AFLP-: A Novel Approach

DNA Preparation

Isolate and purify DNA with an appropriate method for the start material.

AFLP Sample Preparation

Make the reaction steps, the adapters, and the primers of AFLP marker generation the same as Vos and Zabeau, 1993; Vos *et al*., 1995. (We optimized the reaction conditions (Incirli and Akkaya, 2000), by using common *Eco*RI and *Mse*I adapters).

1-Genomic DNA digestion

1. Digest approximately 0.5 µ g of genomic DNA with *Mse*I and *Eco*RI according to the table below.
2. Determine the volume of DNA required obtaining approximately 0.5 µ g of DNA/sample. Aliquot the DNA into a 1.5 mL eppie. If some samples have very concentrated DNA (small volumes) while others have dilute DNA (larger volumes), dilute the concentrated samples in the 1.5 mL tubes with sterile ddH_2O AFTER YOU HAVE ALIQUOTED IT TO THE 1.5 mL tubes (do not dilute the orginal stockes) so that all samples in the 1.5 mL tubes now have the same volume of liquid (this makes the calculations easier). Record the volume value.
3. Make a master mix of the following reagents according to the number of samples to be analyzed plus 1 or 2 extra to allow for carry-over. Add components in the order shown.
   Recipe for digestion (40 µ L total volume/sample):

Reagent	**amount needed/sample**	number of samples	Volume for master mix
10X AFLP DL Buffer [1]	4.4 µ L	X	µ L
*Eco*RI enzyme (20 U/ µ L)	0.25 µ L	X	µ L
Mse I enzyme (4U/µ L)	1.25 µ L	X	µ L
ddH_2O	up to 40 µ L[2]	X	µ L
Volume of master mix to	aliquot per tube =	40 µ L - vol	of DNA (assuming all have the same volume)

   [1] Digestion-Ligation Buffer
   [2] = Remember that you want 40 µ L total volume/digest, which includes the genomic DNA that you will be adding. Be sure to subtract the volume value of genomic DNA from the ddH_2O volume value. That's why its easier if all samples have the same volume!!
4. Vortex the master mix briefly, centrifuge briefly and aliquot the required volume of master mix to each eppie of genomic DNA so that the total final volume is 40 µ L. Mix well by pipetting up and down several times.
5. Place in 37° C water bath for 1 hour.

II. Ligation of double-stranded adapters to the ends of the restriction fragments

1. Near the end of the 1 hour 37° C water bath incubation, make up the following ligation master mix.
   Recipe for ligation (10 µ L total volume/sample):

Reagent	**amount needed/sample**
T4 ligase (1U/µ L)	1.0 µ L
10X AFLP DL Buffer	1.0 µ L
5 µ M EcoRI adaptors	1.0 µ L
50 µ M MseI adaptors	1.0 µ L

Prepared by **Kamel A. Abd-Elsalam** Molecular Markers lab.(MML) Plant Pathology Research Institute (PPathRI)
9-Gamaa St.Giza, Egypt. In 9.10.2002

10 mM ATP	1.0 µ L
ddH_2O	5.0 µ L

2. Aliquot 10 µ L into each 1.5 mL eppie that contains the digestion reaction. Mix well by pipetting up and down several times. Total volume of each tube should now be 50 µ L. Place into 37° C water bath for 3 hours (the total time duration of digestion/ligation should be at least 4 hours).
3. Following incubation, dilute digestion/ligation reaction with approximately 450 µ L ddH_2O (1:9 dilution), vortex and place in -20° C or proceed to next step.

III. Optional DNA preselection of ligated product directed by primers complementary to adapter and restriction site sequences

If your organism contains a simple or small genome, you may want to skip this step. This step is mainly for organisms with large complex genomes and is designed to reduce background smears in the final DNA fingerprint and to provide almost unlimited amount of template. If these things are important to you, you should consider this step.

6. Aliquot 5.0 µ L of diluted digestion/ligation reaction into appropriately labeled 0.5 mL thin-walled PCR tubes.
7. Set up the following PCR amplification master mix.
   Recipe for preselective (PS)-AFLP amplification:

Reagent	**amount needed/sample**
10X PCR Buffer	2.0 µ L
10 mM dNTPs	0.4 µ L
2.75 µM *Eco*RI-PS primer	2.0 µ L
2.75 µ M *Mse*I-PS primer	2.0 µ L
UB *Taq* polymerase	0.25 µ L
ddH_2O	8.35 µ L

8. Vortex briefly, centrifuge and aliquot 15.0 µ L master mix/PCR sample (20 µ L total volume/sample). Mix well by pipetting up and down several times.
9. Place in thermocycler and run the following program (this program is for an MJ PTC-100 thermocycler; you may have to modify the time intervals if you have a different thermocycler):

Step 1	72° C	2 min
Step 2	94° C	30 sec
Step 3	56° C	1 min
Step 4	72° C	1 min
Step 5	Goto Step 2	20X
Step 6	72° C	2 min
Step 7	60° C	30 min
Step 8	4° C	Hold (you only need to do this if the machine is running o/n)
Step 9	END	

5. Once the PCR is done (approximately 2 hr), run out 5 µ L in a 2% agarose gel to confirm amplification. If amplification has occurred, dilute remaining PCR product 1:9 with ddH_2O, vortex and place in -20° C or proceed to next step.

IV. Selective DNA Amplification Of Subsets Of Restriction Fragments Using AFLP Primers And Labelling Of Amplified Products

4. Aliquot 5.0 µ L of diluted PS-AFLP reaction into appropriately labeled 0.5 mL thin-walled PCR tubes.
5. Set up the following PCR amplification master mix.
   Recipe for selective AFLP amplification:

Reagent	**amount needed/sample**

10X PCR Buffer	2.0 µ L
10 mM dNTPs	0.4 µ L
0.46 µ M *Eco*RI-AF primer	2.0 µ L
2.75 µ M *Mse*I-AF primer	2.0 µ L[1]
UB *Taq* polymerase	0.25 µ L
ddH_2O	8.35 µ L

[1] Depending on your organism, you may have to change the selective nucleotides at this primer's (or both this primer and the EcoRI-AF primer's) 3' end. The only way to know is to empirically test and tailor the primers to your orgamism.

6. Vortex briefly, centrifuge and aliquot 15.0 µ L master mix/PCR sample (20 µ L total volume/sample). Mix well by pipetting up and down several times.
7. Place in thermocycler and run the following program (this program is for an MJ PTC-100 thermocycler; you may have to modify the time intervals if you have a different thermocycler):

Step 1	94° C	2 min
Step 2	65° C	30 sec decrease by 0.7° C/cycle
Step 3	72° C	1 min
Step 4	Goto Step 1	12X
Step 5	94° C	30 sec
Step 6	56° C	30 sec
Step 7	72° C	1 min
Step 8	Goto Step 5	23X
Step 9	60° C	30 min
Step 10	4° C	Hold (you only need to do this if the machine is running o/n)
Step 11	END	

8. Run samples on 2% agarose gel to confirm amplification.

AFLP Reagent Recipes and Oligonucleotide Sequences

I. 10X AFLP digestion/ligation (DL) Buffer (10 mL)

reagent	initial concentrations
0.121 g Tris-base	(100 mM)
0.2145 g MgAc	(100 mM)
0.4907 g KAc	(500 mM)
0.077 g DTT	(50 mM)
pH to 7.5 with acetic acid	
add 100 µ L of 10 mg/mL BSA	(100 ng/µ L)
bring up to 10 mL with ddH_2O	

II. *Eco*RI-adaptor Structure

5' CTCGTAGACTGCGTACC	**OLIGO #1**
CATCTGACGCATGGTTAA5'	**OLIGO #2**

III. *Mse*I-adaptor Structure

5'-GACGATGAGTCCTGAG	**OLIGO #3**

Prepared by **Kamel A. Abd-Elsalam** Molecular Markers lab.(MML) Plant Pathology Research Institute (PPathRI)
9-Gamaa St.Giza, Egypt. In 9.10.2002

TACTCAGGACTCAT-5'	**OLIGO #4**

IV. AFLP Primers

AFLP primers consist of three parts; 1) core sequence = corresponds to the adaptors; 2) enzyme-specific = cleavage recognition sequence to the enzymes being used, and; 3) selective sequence = selects which fragments will be amplified. The enzyme-specific and selective sequences can be customized for different enzymes and amplifications, respectively. Below is the basic structure of the *Eco*RI and *Mse*I primers. N = any nucleotide.

Core	Enzyme-specific	Selective
*Eco*RI 5'-GACTGCGTACC	AATTC	NNN-3'
*Mse*I 5'-GATGAGTCCTGAG	TAA	NNN-3'

For the preselective (PS)-AFLP amplification, one *Eco*RI and one *Mse*I primer containing a single selective nucleotide on each are required. For the selective AFLP amplification, at least one *Eco*RI and one *Mse*I primer containing a two to three selective nucleotides on each are required. You may want to order several different primers with different selective nucleotides and determine which ones give you the best results for your organism.

PREPARING AND RUNNING THE POLYACRYLAMIDE GEL

1-Prepare a 7% 19:1 acrylamide/bis-acrylamide gel for running out your labeled samples as follows:

2-Make a 40% sequencing acrylamide stock (wear a mask while making this solution) This will be done for you prior to lab.

Chemical	Dry weight
Acrylamide	19.0g
Bis-acrylamide	1.0g

- Adjust volume to 50 ml with ddH_20 and filter through Whatmann paper.
- Store wrapped in aluminum foil at 4^oC, shelf life is one month.

3. Make solution of 7.0 % acrylamide for the 25cm gel by adding the following to a 100ml beaker (This will be done for you prior to lab):

Chemical	Amount
Urea	13.5g
40% home-made acrylamide	5.25ml
10X TBE	3.0ml

- Adjust volume to 30ml with ddH_20. Mix thoroughly.
- This solution can be made in larger quantities (approx. 150ml volume) and stored as stock at 4^oC in a sealed bottle.

4. Prepare the buffer solution (This will be done for you prior to lab):

- The buffer solution in the gel is a 10X AFLP PAGE buffer. The running buffer, however, is diluted 1X for the running buffer and 0.5X for making the gel.
- Prepare the 10X AFLP buffer by adding the following to a 2L beaker:

Chemical	Amt to use	Conc in 10X
Tris	121.14 g	1M
Boric Acid	61.83 g	1M
0.5 M EDTA	40 ml	20 mM

- Mix until all salts have dissolved.
- Bring to a final volume of 1L with ddH_2O.

5. Prepare the Ammonium Persulfate Solution (APS)

- Make a 10% solution by adding 0.1g of APS to 1.0ml dH2O.

6. Assemble and prepare the 25cm glass plates for the sequencer.

- Rinse both glass plates with isopropanol to clean (only clean the sides of the glass, which will become the inside of the gel sandwich).

- Allow the alcohol to dry and place the 0.25mm spacers ALONG THE LONG EDGES of the rear plate.
- Place the front plate on top of the rear plate and align the spacers with the outside edges of the plates. Make sure the plates are aligned evenly at the bottom.
- Place the left and right rail assemblies over the long edges of the plates. Tighten the knobs against the glass plate until finger tight

7. Prepare and pour the 7.0 % acrylamide gel.
   - Take 30ml of the 7.0 % acrylamide stock solution and put into a 100ml beaker.
   - Add 150 µ L of the 10% APS solution.
   - Add 30 µ L of TEMED.

8. Swirl the solution to mix. (The gel will polymerize only when the APS and TEMED are added. **ONCE THESE ARE ADDED, POUR THE GEL QUICKLY USING A 60 CC SYRINGE SINCE IT WILL START TO POLYMERIZE!!!!**)
   - Draw up the solution using a 60cc syringe with a 14 gauge needle. Angle the glass plates slightly and deliver the acrylamide evenly across the top notch of the front plate. Flow the gel at a steady, even rate until it reaches the bottom of the glass plates. At this point, lay the glass plates flat and check for any air bubbles. Remove any bubbles.
   - Insert the appropriate piece of equipment for forming the wells (the trough to form the base of the wells). Fill the empty space with acrylamide.
   - Put the casting plate in the grooved area in the rails (which will eventually be occupied by the upper buffer chamber) and tighten until finger tight.
   - Allow gel to polymerize for 1.5 hours.

9. Prepare the gel by washing off excess acrylamide, pull and invert the sharktooth combs and pre-run the gel for approximately 45-60 minute. The setting should be set to the following:
1500 V
20 mA
25 W
10. Denature samples for 3-4 minutes at 94? C and place immediately on ice. Load samples and begin electrophoresis.
11. Load your samples and begin electrophoresis.

Silver Staining the Sequencing Gel

Reagents and Equipment

- fix/stop solution
- staining solution
- developing solution, prechilled
- orbital shaker/rocking platform
- 2-3 plastic trays (such as polyethylene sterilizing trays, Fisher Cat.# 13-359-26) with dimensions slightly larger than the glass plate

A. Silver Staining Protocol

Please review Section III before performing the silver staining procedure. **Note:** Handle gel with gloved hands by the edges of the plate to avoid fingerprints.
The staining procedure requires that the gel be incubated in plastic trays. We recommend using a minimum of two trays with similar dimensions to the plate. Rinse the trays with ultrapure water before adding fresh solutions to the trays.
1. **Prepare solutions:**
a. fix/stop solution (10% glacial acetic acid): Add 200ml of glacial acetic acid into 1,800ml of ultrapure or double-distilled water.
b. staining solution: combine 2g (1 packet) of Silver Nitrate ($AgNO_3$) (1 vial) of 37% Formaldehyde in 2L of ultrapure water.
c. developing solution: dissolve 60g (1 packet) of Sodium Carbonate (Na_2CO_3) in 2L of ultrapure water. Chill to 10°C in an ice bath. **Immediately before use** (Step 6a), add 3ml (1 vial) of 37% Formaldehyde and a **400µl aliquot** of the provided Sodium borohydrate (10mg/ml). Discard the remaining Sodium borohydrate in the vial.
2. **Separate the plates:** After electrophoresis, carefully separate the plates using a plastic wedge. The gel should be strongly affixed to the short glass plate.
3. **Fix the gel:** Place the gel (plate) in a shallow plastic tray, cover with fix/stop solution and agitate well for 20 minutes or until the tracking dyes are no longer visible. The gel may be stored in fix/stop solution overnight (without shaking). Save the fix/stop solution to terminate the developing reaction (Step 9). If the developing solution has not yet been chilled, place it on ice at this time.
4. **Wash the gel:** Rinse the gel 3 times (2 minutes each) with ultrapure water using agitation. Lift the gel (plate) out of the wash and allow it to drain 10-20 seconds before transferring it to the next wash.
5. **Stain the gel:** Transfer the gel to staining solution and agitate well for 30 minutes.
6. a. Complete preparation of the developing solution by adding 3ml (1 vial) of the provided 37% Formaldehyde and a **400µl aliquot** of Sodium Thiosulfate (10mg/ml) to the **prechilled** (10°C) sodium carbonate solution. **Pour 1L (half) of the prechilled developing solution into a tray, and set it aside.** Keep the remaining developing solution on ice.
6. b. Remove the gel from the staining solution and set it aside momentarily. Transfer the staining solution into a flask or beaker for silver recovery (see the Note on recovering silver at the end of this section). Rinse the tray and fill it with ultrapure water.
Warning: The timing of the next (rinse) step is very important. Total time from when the gel is placed in ultrapure water to the time it is placed in developing solution should be **no longer than 5-10 seconds**. Longer rinses result in weak or no signal.
7. **Rinse the gel:** Dip the gel *briefly* into the tray containing ultrapure water, drain, and place the gel *immediately* into the tray of chilled developing solution. The time taken to dip the gel in the water and transfer it to developing solution should be no longer than **5-10 seconds**.

Note: If the rinse proceeds too long, repeat Step 5 with the staining solution.
8. **Develop the gel:** Agitate the gel well until the template band starts to develop or until the first bands are visible. Transfer the gel to the remaining 1L of chilled developing solution and continue developing for an additional 2-3 minutes or until all bands become visible.
Note: The developed bands appear fairly light. Prolonged development times result in high background. It is better to stop development early than to over-develop the gel. The sequence ladder will darken upon gel drying and exposure for EDF Film.

Prepared by **Kamel A. Abd-Elsalam** Molecular Markers lab.(MML) Plant Pathology Research Institute (PPathRI)
9-Gamaa St.Giza, Egypt. In 9.10.2002

9. **Fix the gel:** To terminate the developing reaction and fix the gel, add 1L of fix/stop solution (from Step 3) directly to the developing solution and incubate with shaking for
2-3 minutes.
10. **Rinse the gel twice,** for 2 minutes each, in ultrapure water. **Note:** Handle gel with gloved hands by the edges of the plate to avoid fingerprints.
11. **Dry the gel** by placing it at room temperature or using convection heating. View the gel on a light box at visible wavelengths or place it against a bright white or yellow background (e.g., paper). For permanent records, proceed to Section VII on EDF Film development. Discard all solutions according to institutional policies.
Note: Waste silver can be recovered from the used staining solution for recycling. Precipitate silver by adding approximately 10g of NaCl. Collect the AgCl precipitate by filtration or allow it to settle out by gravity.