(MML) TE-AFLP Protocol

Worksheet 1: (MML) notebook worksheet for TE- AFLP-Protocol

Date: / /200
Sample Name----------
Genome Size----------

Digestion-Ligation worksheet:

Day (1)	Volume per reaction µl	Comments
Xbal (6 U/µl)	0.3 µl	6 U (Sometimes 3U)
BamHI (1.25 U/µl)	0.3 µl	1.25 U
Rsal (1 U/µl)	0.3 µl	1 U
Xbal-adapters (4 pmol)	1 µl	
BamHI-adapters (4 pmol)	1 µl	
RL-Buffer (5x)	10 µl	
T4-DNA ligase (1U/µl)	1 µl	
ATP (10 mM)	1 µl	
DNA (10 ng/µl)	15 µl	150 ng minimum concentration; up to 250 ng; decrease ddH2O
dd H2O	10.1 µl	

- work on ice
- use microtiterplate or strips
- make a master mix of the enzyme mix for the number of samples you have plus 10% overage.
- Mix well and centrifuge at 14,000 rpm for 15 seconds **incubate for 2 h at 30°C in a thermocycler**
- dilute in 150µl TE0.1, mix well, spin down 15 sec.
- label the microtiterplate (eg. "rl-reaction"), store at -20°Cthese tubes (or reactions) constitute the template for the preselective amplification step

preparing of adapters for 100 reactions

Xbal-adapter
5µl Xbal- A1 (100pmol/µl stock solution)
5µl Xbal-A2 (100pmol/µl stock solution)
90µl TE0.1 buffer or ddH2O
total 100µl Xbal--adapter (5pmol/µl)

BamHI-adapters
50µl BamHI A1 (100pmol/µl stock solution)
50µl BamHI A2 (100pmol/µl stock solution)
total 100µl BamHI -adapter (50pmol/µl)

After mixing the adapters, heat at **95 °C for 5 mi**n in a thermocycler. Then allow to cool slowly at room temperature. Store at -20°C.
Note: Before adding frozen adapters again to a ligation mix, heat an aliquot of adapters at 95°C for 5 minutes and allow to cool to room temperature over a 10 minute period, spin for 10 seconds.

Amplification

Note: The first PCR reaction uses primers that match the adapter sequence and have one additional "selective" base. This reduces the number of bands that will be amplified. The T4 DNA ligase only ligates one of the strands of the adapter to the fragment. The other is held on by base-pair binding to the other adapter strand. Thus the first step of the +1 PCR reaction is a 72 °C hold that allows the Taq polymerase to ligate the other strand. If you perform a Hot-Start, or place the +1 reactions into a hot thermal-cycler, or omit the initial 72 °C hold, you will loose the second strand and the PCR reaction won't work. **Do not use AmpliTaq Gold for +1 reactions** as the Taq will not be active in the initial 72 °C hold.

It is imperative that all solutions are thawed completely and mixed well when setting up the reactions.

Day (2)	Volume per reaction µl	comments
10x PCR buffer	2	
Diluted restriction/ligation reaction	5	
dNTP (10mM)	0,4	final 200µM each
labelled XbaI-adapter primer (10pmol)	0,6	30 ng final
unlabelled BamHI-adapter primer (10pmol)	0,6	30 ng final
Taq-polymerase (5U/µl)	0,2	1 U (0,5 U is enough)
ddH2O	add to 20µl	

Use the following PCR parameters for amplification

PCR - Parameters	Temp.	Time/cycles	No. of Cycles
1-Heated Lid	105 °C		
2-Block temp.	80 °C		(Forever)
3-Denaturation	95 °C	2.5 min.	1 Cycles
4-Denaturation	95 °C	30 sec.	**10 Cycles**
5- Annealing	70 °C	60 sec.	
6-Primer Extension	72 °C	30 sec.	
7-Denaturation	95 °C	30 sec.	**40 Cycles**
8- Annealing	60 °C	60 sec.	
9-Primer Extension	72 °C	60 sec.	
7-Final Extension	72 °C	20 min	1 Cycles
8-Cooling	4 °C	Stand-by	(Forever) until ready to analyze

- Run 10 µl of the +1 reaction on a 1.5% agarose gel. You should see a smear in the 100 to 1,000 bp range. Sometimes bands are visible through the smear.
- Dilute the other 10 µl in 90 µl of TE 0.1 (somestimes a dilution of 1:15 up to 1:20 is recommend)
- label the microtiterplate (eg. "preamp-reaction"), spin down in centrifuge, store at -20°C

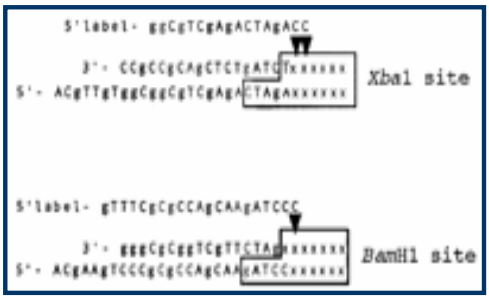

Figure 1. The complementary set of adapters and primer sequences are presented for the restriction sites *Xba*I and *Bam*HI. Boxes indicate genomic sequences and arrowheads indicate selection on nucleotides in the genomic fragments.

Construction of oligonucleotides

About 10 different combinations of endonucleases, primers and adapters were tested (unpublished results). The adapters and primers were selected based on number of bands, even distribution of bands along the gel, minimal background signal and highest level of polymorphism among five springtails from a single forest.

Adapters And Primers	5-.....Oligo.....-3	Comment
*Bam*HI adapters	ACgAAgTCCCgCgCCAgCAA gATCTTgCTggCgCggg.	
*Xba*I adapters	AcgTTgTggCggCgTCgAgA CTAgTCTCgACgCCgCC	
The unlabelled *Bam*HI-C primer sequence	TTTCgCgCCAgCAAgATCCC	
The labelled *Xba*I-CC primer	ggCgTCgAgACTAgACC	

Three endonucleases will generate fragments with six different restriction ends (AA, BB, CC, AB, AC, BB and BC). The expected frequencies of these fragments are, respectively: $a^2/(a + b + c)^2$, $b^2/(a + b + c)^2$, $c^2/(a + b + c)^2$, $2ab/(a + b + c)^2$, $2ac/(a + b + c)^2$ and $2bc/(a + b + c)^2$. In TE-AFLP the fragments with restriction ends from enzyme A are not amplified.