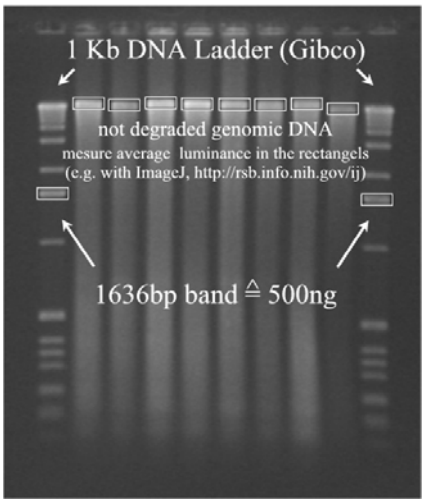
AFLP PROTOCOL

0. Quantification and dilution of DNA



- Prepare $H_2O_{dest/steril}$ in 20-25ml vials (ca. 15 for 500 samples, you will use them to dilute your samples) and in 1.5ml Eppendorf tubes (ca. 10 for 500 samples, you will use them for the master mix preparations). Store Eppendorf tubes in the freezer.
- Control the quality and determine the quantity of the DNA of your samples on an agarose gel (Excel sheet "DNA-Quantification_gel.xls"). Measure the luminance of each band using for Image analysis program (e.g. ImageJ) as shown in the picture to the left. Copy the measurement in the Excel sheet described below.
- Use Excel sheet "DNA-Quantification_form.xls" to quantify the DNA content of your samples and to calculate the dilutions.
- Dilute the DNA to an appropriate (≈ 0.25µg ≈ 45ng/µl) concentration, which depends on the genome size (between 0.5µg [= 90ng/µl] for regular plant genomes and 0.05µg for small plant genomes (see Protocol Applied Biosystems). Use prepared $H_2O_{dest/steril}$ to dilute your samples.
- Always clearly label microtiter plates (use word document "Plate_format.doc") or tubes you use for AFLP analysis. Use the following material for each step:
  1. Dilute your samples in Eppendorf tubes or in yellow Eppendorf microtiter plate.
  2. Use red Eppendorf microtiter plate for restriction-ligation.
  3. Use Microseal skirted (BioLabo) microtiter plates for PCR's.
  4. Use blue Eppendorf microtiter plate to dilute preselective PCR product.
  5. Use Multiplate unskirted (BioLabo) microtiter plate for genetic analyser.

  Work on the sterile bench to keep your samples, solutions, primers and enzymes clean. Only open plastic bags with microtiterplates within the sterile bench and wear gloves to keep the remaining plates sterile.

1. Restriction-Ligation

- Prepare **a**liquots of **R**estriction-**L**igation **M**aster-**M**ix (a-RLMM) in 2ml Eppendorf-tubes ("400 sample units" ⇒ 1720µl). Aliquots don't contain enzymes (wouldn't remain active) and T4 DNA Ligase-buffer 10x (contains ATP, which may degrade).
- Prepare aliquots of T4 DNA Ligase-buffer (115µl = "96-well microtiter plate units"), to avoid repeated defrosting and freezing.

a-RLMM

unit (µl)	*1 sample*	*10 samples*	*410 samples*	
$H_2O_{dest/steril}$	0.617	6.2	255	
NaCl 0.5M	1.1	11	450	
BSA 10x (1g/l)	0.55	5.5	225	
*Mse*1 adaptor (50ρmol/µl = 50µM)	1	10	410	
*EcoR*1 adaptor (5ρmol/µl = 5µM)	1	10	410	
total	4.267	42.7	1750	

- Complete the a-RLMM just before starting the restriction ligation reaction by using an aliquot tube of T4 DNA Ligase-buffer and adding a-RLMM and the enzymes. Mix carefully (with pipette or by flipping, don't Vortex):

RLMM

unit (µl)	*1 sample*	*10 samples*	*"96" samples*	
Aliquot T4 DNA Ligase-buffer 10x	1.1	11	115	
a-RLMM	4.267	42.7	445	
*Mse*1 enzyme (20U/µl)	0.05	0.5	5.2	
*EcoR*1 enzyme (100U/µl)	0.05	0.5	5.2	
T4 DNA Ligase (30weissU/µl)	0.033	0.33	3.5	
total	5.5	55	573.9	
			8 × 71.5µl	

- Prepare for each sample a 0.2ml tube and add *5.5µl* of the RLMM. For 96 Samples use a 96-well PCR microtiter plate and label the plate with red colour.
- Add *5.5µl* of your diluted sample DNA (e.g. 45ng DNA/µl ≈ 0.2–0.3µg DNA/reaction vol.) ⇒ total restriction-ligation product *11µl*.
- Incubate at room temperature over night (PTC-100 thermal cycler, run the RL-program, 14h 20°C then ∞h 4°C), or 2-3 hour at 37°C (but then use a thermal cycler with heated cover).
- Dilute the restriction-ligation product (*11µl*) 20× by adding *189µl* $H_2O_{dest/steril}$ from a vial you prepared. For 96 Samples use red 96-well PCR microtiter plate, put 95*µl* $H_2O_{dest/steril}$ in each hole and add *5µ*l of the restriction-ligation product.
- Freeze the diluted restriction-ligation product for storage.

2. Preselective PCR

- Prepare **p**reselective **M**aster-**M**ix (p-MM) on ice, use 2ml Eppendorf tube.

p-MM

unit (µl)	*1 sample*	*10 samples*	*"96" samples*	
$H_2O_{dest/steril}$	13.3	133	1400	
PCR buffer (10×)	2	20	210	
dNTPs (10mM each)	0.2	2	21	
*Mse*1 presel-Primer (150ng/µl ≈ 29µM)	0.2	2	21	
*EcoR*1 presel-Primer (150ng/µl ≈ 29µM)	0.2	2	21	
Taq Polymerase (5U/µl)	0.1	1	10.5	
total	16	160	1683.5	
			8 × 210 µl	

- Use the Eppendorf PCR-Cooler for sample preparation (after taking the PCR-Cooler out of the freezer, wait 5min to avoid freezing of your samples during preparation).
- Prepare for each sample a 0.2ml tube (or use a 96-well PCR microtiter plate) with *16µl* of the p-MM. Use blue colour to label the PCR plate.
- Add *4µl* of the diluted restriction-ligation product ⇒ total preselective-product *20µl*.
- Put the samples into the PTC-100 thermal cycler and run the AFLPP PCR-program (see page 4 for details), enable heated lid, the length of the program is about 2 hours.
- Check from time to time success of preselective PCR amplification on a small agarose gel by using 12 randomly selected samples (use ca. 8µl undiluted PCR product).
- Dilute the preselective-PCR product 20× (e.g. *5µl* with *95µl* $H_2O_{dest/steril}$). Take $H_2O_{dest/steril}$ from a vial you prepared. Use blue Eppendorf 96-well microtiter plate.
- Freeze the diluted presel-PCR product for storage.

3. Selective PCR

- If you are doing a multiplexing PCR it is possible to mix the EcoR1-primers already in advance (EcoR1-primer Master Mix aliquot, e.g. p¹ 250µl, p² 250µl, p³ 500µl, p⁴ 500µl = 1500µl)
- Prepare **s**elective **M**aster Mix (**s-MM**) on ice. Components between waved lines vary with the primer combinations you have chosen to run your selective PCR. Defrost and incubate the primers or primer Master Mix at 65°C for 5min.

s-MM	**normal**				**multiplexing**			
unit (µl)	*1 sample*	*10 samples*	*"96" samples*		*1 sample*	*10 samples*	*"96" samples*	
$H_2O_{dest/steril}$	12.5	125	1310		8.7	87	915	
$MgCl_2$	—	—	—		1.8	18	189	
PCR buffer (10×)	2	20	210		2	20	210	
dNTPs (10mM each)	0.4	4	42		0.4	4	42	
[1]*EcoR1* fam sel-Primer [AGG] (1µM)	1	10	105		0.5	5	52.5	
[2]*EcoR1*(unlabeled) sel-Primer [AGG] (1µM)	—	—	—		0.5	5	52.5	
[3]*EcoR1* joe sel-Primer [ACC] (1µM)	/	/	/		1	10	105	
[4]*EcoR1* tamra sel-Primer [AGC] (1µM)	/	/	/		1	10	105	
or *EcoR*1-Primer Master Mix aliquot					(3)	(30)	(315)	
*Mse*1 sel-Primer (5ρmol/µl = 5µM)	1	10	105		1	10	105	
Taq Polymerase (5U/µl)	0.1	1	10.5		0.1	1	10.5	
total	17	170	1782.5		17	170	1786.5	
			8 × 222µl				8 × 223µl	

- Use Eppendorf PCR-Cooler for sample preparation (again, wait 5min before using it).
- Prepare for each sample a 0.2 ml tube (or use a 96-well PCR microtiter plate) with *17 µl* of the s-MM.
- Add *3 µl* of the diluted preselective-PCR product ⇒ total selective-product *20 µl*.
- Put the samples into the PTC-100 thermocyler and run the AFLP PCR-program (see page 4 for details), enable heated lid, the length of the program is about 2.45 hours
- Freeze the sel-PCR product for storage

4. Fragment evaluation (ABI PRISM 310 Genetic Analyzer)

- Prepare **L**oading **B**uffer **M**ix aliquots (LBM) in 2ml Eppendorf tubes:
  - Formamid 1500µl (aliquot in 2ml Eppendorf-tube)
  - Gene Scan-500 ROX size standard 4µl
  - 1504µl (max. 60 samples)
- Loading samples:
  - LBM 25µl (96 samples = 2400µl ≈ 8 × 305µl)
  - selective amplification product 2µl
  - 27µl (each sample)
- During analysis check the quality of the individual samples and re-analyse the samples that show problems within the same run. Sometimes more than one repetition is necessary to finally get a satisfactory and usable result.
- Clearly document each sample sheet file and each run file. For each run file indicate which samples had to be reanalysed and which of the repeated analysis gave the best result. Document, which samples had to be re-analysed in a new run and indicate the run.

- If samples give trouble during analysis and if the problem can't be solved by simple re-analysis of the PCR products try to re-do the PCR's (or even start from the beginning with a new restriction-ligation reaction) by diluting your samples more (your isolated DNA is never clean, and some of the remaining substances may inhibit the enzymatic reactions, which may be solved by a higher dilution). If this doesn't help, isolate your back-up plant material or your back-up samples.

Preseltective PCR Program (PTC-100 thermocyler, AFLPP):

Step	1	2min	94°C	(extended initial denaturation step)
	2	20sec	94°C	(denaturation step)
	3	30sec	56°C	(primer annealing step)
	4	2min	72°C	(extension step)
	5	20 times to 2		(step 2 to 4 20×)
	6	30min	60°C	(final extension step)
	7	∞	4°C	
	8	End		

Hold	**Cycle**			**Hold**	**Hold**
	each of 20 cycles				
94°C 2min	94°C 20sec	56°C 2min	72°C 2min	60°C 30min	4°C (forever)

Selective PCR Program (PTC-100 thermocyler, AFLP)

Step	1	2min	94°C	(extended initial denaturation step)
	2	20sec	94°C	(denaturation step)
	3	30sec	66°C –1,0°C/cyc	(primer annealing step, starting at 66°C and reducing each step 1°C till 56°C)
	4	2min	72°C	(extension step)
	5	9 times to 2		(step 2 to 4 9×)
	6	20sec	94°C	(denaturation step)
	7	30sec	56°C	(primer annealing step)
	8	2min	72°C	(extension step)
	9	20 times to 6		(step 6 to 8 20×)
	10	30min	60°C	(final extension step)
	11	∞	4°C	
	12	End		

Further informations

Aplied Biosystems (2000), AFLP™ Plant Mapping Protocol, www.appliedbiosystems.com

Amador et al. (2002), Amplified Fragment Length Polymorphism (AFLP) Workshop, Interdisciplinary Center for Biotechnology Research, University of Florida (http://www.biotech.ufl.edu/WorkshopsCourses/AFLPManual2002b.pdf)

Vos et al. (1995), AFLP: a New Technique for DNA-Fingerprinting, Nucleic Acids Res. 23(21)

Mueller & Wolfenbarger (1999), AFLP genotyping and fingerprinting, TREE 14(10)

Internet: http://www.pcrlinks.com/variants/aflp.htm