AFLP Protocol

This protocol is an amalgamation of protocols from Vos et al., Brent Barrett, Greg Penner, A.S. Reddy, Gibco BRL, Sam Hazen (wheat lab) and changes and additions made in the bean lab (Veronica Vallejo and Judy Kolkman). Please cite: Hazen, S.P., Leroy, P., Ward, R. 2002. AFLP in *Triticum aestivum* L.: patterns of genetic diversity and genome distribution. Euphytica **125** (1): 89-102, 2002.

I. Restriction Digestion of Genomic DNA

	Starting []	Final []	1 reaction (µl)
OnePhorAll (OPA, Pharmacia)	10X	1X	5
Mse I (New England Biolabs)	4U/µl	5U (0.1U/µl)	1.25
EcoRI (GibcoBRL) or Pst I (Promega)	10U/µl	5U (0.1U/µl)	0.5
BSA (comes with Mse I)	10U/µl	0.1 ug/µl	0.5
ddH_2O			32.75
Genomic DNA	50-250 ng/µl		10
Total volume			50

1. Heat one water bath to 70°C and the other to 37°C.
2. Distribute 40 µl of the cocktail into each labeled tube.
3. Add 10 µl of DNA to each tube.
4. Vertex and spin the sample down in the centrifuge.
5. Incubate the tubes at 37°C for 3 hours. Agitate every hour by vortexing. Cetrifugation is not necessary.
6. Inactivate the enzymes at 70°C for 15 min..

II. Adapter Preparation: complete during or before digestion.

EcoRI Adapter (120 reactions):

	120 reactions
EcoRI.1 oligo (1ug/µl)	3.4 µl
EcoRI.2 oligo (1ug/µl)	3.0 µl
OPA	6.0 µl
ddH_2O	107.6 µl

MseI Adapter (120 reactions):

	120 reactions
MseI.1 oligo (0.5 µg/µl)	64.0 µl
MseI.2 oligo (0.5 µg/µl)	56.0 µl
OPA	7.0 µl

Mix the reagents in tubes and run the following PCR profile:
65°C 10 min
37°C 10 min
25°C 10 min
Store tubes at -20°C.

III. Ligation of Adapters.

	1 reaction
EcoRI adapter	1.0 µl
MseI adapter	1.0 µl
T4 DNA ligase 10X buffer	1.0 µl
T4 DNA ligase (3U/µl, Promega)	0.33 µl
ddH_2O	6.7 µl
Total	10.03µl

1. Add 10 µl of the ligation mix to 50 µl of digested DNA. Vortex briefly and spin down if necessary.
2. Incubate at room temperature for 3 hrs. Agitate every hour.

IV. Pre-amplification Reactions:

	1 reaction
EcoRI + A oligo (50 ng/µl)	0.5 µl
MseI + C oligo (50 ng/µl)	0.5 µl
dNTPs (5mM, Gibco as 100mM)	2.0 µl
10X PCR buffer (comes with Taq)	2.0 µl
$MgCl_2$ (comes with Taq)	1.2 µl
ddH_2O	11.9 µl
Template DNA from restriction/ligation	2.0 µl
Taq polymerase (5U/µl, Promega)	0.1 µl
Total	20.2µl

Run following PCR profile:
94°C 2 min
94°C 1 min
56°C 1 min
72°C 1 min
26 cycles
72°C 5 min
4°C soak

1. Add 100 µl of sterile water.

V. Selective Amplification:

	1 reaction
EcoRI + ANN oligo (50ng/µl)	0.5 µl
MseI + CNN oligo (50 ng/µl)	0.6 µl
dNTps (5mM, Gibco as 100mM)	0.8 µl
10X PCR buffer (comes with Taq)	2.0 µl
$MgCl_2$ (comes with Taq)	1.2 µl
ddH_2O	13.82 µl
Diluted template DNA from pre-selective PCR	1.0 µl
Taq polymerase (5U/µl, Promega)	0.08 µl
Total	20 µl

Run the following PCR profile:

94°C 2 min
94°C 30s
65°C 30s
72°C 1 min
} 12 cycles, decrease Tm by 0.7°C each.
94°C 30s
56°C 30s
72°C 1 min
} 23 cycles
72°C 2 min
4°C soak

Add 8 µl of formamide-loading buffer to the PCR product.

Oligo sequences:

EcoRI linker 1 CTC GTA GAC TGC GTA CC
EcoRI linker 2 AAT TGG TAC GCA GTC TAC
EcoRI + A GAC TGC GTA CCA ATT CA

PstI linker 1 CTC GTA GAC TGC GTA CAT GCA
Pst1 linker 2 TGT ACG CAG TCT AC
PstI + A GAC TGC GTA CAT GCA GAC A

MseI linker 1 GAC GAT GAG TCC TGA G
MseI linker 2 TAC TCA GGA CTC AT
MseI + C GAT GAG TCC TGA GTA AC

Bio-Rad Plate Preparation (Bio-Rad Seque Gen GT Sequencing Cell)

Glass preparation: General Note: You must avoid contaminating the IPC with Bind Silane and the outer glass plate with Sigmacote! Therefore, change gloves whenever you are switching between the two pieces of equipment.

1. Wipe IPC with kim-wipe and ethanol across and up and down (vertically and horizontally) – three times. Use a great deal of pressure when wiping with ethanol.. Make sure that the glass is clean. Glass should be free of lint - lint will result in bubbles in the gel and black bubbles.
2. Dry the glass with kim-wipe.
3. IPC should be treated with sigmacote ~every 3-4 gels or until top of gel begins to stick to the IPC. Rule of thumb: Check the IPC by running gloved finger across, if it squeaks, reapply sigmacote.
4. To apply sigmacote: saturate a kim-wipe with sigmacote and wipe both vertically and horizontally. Apply the Sigmacote VERY quickly because it evaporates. Wait 5 minutes to dry.
5. **Change gloves** (to avoid contamination).
6. Clean outer glass plate (the big glass) with ethanol and kim-wipe 3x vertically and horizontally. Use a great deal of pressure when wiping with ethanol.
7. In an eppendorf tube combine 1ml of the ethanol/acetic acid solution (95% ETOH with 0.5% acetic acid) and 3.0µl of bind silane (provided by the Promega silver staining kit). Vortex briefly or mix by inverting.
8. Apply bind silane solution to a Kim-wipe using a pipet and very quickly, wipe the glass plate vertically then horizontally **as fast as you can** because it evaporates. Wait 5 minutes for it to dry. Make sure that you apply the bind silane as evenly as possible. **Note: Bind Silane expires in six months, be sure to write the date on the vial when you receive the kit and place it at 4°C.**

Glass preparation continued:

9. **Change gloves.**
10. Clean IPC 3x with 70% ethanol. Do **not** apply a great deal of pressure when wiping with ethanol.
11. **Change gloves**.
12. Clean the outer glass plate 3x with 95% ethanol (**very light** pressure) by applying 2 ml of 95% ethanol to a kim-wipe and wiping the glass plate vertically then horizontally quickly.

In the event of contamination of either sigmacote or bind silane on the respective glass, soak in 10% NaOH for 30-60 min. Always change gloves between working with bind silane and sigmacote. When soaking the IPC, be sure not to allow the sodium hydroxide solution to enter the buffer chamber.

13. While horizontal, place spacers on the IPC, be sure that the ends of the spacers are flush with the lower edge of the IPC. Place the outer glass plate on top, being careful not to let the surfaces of the outer glass plate and the IPCs touch.
14. Erect vertically and place the clamps on each side. **Note:** The tension may be adjusted by twisting the levers located on each clamp. Then press the black levers on the clamps to fasten the clamps. Try to push the levers at the same time if possible. **Note:** Be sure that the bottom of the clamps are flush with the bottom edges of the plates. This is CRUCIAL! If the clamps are not flush the gel could leak out of the rig. Once the clamps are in place this is now called the "assembled unit".
15. Slide the assembled unit into the caster base. Insert pegs and turn both pegs at the same time. **Note:** Be sure to turn the pegs while the assembled unit is vertical. Check to see if the plates are centered with the hole in the bottom of the caster base. Also check to be sure that plates and clamps are making an indentation in the rubber mat on the bottom of the caster base indicating that a seal has been made.
16. Check to see if comb will easily insert between glass. If not, adjust the clamps.

Acrylamide gel solution (6%)

17. Make **fresh** 10% APS solution: In an eppendorf tube combine 0.1g of Ammonium Persulfate (Promega) and 1000 µl of MilliQ sterile water and mix by inverting or briefly vortex. **Note:** The amount of APS in the solution will control how fast the polymerization reaction will occur, so it is **better to be under 0.1g of APS rather than over!**
18. Pour 100ml of 6% Acrylamide premix (recipe located at the end of protocol) into a beaker and add 100µl TEMED (Invitrogen) and 500µl 10% fresh APS. **Note:** The polymerization reaction occurs only when all 3 of the reagents are in a solution together. It is best to add the TEMED first and then add the APS. **Note:** The polymerization reaction is temperature dependant, the colder the acrylamide solution the slower the polymerization reaction will occur.
19. Draw the solution into syringe immediately after adding the APS. Fill syringe without lifting out of the acrylamide solution to avoid bubbles.
20. Place tube on syringe and turn it upward. Knock on the syringe a few times to dislodge the bubbles. Push air out of the syringe and then turn the syringe horizontal. Continue to push the air out of the rubber tube until there are no bubbles in either the syringe or the tube. **Note:** The horizontal position helps the bubbles out of the tube without losing so much acrylamide solution.
21. Insert the tube into Caster base and inject gel solution with moderate pressure. If bubbles appear, slightly tip gel rig up, or gently knock on the glass.
22. Insert comb upside-down (with the teeth facing up). Be sure to insert far enough as to create a deep enough well, but not so deep that the wells are inaccessible by the micropipette tip (ie., only about half the depth of the teeth on the comb).
23. Allow the gel to polymerize for at least 1 hour. Cover opening with cling film if you are leaving the gel overnight.

Gel Loading

1. Make 2 liters 1x TBE (200 ml 10x TBE + 1800 ml MilliQ water). Optional - microwave TBE for 5 minutes and skip step 8.
2. Fill bottom tray (universal base) with 1x so about 1 inch of the bottom of the unit is submerged or 1600 ml of solution is left in your beaker/cylinder.
3. Remove the assembled unit from the caster base.
4. Insert the assembled unit into the universal base so that it fits into the groove.
5. While holding the assembled unit in the universal base with one hand, slide the stabilizer bar in between the assembled unit and the universal base with the other hand.
6. Fill gel IPC buffer chamber with 1x TBE until ~1inch above the IPC. **Note: If the buffer level is too low sparks can occur at the top of the rig and damage to the IPC can also occur.**
7. Remove the comb, flush out the gel the well with buffer using a needle syringe filled with 1xTBE.
8. Warm up gel at 80-90W for 1hour. **Note:** Another commonly used, and preferred, option is to heat the buffer (2000ml) in the microwave on high power for 5 min. **Note:** This is a good time to make fix/stop, silver staining and developing solutions and place them in the FREEZER (-20ºC); the colder that the solutions are when you stain the better the quality of your bands.
9. Denature the PCR reactions for 5 min at 95ºC or boiling and **immediately** put into ice
10. Add 8µl of formamide loading buffer (FLB) to each PCR tube (if each tube contains 20µl of reaction solution)
11. Before loading gel, use the needle syringe to flush well again. **Note:** It is VERY important to be certain that the well is **completely** clean. **Any** residue in the well will effect the migration of the DNA.
12. Insert the comb teeth-side down. **Note:** you may pierce the gel SLIGHTLY to ensure proper separation of the wells. Also, black binder clips are recommended to keep the comb in place while loading.
13. Load 4.5 µl of sample.
14. Run gel (80-90W) for 10 min and remove comb. [Make fix/stop developing and staining solutions if you haven't already and place them in the freezer.]
15. Run gel until the light blue dye migrates 1 inch below bottom rib of IPC.
16. Insert tube into the drainage valve on the IPC to drain buffer from plates. Pour remaining buffer into sink.
17. After draining, place gel rig horizontally on counter. Remove side clamps. Starting at an upper corner, gently pry plates apart checking to be sure that gel is sticking to the outer glass plate (it should be). Wash clamps and IPC **IMMEDIATELY**.

Silver Staining for AFLPs: Uses Promega's Silver Sequence™ DNA Sequencing System. The following protocol is adapted from the manufacturers recommended procedures and optimized for use with the Bio-Rad Seque Gen GT system.

1) Separate plates while keeping the gel attached to outer glass plate.

2) **Fix the gel**: place the gel in tray with cold fix/stop solution (do not pour directly onto the gel) and agitate gently (place on the orbital shaker) for 20 minutes. Gel may be stored in fix/stop solution overnight (however, bands get fatter when you do this). **Save fix/stop solution and place back in the freezer.**

3) **Wash the gel:** Rinse the gel 3x for 2-3 min each in ddH_2O using agitation. Lift gel from solution and allow it to draining 10-20 seconds. Stand the gel upright (by leaning it against the cabinet, gel side facing toward you) while you wash the tray with ddH_2O.

4) **Stain the gel:** Pour the staining solution into the tray. Transfer the gel to staining solution and agitate for 30 min. In the last 10 min, add the final reagents to the developing solution. Remove gel from tray and stand upright. Transfer staining solution to a beaker for proper disposal. **Do not dispose of silver nitrate solution in the sink!**

5) **Rinse the gel:** Fill tray with ddH_2O. Submerge the gel and agitate **for 3-7 seconds ONLY**. **Note:** Too much time in the rinse will result in weak staining. Remove the gel from the tray and stand upright. Dispose of the water down the sink.

6) **Develop the gel:** Pour 1L of developing solution into the tray. Place the gel in the tray and agitate until bands begin to appear. **Note:** This is a temperature sensitive reaction. The colder the solution the longer it will take for the bands to appear but the clearer they will be; a slushy consistency produces the sharpest bands. Pour in the rest of the chilled developing solution and keep agitating until band are clearly visible **Note:** Do not forget the gel will continue to get darker until you neutralize the developing solution, therefore, you must carry out step 7 just **BEFORE** the gel has reached the desired degree of developing.

7) **Neutralize the gel:** Just before the desired degree of developing has been achieved, you must fix the gel: add 2L of fix/stop solution directly to developing solution and agitate for 2-3 min. (use your hands and pull the plate up and down to more quickly neutralize the developing solution will prevent the gel from getting too dark).

8) Rinse gel 2x for 2-3 min. each in ddH_2O using agitation.

9) Dry gel on glass by standing the plate upright.

Solutions:

Fix/Stop Solution

200ml of glacial acedic acid
1,800ml of ddH_2O
Freeze (-20°C) for about 90 min.
If necessary, stir to break ice before using.

Developing Solution

2,000ml of ddH_2O
60g (1 packet) of sodium carbonate (Na_2CO_3) (included in Promega staining kit)
Freeze (-20°C) for about 90 min. If necessary, stir to break ice before using.
Immediately before using add:
3ml (1 vial) of 37% formaldehyde (included in Promega staining kit)
400µl of sodium thiosulfate (included in Promega staining kit)

Note: the sodium thiosulfate **vial provided with the kit contains more than 400 µl therefore, use a pipettor to measure the appropriate volume into the solution. Also, a 1000 µl pipettor tip does not fit into the opening of the vial, therefore, you should use the 200 µl pipettor and measure 200 µl 2x to get 400 µl.**

Staining Solution

2,000ml of ddH_2O
2g (1 packet) of silver nitrate ($AgNO_3$) (included in Promega staining kit)
3ml (1 vial) of 37% formaldehyde (included in Promega staining kit)
After using, dispose of the solution in a container. Add an indiscriminate amount of sodium chloride crystals to precipitate the silver nitrate. The next day when the silver is completely precipitated (solution is clear), decant the supernatant into the sink with running water. The remaining precipitate is removed by the ORCBS chemical disposal service after 90 days.

10x TBE Buffer (1L)

Trisbase	108g
Boric acid	55g
0.5M EDTA pH 8.0	40ml

Add ddH_20 to total volume of 1000ml (Stir)

To make 1X TBE: 900 ml ddH_20
100 ml 10X TBE

6% Acrylamide Solution For one gel

42g Urea
15ml Acrylamide 40% - plusone (ReadySol DNA/PAGE from Amersham Pharmacia)
10ml 10x TBE
Add MilliQ H_20 to total volume of 100ml

1) Add urea, TBE, and approx. 25ml water in a beaker. Stir with heat until urea dissolves.
2) Transfer solution to 100ml-graduated cylinder and add water up to 85ml. Transfer to vacuum flask.
3) Add acrylamide to flask and degas to appox. 10min.

Gel: 100ml Acrylamide (6%)
500µl 10% fresh APS
100µl TEMED
Pour acrylamide into beaker and add 10% APS and TEMED to the acrylamide. Do not mix APS and TEMED prior to adding acrylamide - solution will not polymerize.

Pre-mix acrylamide gel solution (10.5 gels):

1. Measure out 441 g of Urea.
2. Place into a large beaker and add 105 ml 10X TBE and Milli Q water just to cover.
3. Put on magnetic stirrer; stir and set heat at 3 or 4. Allow to dissolve.
4. Pour dissolved solution into a 1 liter graduated cylinder and add Milli Q water until the volume reaches 892.5 ml.
5. Measure out 157.5 ml of acrylamide.
6. Pour the Urea solution into a brown glass bottle or a bottle covered with aluminum foil. Add the acrylamide solution to the bottle and stir gently a few time.
7. Keep at 4ºC.

Note: This protocol makes solution for 10.5 gels. This is to account for measuring errors and spills.

Note: When Urea dissolves in water, this is an endothermic reaction, that is why there is a need to add heat to the reaction, so that the urea dissolves faster.

Note: Do not add too much water to the urea to dissolve it because the FINAL volume should not exceed 892.5 ml!!!!! This is critical!

Note: Solution does not require filtration.

10% APS:

0.1g APS (Ammonium Persulfate, Promega)
1000µl ultra-pure water
Dissolve APS in water (vortex)

Formamide Loading Buffer (either one works the same):

		10ml	50 ml
Formamide		add up to 10 ml	49 ml
EDTA pH 8.0 (stock 0.5M)	10 mM	200 µl	1 ml
Xylene Cyanol	1 mg/ml	10 mg	50mg
Bromophenol Blue	1 mg/ml	10 mg	50mg

Mix all components and aliquot 500 ml/eppendorf, then freeze (-20ºC).

DNA Ladder Mix
137µl of ddH_20
58µl of Formamide loading buffer
5µl of DNA ladder (Invitrogen 50µg (1µg/µl))

EDTA 0.5M pH 8.0
186.1g of disodium EDTA.2 H_20
800ml of H_20 and stir vigorously
Adjust the pH to 8.0 with NaOH pellets (~ 20g)
Note: EDTA will not dissolve until proper pH is attained.
Autoclave

10% NaOH:
100 g NaOH pellets
800 ml ddH_2o

Place beaker in an ice bath over a magnetic stirrer, then dissolve pellets in water. Once the pellets are dissolved, adjust volume to 1000 ml.

Gel Scoring and Scanning

1) Scan the gel and save as compressed tif and jpg files.

2) Score gel while still on glass and make note on printout of scanned image.

3) Keep original score sheet and gel printout in folder.

Front Plate Cleaning

1) Soak gel in 3% NaOH for 30min.

2) Carefully use a razorblade to scrape off the gel toward one end and collect the acrylamide to dispose in appropriate container.

3) Scrub the front side of the plate with powdered SequeSoap.

4) Rinse with dH_2O and wipe with sponge.

5) Scrub front and back of plate with liquid SequeSoap using the sponge.

6) Rinse well with dH_2O.

7) Clean front and back of plate with ethanol and kimwipe.

8) Mark back of plate with a “B”.

Proper way to wipe the glasses: