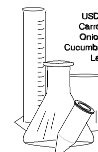


AFLP
Restriction Digestion of Genomic DNA

1. Make a master mix as follows:

Component	Per $\frac{1}{4}$ reaction	Per × $\frac{1}{4}$ reactions
5× Reaction buffer	1.25 µl	
*Eco*R I + *Mse* I (1.25 u each/ µl)	0.5 µl	
AFLP-grade H_2O	1.5 µl	
Total volume	3.25 µl	

2. Mix 3 µl DNA (60 ng) and 3.25 µl of master mix per $\frac{1}{4}$ reaction.

3. Incubate 2 hours at 37°C, and then heat inactivate enzyme for 15 minutes at 70°C.

Ligation of Adapters

1. Make a master mix as follows:

Component	Per $\frac{1}{4}$ reaction	Per × $\frac{1}{4}$ reactions
Adapter ligation solution	6 µl	
T4 DNA Ligase	0.25 µl	
Total volume	6.25 µl	

2. To the digestion (6.25 µl), add 6.25 µl of master mix. Incubate at 20°C ± 2° for 2 hours.

3. Dilute reaction 10×: take 2.5 µl of reaction, and add 22.5 µl of TE. Store diluted ligation mix, and remaining undiluted ligation mix at -20°C.

Preamplification

1. Make a master mix as follows:

Component	Per $\frac{1}{10}$ reaction	Per × $\frac{1}{10}$ reactions
Preamplification primer	4 µl	
10× PCR buffer	0.5 µl	
Taq polymerase (5 u/µl)	0.022 µl	
Water	0.078 µl	
Total volume	4.6 µl	

2. Place 0.5 µl of diluted ligation mixture into a PCR tube. Add 4.6 µl of master mix. Run a PCR program of 20 cycles of [94°C for 30 seconds, 56°C for 1 minute, 72°C for 1 minute].

3. Dilute entire reaction by 50× by adding 249.9 µl of TE, then transfer to a 0.5 ml tube for storage at -20°C.

Primer Labelling

1. Make a primer labelling reaction as follows:

Component	Per $\frac{1}{4}$ reaction	Per $\times\frac{1}{4}$ reactions
*Eco*R I primer	0.045 µl	
Water	0.021 µl	
Kinase buffer	0.025 µl	
^{33}P	0.025 µl	
PNKinase	0.00875 µl	
Total volume	0.125 µl	

2. Incubate at 37°C for 1 hour, then heat inactivate at 70°C for 10 minutes.

Selective Amplifications

1. Make mix "**A**" as follows:

Component	Per $\frac{1}{4}$ reaction	Per $\times\frac{1}{4}$ reactions
Water	1.991 µl	
PCR Buffer	0.5 µl	
Taq polymerase (5 u/µl)	0.00875 µl	
Total volume	2.5 µl	

2. Using the tube of ^{33}P-labelled primer, make mix "**B**" as follows:

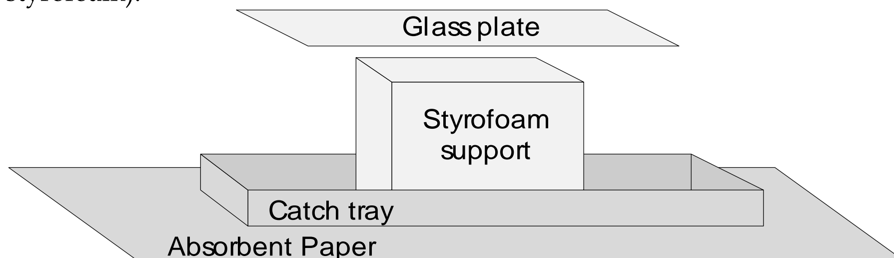
Component	Per $\frac{1}{4}$ reaction	Per $\times\frac{1}{4}$ reactions
Labelled *Eco*R I primer	0.125 µl	
Mse I primer	1.125 µl	
Total volume	1.25 µl	

3. Into each PCR tube, add 2.5 µl of mix "**A**". Next add 1.25 µl of diluted pre-amplified DNA. Finally add 1.25 µl of mix "**B**". Cover, hand-centrifuge, and wipe caps with damp towel to reduce static electricity. Run with PCR program #27.

4. Add an equal volume (5 µl) of formamide buffer.
(10 ml deionized formamide, 10 mg xylene cyanol FF, 10 mg bromophenol blue, 200 µl 0.5 M EDTA, pH 8.0)

Preparing an acrylamide gel

1. Set up work area: White absorbent paper, spill catch tray, plate support (hunk of styrofoam).



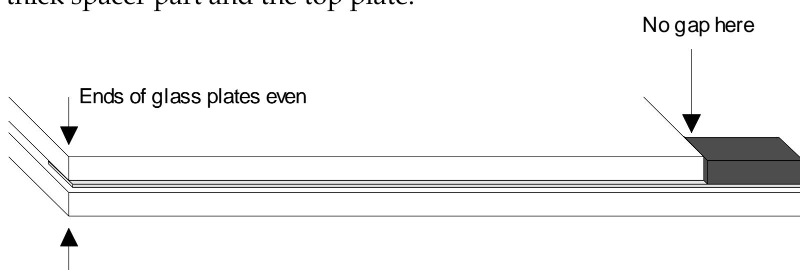
Printed January 2, 2014 © 1995 Douglas Senalik dsenalik@facstaff.wisc.edu

2. About every 10 runs, the long plate is cleaned with 2N NaOH, and the short plate is treated with Rain-X.

3. In sink, wash the long glass plate with Alconox and water, using just a gloved hand to rub. Rinse with tap distilled water. Dry the non-gel side with a paper towel, and set on styrofoam support. Dry the gel side with Kimwipes. Rinse with some 95% ethanol (pure grade from glass bottles) and some water, and dry with Kimwipes. Rinse again with just 95% ethanol, and dry again.

4. Repeat procedure with short plate.

5. Place spacers on long plate, and place short plate on top. Leave no gap between the thick spacer part and the top plate.



6. Place 7 binder clips on each side, centering the clip over the spacer. Binder clips are right next to each other.

7. Place a 3rd spacer at the bottom, and add 6 more binder clips.

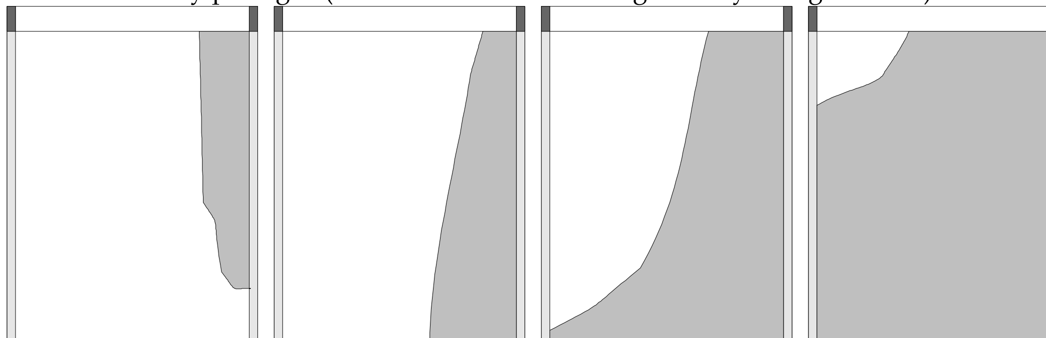
8. For a 60 ml gel, mix 12 ml 5x TBE and 13 ml dH_2O. Place on hot plate, warm the solution, and add 27 g urea and a stir bar. Stir until dissolved.

9. Bring to 51 ml volume with dH_2O.

10. Filter through a 0.45 µm 25 mm diameter membrane filter using the 60 ml "Urea" syringe. Filter directly into the acrylamide squirt bottle.

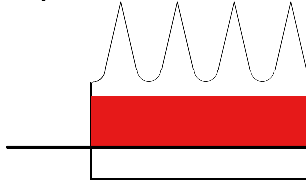
11. Add 9 ml of 40% acrylamide stock.

12. When everything is ready, add 320 µl of 10% APS and 30 µl TEMED. Swirl to mix, and immediately pour gel. (APS amount is increased gradually as it gets older)



© 1995 Douglas Senalik dsenalik@facstaff.wisc.edu Printed January 2, 2014

13. Insert combs until orange part is even with top of plate. Cover with extra acrylamide solution.



14. Add 2 binder clips, one over each half of comb. Tilt gel to 15° angle, and allow to polymerize for 1 to 1.5 hours.

15. Pour excess acrylamide solution into marked waste bottle, and clean acrylamide squirt bottle immediately.

16. Gel may be left overnight. Add moistened Kim-Wipes and cover with plastic film to prevent evaporation.

17. Remove combs and excess acrylamide. Wipe off any residue.

18. Close drain.

19. Clamp gel in place in running apparatus. Tighten clamps "until your fingers fall off."

20. Mix 800 ml 1x TBE. (160 ml of 5× TBE, bring to 800 ml with water) Keep the volume to this minimum to reduce the amout of radioactive waste. Add buffer to top reservoir to just cover the short plate.

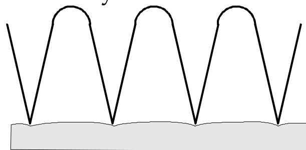
21. Use the 60 ml "Acrylamide" syringe to rinse out well area. Pull up some buffer, and squirt it into well area.

22. Pour remaining TBE into bottom reservoir. Fill from very right end to avoid bubbles between glass plates. If bubbles are caught, force out somehow with syringe.

23. Pre-run at 60 watts for about 30 minutes.

24. Disconnect power cables. Clean out well area again with 60 ml syringe.

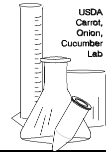
25. Carefully insert combs so that teeth touch but do not puncture top of the gel.



26. Load samples. Samples are denatured for 3 minutes at 90°C (Program #90) and then kept on ice.

27. Run for 2 hours at 60 watts.

Printed January 2, 2014 © 1995 Douglas Senalik dsenalik@facstaff.wisc.edu



While gel is running

28. Turn on cold trap at least 1 hour before use.

29. Put ice in pre-trap.

30. Cut piece of Whatman 3MM paper about 13 1/2 × 16 1/2 inches. Cut off corner to line up with lower left side of gel.

Disassembling the gel

31. Turn off power and remove cables.

32. Drain upper reservoir by opening valve.

33. Remove glass plates, blot off any surface liquid, and set on ice to cool. Let cool for 2 to 3 minutes.

34. Remove combs, and slide spacers out from the sides. Pry plates apart. Gel should stick only to the long plate.

35. Place a piece of Whatman 3MM paper (about 13 1/2 × 16 1/2 inches) on top of the gel. Smooth it with your fingers. Carefully peel the gel off, which will stick to the paper.

36. Place gel+paper on the drying machine, gel side up. Cover with plastic wrap, and dry under vacuum with heat set to 80°C for 50 minutes.

37. Dump liquid waste into appropriate radioactive waste bottle. Wash plates, spacers, gel apparatus, etc.

38. When gel is dry, place gel side in contact with X-ray film for 36 to 72 hours in a room temperature cassette.

© 1995 Douglas Senalik dsenalik@facstaff.wisc.edu Printed January 2, 2014