Southern hybridization of RT-PCR clone
(antibody light chain)

OBJECTIVE OF SOUTHERN BLOTTING: To confirm the RT-PCR clone (white colony growing on kanamycin) contains the antibody light chain gene.

13A CONCEPT OUTLINE

- We will probe plasmid DNA, so not much DNA is required; it will be digested with only one restriction enzyme (EcoRI). When you do a Southern of a higher organism’s genomic DNA, at least 10 µg is cut, often with several restriction enzymes in parallel (typically 6-cutters to generate large DNA fragments).
- On an agarose gel, digested eukaryotic genomic DNA will produce a smear because of the thousands of bands of various sizes which are generated. The Southern will be able to detect a single small fragment of DNA in the smear. In our experiment, the pattern will be infinitely simpler.

START: Miniprep plasmid DNA using Qiagen miniprep kit protocol

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Digest with EcoRI (12.5 uL DNA; 1.5uL buffer; 1uL EcoRI; 30-60 minutes)

13B BASIC EXPERIMENTAL OUTLINE

- For a Southern blot, tight, well-resolved bands are necessary for a clean result. Therefore gels are typically run at a low voltage for a long time.
- Before transfer to the membrane, DNA in the gel must be denatured using NaOH. ssDNA is required to allow probe hybridization.
- WEAR GLOVES to prevent oils from your hands getting on the membrane or gel

I. RUN GEL (With ladder, positive and negative controls)

Pour 1.6% agarose gel

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Aliquot ladders and control plasmid

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Add 1 µl loading dye to all samples (controls and digest)

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Load 10 µl from each tube

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Electrophorese at 60 volts for approximately 2 hours

II. DENATURE AND NEUTRALIZE DNA

Transfer gel to container with denaturing buffer

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Agitate for 15 minutes

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Pour off denaturing buffer and repeat
Pour off denaturing buffer and add distilled water to rinse briefly
Pour off water and add **neutralizing buffer**
Agitate for 15 minutes
Pour off neutralizing buffer and repeat

**III. SET UP BLOTTING APPARATUS AND TRANSFER DNA TO NYLON MEMBRANE** {see diagram at end of handout}

**DO NOT touch** membrane with bare hands! Obtain membrane, label it (ballpoint pen only) with name & mark a corner for orientation; wet it by briefly floating in distilled water
Put glass plate on rubber stoppers in bottom of blotting container; add 10X SSC buffer to fill
Place two filter papers together and soak in SSC, then place on tray top. Ends of this paper MUST be submerged in SSC buffer as this is the “wick” which pulls buffer up into the gel.
Remove gel from neutralizing buffer and place face down on the wet filter papers
Place membrane on top of gel, align carefully and rub out air bubbles
Put 4 strips Parafilm sheet wax around membrane edge to prevent short-circuit
Center two pieces of filter paper on top of membrane
Center a stack of paper towels on top of filter paper
Add weight (flask full of water) and leave overnight

**IV. WASH AND BAKE NYLON MEMBRANE**

Remove the top layer of towels, filter paper and Parafilm
Cut off bottom right corner of gel+membrane
Peel the membrane from the gel carefully
Transfer to 2x SSC buffer and agitate for 1 minute
Put membrane between two pieces of filter paper and tape around edges
Label with your name and date
Bake at 70 °C for 30 minutes
13C CONCEPT OUTLINE

- Pre-hybridization, or blocking, is a crucial step. It minimizes nonspecific “sticking” of the probe to unoccupied sites on the membrane.
- Blocking typically involves a generic cheap protein (such as casein in powdered milk, or bovine serum albumin) and fragmented salmon sperm DNA.
- Hybridizations (exposing the membrane to probe DNA) are usually carried out at 68 °C in an aqueous solution
- Use the smallest volume of buffer needed to cover the membranes (to reduce amount of probe and hybridization time). Heat-sealed plastic bags are often used to accomplish this.
- Probes can be restriction fragments, plasmids or oligonucleotides. Although some people use radioactive ³²P, most people now use nonradioactive (colorimetric or chemiluminescent) probes.
- Our probe is part of an antibody light chain gene, labeled for colorimetric detection.

13C BASIC EXPERIMENTAL OUTLINE

I. PREHYBRIDIZE MEMBRANE

Carefully remove membrane from filter paper and transfer to container with prehybridization buffer
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Cover and agitate at 50 °C for an hour

II. HYBRIDIZE MEMBRANE WITH PROBE

Transfer membrane to a container with hybridization buffer
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Cover (plastic wrap) and agitate at 50 °C overnight

13D CONCEPT OUTLINE

- Wash the membranes under very stringent conditions (high temp and low salt) to remove unbound probe.
- Some blocking reagent may be stripped from the nylon membrane during the wash, so reblock the membrane before adding antibodies.
- Probe is labeled with digoxigenin. It is detected using anti-digoxigenin antibodies which are covalently linked to the enzyme alkaline phosphatase (AP). AP acts on a detection reagent substrate to produce purple/indigo precipitates. The amount of colored precipitate is proportional to the mass of labeled DNA present.

13D BASIC EXPERIMENTAL OUTLINE
Numbered buffers refer to Boehringer-Mannheim Genius Nucleic Acid Detection Kit

I. WASH UNBOUND PROBE OFF MEMBRANE

Remove membrane from hybridization solution and transfer to container with wash buffer - incubate for 5 minutes at room temp (2X SSC / 0.1% SDS)
Pour off first wash and repeat with agitation
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Pour off second wash and repeat at 50 °C
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Pour off third wash and add buffer 1 (0.1 M Tris, 0.15 M NaCl)
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Agitate for 5 minutes at room temp
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Pour off buffer 1 and add buffer 2 (buffer 1 plus blocking reagent)
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Agitate for 15 minutes at room temp

II. ADD ANTIBODY TO PROBE

Pour off buffer 2 and add antibody/enzyme solution (20 mL)
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Agitate for 15 minutes at room temp

III. WASH OFF UNBOUND ANTIBODY

Pour off antibody solution and add buffer 1
↓
Agitate for 5 minutes at room temp
↓
Pour off buffer 1 and repeat
↓
Pour off buffer 1, add 10 mL buffer 3 and agitate for 5 minutes at room temp
(Buffer 3: 0.1 M Tris 9.5; 0.1 M NaCl; 0.05 M MgCl₂)

IV. DEVELOP BOUND ANTIBODY FOR VISUALIZATION

Transfer membrane to container (DNA side UP) and add color solution
(45 µL NBT + 32 µL x-phos (BCIP))
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Incubate in the dark from 10 minutes to 1 hour
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When you can see a signal, pour off solution and add TE buffer
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Agitate for 30 minutes at room temp
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Dry the membrane on filter paper and store in the dark.
Appearance of a typical Southern blot:
Digested genomic DNA smears in agarose gel stained with ethidium bromide; and final result showing specific bands detected on membrane.