Andrew C. Clarke and Heidi M. Meudt
Allan Wilson Centre for Molecular Ecology and Evolution
Massey University
NEW ZEALAND

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AFLP®[1] (AMPLIFIED FRAGMENT LENGTH POLYMORPHISM)

for multi-locus genomic fingerprinting

Protocol based on:

Vos *et al.* 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407-4414.

For additional information see:

Meudt & Clarke. *in press.* Almost Forgotten or Latest Practice? AFLP applications, analyses, and advances. *Trends in Plant Science.*

1. Preliminary Considerations

Time Frame for Lab Work:

We have found it is best to complete the DNA digestion, ligation and pre-selective PCR amplification in one day. This makes for a long day, so if these steps need to be done over several days use the appropriate storage conditions listed at the end of each step as overnight storage conditions i.e., do not store digestion and restriction products overnight at +4°C (the restriction enzymes might display star activity and the ligation reactions may degrade).

Number of Selective Bases on Selective Primers:

The complexity of the AFLP profiles is correlated with the genome size of the species i.e., larger genomes produce more bands. For plant genomes we have found Eco+3, Mse+3 good for small plant genomes (500-1000 Mbp) and Eco+3, Mse+4 good for medium-sized plant genomes (1000-3000 Mbp).

The Royal Botanic Gardens, Kew website maintains a useful database (http://www.rbgkew.org.uk/cval/homepage.html), which lists the genome sizes of many plants species.

As Vos *et al.* (1995) noted, the addition an extra selective base does not reduce the number of fragments by a ¼ as would be expected, but only about ½. This suggests there is some mispriming, or non-specific amplification, in the PCR. Indeed, experiments with two different primer combos that differed only in the 2nd selective

[1] AFLP® technology is covered by patents and patent applications owned by Keygene N.V.; AFLP is a registered trademark of Keygene N.V.

base of the Eco+4 selective primer (Eco+AATA versus Eco+ATTA) produced nearly identical profiles, suggesting substantial mispriming at the 2nd selective base.

Also, for fluorescent AFLPs only one strand (the labelled strand) is detected, whereas both strands are detected in methods such as silver staining. So, for a given primer combination far fewer bands will be observed with fluorescent labelling as with silver staining — this should considered when choosing the length of the selective primers.

2. Genomic DNA Quantification

DNA should be extracted using a method which yields high molecular weight DNA that is free of contaminants. For plants, the Qiagen DNeasy Plant Kit gives excellent results.

Run aliquots of genomic DNA on a 1% agarose gel to assess both the DNA quantity and quality (the DNA should exist as a single, intact, high molecular weight band). It is not sufficient to simply measure the DNA concentration by spectrophotometry, as the DNA may be poor quality (degraded) even though there is a lot of it.

3. Restriction Endonuclease Digestion Reaction

Sample Replicates
To ensure robustness of the technique, it is necessary to include a number of replicate samples, preferably from the digestion step. For further information, see Meudt & Clarke (*in press*).

Digest 60 ng-1 µg of DNA in a total volume of 25 µL.

Variable Amounts of DNA for Digestion
We have found 250 ng to be a good amount to aim for. Do not worry if different DNA digests have slightly different amounts of DNA (as long as it's in the above range) as the two consecutive PCR reactions even out any differences that might otherwise appear in the intensity of the profiles.

Set up the restriction endonuclease digestion reaction (25 µL volume).

	×1	
Milli-Q H_2O	to 25 µL	
5× Reaction Buffer*	5.0	(1×)
*Eco*R I (10 U/µL) (Roche)	1.0	
Mse I (10 U/µL) (NEB)	1.0	
Genomic DNA	?	(~250 ng)
TOTAL	**25 µL**	

* 250 mM potassium acetate (KOAc) (Sigma), 50 mM magnesium acetate (MgOAc) (Sigma) and 50 mM Tris-HCl [pH 7.5]

Incubate at 37°C for 2 hours, then 70°C for 15 min to irreversibly denature the restriction enzymes.

Run 5 µL of digested DNA on a 1% agarose gel (next to an equivalent amount of undigested DNA as a control) to check that digestion is complete. The high molecular weight band should have completely disappeared, and a smear should be present up to ~750bp.

Problems with Incomplete Digestion

If the digestion appears incomplete the reaction can be incubated at 37°C for an additional hour, but only if the denaturation step above has been omitted. If the enzymes have already been denatured, additional enzyme would need to be added. Digestion should not proceed for more than 3 hours total as *Eco*R I may display star activity and non-specifically digest the DNA.

Store digested DNA at −20°C.

4. Linker Ligation Reaction

Set up the DNA ligation reaction (20 µL volume).

	×1	
Milli-Q H_2O	10.0	
10× Ligation Buffer (Roche)*	2.0	(1×)
Linker — Eco†	1.0	
Linker — Mse†	1.0	
T4 DNA Ligase (1 U/µL) (Roche)	1.0	
Eco/Mse Digested DNA	5.0	
TOTAL	**20 µL**	

Ligation Buffer

The ligation buffer contains ATP, which is damaged by repeated freeze–thawing. Avoid unnecessary freeze–thaw cycles by aliquoting the ligation buffer into single-use aliquots (e.g., 50 µL).

* If using Invitrogen T4 DNA Ligase also use 1 µL of the enzyme, but the Invitrogen Ligation Buffer is supplied at a 5× stock so 4.0 µL will be required in the reaction (reduce the water to 8.0 µL to retain the overall volume at 20 µL).

† See Appendix 1 for linker sequences and Appendix 2 for instructions for preparing the linkers.

Incubate at 37°C for 3 hours. Store ligation reactions at −80°C. **N.B.** It is not sufficient to store the ligation reactions at −20°C; we have found they slowly degrade at this temperature.

5. Pre-Selective PCR Amplification Reaction

Set up the pre-selective PCR reaction (20 µL volume).

	×1	
Milli-Q H_2O	8.3	
Betaine (5 M)	4.0	(1 M)
dNTPs (2 mM)	2.5	(0.25 mM)
10× PCR Buffer (Roche)*	2.0	(1×)
Eco+A Primer (10 pmol/µL)†	1.0	(0.5 µM)
Mse+C Primer (10 pmol/µL)†	1.0	(0.5 µM)
Taq Polymerase (5 U/µL) (Roche)	0.2	(1 U)
Ligated DNA	1.0	
TOTAL	**20 µL**	

* Contains 15 mM $MgCl_2$.

† See Appendix 1 for primer sequences.

Use the following low stringency program:

Step			
1	94°C	30 sec	×20 cycles
2	56°C	1 min	
3	72°C	1 min	
4	10°C	HOLD	

Limit ramping speed to 1°C/s.

Although not really necessary, 5 µL aliquots of pre-selective amplification PCR products can be electrophoresed on a 1% agarose gel to check the PCR has worked. A faint smear should be observed.

6. Selective PCR Amplification Reaction

Diluting Pre-Amp PCR Products

If conducting primer screening with many different selective primer combos from a single pre-amp product (pre-amp) there is a chance you will use up all of your pre-amp. Although more pre-amp can be generated by going back to the ligations (which have been stored at −80°C) this is a nuisance. An easier alternative if you think you will use up the pre-amps is simply to dilute them 10× with MilliQ-H_2O and use 1 µL of the dilution (instead of 1 µL neat) in the selective amplification. There is no noticeable difference in the intensity of the selective amplification products when using diluted pre-amps as opposed to undiluted pre-amps.

Selective Primer Screening

Before embarking on the full project, it is necessary to screen a number of selective primer combinations on a small number of samples (~10) to obtain combinations which give high quality profiles. See Meudt & Clarke (*in press*) for further information.

Recommended Fluorophores for AFLP

Colour	Fluorophore
BLUE	6FAM™
GREEN	VIC®
YELLOW	NED™
RED	PET™
ORANGE	LIZ™

If detecting the AFLP products on an ABI 3730 Genetic Analyzer with the G5 filter, up to 4 selective amplification products (each labelled with a different dye or fluorophore) can be poolplexed (see Step 7). The fifth dye (LIZ) is reserved for the size standard (ladder). VIC, NED, PET and LIZ are proprietary dyes and are available from ABI only; 6FAM can be obtained from ABI, but is cheaper from Sigma, Invitrogen, etc. Alternative dyes (e.g., HEX for green, TAMRA for yellow) cannot be used with the G5 filter set — they possess spectral overlap with the recommended dyes (causing bleed-through and a mixed signal at some wavelengths) or fluoresce too weakly to be detected properly.
The size of the circle represents the relative intensity of each fluorophore (from http://depts.washington.edu/genomelb/DataAnalysisV2.html; see Step 7).

Set up the selective PCR reaction (20 µL volume).

	×1	
Milli-Q H_2O	9.8	
dNTPs (2 mM)	2.5	(0.25 mM)
$MgCl_2$ (25 mM)	2.5	(3.125 mM)
10× PCR Buffer (Roche)*	2.0	(1×)
•XXX-Eco+ANN Primer (10 pmol/µL)†	1.0	(0.5 µM)
Mse+CNN Primer (10 pmol/µL)†	1.0	(0.5 µM)
Taq Polymerase (5 U/µL) (Roche)	0.2	(1U)
Pre-amp PCR Product	1.0	
TOTAL	**20 µL**	

* Contains 15 mM $MgCl_2$.

• Denotes fluorescently-labelled primer. XXX denotes fluorophore (e.g., 6FAM, VIC, NED or PET).

† See Appendix 1 for primer sequences.

Magnesium Concentration

We have trialled different final Mg^{2+} concentrations of 1.5-4.625 mM at approx. 0.5 mM increments and have found that above 2.75 mM there is no observed difference in the profiles (below 2.75 mM Mg^{2+} the overall intensity of the profiles is less). We use a Mg^{2+} concentration of 4.625 mM (1.5 mM from 1× PCR buffer + 3.125 mM from additional $MgCl_2$).

Use the following touchdown program:

Step				
1	94°C	2 min		×1
2	94°C	30 sec		
3	65°C	30 sec	↓1°C/cycle	×10
4	72°C	1 min		
5	94°C	30 sec		
6	56°C	30 sec		×30
7	72°C	1 min		
8	72°C	30 min		×1
9	10°C	HOLD		

Limit ramping speed to 1°C/s.

Touchdown Program

We have experimented with final annealing temperatures of 56°C and 52°C and found no difference between the profiles generated using these two programs. The technique appears very robust to changes in this PCR program.

Prolonged Incubation at 72°C

Incubation for 30 min ensures the non-templated adenosine (A) is added to the 3′ end of the synthesised strands. The efficiency of the addition of the non-templated A to the 3′ ends of the PCR product depends on the nucleotides preceding it (Brownstein *et al.*, 1996). A mixture of adenylated and non-adenylated products in the selective amplification PCR reaction due to inadequate incubation at 72°C can lead to 'stutter' or 'split' peaks in the profiles. To drive the adenylation to completion it is recommended that the PCR is incubated at 72°C for 30 min at the end of the PCR program. We have trialled final 72°C steps of 2 min, 5 min, 30 min and 45 min, and although we observed no difference in the degree of stutter between these extension times we would recommend using 30 min as this is the accepted standard for genotyping in the literature.

7. PCR Product Pooling and Capillary Electrophoresis

Purification of Selective Amplification PCR Products

Some protocols recommend cleaning the selective amplification PCR products prior to capillary electrophoresis to remove excess primers, dNTPs and salts — in fact Mark Berres (http://ravel.zoology.wisc.edu/sgaap/), who uses Agencourt CleanSEQ magnetic beads, claims this is essential for capillary electrophoresis. We have compared the profiles of non-cleaned, CleanSEQ cleaned, and ethanol precipitated products and found the clearest profiles are actually from non-cleaned products. Therefore, we would **not** recommend cleaning the products prior to capillary electrophoresis.

Ratios of Different Fluorophores

As noted in Step 6 above under 'Recommended Fluorophores for AFLP', the different fluorophores fluoresce with different intensities. To compensate for this, differently-labelled selective amplification products can be poolplexed in different ratios. For 6FAM, VIC, NED and PET we use a pooling ratio of 1:1:1:2 respectively (see below). The optimal ratio of each fluorophore should be determined prior to high-throughput genotyping.

Pool up to four differently-labelled selective amplification PCR products (e.g., 6FAM, VIC, NED and PET) for capillary electrophoresis (this is called post-PCR multiplexing, or preferably poolplexing). For each sample, pool 2 µL of 6FAM product, 2 µL of VIC product, 2 µL of NED product, and 4 µL of PET product (total of 10 µL) and mix.

If using the Allan Wilson Centre Genome Service (http://awcmee/genome-service.htm), submit the 10 µL mixture for capillary electrophoresis. The AWCGS will combine a 1 µL aliquot of the mixture with GeneScan-500 LIZ size standard and formamide prior to electrophoresis on a 3730 Genetic Analyzer (Applied Biosystems).

For non-ACWGS customers, please consult your capillary electrophoresis provider and/or Applied Biosystems (http://www.appliedbiosystems.com/) for instructions on preparing and electrophoresing your samples.

8. Data Analysis

For the AWCGS, AFLP profiles will be supplied electronically (in *.fsa format). For a list of software appropriate for scoring AFLP data, and appropriate scoring techniques, see Meudt & Clarke (*in press*). Scoring methods will also be covered in Holland *et al.* (*in prep.*).

References

Brownstein, M. J., Carpten, J. D., and Smith, J. R. 1996. Modulation of non-templated nucleotide addition by *Taq* DNA polymerase: Primer modifications that facilitate genotyping. *BioTechniques* **20**: 1004-1010.

Holland, B. R., Clarke, A. C., and Meudt, H. M. *in prep.* Exploring AFLP parameter space: Tuning parameters to improve phylogenetic resolution.

Meudt, H. M., and Clarke, A. C. *in press.* Almost Forgotten or Latest Practice? AFLP applications, analyses, and advances. *Trends in Plant Science*.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407-4414.

Appendix 1 — Oligonucleotide Sequences

Class	Oligo Name	Sequence
Linker	*Eco*R I Linker:	
	Eco Linker I	5′ CTCGTAGACTGCGTACC 3′
	Eco Linker II (3′-5′)	3′ CATCTGACGCATGGTTAA 5′
	Eco Linker II (5′-3′)	5′ AATTGGTACGCAGTCTAC 3′
	Mse I Linker:	
	Mse Linker I	5′ GACGATGAGTCCTGAG 3′
	Mse Linker II (3′-5′)	3′ TACTCAGGACTCAT 5′
	Mse Linker II (5′-3′)	5′ TACTCAGGACTCAT 3′
Pre-amp	*Eco*R I Primer:	
	Eco+A	5′ GACTGCGTACCAATTC**A** 3′
	Mse I Primer:	
	Mse+C	5′ GATGAGTCCTGAGTAA**C** 3′
Selective	*Eco*R I Primers:	
	•Eco+ANN	5′ •GACTGCGTACCAATTC**ANN** 3′
	Mse I Primers:	
	Mse+CNN	5′ GATGAGTCCTGAGTAA**CNN** 3′

Bold type denotes selective sequence.

† Denotes fluorophore (e.g., 6FAM, VIC, NED or PET)

• Denotes fluorescently-labelled primer.

Purification of Oligos

A small fraction of oligos of incorrect length are synthesised along with the correct length oligo as a by-product of the synthesis reaction. If these spurious oligos are incorporated into the selective amplification PCR they may lead to stutter in the profiles. Some researchers (and oligo-synthesising companies!) recommend high-performance liquid chromatography (HPLC) purification of oligos to purify the correct length oligo, although many consider it unnecessary and purchase all oligos de-salted only. We have only purchased only one HPLC-purified oligo and found it no better than its de-salted equivalent. Also, HPLC-purified oligos can add considerable cost to AFLPs. Therefore, we would consider HPLC-purification unnecessary.

Appendix 2 — Annealing of AFLP Linkers

$T_{10}E_1$ = 10 mM Tris, 1 mM EDTA

Eco Linker I (1 nmol/µL)	1.0
Eco Linker II (1 nmol/µL)	1.0
Milli-Q H_2O	108.0
$T_{10}E_1$	90.0
TOTAL	**200 µL**

Mse Linker I (1 nmol/µL)	10.0
Mse Linker II (1 nmol/µL)	10.0
Milli-Q H_2O	90.0
$T_{10}E_1$	90.0
TOTAL	**200 µL**

The Mse linker is required at a much higher concentration in the ligation reaction because of the greater proportion of *Mse* I ends in the reaction compared to *Eco*R I ends.

In a thermal cycler, heat to 95°C for 5 min, followed by cooling to 5°C over 30 min (Δtemp = 90°C = 0.05°C/sec). Slow cooling of the linkers favours perfect annealing.

Store AFLP linkers at −20°C.