Amplified Fragment Length Polymorphism (AFLP) Protocol
Introduction taken from
http://biologi.uio.no/FellesAvdelinger/DNA_KAFFE/Kaffe_Resources/AFLP.html;
Protocol modified from Vos et al. (1995) NAR 23:4407-4414;
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The AFLP technique, originally known as selective restriction fragment amplification (SRFA) (Zabeau and Vos 1993), produces highly complex DNA profiles by arbitrary amplification of restriction fragments ligated to double-stranded adaptors with hemi-specific primers harboring adaptor-complementary 5' termini (Vos et al. 1995). The technique has been widely used in the construction of genetic maps containing high densities of DNA marker loci. The AFLP protocol amplifies restriction fragments obtained by endonuclease digestion of target DNA using "universal" AFLP primers complementary to the restriction site and adapter sequence. However, not all restriction fragments are amplified because AFLP primers also contain selective nucleotides at the 3' termini that extend into the amplified restriction fragments. These arbitrary terminal sequences result in the amplification of only a small subset of possible restriction fragments. The number of amplified fragments (generally kept around 50-100) can therefore be "tailored" by extending the number of arbitrary nucleotides added to the primer termini. Alternatively, the use of endonuclease combinations that vary in their restriction frequency can also be used to tune the number of amplicons. Generally, the abundant restriction fragments produced from complex genomes require AFLP primers with longer selective regions. Conversely, analysis of small genomes require only few arbitrary nucleotides added at the primer 3' termini. The resulting AFLP fingerprints are usually a rich source of DNA polymorphisms that can be used in mapping and general fingerprinting endeavors.

The AFLP protocol can be divided into the following steps: (1) DNA digestion with two different restriction enzymes (generally a rare and a frequent cutter), (2) ligation of double-stranded adapters to the ends of the restriction fragments, (3) optional DNA pre-amplification of ligated product directed by primers complementary to adapter and restriction site sequences, and (4) DNA amplification of subsets of restriction fragments using selective AFLP primers and labeling of amplified products. Amplification of very small "genomes" (plasmids, cosmids, BACs) requires primers with no selective nucleotides. AFLP fingerprinting of bacteria and fungi generally requires primers with 2 selective bases. Complex genomes require the use of more than 2 selective bases in one or both primers. In the case of complex genomes it is recommended to carry the amplification in two consecutive steps (preamplification and selective amplification) to increase specificity and the amount of initial template. The AFLP fragments are usually detected by labeling one of the two AFLP primers. For example, radioactively labeled primers can be obtained by phosphorylating the 5' ends with g-33P-ATP and polynucleotide kinase or by the use of dye labeled primers. Do not label the two primers if the generation of doublets resulting from the different mobility of complementary strands in sequencing gels wants to be avoided. Finally, the labelled reaction products are separated by electrophoresis using denaturing polyacrylamide gels and exposed to X-ray

films to visualize the AFLP fingerprints (if radioactive) or by running out the products on an automated DNA sequencer (i.e. the Li-Cor).

CONSTRUCTION OF AFLP ADAPTORS

1) Order all of the oligonucleotides listed at the end of this protocol or, if you have a different combination of oligos that you will be using, order those. This protocol is designed to use the *Mse*I/*Eco*RI combination. Dilute the oligos to 100 μM concentrations in ddH_2O.

2) Recipe for making the adaptors:

*Mse*I adaptors (50 μM [conc])	*Eco*RI adaptors (5μM [conc])
100 μL 100 μM *Mse*I.1	10 μL 100 μM *Eco*RI.1
100 μL 100 μM *Mse*I.2	10 μL 100 μM *Eco*RI.2
2 μL 1 M Tris-HCl (pH 8.0)	2 μL 1 M Tris-HCl (pH 8.0)
2 μL 5 M NaCl	2 μL 5 M NaCl
0.4 μL 0.5 M EDTA	0.4 μL 0.5 M EDTA
	175.6 μL ddH_2O

Briefly vortex. Final volume for both adaptor mixtures should be approximately 200 μL.

3) You will need to heat the mixtures to 95°C and slowly cool them to room temperature so that the two oligos in the mixture will hydrogen bond and form the adaptors. I find the easiest way to do this is by using a thermocycler. Aliquot the above mixtures into 50 μL volumes in thin wall PCR tubes. Place in a thermocycler and run the following program.

Step 1:	95°C 2 minutes
Step 2:	95°C decreasing to 25°C by 1°C increments per 1 minute intervals
Step 3:	END

4) Store the aliquots at -20°C.

DNA DIGESTION WITH TWO DIFFERENT RESTRICTION ENZYMES

5) Make up 10 mL AFLP Digestion/Ligation (DL) Buffer (recipe is at the end of this protocol). This can be made up ahead of time and stored in at -20°C.

6) Genomic DNA can also be prepared ahead of time. You should have approximately 100-250 ng of DNA/sample that you want to generate AFLPs for. The DNA should be of good quality and clean. Phenol-chloroformed isolated DNA works well. Resuspend the DNA in the lowest volume of liquid possible to keep the concentration high.

7) Determine the volume of liquid required to obtain approximately 100-250 ng 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 so that all samples have the sample volume of liquid (this makes the calculations easier). Record the volume value.

8) Mix the following reagents together into a master mix.

Recipe for digestion (40 μL total volume/sample):

Reagent	**amount needed/sample**
*Eco*RI enzyme	5 U[1]
Mse I enzyme	5 U[1]
10X AFLP DL Buffer	4.4 μL
ddH_2O	up to 40 μL[2]

[1] = Different companies pack their restriction enzymes at different unit concentrations so volumes will differ.
[2] = Remember that you want 40 μL 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!!

9) 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 μL. Mix well by pipetting up and down several times.

10) Place in 37°C water bath for 1 hour.

LIGATION OF DOUBLE-STRANDED ADAPTERS TO THE ENDS OF THE RESTRICTION FRAGMENTS

11) Near the end of the 1 hour 37°C water bath incubation, make up the following ligation master mix.

Recipe for ligation (10 μL total volume/sample):

Reagent	amount needed/sample
T4 ligase	1 U[3]
10X AFLP DL Buffer	1.1 μL
5 μM *Eco*RI adaptors	1.0 μL
50 μM *Mse*I adaptors	1.0 μL
10 mM ATP	1.0 μL
ddH_2O	up to 10 μL

[3] = Different companies pack their ligase at different unit concentrations so volumes will differ.

12) Aliquot 10 μL 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 μL. Place into 37°C water bath for 3 hours (the total time duration of digestion/ligation should be at least 4 hours).

13) Following incubation, dilute digestion/ligation reaction with approximately 450 μL ddH_2O (1:9 dilution), vortex and place in -20°C or proceed to next step.

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.

14) Aliquot 5.0 μL of diluted digestion/ligation reaction into appropriately labeled 0.5 mL thin-walled PCR tubes.

15) Set up the following PCR amplification master mix.

Recipe for preselective (PS)-AFLP amplification:

Reagent	**amount needed/sample**
10X 1.5 mM $MgCl_2$ PCR Buffer	2.0 µL
10 mM dNTPs	0.4 µL
2.75 µM *Eco*RI primer	2.0 µL
2.75 µM *Mse*I primer	2.0 µL
UB *Taq* polymerase	0.25 µL
ddH_2O	8.35 µL

16) Vortex briefly, centrifuge and aliquot 15.0 µL master mix/PCR sample (20 µL total volume/sample). Mix well by pipetting up and down several times.

17) 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 15 min
Step 8:	Hold 4°C (you only need to do this if the machine is running o/n)
Step 9:	END

* if the sample had less than 25 ng total genomic DNA added, you may have to increase the number of cycles to increase yield.

18) Once the PCR is done (approximately 2 hr), run out 5 µL PCR product in a 2% agarose gel to confirm amplification next to 5 µL of the dilution used as template. If amplification has occurred (evident by a smear when the two lanes are compared), dilute remaining PCR product 1:9 with ddH_2O, vortex and place in -20°C or proceed to next step. If PCR was weak, increase the number of cycles (see above).

SELECTIVE DNA AMPLIFICATION OF SUBSETS OF RESTRICTION FRAGMENTS USING AFLP PRIMERS AND LABELLING OF AMPLIFIED PRODUCTS

19) Aliquot 5.0 μL of diluted PS-AFLP reaction into appropriately labeled 0.5 mL thin-walled PCR tubes.

20) Set up the following PCR amplification master mix.

Recipe for selective AFLP amplification:

Reagent	amount needed/sample
10X 1.5 mM $MgCl_2$ PCR Buffer	2.0 μL
10 mM dNTPs	0.4 μL
0.46 μM *Eco*RIAF labeled primer	2.0 μL
2.75 μM *Mse*IAF primer	2.0 μL[4]
UB *Taq* polymerase	0.25 μL
ddH_2O	8.35 μL

[4] = this is the selective primer. Depending on your organism, you may have to change the selective nucleotides at this primer's (or both this primer and the *Eco*RIAF primer's) 3' end. The only way to know is to empirically test and tailor the primers to your organism.

21) Vortex briefly, centrifuge and aliquot 15.0 μL master mix/PCR sample (20 μL total volume/sample). Mix well by pipetting up and down several times.

22) 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 30 sec
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:	Hold 4°C (you only need to do this if the machine is running o/n)
Step 11:	END

23) Add 20 μL of formamide loading buffer (LB). If your samples still look overloaded (evident by "blob"-like bands, dilute PCR with 10 μL ddH_2O and then add 30 μL LB.

AFLP PAGE USING Li-COR AUTOMATED SEQUENCER

24) Prepare a 6.5% acrylamide gel for running out your labeled samples I have used Li-Cor's KB^{Plus} gel matrix with great success. You may also try the following recipe for a 7% 19:1 acrylamide/bis-acrylamide gel.

Reagent	amount needed
40% stock 19:1 acrylamide/bis-acrylamide	5.25 mL
10X AFLP PAGE Running Buffer	1.5 mL
Urea	13.5 g

25) Dissolve and mix above reagents well, measure in graduate cylinder and top off to 30 mL with ddH_2O.

26) Clean glass plates and assemble gel rig according to Li-Cor directions. If using a 32 well-square tooth comb (recommended by Li-Cor) or any other square tooth comb, apply bind silane to both plates before putting the rig together. γ-methacryloxypropyltrimethoxysilane (bind silane) working stock is made by mixing 50 μL bind silane dissolved in 10 mL 100% EtOH. Add 100 μL of the working stock with 100 μL 10% acetic acid in 1.5 mL eppie, vortex well, apply to both plates where comb will be mounted. Let dry for 3 minutes, **DO NOT** wipe area with alcohol after applying.

27) When ready to pour gel, add 225 μL freshly-made 10% APS (0.1 g APS/1 mL ddH_2O) and 22.5 μL TEMED to the gel matrix solution. **ONCE THESE REAGENTS ARE ADDED, POUR THE GEL QUICKLY USING A 60 CC SYRINGE SINCE IT WILL START TO POLYMERIZE!!!!**

28) Let the gel polymerize for at least 1.5 hours before using.

29) Prepare the gel by washing off excess acrylamide, pull the 32 well-square tooth comb (recommended by Li-Cor) and pre-run the gel for approximately 8 minutes (see Li-Cor manual if you have questions on how to do this). The setting should be set to the following:

1500 V
40 mA
40 W
45°C

30) Denature samples for 3-4 minutes at 94° C and place immediately on ice. Load samples and begin electrophoresis.

AFLP Data Sheet

Date:_____

Determine the volume of liquid required to obtain approximately 0.5 μg of DNA/sample. Aliquot the DNA into a 1.5 mL eppie. Dilute concentrated samples so that all have the sample volume of liquid (this makes the calculations easier). Record the volume value.

Notes for DNA samples and concentrations:

_____
_____
_____
_____

I. DIGESTION W/ *ECO*RI AND *MSE*I.

Reagent	Amount needed/sample	Amount used/sample	# Samples	Total
10X Buffer	4.4 μL	_____	_____	_____
*Eco*RI[1]	5 U	_____	_____	_____
*Mse*I[1]	5 U	_____	_____	_____
Genomic DNA	0.5 μg	_____	_____	_____
ddH_2O[2]	up to 40 μL	_____	_____	_____

[1] = Different companies pack their restriction enzymes at different unit concentrations so volumes will differ.
[2] = Remember that you want 40 μL 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!!

II. Add master mix to eppies with DNA so that total volume is 40 μL.

III. Incubate @ 37°C for approximately 1 hour.

IV. Digestion/ligation with *Eco*RI, *Mse*I and T_4 ligase.

Reagent	Amount needed/sample	Amount used/sample	# Samples	Total
10X Buffer	1.1 μL	_____	_____	_____
T_4 ligase[3]	1 U	_____	_____	_____
*Eco*RI adaptors	1 μL 5 μM	_____	_____	_____
*Mse*I adaptors	1 μL 50 μM	_____	_____	_____
ATP	1 μL 10 μM	_____	_____	_____
ddH_2O	up to 10 μL	_____	_____	_____

[3] = Different companies pack their ligase at different unit concentrations so volumes will differ. You may have to adjust water volume/sample.

V. Add 10 μL of above master mix to each tube (tubes will now have 50 μL volume).

VI. Incubate @ 37°C for approximately 3 hour (4 hour total incubation). Place @ -20°C.

AFLP Reagent Recipes and Oligonucleotide Sequences

10X AFLP digestion/ligation (DL) Buffer (10 mL) (initial concentrations in parenthesis after reagent)

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 µL of 10 mg/mL BSA	(100 ng/µL)
bring up to 10 mL with ddH_2O	

*Eco*RI-adaptor Structure

5'-CTCGTAGACTGCGTACC	**OLIGO #1**
CATCTGACGCATGGTTAA-5'	**OLIGO #2**

*Mse*I-adaptor Structure

5'-GACGATGAGTCCTGAG	**OLIGO #3**
TACTCAGGACTCAT-5'	**OLIGO #4**

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.

10X PAGE Running Buffer (1000 mL)
(initial concentrations in parenthesis after reagent)

10X TBE

Use at 1X strength for running buffer and gel matrix

40% Stock 19:1 acrylamide/bis-acrylamide

19 g acrylamide
1 g bis-acrylamide
bring up to 50 mL with ddH_2O
shelf life is one month at 4°C