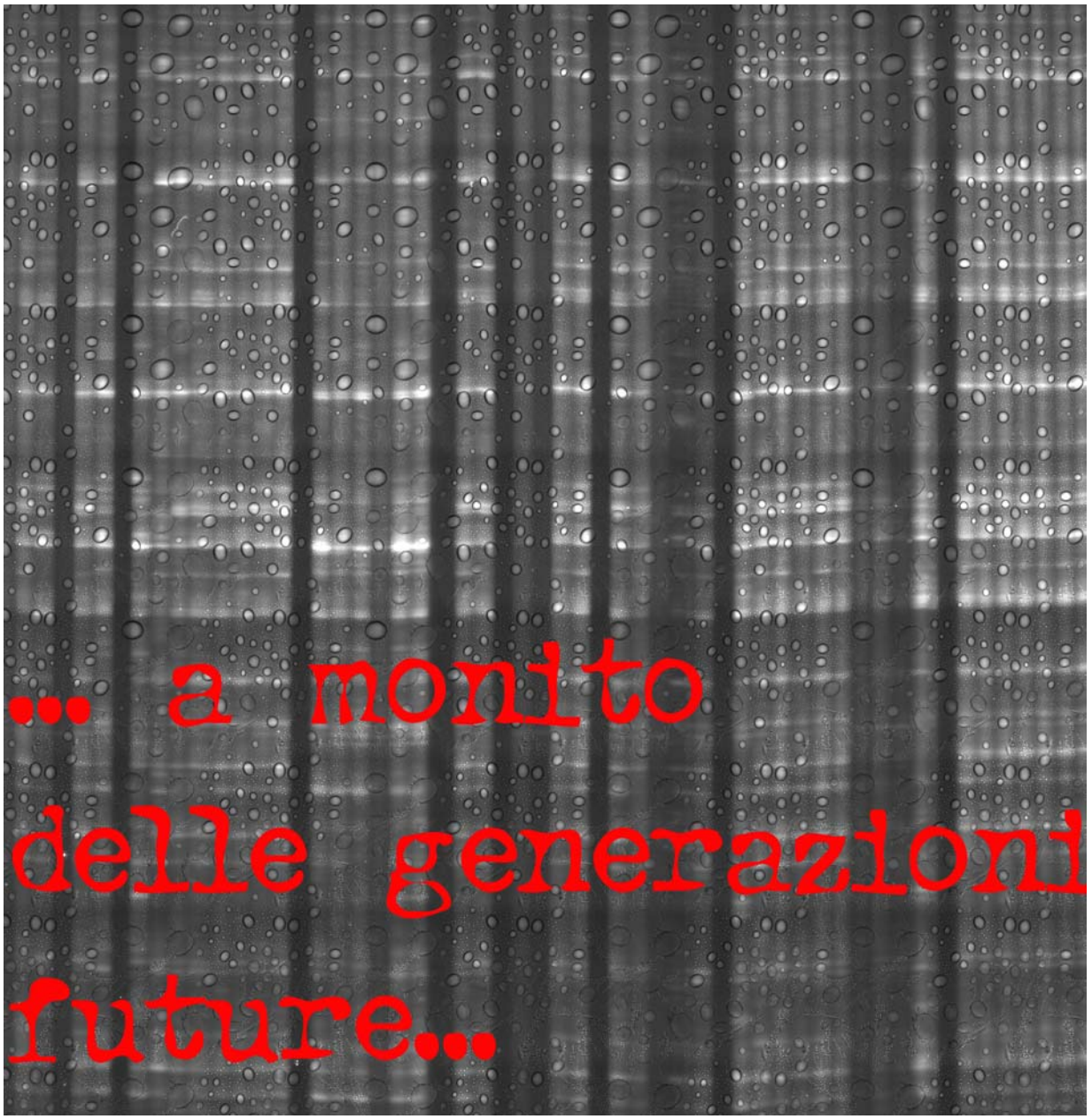
AFLP protocol



1. PREPARATION OF MATERIALS

- ✓ Genomic DNA: 500ng in 30µl.
- ✓ Adapters preparation: see Figure 1 and Table1. Dilute lyophilized adapters to 200pmol/µl (Stock solution) and see Table 2 and Table 3. Heat the adapter mixes at 94°C for 4' and then let the temperature low down at room temperature.
- ✓ Prepare the 5x RL Buffer as reported in Table 4.

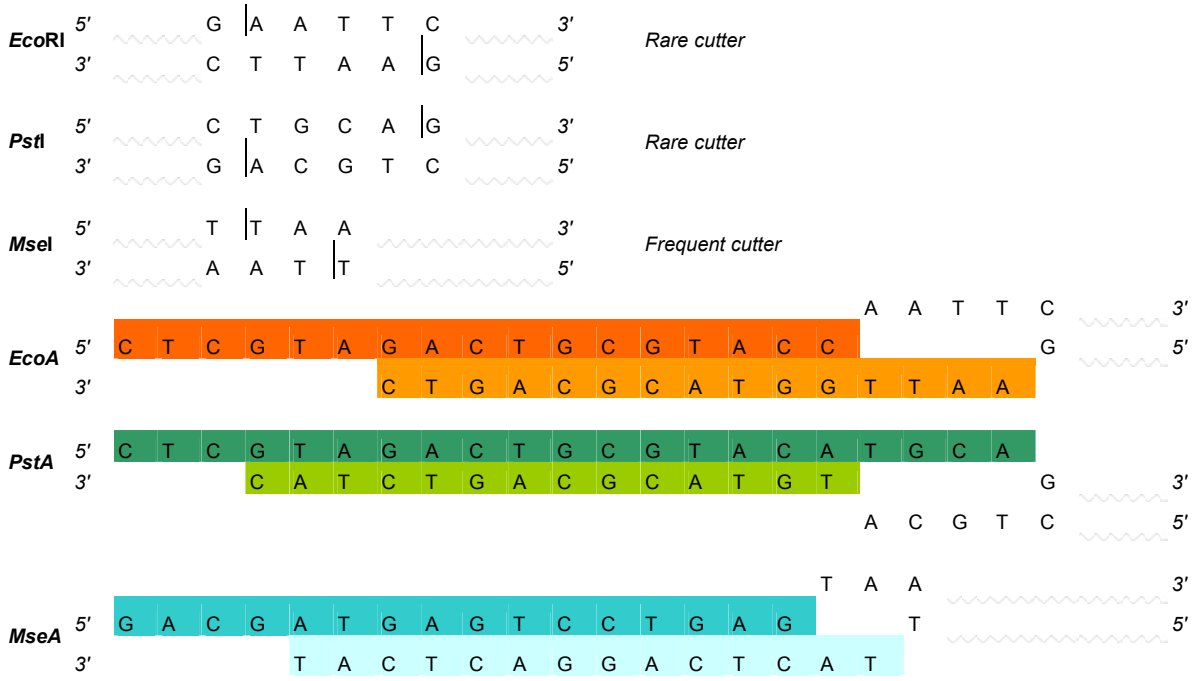


Figure 1 Cutting sites of restriction enzymes and structures of adapters

Table 1 Sequences of adapters (5'→3')

Adapter	Sequence
*Eco*RI-A 1	5' CTC GTA GAC TGC GTA CC 3'
*Eco*RI-A 2	5' AAT TGG TAC GCA GTC 3'
*Pst*I-A 1	5' CTC GTA GAC TGC GTA CAT GCA 3'
*Pst*I-A 2	5' TGT ACG CAG TCT AC 3'
*Mse*I-A 1	5' GAC GAT GAG TCC TGA G 3'
*Mse*I-A 2	5' TAC TCA GGA CTC AT 3'

Table 2 Preparation of *Eco*RI- or *Pst*I- adapters

Reagent	µl	Final concentration
*Eco*RI-/*Pst*I A1 (200pmol/µl)	1.5	5pmol/µl
*Eco*RI-/*Pst*I A2 (200pmol/µl)	1.5	5pmol/µl
H_20	57.0	-
Final volume	60.0	-

Table 3 Preparation of *Mse*I-adapters

Reagent	µl	Final concentration
*Mse*I-A1 (200pmol/µl)	15	50pmol/µl
*Mse*I-A2 (200pmol/µl)	15	50pmol/µl
H_20	30	-
Final volume	60	-

Table 4 Preparation of 5x RL Buffer

Reagent	Working dilution	µl	Final concentration
One Phor All (OPA)	10x	500	100mM
DTT	1M	25	25mM
BSA	10ng/µl	25	0.25µg
H_20	-	450	-
Final volume	-	1000	-

2. RESTRICTION AND LIGATION OF ADAPTERS

a. Prepare RL Mix as indicated in Table 5.
b. Add 20μl of RL mix to the 30 μl of genomic DNA.
c. Incubate at 37°C for 4hr.
d. Place restricted/ligated samples on ice.
e. Agarose gel electrophoresis of 5μl of reaction (agarose 1%, 90', 150V) (Figure 2).
f. Dilute 1/10 the restricted/ligated products.

Table 5 Preparation of RL mix

Reagent	Working dilution	μl	Final concentration
RL Buffer	5x	10.000	1x
*Mse*I	4U/μl	1.250	5U
*Eco*RI/*Pst*I	20U/μl	0.250	5U
*Mse*I A	50pmol/μl	1.000	50 pmol
*Eco*RI/*Pst*I A	5 pmol/μl	1.000	5 pmol
ATP	10mM	1.000	0.2mM
T4 DNA Ligase	7.5U/μl	0.133	1U
H_20	-	5.367	-
Final volume	-	20.000	-

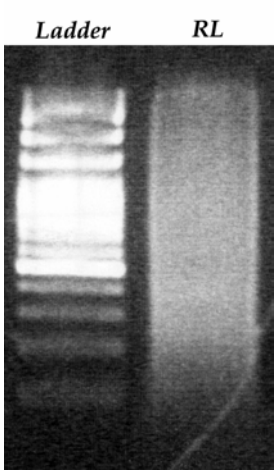


Figure 2 Agarose gel electrophoresis of an RL reaction

3. PRE-AMPLIFICATION PCR

a. Perform the PRE-AMPLIFICATION PCR reactions (Figure 3, Table 6, and Table 7) with temperature profile as given in Table 8.
b. Agarose gel electrophoresis of 5µl of the PREAMP reaction (agarose 1%, 90', 150V) (Figure 4).
c. Dilute 1/10 the PREAMP products

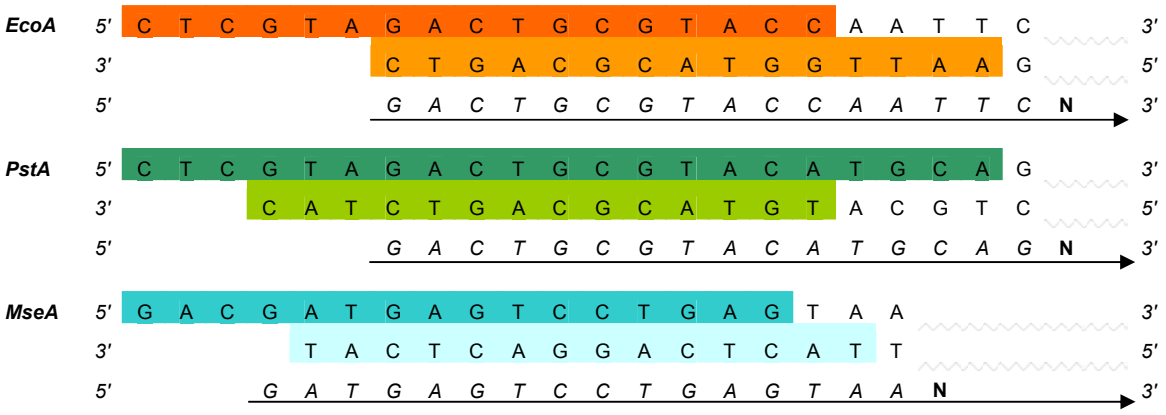


Figure 3 Structures of EcoRI- *Pst*I-, and *Mse*I-adapters and the respective primers

Table 6 *Eco* and *Mse* primer sequences for PREAMP reactions

Primer	Sequence§
Eco +1	5' GAC TGC GTA CCA ATT C N 3'
Pst + 1	5' GAC TGC GTA CAT GCA G N 3'
Mse +1	5' GAT GAG TCC TGA GTA A N 3'

§Ns are the selective nucleotides chosen for each primer.

Table 7 Preparation of PREAMP mix

Reagent	Working dilution	µl	Final concentration
Eco +1/*Pst* +1 Primer	50ng/µl (~6pmol/µl)	1.5	75ng
Mse +1 Primer	50ng/µl (~6pmol/µl)	1.5	75ng
dNTPs	5mM	2.0	0.2mM
PCR Buffer	10x	5.0	1x
Taq polymerase	5U/µl	0.2	1U
H_20	-	34.8	-
Diluted RL reaction	-	5.0	-
Final volume	-	50.0	-

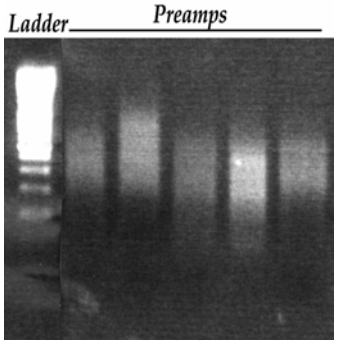


Figure 4 Agarose gel electrophoresis of some PREAMP reactions

Table 8 Temperature profile for PREAMP and SELECTIVE PCRs

Cycle name	Temp (°C)	Duration	No of Cycles	*Touch-down profile
				64.3
Pre-PCR	94.0	45"	1	63.6
	65.0	30"		62.9
	72.0	1'		62.2
Touch-down PCR*	94.0	30"	12	61.5
	64.3	30"		60.8
	72.0	1'		60.1
PCR	94.0	30"	20	59.4
	55.9	30"		58.7
	72.0	1'		58.0
Elongation	72.0	10'	1	57.3
				56.6

4. SELECTIVE AMPLIFICATION PCR

a. Perform SELECTIVE AMPLIFICATION PCR (Table 9 and Table 10) with the temperature profile as in the PREAMP reaction (Table 8).
b. PAGE (See PAGE).

Table 9 *Eco* and *Mse* primer sequences for SELECTIVE AMPLIFICATION reactions

Primer	Sequence§
Fluo-*Eco* +3	Fluor- 5' GAC TGC GTA CCA ATT C NNN 3'
Fluo-*Pst* +3	Fluor- 5' GAC TGC GTA CAT GCA G NNN 3'
Mse +3	5' GAT GAG TCC TGA GTA A NNN 3'

§Ns are the selective nucleotides chosen for each primer, with the first (5') N the same as in the PREAMP primer.

Table 10 Preparation of the SELECTIVE AMPLIFICATION mix

Reagent	Working dilution	µl	Final concentration
Fluorecinated *Eco*+3/*Pst*+3 Primer	50ng/µl (~6pmol/µl)	0.60	30ng
Mse+3 Primer	50ng/µl (~6pmol/µl)	0.60	30ng
dNTPs	5mM	0.80	0.2mM
PCR Buffer	10x	2.00	1x
Taq polymerase	5U/µl	0.08	0.4U
H_20	-	10.92	-
Diluted PREAMP reaction	-	5.00	-
Final volume	-	20.00	-

5. PAGE (POLYACRILAMIDE GEL ELECTROPHORESIS)

a. Mix in a beker urea, acrylamide, methylenbisacrilamide, TBE 10x, 135ml H_20 and stir until completely dissolved (almost 2 hr) (Table 11).
b. Adjust to the final volume with H_20.
c. Filter and de-gas the 19:1mixture.
d. Prepare 80ml of PAG: 80ml of 19:1 mixture, 80µl of TEMED, 300-500µl APS 10%.
e. Add 6µl of Dextran Blue Formamide dye (Table 12) to SELECTIVE AMPLIFICATION reactions.
f. Denaturation step at 94°C.
g. Cool immediately the denatured samples on water-ice.
h. Load 1-1.5µl of the reactions from previous step into the polyacrylamide gel.
i. Run at 100W and 2700V for 2.5 hr.

Table 11 Preparation of 300ml of 6% (w/v) denaturing Polyacrylamide (19:1 Acrylamide/Methylenbis-acrylamide) Gel

Reagent	Working dilution	Quantity	Final concentration
Urea	-	135g	7.5M
Acrylamide	-	18g	6%
Methylenbisacrylamide	-	0.75g	
TBE	10x	15ml	0.5x
H_20	-	to final volume	-
Final volume	-	300ml	-

Table 12 Preparation of Dextran Blue Formamide dye

Reagent	Working dilution	Quantity
Formamide	98%	14.7ml
Dextran Blue	2%	300mg
EDTA	0.250mM	7.5µl
H20		292.5µl
Final volume		15ml