy duty household drill with keyless chuck (so pestles can be easily replaced) and plastic drill bit/pestles (VWR Scientific, catalog#KT95050-99)
Microcentrifuge: with a fixed angle rotor, capable of 10,000 rpm and holding as many samples as possible.

(see Recipes at the end of this manual)

Microprep Buffer: 2.5 parts DNA Extraction Buffer, 2.5 parts Nuclei Lysis Buffer, 1.0 part 5% Sarkosyl. Add 0.3-0.5g Sodium Bisulfite/100 mLs to buffer immediately before use (can be increased to avoid color in final product).

For 75 extractions:
25 mLs DNA Extraction Buffer
25 mLs Nuclei Lysis Buffer
10 mLs Sarkosyl
60 mLs Microprep Buffer, add 0.2g Sodium Bisulfite

Troubleshooting guide

Problem	Possible solution
Low concentration of DNA	Grind longer Use younger tissue
DNA does not form firm pellet	After spin, remove replace 500 µL isoprop. with 70% EtOH, gently mix, respin
DNA does not digest	Dry pellet longer before resuspension (remove alcohol residue) Take only the aqueous layer of the chloroform gradient, do not take any interface (avoid chloroform residue)
DNA does not PCR	See solutions for not digesting Use different amount of DNA (probably less)

Agarose gel assessment of DNA concentration

There are two simple methods of assessing DNA concentration, one is to take a dilution of your DNA in water and make a spectrophotometric reading at 260 nm, the other is to run a sample on agarose gel, stain with ethidium bromide and compare the concentration against known standards. The preferred method for this course is using agarose gels as this is more sensitive to low concentrations of DNA - typically 10 ng can be detected by this method. This assessment method will, after staining with ethidium bromide:-

- show if the DNA is sheared,
- show if there is RNA remaining,
- allow the DNA concentration to be calculated.

1. Using a 500 mL Duran bottle: make up 250 mL of 0.8% agarose in 1xTBE buffer; heat in a microwave oven on medium power until boiling and dissolved, leave the lid loose the whole time so that pressure cannot build up in the bottle; leave to cool to 65-80ºC.
2. Prepare the gel casting tray with the desired number of wells and tape the ends. Pour the gel: not too deep - until the whole base is just covered with molten agarose. Allow the gel to set for 1 hour.
3. Take 2-3 of each DNA preparation to 4 of SDW (sterile distilled water) and add 5 of Orange G loading dye or some other appropriate agarose gel loading dye.
4. Prepare 4 standards - of 0.5, 0.25, 0.1 and 0.05 µg - using the lambda DNA at 0.5 µg/ in the same way as in 3 above. Dilute in SDW.
5. Remove the tape from the ends of the casting tray and place the tray in the electrophoresis tank. Pour in 1 x TBE buffer, and remove the comb.
6. Load samples and standards, segregate the samples into blocks of 15 or so separated by a DNA ladder; run at 100V for 1-2 hour.
7. Remove the gel from the casting tray to a deep tray containing 5 of 10 mg/mL ethidium bromide solution in 150 mL of 1 x TBE and stain for 20-30 minutes; rinse the gel briefly with distilled water.
8. Visualise under UV and photograph.
9. Assess the DNA concentration of each sample against the standards.

Wear gloves when handling the gel at all subsequent stages. Care should be taken when handling ethidium bromide - wear gloves. Ethidium bromide is a mutagen and a suspected carcinogen.

1) Allow the gel to set. It will become translucent all over when completely set. You will be able to notice the change from transparency to opaqueness at the edges first, but wait until the whole gel has set before removing the comb.

2) Remove the gel tray from the casting tray and place in the gel box. Take care that the gel does not slip off the gel tray.

3) Place the tray on the center support and add more 1 x TBE buffer until the gel is just covered.

4) Carefully remove the comb from the gel. Convention dictates that the "top" of a gel is where the wells are. The gel is now ready! Make sure that you have placed the gel in the tray in the correct orientation – DNA is negatively charged and runs towards the positive electrode (Red)

DNA Quality and quantity check

To test for the quality and amount of isolated DNA, a sample of isolated DNA (5 µL) is run on the 1% agarose gel in TBE buffer (0.04 M Tris-acetate; 1 mM EDTA, pH 8) that was prepared as described above.

Place 5 µL of your DNA in a microfuge tube, add 5 µL of water and 2 µL of loading dye and place all the solution into one of the wells in the gel. In one lane place a DNA concentration standard (usually a known amount of lambda DNA). A range of lambda DNA amounts (50ng, 100ng, 250ng and 500ng) can be used to get a more accurate estimation of the DNA concentration. We will use these 4 concentration standards

Run the gel at 100V for 90 minutes
Determine the DNA quality on a UV-transilluminator, photograph if possible and determine the DNA concentration of samples visually using the λ DNA amounts for comparison.

Dilute an aliquot of your DNAs to a concentration of 10ng/µL for the PCR reactions.

Polymerase chain reaction (PCR): SSRs

The polymerase chain reaction is a three-step process, which is repeated in several cycles. The three steps are ***denaturation*, *annealing*** and ***extension*.** In the first of the chain reaction, *denaturation*, the two DNA strands are separated by heating the DNA to 94^{o}C. Heat treatment breaks the relatively weak bonds between the DNA bases yielding two long single strands. In the second step, *annealing*, two primers attach themselves to the single strands. These primers are small, synthetic single-stranded DNAs each about 20 base pairs long. They are selected so that they are complementary to the two single strands. The primers track down and bind themselves to the region being sought by framing the target sequence within the long DNA strand. The annealing temperature is usually in the range of 50-65^{o}C depending on the length and composition of the primer. Once the primers have attached themselves, two short stretches of double-stranded DNA are generated. In the third step, *extension*, these short stretches serve as starting blocks for the enzyme *Taq polymerase*. Starting at the 3'-end of the primers, the enzyme adds the nucleotides complementary to the template at about 72^{o}C, linking them together. It extends the primers in the direction of the target sequence, thereby making a double strand out of the two single strands. From the original double strand, there are now, at the end of this first cycle, two new DNA double strands identical with the first one. The three-step cycle can now be repeated as often as necessary where only the sequence flanked by the two primers is amplified. The new DNA sequences provide the template in the next cycle for the creation of new strands, resulting in four in the next cycle and then in 8, 16, 32 copies and so on.

All the enzyme used in this experiment are very expensive! Always keep the enzyme on ice and remove the enzyme from the freezer only before starting the required step of your experiment!

To carry out the PCR reaction, use small PCR tubes with your genomic DNA as template and the primer(s) that will amplify your DNA sequence. The PCR reaction is carried out in 50 µL volumes, which contains your genomic DNA, 15 ng of primer, 100 mM of each dNTP, 10 mM Tris-HCl, pH 8.3, 2 mM $MgCl_2$ and 2 units of Taq polymerase.

Each of you will use the 2 cowpea DNA samples that you extracted (one using the CTAB procedure and one using the FTA procedure) and one microsatellite primer set.

Recipe:

Stock	**Final conc**	**µL Single Reaction**	**µL Cocktail**
Genomic DNA			
Primer 1			
Primer 2			
dNTP			
Tris-HCl			
$MgCl_2$			
Taq			

Place all tubes into the thermal cycler and use the following DNA amplification programme:

(i) 94°C for 5 minutes x 1 cycle;

(ii) 94°C for 1 minute;

(iii) 55°C for 1 minute, 72°C for 1 minute x 40 cycles;

(iv) 72°C for 7 minutes x 1 cycle;

(v) hold at 4°C until ready to load onto gel.

The amplification will last about 4 hours!

SSR product visualization

For the SSR technique pour a 3% super-fine agarose gel (50 or 100 mL using 1x TAE buffer, ask your supervisor for the exact ml to be used which depends on the gel size). The demonstrator will show how to melt the agar and to pour the gel. Before pouring the gel add 2 µL ethidium bromide and mix gently the agar with the ethidium bromide (10mg/mL).

Add 5 µL of the loading dye to your PCR reaction. Load 20 µL of the mixture into one of the wells in the gel. All the reactions with the same set of primers should be run in adjacent lanes to make size comparisons easier. Run the gel at 120mV for 90 minutes. Don't let the loading dye run out of the gel. Wearing gloves, place gel on UV screen (why are gloves necessary?) and write down your observation. Also take a photo from your gel for your protocol; the demonstrator will help you in making a photo.

LIGATION TECHNIQUE

pGEM®-T and pGEM®-T Easy Vector Systems
INSTRUCTIONS FOR USE OF PRODUCTS A1360, A1380, A3600 AND A3610.

Cloning PCR Products with pGEM®-T and pGEM®-T Easy Vectors

Ligation Using 2X Rapid Ligation Buffer

1. Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect contents at the bottom of the tube.

2. Set up ligation reactions as described below. Vortex the 2X Rapid Ligation Buffer vigorously before each use. Use 0.5ml tubes known to have low DNA binding capacity.

Reagents	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer, T4 DNA Ligase	5µl	5µl	5µl
pGEM®-T or pGEM®-T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	Xµl	-	-
Control Insert DNA	-	2µl	-
T4 DNA Ligase (3 Weiss Units/µl)	1µl	1µl	1µl
Deionized water to a final volume of	10µl	10µl	10µl

3. Mix the reactions by pipetting. Incubate the reactions 1 hour at room temperature.

Alternatively, incubate the reactions overnight at 4°C for the maximum number of transformants.

Transformation of JM109 High Efficiency Competent Cells

1. Prepare LB/ampicillin/IPTGIX-Gal plates.
2. Centrifuge the ligation reactions briefly. Add 2µl of each ligation reaction to a sterile 1.5ml tube on ice. Prepare a control tube with 0.1ng of uncut plasmid.
3. Place the JM109 High Efficiency Competent Cells in an ice bath until just thawed (5 minutes). Mix cells by **gently** flicking the tube.
4. Carefully transfer 50µl of cells to the ligation reaction tubes from Step 2. Use 100µl of cells for the uncut DNA control tube. **Gently** flick the tubes and incubate on ice for 20 minutes.
5. Heat-shock the cells for 45-50 seconds in water bath at exactly 42°C. DO NOT SHAKE. Immediately return the tubes to ice for 2 minutes.
6. Add 950µl room temperature SOC medium to the ligation reaction transformations and 900µl to the uncut DNA control tube. Incubate for 1.5 hours at 37°C with shaking (~150rpm).
7. Plate 1 OOµI of each transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates. For the uncut DNA control, a 1:10 dilution with SOC is recommended.
8. Incubate plates overnight at 37°C. Select white colonies.

Recipes

Make all buffers and solutions with distilled water.

Prepare them well in advance of your experiments. This is particularly relevant to the acrylamide gel mix which must be pre-made the day before you need it and stored at 4°C

DNA Extraction Buffer

Sorbitol	0.35M	63.75g
Tris Base	0.1M	12.1g
EDTA	0.005M	1.86g
		-----------
		1 L

Adjust PH to 8.25 with HCL

*Add NaBisulfite 3.8-5.0g/L before using

Nuclei Lysis Buffer

		Final
1M Tris (PH=8)	200mL	200mM
0.25M EDTA	200mL	50mM
5M NaCl	400mL	2M
CTAB	20g	2%
DdH2O	200mL	
	-----------	
	1 L	

*Dissolve CTAB in heated water separately then mix with the rest.

Sarkosyl

5% Sarkosyl 50g/L

$TE_{0.1}$ pH 8.0

10 mM Tris pH 8

0.1 mM EDTA pH 8

Make from heat sterilised components. Store at room RT

10xPCR buffer

500 mM KCl

100 mM Tris-HCl pH 8.5

15 mM $MgCl_2$

1 mg/mL Gelatin

Add all components together; dispense into 20 mL volumes and heat sterilise.

Store at -20ºC

10xTBE

Tris Base 121 g

Boric Acid 51.3 g

EDTA 3.7 g

Distilled Water to 1 litre

Dissolve components, sterilise by heating.

Store at room temperature (RT)

Acrylamide Mix 4.5%

Urea 420 g

10XTBE 100 mL

Acrylamide mix 40% (19:1) 115 mLs

Distilled Water to 1 litre

Dissolve the Urea in 400 mLs of water by heating gently (1 minute/medium in a microwave oven) stir till the crystals completely dissolve; while stirring add 100 mL of 10XTBE and then add the 115 mLs of acrylamide; make up to 1 litre with water. Store at 4ºC **as this must be used chilled.**

NB make less, e.g. 250 mLs, if you plan to pour only 2 to 3 gels. Each gel requires 60 mls.

20 x SSC

NaCl 175.32 g

Trisodium citrate 88.23 g

Distilled water to 1 litre

Store at RT

Acrylamide gel loading dye

98% Formamide

0.025% Bromophenol blue

0.025% Xylene cyanol

10 mM EDTA pH 8

Store at RT

Silver staining solutions

Note: we will be using the BioRad kit; these are for your reference only

1.Developer:
Dissolve 60 g sodium carbonate (make sure it is anhydrous and not too old)

in 2 litre of distilled water and place at 4ºC, for at least 4 hours before required.

2.Fixer:
10% acetic acid (200 mL glacial acetic acid added to 1.8 litre distilled water).

3. Silverstain solution:
12 mL 1.010 N silver nitrate solution in 2 litre of distilled water; add 3 mL formaldehyde (40% solution) and mix.

4. Developing solution:

Immediately prior to developing the gel, add 300 µL of sodium thiosulphate solution (0.1001 N) and 3 ml of formaldehyde (40% solution) to the pre-chilled sodium carbonate solution.

XI.C. <u>Composition of Buffers and Solutions</u>

IPTG stock solution (0.1M)
1.2g IPTG
Add water to 50m1 final volume. Filter-sterilize and store at 4°C.

X-Gal (2ml)
100mg 5-bromo-4-chloro-3indolyI-ß-D-galactoside
Dissolve in 2mI N,N' –dimethyl-formamide. Cover with aluminum foil and store at -20°C.

LB medium (per liter)

10g	Bacto®-tryptone
5g	Bacto®-yeast extract
5g	NaCI

Adjust pH to 7.0 with NaOH.

LB plates with ampicillin

Add 15g agar to 1 liter of LB medium. Autoclave. Allow the medium to cool to 50°C before adding ampicillin to a final concentration of 100µg/ ml. Pour 30-35ml of medium into 85mm petri dishes. Let the agar harden. Store at 4°C for up to 1 month or at room temperature for up to 1 week.

LB plates with ampicillin/lPTG/X-GaI
Make the LB plates with ampicillin as above; then supplement with 0.5mM IPTG and 80µg/ ml X-Gal and pour the plates. Alternatively, 100µl of 100mM IPTG and 20µl of 50mg/ ml X-Gal may be spread over the surface of an LB-ampicillin plate and allowed to absorb for 30 minutes at 37°C prior to use.

SOC medium (100ml)

2.0g	Bacto@-tryptone
O.5g	Bacto@-yeast extract

1ml	1M NaCl
0.25ml	1M KCI
1ml	2M Mg^{2+} stock, filter-steri1ized
1ml	2M glucose, filter-sterilized

Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{2+} stock and 2M glucose, each to a final concentration of 20mM. Bring to 100m1 with sterile, distilled water. The final pH should be 7.0.

2M Mg^{2+} stock

20.33g	$MgCl_2$ $6H_2O$
24.65g	$MgSO_4$ $7H_2O$

Add distilled water to 100ml. Filter sterilize.

2X Rapid Ligation Buffer, T4 DNA Ligase (provided)

6OmM	Iris-HCI (PH 7.8)
20mM	$MgCl_2$
20mM	DTT
2mM	ATP
10%	polyethylene glycol (MW8000, ACS Grade)

Store in single-use aIiquots at -20°C.
Avoid multiple freeze-thaw cycles.

TYP broth (per liter)

16g	Bacto®-tryptone
16g	Bacto®-yeast extract
5g	NaCI
2.5g	K_2HPO_4

TOTAL PROTEIN ELECTROPHORESIS

Sample Preparation Buffer

1. About 0.2gm of the washed plant tissue eg leaves is taken.

2. Add about 500ul of Tris-Citric Buffer pH 7.0 (0.2M Tris (Trizma base) ie 2.42g/100mls titrated to pH 7.0 with 1M Citric acid).

3. Grind in mortar with pestle.

4. Centrifuge at 15,000g for 6 min.

Sample Buffer (SDS Reducing Buffer)

3.55 ml	deionized water
1.25 ml	0.5 M Tris-HCl, pH 6.8
2.5 ml	glycerol
2.0 ml	10% (w/v) SDS
0.2 ml	0.5% (w/v) bromophenol blue
9.5 ml	Total Volume

Store at room temperature.
Use: Add 50µl ß-Mercaptoethanol to 950 µl sample buffer prior to use. Dilute the sample at least 1:2 with sample buffer and heat at 95ºC for 4 minutes.

Stock Solutions and Buffers

1. Acrylamide/Bis (30% T, 2.67 % C)

87.6 g	acrylamide	(29.2 g/100 ml)
2.4 g	N'N'-bis-methylene- acrylamide	(0.8 g/100 ml)

Make to 300 ml with deionized water. Filter and store at 4ºC in the dark (30 days maximum)
or use;
Preweighted Acrylamide/Bis, 37.5:1 mixture (30%T, 2.67% C)
(Bio-Rad catalog number 161-0125, 150 g)

✓ 30% Acrylamide/Bis Solutions, 37.5:1 mixture (30%T, 2.67% C)
(Bio-Rad catalog number 161-0158, 500 ml)
(Bio-Rad catalog number 161-0159, 2 x 500 ml)

2. 10% (w/v) SDS
Dissolve 10g SDS in 90 ml water with gentle stirring and bring to 100 ml with deionized water. Alternatively 10% SDS solution (250 ml) can be used (Bio-Rad catalog number 161-0416)

3. Gel buffer: 1.5 M Tris-HCl, pH 8.8
    27.23 g   Tris base   (18.15 g/100 ml)
    80 ml   deionized water

Adjust to pH 8.8 with 6 N HCl. Bring total volume to 150 ml with deionized water and store at 4ºC. Alternatively 1.5 M Tris-HCl, pH 8.8 (1 L) premised buffer can be used (Bio-Rad catalog number 161-0798).

4. Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8
    6 g   Tris base
    60 ml   deonized water

Adjust to pH 6.8 with 6 N HCl. Bring total volume to 100 ml with deionized water and store at 4ºC. Alternatively, 0.5 M Tris-HCl, pH 6.8 (1 L) premixed buffer can be used (Bio-Rad catalog number 161-0799).

5. 10x Electrode (Running) Buffer, pH 8.3 (makes 1L)
    30.3 g   Tris base
    144.0 g   Glycine
    10.0 g   SDS

Dissolve and bring total volume up to 1,000 ml with deionized water. Do not adjust pH with acid or base. Store at 4ºC. If precipitation occurs, warm to room temperature before use. Alternatively, electrophoresis running buffer 10x Tris/Glycine/SDS, 5 L cube (Bio-Rad catalog number 161-0772) can be used.

Use: Dilute 50 ml of 10x stock with 450 ml deionized water for each electrophoresis run. Mix thoroughly before use.

6.10% APS (fresh daily)
    100 mg   ammonium persulfate
    Dissolved in 1 ml of deionized water.

Gel Formulations (10 ml)

	10%	12.5%	15%	17%
Distilled $H_2 0$.	4.1ml	3.4ml	2.4ml	1.7ml
30% Acrylamide stock	3.3ml	4.0ml	5.0ml	5.7ml
Gel Buffer stock (pH 8.8)	2.5ml	2.5ml	2.5ml	2.5ml
10% Ammonuim persulphate (fresh)	50µl	50µl	50µl	50µl
TEMED	10µl	10µl	10µl	10µl

1. Prewash the plated in 6M HCl, and rinse several times in distilled $H_2 0$.

2. Inset the comb and mark 1 cm below the comb with a marker.

3. Prepare separating gel by mixing as stated above.

4. Fill plates set with above solution to about the mark and top with drops of n-butanol.

5. Allow to polymerise for about 40mins.

6. Prepare stacking gel by mixing,

Distilled H_20	5.7
30% Acrylamide stock	1.7
Buffer stock	2.5
10% SDS	0.1
10% Ammonium persulphate (fresh)	50µl
TEMED	5µl

7. Wash off n- butanol with distilled H_20.

8. Fill the space left to about the brim and immediately inset the comb.

9. Allow to polymerise for about 30mins

Running The Electrophoresis

10. The set-up in '9' is removed and set in place in the electrophoresis tank.

11. Electrode Buffer/Running pH 8.3.

12. The Electrode Buffer is poured into the bottom and top compartments to make contact with ends of the gels and form a circuit.

*NOTE: Precooling of the buffer is necessary.

13. Remove the comb and check the wells.

*NOTE: This is the time the comb should be removed and not earlier

14. The gel wells are loaded with 15µl of the extracts.

15. The power leads are connected to make an anionic contact (Black lead) to the top and cathodic contact (Red lead) to the bottom.

16. Run system at constant voltage of 50V for about 15 min or until the front/tracking dye moves into the separating gel.

17. The voltage is then adjusted to 80V for about 1 hour or until the front/tracking dye moves within 2mm to the bottom of the separating gel.

18. STAINING THE GEL SOLUTION

Coomassie blue	0.25g
50% Methanol	90ml
Glacial acetic acid	10ml

19. The gel is removed and stained in the solution above for 45mins to 3hrs.

20. DESTAINING SOLUTION

Methanol	300ml
Glacial acetic acid	100ml
Distilled H_2O	1L

The gels are destained by washing in destaining solution at 3 hourly until the background became clear with only protein bands taking up the stain.

21. Store in 20% Glycerol solution.

AFLP MOLECULAR MARKER PROCEDURE

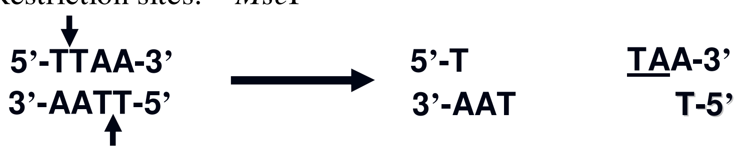
Introduction

This PCR (Polymerase Chain Reaction) based marker method requires no prior sequence knowledge and provided some diversity exists at the molecular level, a reasonable number of polymorphic markers should be obtained from a series of primer combinations. The other main advantage of using AFLP is that it incorporates a series of molecular techniques that are relevant to many other molecular marker types, e.g. RFLP (Restriction Fragment Length Polymorphism), SSAP (Sequence Specific Amplified Polymorphism), SNP (Single Nucleotide Polymorphism), SSR (Simple Sequence Repeat) to name a few.

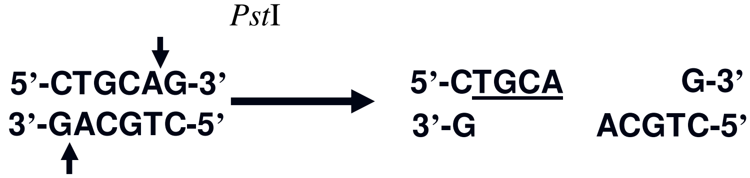
In summary, the AFLP method reduces the whole genome into amplifiable fragments of less than 800 base pairs in length so that a subset of them can be examined on denaturing polyacrylamide gels. The series of procedures uses a range of DNA modifying enzymes and finally relies on fragment amplification by PCR.

Firstly, genomic DNA is double digested using two restriction enzymes, for example, a six base rare cutter, *Pst*I and a four base frequent cutter, *Mse*I.

Restriction sites: *Mse*I



*Pst*I



Note that the overhangs are 5' for MseI, and 3' for PstI. This will give three classes of restricted fragments on double digestion as shown below:

Mse…………………………..Mse

Mse………………………… .Pst
Pst…………………………………………………………..Pst

The fragments generated will be of varying lengths depending on where the restriction sites are situated in the genome. Generally, the Mse/Mse fragments will be the shortest in length as 5'TTAA3' is a reasonably common site in most genomes, compared to the rarer 5'CTGCAG3' site of *Pst*I.

Using T4 DNA ligase, double stranded adapter sequences are then ligated onto the overhangs made by the restriction enzymes.

MseI adapter:

5' GACGATGAGTCCTGAG 3'
3' TACTCAGGACTCAT 5'

PstI adapter:
5' TGTACGCAGTCTAC 3'
3' ACGTACATGCGTCAGATGCTC 5'

In the sequences above, each double stranded adapter is made up from two short, single stranded complementary sequences (the upper strand 1, and the lower strand 2).

The adapters provide a known sequence from which amplification can be primed using a DNA polymerase and specially designed primers complementary to the lower of the two strands. A subset of fragments is amplified. This is achieved by adding ***selective bases*** on the 3´ end of the primers. The figure below shows an *Mse*I/*Pst*I fragment with the adapters in positions adjacent to the restricted fragment to which they become ligated. The primers, which are almost identical to the upper strand and complementary to the lower strand of the adapter, are also shown, with **XXX** representing the 3 selective bases on the Mse primer, and **XX** the 2 selective bases on the Pst primer.

MseI primer

5' GATGAGTCCTGAG**TAAXXX** 3'
GACGATGAGTCCTGAG**TAA**__________**CTGCA**TGTACGCAGTCTAC
TACTCAGGACTCAT **T**__________**G**ACGTACATGCGTCAGATGCTC
3' **XXG**ACGTACATGCGTCAG 5'

PstI primer

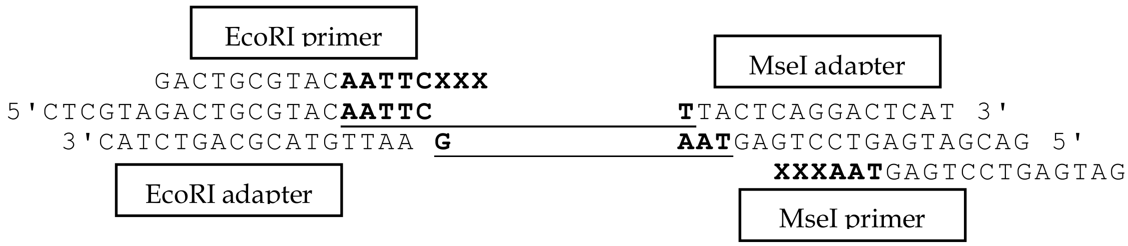
If *Eco*RI (5'G*AATTC3') is used instead of *Pst*I, the adapter is:

5'CTCGTAGACTGCGTAC 3'

3'CATCTGACGCATGTTAA 5'

The figure below shows the EcoRI adapter ligated to a fragment, the primer above designed to the upper strand of the adapter plus three bases of selection.

EcoRI primer is shown as the top strand aligned to its double stranded adapter and the lower most strand represents the MseI primer aligned to its double stranded adapter.



Amplification of the subset of fragments is carried out by PCR (Polymerase Chain Reaction), using *Taq* DNA polymerase. There are various ways to visualize and quantify AFLP fragments, but most use denaturing polyacrylamide gels as the medium for fragment separation and electrophoresis. Some rely on labelling the primer of the rare cutter (*Pst*I or *Eco*RI), with either a radioactive isotope or with a fluorescent tag. Radiolabelled amplified fragments are examined after exposure of the dried gel to a phosphorimage plate and then scanned, e.g. using Amersham Pharmacia Typhoon, or to normal X-ray film. The fluorescence method requires analysis of fragments with sophisticated software, e.g. ABI fragment analysis programmes. Silver staining generally give the same banding pattern as would be seen with a radiolabelled reaction but silver-staining is less sensitive, so more PCR product needs to be loaded on to gels. Silver staining of fragments in polyacrylamide gels gives results shortly after the gels are run; it is a straightforward, reliable and relatively inexpensive procedure.

SUMMARY OF STEPS IN AFLP ANALYSIS

1. Prepare DNA Sample → Agarose gel assessment of DNA quality

2. Prepare/anneal adapters

3. Digest mix for AFLP template – prepare and incubate 37°C, 2-3 hours

4. Ligate adapters to digested DNA at 37°C, overnight

5. Amplification of AFLP templates
- Preamplification ~ 1.5 hours (dilute with SDW)
- Reamplification ~ 2 hours (add loading dye and denature)

6. Visualistation of AFLP products by polyacrylamide gel electrophoresis and silver staining
- Prepare gel, insert comb, polymerise ~2 h
- Load samples into preflushed wells, run gel ~1-2 h
- Silver stain gel ~ 0.5 hours, allow to dry overnight

7. Score directly from dried gel on plate, or;
Photocopy or scan image on the plate

AFLP Method

Preparation of the double stranded adapter:

Single stranded adapter sequences need to be annealed together. Both the PstI and the EcoRI adapters are used at 5 pmol/µL and the MseI adapter at 50 pmol/µL.

1. Prepare a dilution of the PstI adapter sequences, 1 and 2, each at 10 pmol/µL in water.
2. Pipette equal volumes of adapter sequence 1 and 2 into a fresh tube to combine, to finally give a concentration of 5 pmol/µL.
3. Repeat steps 1 and 2 above for EcoRI adapter sequence at 5 pmol/µL final concentration.
4. Repeat steps 1 and 2 for MseI adapter sequence at 50 pmol /µL final concentration; but remember that MseI is used at 10 fold higher concentration than PstI and each sequence 1 and 2 needs to be at 100 pmol/µL before they are combined.
5. Incubate at 65ºC for 15 minutes, transfer to 37ºC for 30 minutes and finally leave at room temperature for 1 hour.
6. Store the annealled double stranded adapters at -20º

AFLP template preparation:

Double Digest: The reaction is typically done in 0.5ml or 1.5ml tubes and incubated at 37ºC for 2 to 3 hours. The digest components are listed in Table 1.

Table 1:

Digest mix	x 1 (µL)	Master mix (µL)
0.5 µg of DNA	5	
5 x RL Buffer	10	
10 U *Pst*I or *Eco*RI (10 U/)	1	
4 U *Mse*I (4 U/)	1	
SDW to 50	33	
Total	50	

Points to note:

1. Each DNA is made to 5 µL in water, ideally aiming for DNA preparations at 0.5 µg/ but they will not all be at this concentration, some will be more dilute. To each tube add the SDW then add the appropriate volume of DNA.
2. Before putting the remaining digest components together check the stock enzyme concentration, it may be necessary to adjust the volumes needed.
3. Table 1 shows the example for 20 reactions, so that from the master mix 45 will be added to each DNA sample.
4. Mix the contents of the tubes, give a brief spin and place in the incubator.
5. Always overestimate the number of reactions to allow for pipetting errors.

Ligation of adapters: The 50 digest reaction mix from Table 1 has a series of components added to it, see Table 2 below (Note: ATP and DNA Ligase supplied require dilutions to be made to 10 mM ATP and 1 U/µL DNA Ligase).

Table 2:

Ligation mix	x 1 (µL)	Master mix (µL)
Digested DNA (see Table 1)	50	
5xRL buffer	2	
5 pmol PstI or EcoRI adapter (5 pmol/µL)	1	
50 pmol MseI adapter (50 pmol/µlL)	1	
1 mM ATP (10mM)	1	
1 U T4 DNA Ligase (1 U/µL)	1	
SDW to 60	4	
Total	**60**	

For convenience, the ligation is left to incubate at 37ºC overnight. Digestion also continues, at this stage it is a digestion/ligation reaction. This comes about by the clever design of the adapters. In the figure below, the **T** to the left of the cleavage point (*) of *Mse*I is replaced in the adapter sequence by any base other than T. In the example below, it is shown replaced by a G; similarly for the **G** to the right of the cleavage point in *Pst*I. So that ligation takes place and digestion can continue simultaneously.

```
MseI                            T*TAA   cutting site
        GACGATGAGTCCTGAG TAA   adapter strand 1

PstI    cutting site         CTGCA*G
        adapter strand 1     CTGCA TGTACGCAGTCTAC
```

Dilute the digest/ligated AFLP template with the addition of 440 µl of $TE_{0.1}$ pH 8; store at -20ºC.
(General rule: use 1 or 2 µL of this template in a 10 µL pre-amplification PCR volume).

Amplification of AFLP templates:

Two amplifications are usually required to optimise the AFLP. An initial round of PCR (preamplification) is carried out to enrich a subset of the AFLP template, this also helps reduce background interference with the real AFLP bands in future polyacrylamide gels. The preamplified products are diluted and reamplification follows. It is important to use ***normal*** *Taq* DNA polymerase for AFLP and ***not*** a ‘hot start’ version. Adapters are not phosphorylated and only strand 1 of each adapter is ligated onto the restriction fragments. Adapter strand 2 is annealed by homology and relies on the extension by *Taq* DNA polymerase in the first round of PCR to anchor the adapter strand to the fragment.

Preamplification is a PCR with each primer having zero or one base of selection. The base of selection chosen depends on the final primer combination for reamplification.
For example, PstI+C/MseI+T (Table 3) preamplification for a final reamplification with PstI+CC/MseI +TGC (Table 4), the PCR examples in Tables 3 and 4 below uses these primer combinations, both scaled up for 20 reactions.

Table 3: Preamplification PCR

PCR Preamplification	x 1 (µL)	Master Mix (µL)
PstI +C (7.5 ng/µL)	2	
MseI +T (7.5 ng/µL)	2	
1 mM each dNTP	2	
10 X PCR buffer	1	
1 U *Taq* Polymerase (5 U/µL)	0.2	
SDW to 9 µL	1.8	
Sub-Total	9	
Template (PstI/MseI dig/adapt)	1	
Total PCR volume	10	

1. Dispense the master mix 9 µL volumes into the 0.2 mL microtubes (strips of 8).
2. Note that each dNTP is at 200 µM concentration in both the pre- and reamplification PCR (dNTPs supplied require dilution in SDW to 1mM each dNTP; prepared as a mixture of all 4 dNTPs).
3. To each add the 1 µL of PstI/MseI digest/adapter template.
4. Ensure all contents of each tube are at the bottom of the tube and the strip of caps firmly in place, if necessary spin briefly in the plate holding rotor of the Sigma centrifuge.
5. Carry out PCR using the program: (94ºC/ 30 secs; 56ºC/1 min; 72ºC/1 min) times 20 cycles; 10ºC/30 mins.

<u>Dilution of the preamplified products:</u>

A tenfold dilution is made by adding 90 µL of $TE_{0.1}$ pH 8 to each sample.
Store all PCR amplifications and dilutions at -20ºC.

Table 4: Reamplification PCR

PCR Reamplification	x 1 (µL)	Master Mix (µL)
PstI +CC (7.5 ng/µL)	2	
MseI +TGC (7.5 ng/µL)	2	
1 mM each dNTP	2	
10 X PCR buffer	1	
1 U *Taq* Polymerase (5 U/µL)	0.2	
SDW to 9	0.8	
Sub-Total	8	
Template (preamp. dilution)	2	
Total PCR volume	10	

1. Dispense the master mix 8 µL volumes into the 0.2 mL microtubes (strips of 8).
2. To each add the 2 µL of the tenfold dilution of preamplification template.
3. Ensure all contents of each tube are at the bottom of the tube and the strip of caps firmly in place. If necessary spin briefly in the plate holding rotor of the Sigma centrifuge.
4. Carry out PCR using the touchdown AFLP program:
   (94ºC/ 30 secs; 65ºC/30 secs reducing by 0.7ºC each subsequent cycle to 56ºC; 72ºC/1 min) times 11 cycles; (94ºC/ 30 secs; 56ºC/30secs; 72ºC/1 min) times 24 cycles; 10ºC/30 mins.

<u>Denaturation of AFLP products:</u>

1. Dispense 8 µL of the stop/loading dye (SLD) to each sample.
2. Denature by heating at 94ºC for 5 minutes, then cool to 10ºC for 5 minutes.
3. Remove samples to ice for loading onto a gel immediately or to -20ºC for loading at a future date.

There is no need to denature the samples again provided they are kept cool/frozen. The SLD has three roles:-

- the EDTA stops the activity of the *Taq* Polymerase,
- the formamide helps to keep the sample denatured, and
- the formamide acts as a heavy viscous liquid taking the sample down into the well when loading the polyacrylamide gel.

Visualising AFLP products

Polyacrylamide Gel preparation:

A 4.5% polyacrylamide stock solution can be prepared in advance and stored at 4ºC (see Buffer List). The glass plates need to be thoroughly cleaned and silanised before pouring the polyacrylamide gel.

Plate preparation:

1. Both of the plates are cleaned using detergent, rinsed with de-ionised water and dried with paper towels. If you are preparing plates for the first time[1] decide on the working side, i.e. the side that will come into contact with the gel and keep to this by marking the outer surface with a diamond marker or some sticky tape.
2. Repelcote silane treatment: take the larger plate to the fumehood, tip a small amount of Repelcote onto a paper towel and spread, working in even sweeps across the whole plate. Leave for a few minutes to dry. Give an ethanol wipe over the whole surface to evenly spread the silane and a second ethanol wipe to thoroughly polish the plate; give a final polish with a dry piece of paper towel. Rinse with de-ionised water and dry with paper towels.
3. Bind silane treatment: prepare this solution as instructed by the company (See Buffer & Solutions section). The smaller of the two plates will be given this treatment. Dispense 500 µL of the working solution into the middle of the plate and spread evenly over the whole surface, working in even sweeps. Allow to dry. This can be done on a work bench; as this is less volatile compared to the repel silane a fumehood is not necessary. (Note that fresh gloves need to be worn each time so that there is no cross contamination of silane solutions).
4. Ensure spacers are clean and dry. Align to the edges of the larger plate and place the smaller plate on top (take care not to touch the working side with gloves). Square off the corners and secure both plates together at the sides with thermostable tape at 2 to 3 places down each edge.

Pouring the Polyacrylamide gel and preparation for sample loading:

1. Dispense 60 mL of 4.5% polyacrylamide gel mix from the chilled stock (see Buffer List) into a pouring bottle, add 30 µL of chilled TEMED (N,N,N'N' – Tetramethyl ethylenenediamine) and 400 µL of chilled 10% ammonium persulphate. Mix by inverting the bottle gently.
2. Open the nozzle and allow the polyacrylamide to flow slowly and gently between the plates; you have about 10 minutes (depends on ambient temperature) before the acrylamide begins to polymerise.
3. Place the comb into position immediately after pouring the gel; ensure that air bubbles

[1] New plates need to be seasoned - this means silanising the plates and pouring/setting polyacrylamide gel a couple of times before using with important reactions.

are not trapped; clip the plates at the comb end:
Choice of comb:
(a) a castellated well forming comb is put in place; it is removed once the gel has polymerised and the pre-formed wells flushed free of urea just before pre-running the gel and before loading samples.
(b) a sharkstooth comb is placed into position with the straight edge in contact with the gel during polymerisation. This comb is inverted to form the wells and left in place while loading samples and running the gel.

4. Allow the gel to polymerise for 1.5 hours. If using "home-made" silver staining, then this time can be used to make the **DEVELOPER** as follows: **Dissolve 60 g sodium carbonate (make sure it's anhydrous and not too old) in 2 L distilled water and refrigerate at 4°C.**
5. Rinse the well/comb region carefully under a flow of de-ionised water, and rinse away the gel on the outside of the plate.
6. Remove the comb by sliding it out, continue to rinse the well area.
7. Dry the plates and mount onto the electrophoresis unit, the longer plate outermost; pour 500 ml of 1x TBE buffer (See Buffer List) into the top reservoir; flush out the well area using a syringe to remove any loose acrylamide. This is very important as acrylamide stuck along the edge of the smaller plate will interfere with sample loading.
8. If using a sharkstooth comb, place it into position so that the points of the comb just touch the gel, placing it level with the edge of the longer plate and fill the bottom reservoir with 500 ml of 1xTBE buffer.
9. ***IMPORTANT SAFETY NOTE:*** **If you have poured a sub-standard polyacrylamide gel, i.e. has too many bubbles, then leave the gel to polymerise before discarding the acrylamide**

Sample loading:

1. Load 3 μL of each sample (keep these on ice while loading) take care not to touch/move the comb.
2. Run at 1600 V for 1 to 2 hours until the darker blue (bromophenol blue) dye runs off.
3. Unmount the gel/plates, remove the tape; using the plastic Wonder Wedge to separate the two plates while still hot. The gel should remain in complete contact with the smaller plate treated with bind silane.

NOTE: We will be using the BioRad Silver Stain Plus kit, but the following is included for your reference:

"Home-made" Silver staining of the gel:

1. Make up the fixer: (10% acetic acid) 1.8 litre distilled water and 200 mL glacial acetic acid in 2 litre container. Pour into a shallow tray and immerse the gel/plate to

fix for half an hour, shaking gently.

2. Meanwhile make the silver stain solution: 12 mL 1.01 N silver nitrate solution in 2 litre of distilled water; add 3 mL formaldehyde (40% solution) and mix.
3. Tip the fixer back into its container (it can be recycled a few times!); wash the gel 3 times in fresh distilled water. Carry out the 4th rinse on a shaker for approximately 10 minutes or until "greasiness" has gone from the gel.
4. Pour off the rinse water and add the silver stain solution. Leave on a shaker for half an hour.
5. Set up a tray containing approx 3 litre of distilled water, have a timer and a piece of A4 white paper in a clear plastic bag close at hand.
6. Immediately prior to developing the gel add 300 µL of sodium thiosulphate solution (0.1001 N) and 3 mL of formaldehyde (40% solution) to the pre-chilled sodium carbonate solution. Pour this developing solution into a tray.

*****THE NEXT FEW STEPS HAVE TO BE FOLLOWED QUICKLY AND CAREFULLY SO MAKE SURE YOU HAVE EVERYTHING SET UP AND READY*****

7. Remove the gel from the silver stain and rest it ***ON*** the tray containing the 3 litres of water (do not put it into the water yet). Tip the stain back into its container; it can be used for up to five more gels. Rinse remnants of stain from the tray with de-ionised water.
8. Set a timer for 10 seconds. Start the timer and quickly lower the gel into the water. Agitate several times so that all excess silver stain is removed from the gel surface. When 10 seconds is up, ***QUICKLY*** drain the gel and place it in the developing solution. Initially, tip the tray to ensure developer covers the gel evenly; then as the larger fragments begin to develop immerse up and down holding the top end of the plate. This way the middle / smaller sized fragments remain in the developer longer. The pre-cooling of the sodium carbonate also greatly slows down the rate of development.
9. Use the piece of white paper to check for the progress of band development. Stop the reaction when bands near the bottom of the gel start to show by adding the 2 litre of fixer saved from earlier. Agitate the gel plate vigorously until bubbling ceases.
10. Soak the gel in a tray of distilled water for 10 mins and leave to dry overnight, standing vertically.

$CaCl_2$ Competent Cell preparation and transformation
(Helsinki Lab, adapted by SE Mitchell)

<u>Cell preparation</u>

1. Dilute 150 uls of an O/N culture into 30 ml of media. Grow to an OD_{600} = 0.6 (approx 2 hours).

2. Put on ice for 10 minutes. Spin 5K, 4C, 5 min.

3. Suspend gently in 15 ml of 50 mM $CaCl_2$. Incubate on ice for 15 min.
The Ca++ treatment is very bad for the cells, so treat them gently after this.

4. Spin 5K, 4C, 5 min.

5. Suspend in 3 ml of 50 mM $CaCl_2$, 10 mM Tris-HCl pH 8.

6. Use immediately or freeze in 200 ul aliquots in 15% glycerol at -80C. (Cells may be refrigerated for 12-24 hours before using. Transformation efficiency will increase up to that time and then decrease.)

<u>DNA transformation</u>

1. Add 10 uls of DNA to 200 uls of competent cells.

2. Put on ice for 30-60 min.

3. Shock at 42C for 2 min.

4. Add 1 ml of media and incubate at 37C for 1 hour.

5. Take a portion and spread on appropriate plate by suspending cells in 50-100 ul of 100 mM Tris 7.6 and spreading the cells directly on the plate.