

## CHAPTER TWENTY SIX

# RFLP analysis of insect genomes

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### 26.1 INTRODUCTION

Genetic markers are useful for identifying and tracking genes in segregating populations and for comparing genetic diversity among populations. Traditionally, genetic linkage maps were generated by combining data from many populations, because only a limited number of markers segregated in any one population. Restriction fragment length polymorphism (RFLP) analysis employs cloned DNA sequences that hybridize to specific regions of the genome, with genetic polymorphisms defined as differences in the length of DNA fragments observed following digestion with restriction endonucleases and Southern blotting procedures. Since the demonstration of their utility for genetic mapping (Botstein *et al.*, 1980), RFLP markers have been widely used for constructing comprehensive linkage maps for many plant and animal species.

RFLP analyses of insects can be difficult because of their small size and their correspondingly small amount of total genomic DNA. Still, useful Southern blots can be prepared from insects with as little as 0.5  $\mu\text{g}$  of extractable DNA per individual. With these smaller insects, RFLP analysis is generally limited to a single restriction enzyme digest and a single Southern blotting effort for each individual. The individual Southern blots can, however, routinely be regenerated for 15–25 rounds of hybridization. A detailed RFLP linkage map exists for the yellow fever mosquito, *Aedes aegypti* (Severson *et al.*, 1993), and these markers have been used to identify genome regions carrying genes determining mosquito competence to transmit some malarial (Severson *et al.*, 1995a) and filarial worm (Severson *et al.*, 1994; Beerntsen *et al.*, 1995) parasites to their vertebrate hosts. With *Anopheles gambiae* mosquitoes, Romans *et al.* (1991) used RFLP markers to investigate the genetic relationship between a putative diphenol oxidase gene and an esterase locus previously associated with resistance to some malarial parasites. RFLP markers have also been linked with insecticide resistance in the German cockroach, *Blattella germanica* (Dong and Scott, 1994), and with genes for diapause hormone and a neuropeptide in the silkworm, *Bombyx mori* (Pinyarat *et al.*, 1995).

RFLP markers do offer several advantages for genome analysis in insects. We prefer to develop cDNA clones as RFLP markers because they typically represent single or low copy number sequences for which putative gene functions can frequently be determined with partial sequence information (Severson and Zhang, 1996).

Transcribed sequences also reflect sufficient evolutionary conservation, even among distantly related species, for developing comparative linkage maps. Comparative maps, based on common RFLP markers, have been developed for maize with rice (Ahn and Tanksley, 1993), sugarcane (D'Hont *et al.*, 1994), and sorghum (Pereira *et al.*, 1994), for tomato with potato (Bonierbale *et al.*, 1988) and pepper (Tanksley *et al.*, 1988), for garden pea with lentil (Weeden *et al.*, 1992), for human with mouse (Nadeau *et al.*, 1992) and for the mosquito, *Aedes albopictus* with *Ae. aegypti* (Severson *et al.*, 1995b). The methods described here were developed for use with *Ae. aegypti* and should, therefore, be applicable to most insect species of this general size or greater.

## 26.2 MATERIALS

### 26.2.1 DNA isolation and purification

1. DEB lysis buffer: 1% SDS, 0.2 M NaCl, 25 mM EDTA, 10 mM Tris, pH 8.0 (prepare fresh).
2. Proteinase K: 20 mg/mL (store at 4°C).
3. RNase A: 10 mg/mL (store at -20°C; Sigma R-5125).
4. 1 M Tris, pH 9.5.
5. Phenol: Equilibrated in 1 M Tris, pH 9.5 containing 0.1% 8-hydroxyquinoline (store in dark at -20°C; GibcoBRL #15509-011) (*avoid contact with skin and wear safety glasses*).
6. 8-Hydroxyquinoline: (Sigma H-6878).
7. Chloroform.
8. 95% Ethanol.
9. Restriction enzyme and appropriate buffer.
10. BSA: 1 mg/mL (GibcoBRL 11018-025).
11. Spermidine: 100 mM (Sigma S-2501).
12. Plastic pellet pestles: (Kontes #749520-0000).
13. Microcentrifuge tubes: 0.5 and 1.5 mL.

### 26.2.2 DNA separation

1. Horizontal slab gel apparatus and power supply.
2. Agarose: (TreviGel™ 5000; Trevigen #9806-50-P).
3. 50× TAE buffer: 242 g Tris, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA, pH 8.0. Adjust volume to 1 L with ddH<sub>2</sub>O.
4. Ethidium bromide: 10 mg/mL (store in dark at RT). *This is a mutagen, avoid contact with skin.*
5. Agarose dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in ddH<sub>2</sub>O.
6. UV transilluminator.

### 26.2.3 Southern blotting with nylon membranes

1. Gel denature solution: 150 mM NaOH, 3 mM EDTA, pH 8.0.
2. Gel neutralize solution: 150 mM sodium phosphate buffer, pH 7.8 (below).

3. Sodium phosphate buffer stock solution: 0.5 M, pH 7.8.
4. Sodium pyrophosphate stock solution: 0.1 M (Sigma P-8010).
5. Whatman chromatography paper #3030917.
6. 254<sub>nm</sub> germicidal lamp.
7. Nylon membrane, positively charged.

#### 26.2.4 DNA insert preparation

1. *Taq* DNA polymerase with 10× reaction buffer.
2. 25 mM MgCl<sub>2</sub>.
3. 5 mM dNTP mix.
4. Primers: Specific primers at ~10 ng/μL (1.5 μM).
5. Mineral oil.
6. Chloroform.
7. Thermal cycler.
8. Low melting point agarose (GibcoBRL #15517-014).
9. Phenol.
10. 4 M Ammonium acetate.
11. 95% Ethanol.
12. 70% Ethanol.
13. TE, pH 7.4: 10 mM Tris, pH 7.4, 1 mM EDTA.

#### 26.2.5 Probe radiolabelling

1. *Taq* DNA polymerase with 10× reaction buffer.
2. 25 mM MgCl<sub>2</sub>.
3. 5 mM dATP, dGTP, dTTP mix.
4. Primers: Specific primers at ~10 ng/μL (1.5 μM); Random nine-mer at 1 mg/mL.
5. Thermal cycler.
6. α<sup>32</sup>P-dCTP: (3000 Ci/mMole, Dupont NEN-013H).
7. Biogel elution buffer: 0.5% SDS, 1 mM EDTA, 20 mM Tris, pH 7.4, 5 μg/mL yeast RNA.
8. Biogel suspension: Slowly add 1 g Biogel P60 (Bio-Rad #150-4160) to 28 mL Biogel elution buffer using a stirplate (this equals about 10 bed volumes of buffer). Allow to equilibrate for several hours (preferably overnight) before use. Store at 4°C.
9. Yeast RNA: 10 mg/mL (Sigma R-6625).
10. Orange G: 0.5% (w/v) Orange G in ddH<sub>2</sub>O (Sigma O-7252).

#### 26.2.6 λ *Hind*III marker radiolabelling

1. λ phage DNA: (Boehringer Mannheim Biochemicals #745-782).
2. *Hind*III restriction enzyme and 10× reaction buffer.
3. Phenol.
4. Chloroform.
5. 95% Ethanol.
6. 70% Ethanol.

7. TE pH 8.0: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
8. 10× Nick translation buffer: 0.5 M Tris, pH 7.4, 0.1 M MgSO<sub>4</sub>, 1 mM DTT, 0.5 mg/mL BSA, 0.2 mM each dATP, dGTP, dTTP (store at -20°C).
9. α<sup>32</sup>P-dCTP: (3000 Ci/mMole, Dupont NEN-013H).
10. Klenow (DNA polymerase I large fragment).

### 26.2.7 Blot hybridization and regeneration

1. Blot preblock solution: 2% SDS, 0.5% BSA, 1 mM EDTA, 1 mM *o*-phenanthroline.
2. Hybridization solution: 0.1 M NaPO<sub>4</sub>, pH 7.8, 20 mM sodium pyrophosphate, 5 mM EDTA, 0.1% SDS, 10% (w/v) sodium dextran sulfate, 1 mM *o*-phenanthroline, 0.5 mg/mL heparin sulfate, 50 μg/mL sheared salmon sperm DNA, 50 μg/mL yeast RNA. To prepare 100 mL: Warm about 30 mL ddH<sub>2</sub>O, the sodium phosphate buffer and the sodium pyrophosphate in a beaker on a hot plate at a medium setting with stirring. Gradually add the sodium dextran sulfate until completely in solution. Turn off heat and add remaining ingredients. Bring to a final volume of 100 mL with ddH<sub>2</sub>O. Store at 4°C.
3. 1 M *o*-Phenanthroline: prepare in 95% ethanol; Sigma P-9375.
4. Sodium dextran sulfate: (Dextran Products Ltd, Scarborough, Ontario, Canada.).
5. Heparin sulfate: (Sigma H-7005).
6. Sodium phosphate buffer stock solution: 0.5 M, pH 7.8.
7. Sodium pyrophosphate stock solution: 0.1 M (Sigma P-8010).
8. Salmon sperm DNA: (Sigma D-1626). To prepare: Dissolve 1 g of DNA in 100 mL 0.1 M NaCl. Extract with phenol/chloroform. Shear DNA by drawing supernatant through an 18½ G needle about 12 times. Precipitate with ethanol and resuspend in ddH<sub>2</sub>O. Check concentration by spectrophotometer A<sub>260</sub> readings and adjust to final concentration of about 10 mg/mL. Store in aliquots at -20°C.
9. Yeast RNA: 10 mg/mL (Sigma R-6625).
10. 20×SSC: (1×SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0).
11. 10% SDS.
12. Wash solution I: 2×SSC, 0.1% SDS.
13. Wash solution II: 0.2×SSC, 0.1% SDS.
14. Hybridization oven.
15. Plastic sheet savers: (20th Century Plastics #NRPB30-00).
16. X-ray film: (Kodak XAR-5).
17. Staticide: (ACI Inc. #2005).
18. 0.4 N NaOH.
19. Blot neutralizer solution: 0.1×SSC, 0.1% SDS, 0.2 M Tris, pH, 7.4.

## 26.3 METHODS

### 26.3.1 DNA isolation and purification

This method was developed to produce DNA suitable for restriction enzyme digestion with a minimum of handling and is amenable to rapidly processing large numbers of individual mosquitoes (Severson and Kassner, 1995). It can be used to isolate genomic DNA from fresh, frozen or ethanol-preserved insects. With ethanol-pre-

served tissue, blot the individuals on paper towelling to remove excess ethanol, then allow them to air-dry overnight at RT before proceeding (D. Zaitlin, unpublished).

1. In a 1.5 mL microcentrifuge tube and using a plastic pellet pestle, homogenize a single mosquito in 120  $\mu$ L of freshly prepared DEB and 1.5  $\mu$ L of RNase A. With ethanol preserved individuals, grind dry to a powder, add the DEB and RNase A, then rehomogenize. Incubate at 37°C for 60 min.
2. Add 3  $\mu$ L of proteinase K and incubate at 50°C for 60 min.
3. Add 60  $\mu$ L each of phenol and chloroform. Vortex and centrifuge at full speed in a microfuge for 10 min.
4. Transfer the supernatant to a 0.5 mL microcentrifuge tube and add 300  $\mu$ L of 95% ethanol. Use care to avoid transferring any of the organic phase as restriction enzymes are extremely sensitive to inactivation by phenol. Mix well by laying tubes horizontal on a platform shaker and shaking for 10–15 min. Incubate at -20°C overnight.
5. Centrifuge at full speed in a microfuge for 10 min. Discard supernatant, remove as much ethanol as possible with a 10  $\mu$ L micropipette. Incubate tubes at 37°C for ~10 min with the cap open to evaporate remaining ethanol.
6. Resuspend DNA pellet in 11.1  $\mu$ L ddH<sub>2</sub>O. Incubation at 65°C will facilitate DNA resuspension. The DNA must be completely resuspended or it will not digest with restriction enzymes.
7. Restriction digests can be set up from a master mix containing for each DNA preparation: 0.2  $\mu$ L restriction enzyme (1–2 U), 1.5  $\mu$ L 10 $\times$  reaction buffer, 0.1  $\mu$ L RNase A, 1.5  $\mu$ L BSA, and 0.6  $\mu$ L spermidine. Add 3.9  $\mu$ L of the master mix to each tube and incubate at 37°C for 1 h minimum. After incubation, proceed with DNA separation (Section 26.3.2) or store at -20°C.

### 26.3.2 DNA separation

1. Prepare a 0.9% agarose gel of ~5 mm thickness. We use a fine-toothed comb to provide a well capacity of ~20  $\mu$ L. This concentrates the DNA in a small area and enhances resolution of restriction fragments, particularly when small quantities of DNA are size-fractionated.
2. Add 1/10 vol agarose dye to each restriction digest. Mix well and centrifuge briefly to pellet sample.
3. Load samples into gel. Load  $\lambda$  *Hind*III marker DNA at 15–20 well intervals. Perform electrophoresis in 1 $\times$  TAE buffer with buffer recirculation at ~1 V/cm until the bromophenol blue dye just runs off the gel.
4. Following size fractionation, stain the gel for ~1 h with 0.5  $\mu$ g/mL EtBr in 1 $\times$  TAE buffer, followed by a ~1 h destain in ddH<sub>2</sub>O. Visualize the DNA under UV light and photograph the gel for a permanent record.

### 26.3.3 Southern blotting with nylon membranes

The general method of Southern (1975) remains as the procedure of choice for permanently transferring DNA restriction fragments to a solid matrix (Chapter 20). Various modifications of the procedure are currently in practice. However, for maximizing the amount of information obtainable from individual Southern blots, the use

of positively charged nylon membranes and capillary transfer of the DNA with an alkaline buffer (Reed and Mann, 1985) is preferred. This procedure covalently binds the DNA to the membrane. The method described here is adapted from the literature (Murray *et al.*, 1992; Zaitlin *et al.*, 1993) and routinely provides 15–20 rounds of hybridization on membranes carrying genomic DNA digests from individual mosquitoes. Additionally, 2 or 3 different probes can frequently be hybridized simultaneously to the same blot, to increase the total number of markers evaluated for each individual (Severson *et al.*, 1993).

1. Place the agarose gel carrying the size-fractionated genomic DNA about 6–7 cm from a 254<sub>nm</sub> germicidal lamp with the high-molecular weight end of the gel centred directly under the lamp. Photo-nick the DNA by exposure to the light for 1 min.
2. Transfer the gel to a clean plastic or glass container with sufficient gel denature solution to cover the gel. Shake gently at RT on a platform shaker for 15 min (minigels) or 25 min (larger gels). Carefully pour off the gel denature solution and rinse the gel 3× with ddH<sub>2</sub>O.
3. Add sufficient gel neutralizer solution to cover the gel. Shake gently at RT on a platform shaker for 20 min (minigels) or 30 min (larger gels).
4. Cut a piece of nylon membrane exactly the size of the gel and prewet in 25 mM sodium pyrophosphate for at least 15 min before setting up the transfer. Additionally, cut 6 pieces of Whatman filter paper and a 4–5 inch stack of single-fold paper towels the same size as the gel.
5. Set up transfer of the DNA from the gel to the nylon membrane by capillary action using 25 mM sodium pyrophosphate as the transfer solution. Start with an ~1 inch pad of the pre-cut paper towels in a clean glass container and add transfer solution sufficient to completely saturate the towelling. Gently compress the towel pad to remove air bubbles. Place three pieces of the Whatman filter paper on the towel pad and carefully invert the gel and place it on the filter paper. Gently remove air bubbles by sliding a finger across the gel surface. Next, place the nylon membrane, labelled with a black ball-point pen, on the gel and again gently remove air bubbles. Place three pieces of the Whatman filter paper on the membrane, followed by a 2–2.5 inch stack of the paper towelling. Finally, place a glass plate on top of the stack. Add transfer solution to the container to a level just under the gel. Allow to transfer overnight.
6. Remove the membrane from the transfer stack and allow it to air-dry at RT. Place membrane between Whatman filter paper and bake *in vacuo* at 80°C for 3–5 h. Wrap with aluminium foil and store at RT.

#### 26.3.4 DNA insert preparation

Preparation of clone insert DNA for radiolabelling is efficiently accomplished by using a polymerase chain reaction (PCR) protocol (Saiki *et al.*, 1988; Chapter 21). Most cloning vectors contain defined primer annealing sites that facilitate PCR amplification of the insert with a small amount of associated vector sequence. This procedure is extremely useful for rapidly isolating insert DNA from random  $\lambda$  phage cDNA clones to screen for useful RFLP polymorphisms (Severson *et al.*, 1993). Specific directions are presented for amplifying inserts from vectors with SP6 and T7 promotor

primers. Other primer combinations may require appropriate adjustment of the anneal temperature in the PCR programme.

1. Set up the PCR reaction in 0.5 mL microcentrifuge tubes as follows:
  - 5  $\mu$ L 10 $\times$  Reaction buffer.
  - 3  $\mu$ L 25 mM MgCl<sub>2</sub>.
  - 2  $\mu$ L 5 mM dNTP mix.
  - 4  $\mu$ L SP6 primer at  $\sim$ 10 ng/ $\mu$ L.
  - 4  $\mu$ L T7 primer at  $\sim$ 10 ng/ $\mu$ L.
  - $\sim$ 1 unit *Taq* DNA polymerase.
 Template DNA for amplification use the following:
  - $\lambda$  phage plug preparations: add 5  $\mu$ L of a 100  $\mu$ L preparation.
  - plasmid minipreps: add 1 ng.
  - bacterial colonies or  $\lambda$  phage plaques: scrape the surface with a sterile toothpick and swirl the toothpick in the reaction mixture.
  - ddH<sub>2</sub>O to a final volume of 50  $\mu$ L.
2. Mix gently and overlay with mineral oil.
3. Place tubes in the thermal cycler and run the following programme for 30 cycles:
  - 94°C for 1 min
  - 50°C for 1 min
  - 72°C for 2 min
4. When the programme is completed, remove most of the mineral oil with an Eppendorf pipette, add 50  $\mu$ L chloroform, vortex, and centrifuge for 2 min.
5. Transfer the aqueous supernatant to a clean 0.5 mL microcentrifuge tube. Check a 5  $\mu$ L aliquot on a 0.6% agarose gel. Store at  $-20^{\circ}$ C.
6. Size-fractionate the reaction products on a 1.2% low melting temperature (LMT) agarose gel in 1 $\times$  TAE running buffer.
7. Place the EtBr-stained gel on UV transilluminator and excise the slice of LMT containing the fragment of interest using a clean razor blade. Make cuts around the band as tight as possible to minimize the amount of associated LMT agarose. *Use adequate shielding and minimize skin exposure to ultraviolet light while performing this procedure as you can receive serious burns, including eye damage.*
8. Place gel slice in a 1.5 mL microcentrifuge tube and centrifuge briefly to pellet the slice. Adjust the total volume to  $\sim$ 400  $\mu$ L with TE, pH 8.0, and incubate the tube at 65°C for 5 min. Vortex the tube briefly and immediately add 400  $\mu$ L of phenol prewarmed to 37°C. Vortex well and centrifuge at high-speed for 5 min, extract the supernatant with 400  $\mu$ L chloroform, and precipitate the DNA overnight at  $-20^{\circ}$ C with 40  $\mu$ L 4 M ammonium acetate and 1 mL 95% ethanol.
9. Centrifuge at high-speed for 10 min, decant off the supernatant and remove as much of the ethanol as possible with a 10  $\mu$ L micropipette. Incubate the tube at 37°C with the cap open for  $\sim$ 10 min to remove remaining ethanol. Resuspend the pellet in 20–25  $\mu$ L TE, pH 7.4. Estimate fragment concentration by checking a 2–4  $\mu$ L aliquot on a standard agarose gel.

### 26.3.5 Probe radiolabelling

DNA hybridization probes can be radiolabelled with <sup>32</sup>P to very high specific activities using a simple PCR protocol (Severson and Kassner, 1995). This procedure is

complementary to PCR-based insert isolation (Section 26.3.4). Specific directions are presented for radiolabelling inserts generated with SP6 and T7 promoter primers. Inserts prepared with other primer combinations may require appropriate adjustment of the anneal temperature in the PCR programme. For insert DNA prepared by standard restriction enzyme digestion and isolation from the cloning vector, we use a completely random nine-base oligonucleotide mix (RP9) with an anneal temperature of 37°C to prime the PCR labelling reaction.

1. Place 50 ng of insert DNA into a 0.5 mL microcentrifuge tube and add ddH<sub>2</sub>O to a final volume of 28.6 µL.
2. To set up a number of labelling reactions at one time, on ice prepare a master mix containing for each reaction: 5 µL 10× reaction buffer, 3 µL 25 mM MgCl<sub>2</sub>, 4 µL SP6 primer, 4 µL T7 primer, 0.2 µL 5 mM mixture of dATP, dGTP, dTTP, 0.2 µL (~1–2 U) *Taq* DNA polymerase and 5 µL (~50 µCi) α<sup>32</sup>P-dCTP. Add 21.4 µL of the master mix to each tube containing insert DNA and mix gently. *Use proper safety procedures for handling radioactive materials.* This reaction can be set up with any known primer combination for your insert. For labelling undefined inserts or inserts too long to efficiently prepare using a PCR procedure, use 9 µg of the RP9 mixture in each reaction. No mineral oil overlay is necessary.
3. Place tubes in the thermal cycler and run the following programme for one cycle:
  - 94°C for 2 min
  - 50°C for 3 min
  - slow ramp up to 72°C
  - 72°C for 5 min
4. Prepare a small Biogel chromatography column for each probe: Plug the tip of a 1 mL Eppendorf pipette tip with a small amount of glass wool. Do not pack the glass wool too firmly. Place the tips in a holder that will maintain them in position directly over 1.5 mL microcentrifuge tubes. To set up a Biogel column in each tip, the holder can initially be held over small plastic tub. Swirl the Biogel preparation to resuspend the Biogel. Immediately add 1 mL of the Biogel to each tip. Allow the buffer to drip through, forming a gel matrix, and discard the flow-through. This will take ~15 min. Next, place a rack containing a labelled, empty 1.5 mL microcentrifuge tube directly under each tip.
5. Add 10 µL Orange G to each labelling reaction, mix, and centrifuge briefly to pellet the contents. Slowly add the reaction mixture to the surface of the Biogel matrix in the tip. Allow ~1–2 min for the mixture to penetrate the matrix.
6. Add 330 µL of Biogel elution buffer to the surface of the matrix. Collect the flow-through (containing the probe) until the Orange G dye front reaches the glass wool plug. Discard the used tip in an appropriate radioactive waste container.
7. Determine probe activity by Cerenkov counting in a liquid scintillation counter.

### 26.3.6 λ *Hind*III marker radiolabelling

λ *Hind*III DNA markers, included on most Southern blots, can be directly visualized on autoradiograms by including a <sup>32</sup>P-labelled λ *Hind*III mixture during probe hybridization. This makes RFLP allele identifications much easier to determine, particularly when comparing genotypes across more than one Southern blot. The λ *Hind*III DNA can be labelled with a simple fill-in reaction.



1. Digest  $\lambda$  phage DNA with *Hind*III: 50  $\mu$ g  $\lambda$  DNA, 25  $\mu$ L 10 $\times$  *Hind*III buffer, 10  $\mu$ L *Hind*III, ddH<sub>2</sub>O to 250  $\mu$ L total volume. Incubate at 37°C for 2–3 h.
2. Add 125  $\mu$ L each of phenol and chloroform, vortex briefly, and centrifuge at high-speed for 2 min. Extract the supernatant with 250  $\mu$ L chloroform, and precipitate with 625  $\mu$ L 95% ethanol.
3. Centrifuge at high speed for 10 min, decant the supernatant, and wash the DNA pellet with 1 mL ice-cold 70% ethanol. Remove as much of the ethanol as possible with a 10  $\mu$ L micropipette and resuspend the DNA in 400  $\mu$ L of TE, pH 8.0. Store in aliquots at –20°C.
4. To prepare labelled marker: 2.5  $\mu$ L 10 $\times$  nick translation buffer, 2.5  $\mu$ L  $\lambda$  *Hind*III DNA, 2.5  $\mu$ L (~25  $\mu$ Ci)  $\alpha$ -<sup>32</sup>P-dCTP, 1  $\mu$ L Klenow, ddH<sub>2</sub>O to 25  $\mu$ L total in a 1.5 mL microcentrifuge tube. Mix gently and incubate at RT for 30 min. *Use proper safety procedures for handling and disposal of radioactive materials.*
5. Remove unincorporated <sup>32</sup>P using Biogel column chromatography (Section 26.3.5). Determine probe activity by Cerenkov counting in a liquid scintillation counter. A single  $\lambda$  *Hind*III labelling reaction can be used in hybridizations for about 3 weeks.

### 26.3.7 Blot hybridization and regeneration

Blot hybridizations can be efficiently performed in glass bottles in a commercial hybridization oven (Chapter 20). These instruments facilitate hybridizations with a minimum of hybridization solution and provide uniform contact of the membrane with probe DNA. Also, prehybridization, hybridization with probe, and posthybridization membrane washes can all be performed while the membrane remains in a glass bottle. This reduces the amount of handling and minimizes exposure to radioactive materials by the researcher. *Use proper safety procedures for handling radioactive materials.*

1. Prior to the initial hybridization, newly prepared Southern blots should be incubated in 200–300 mL of preblock solution with gentle shaking at RT for 3–5 h. Once preblocked, the membrane can be wrapped in Saran Wrap and stored at –20°C or you can immediately proceed with hybridization. The preblock solution can be re-used for several blots. It should be stored at 4°C and prewarmed at 65°C to redissolve the SDS prior to re-use.
2. Prewarm the hybridization oven and the hybridization solution to 65°C. Various sized glass bottles are available for use with hybridization ovens. Select a bottle appropriate for the size of your Southern blot and add hybridization solution sufficient to just cover the membrane as the bottle rotates in the oven. The same solution is used for both prehybridization and hybridization. For 300  $\times$  35 mm bottles, we use 10–16 mL and for 150  $\times$  35 mm bottles we use 5–7.5 mL. Return the bottles to the oven for several minutes before adding membranes. Roll the membrane with the DNA-containing side toward the inside, slide it into the bottle, and allow it to unroll against the side of the bottle. Return the bottle to the oven for 3–5 h minimum, with constant rotation. We prefer an overnight prehybridization.
3. Incubate the tubes carrying probe DNA and  $\lambda$  *Hind*III probe at 100°C for 5 min and then immediately place them on ice. Centrifuge the tubes briefly before opening them to bring the probe solution to the bottom of the tube. For

hybridizations to Southern blots for individual mosquitoes, add  $^{32}\text{P}$ -labelled probe at  $\sim 750\,000$  cpm/mL of hybridization solution. For  $\lambda$  *Hind*III probe, add  $\sim 1000$  cpm/mL of hybridization solution.

4. Return bottles to the oven overnight, with constant rotation.
5. Remove bottles and carefully pour the hybridization solution into an appropriate waste container. The membranes can then be washed directly in the bottles. Again, discard the wash solutions appropriately. For each wash, fill the bottle  $\sim 1/2$  full with wash solution as follows: (i) Add wash solution I at RT and return bottle to oven for 15 min. (ii) Discard the first wash and add wash solution I prewarmed to  $65^\circ\text{C}$ . Return bottle to oven for 15 min. (iii) Discard the second wash and add wash solution II prewarmed to  $65^\circ\text{C}$ . Return bottle to oven for 15 min. (iv) Discard the third wash and add wash solution II prewarmed to  $65^\circ\text{C}$ . Return bottle to oven for 15 min.
6. Carefully remove the membrane from the bottle with forceps and place in a plastic tub with wash solution II at RT.
7. Trim the edges of a plastic sheet saver, leaving the folded edge intact. Place a membrane within the plastic sheet saver and press it flat to remove all bubbles and excess fluid. A rolling pin works well. Be sure that at least a 0.5 inch edge exists between the membrane and the periphery of the plastic sheet. It is important that the membrane does not dry because the probe DNA would become irreversibly bound. Wipe the exterior surface of the plastic sheet with Staticide using a Kimwipe.
8. Place the plastic sheet containing the membrane in a film cassette containing an intensifying screen. Expose the membrane to X-ray film at  $-80^\circ\text{C}$ . Time of exposure can be estimated with practice by the intensity of signal detected with a Geiger-Mueller counter. The plastic sheets can be reused indefinitely.
9. Membranes are regenerated for additional hybridizations by incubation with gentle shaking in 0.4 N NaOH at  $42^\circ\text{C}$  for 30 min followed by incubation in blot neutralizer solution at  $42^\circ\text{C}$  for 30 min. The membranes can be wrapped in Saran Wrap and stored at  $-20^\circ\text{C}$  between hybridizations.

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#### NOTES

1. It is important that the DEB extraction buffer be freshly prepared. The SDS seems particularly vulnerable to precipitating out if the buffer sits for any length of time. The restriction digest may fail with DNA extracted using old buffer. It is also important that the insect tissue be thoroughly ground in the buffer before proceeding with the extraction process.
2. Acid depurination of the DNA prior to Southern blotting is not recommended because the physical degradation of purine bases can reduce the number of rounds a blot can be rehybridized.
3. Cloned inserts larger than  $\sim 2$  kb are not efficiently amplified with standard PCR conditions. For larger clones, conventional plasmid miniprep, restriction enzyme digestion, and LMT agarose purification of inserts for radiolabelling is recommended.
4. Excellent positively charged nylon membranes are available from a variety of suppliers. However, there can be extreme variability in membrane performance

and some lots and portions of specific lots will fail completely. It is helpful to record the lot number for each Southern blot, because it can help with troubleshooting.

5. We prefer baking filters in preference to UV crosslinking because there is some evidence that baked filters will retain target DNA better and, subsequently, increase the number of rounds a blot can be rehybridized.

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