INVERSE PCR

We will amplify genomic DNA sequences that lie adjacent to transposon insertions by a combination of techniques called "inverse PCR". Basically this method consists of restricting total genomic DNA from the transposon strain with a restriction endonuclease that cuts inside the transposon as well as in the flanking genomic DNA sequence, then ligating the restricted DNA sample to permit formation of DNA circles of the DNA between the two restriction sites. The ligated DNA mixture is then used as template for PCR, using primers that will anneal with transposon sites and specifically amplify the ligated fragment. The protocol we will use has been developed at the Berkeley Drosophila Genome Project (BDGP) for routine analysis of transposon insertions. You should make a copy of the protocol from the BDGP website (http://www.fruitfly.org/about/methods/inverse.pcr.html) for use in the following experiments.

Restriction of genomic DNA

1. Place 3 μl of your genomic DNA sample (~3 fly equivalents) into each of two microtubes. Digest one with *Sau3AI* and the other with *HinP1I* in a total volume of 25 μl, following the directions provided in the BDGP protocol under "II. Digestions". Incubate at least 2.5 hrs at 37°C, then 20 min. at 65°C.

Ligation of restricted DNA

2. Place 10 μl of each restricted DNA sample into a microtube and carry out ligation of the DNA following the directions in the BDGP protocol under "III. Ligations".

PCR

3. Label four PCR microtubes "1" – "4" and follow directions provided in the BDGP protocol under "III. PCR" to prepare reactions mixtures in each tube containing:

Tube	DNA template	Forward primer	Reverse primer
1	ligated *Sau*3AI	Plac4	Plac1
2	ligated *Sau*3AI	Pry2	Pry1
3	ligated *Hin*P1I	Plac4	Plac1
4	ligated *Hin*P1I	Pry2	Pry1

Perform PCR using the cycling program recommended at the BDGP site.

Gel analysis and purification of PCR products

4. Prepare a 1.6% agarose gel. Load 20 μl of each sample (each plus 5 μl of loading dye), plus a marker lane. Electrophorese.

5. Ethidium bromide-stain and photograph your gel. Study the sizes of bands and excise gel blocks containing desired DNA fragments, placing each fragment in a microtube.

6. Dissolve the gel by adding 600 μl of 6M NaI solution and incubating at 50°C for 5 min. Mix the tube contents occasionally during the incubation, until the gel block is visibly dissolved. *Caution: NaI is a chaotropic agent; it should be handled with care and disposed of as a hazardous agent.*

7. Add 5 μl suspended glassmilk to the tube, cap and mix by inverting. Place the tube on ice for 5 min. to promote binding of DNA to the silica beads.

8. Centrifuge the tube for 10 sec. Remove the solution, avoiding disturbing the white pellet. Add 600 μl Wash solution and resuspend the silica beads by vigorous vortexing.

9. Repeat step #8 two more times (a total of three washes).

10. After removing the solution from the last wash, centrifuge the tube again for 20 sec. Use a micropipet tip to carefully remove the last residue of liquid.

11. Gently resuspend the silica beads in 10 μl TE buffer, using the micropipet tip. Place the tube in the 50°C waterbath to promote release of DNA from the beads.

12. Centrifuge the tube for 20 sec. Carefully remove the solution (containing DNA) to a new, labeled microtube.

Washing and autoradiography of the blot

To remove unhybridized probe from the blot, it is necessary to carry out a series of washes that will leave only stably attached radioactive probe on the membrane. After washing, the moist membrane can be placed against a piece of X-ray film to permit autoradiography of the membrane; the developed film can be analyzed to determine the location on the gel of bands that hybridized to the probe.

13. Carefully decant and discard the radioactive hybridization mixture from the hybridization tube. Add 50 ml of 2xSSC + 0.1% SDS to the tube, cap tightly, and return to the rotator for 15 min.

14. Decant and discard the solution in the tube. Replace it with another 50 ml of 2xSSC + 0.1% SDS and repeat the washing. Repeat this washing a third time.

15. Remove the blot from the tube and carefully place it into a sealable bag. Add 50 ml of 2xSSC + 0.1% SDS to the bag, seal it, and place it in a 42°C waterbath for 15 min.

16. Decant and discard the solution in the bag. Replace it with another 50 ml of 2xSSC + 0.1% SDS and repeat the 42°C washing (15 min.).

17. Remove the membrane from the bag and lightly blot it. Mount it, cover it with Saran wrap, and place it in a cassette against X-ray film. These steps will be demonstrated. Autoradiograph at -80°C using an appropriate amplifier screen.

18. Develop and analyze the exposed film.

Prehybridization solution:
5x SSC
50% formamide
4x Denhardt's solution
100 μg/ml denatured DNA
0.1% SDS

50x Denhardt's solution:
1% polyvinylpyrrolidone 25
1% Ficoll 400
1% nuclease-free bovine serum albumin