Southern Blotting

(Technical Review)

Lim Wei Chun
• **Professor Sir Edwin Mellor Southern (1975):** Detection of specific sequences among DNA fragments in a large, complex sample of DNA separated by gel electrophoresis.
Function of Southern Blot

• determine the molecular weight of a restriction fragment and to measure relative amounts in different samples
• detect the presence of a particular bit of DNA in a sample
• analyze the genetic patterns which appear in a person's DNA.
• analyze restriction digestion fragmentation of DNA or a biological sample
• definitive test to ensure that a particular section of DNA of known genetic sequence has been successfully incorporated into the genome of the host organism
• detecting large gene rearrangements/deletions and large trinucleotide repeat expansions
Overview of Southern Blotting

Target DNA
- Digest with restriction endonuclease
- Apply to individual wells on an agarose gel

Probe DNA
- Add label e.g. radioactive label (*)
- Denature by heat

Migration
- High mol. wt
- Low mol. wt

Denature in alkali
- Apply a nitrocellulose or nylon membrane

Transfer DNA to membrane

Hybridize to immobilized target DNA

Wash off excess probe DNA
- Apply X-ray film

Develop film
# Type of Membrane

<table>
<thead>
<tr>
<th>Membrane Type</th>
<th>Nitrocellulose</th>
<th>Nitrocellulose with Reinforcing Fleece</th>
<th>Nylon Membrane</th>
<th>Highly Positive charged Nylon Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FEATURE</strong></td>
<td>Superior for chemiluminescence detection using horseradish peroxidase.</td>
<td>Suitable for repeated probing.</td>
<td>For nucleic acid applications. Easier to block than highly positively charged membrane.</td>
<td>For nucleic acid applications. Highest binding capacity available.</td>
</tr>
<tr>
<td><strong>MEMBRANE TYPE</strong></td>
<td>Nitrocellulose, 100% pure</td>
<td>Nitrocellulose, reinforced</td>
<td>Nylon, moderately positively charged</td>
<td>Nylon, highly positively charged</td>
</tr>
<tr>
<td><strong>APPLICATIONS</strong></td>
<td>Western, Southern, Northern blotting</td>
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<td>Southern, Northern blotting</td>
<td>Southern, Northern blotting</td>
</tr>
<tr>
<td><strong>BINDING</strong></td>
<td>75-110 µg/cm²</td>
<td>75-90 µg/cm²</td>
<td>&gt;400 µg/cm²</td>
<td>&gt;600 µg/cm²</td>
</tr>
<tr>
<td><strong>PORE SIZES</strong></td>
<td>0.45 µm</td>
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<tr>
<td></td>
<td>0.2 µm</td>
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<td></td>
<td>0.1 µm</td>
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<tr>
<td><strong>TRANSFER METHODS</strong></td>
<td>Semi-dry Blotting ++</td>
<td>++</td>
<td>++</td>
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<tr>
<td></td>
<td>Tank Blotting ++</td>
<td>++</td>
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<td>++</td>
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<tr>
<td></td>
<td>Vacuum Blotting ++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Capillary Blotting ++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>IMMOBILIZATION</strong></td>
<td>UV-crosslinking, DNA, RNA ++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Baking (80 °C), DNA, RNA ++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Drying, DNA, RNA -</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Drying, Protein ++</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>DETECTION METHODS</strong></td>
<td>Colorimetric ++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Chemiluminescent ++</td>
<td>++</td>
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<td>++</td>
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<tr>
<td></td>
<td>Isotopic ++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>Fluorescent ++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>REPROBING</strong></td>
<td>limited</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

++ Recommended
+ Satisfactory
<table>
<thead>
<tr>
<th>Comparison of membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nitrocellulose</strong> – high ionic strength – usually 10x SSC</td>
</tr>
<tr>
<td><strong>Nylon membrane</strong> - able to bind DNA under a variety of conditions (acid, neutral, alkaline, high or low ionic strength), but a high-salt buffer such as 20× or 10×SSC appears to be beneficial</td>
</tr>
<tr>
<td><strong>Positively charged nylon membrane</strong> – alkaline buffer enable covalent bonding with membrane but will provide high background if use chemiluminescent detection system. Not work as well with uncharged nylon membrane</td>
</tr>
</tbody>
</table>
Handling of Membrane

- Wear non-powdered gloved to avoid contamination
- Cut membranes only with clean blunt-ended forceps
Types of Southern Blotting

- Capillary blotting
  - Upward
  - Downward

- Vacuum blotting

- Semi-Dry blotting / Electroblotting
Capillary Blotting

Upward capillary transfer

Backward capillary transfer
Vacuum Blotting

(Example from Amersham)
Semi-dry blotting

An exploded view of the Trans-Blot SD cell: 1, safety lid; 2, cathode assembly with latches; 3, filter paper; 4, gel; 5, membrane; 6, filter paper; 7, spring-loaded anode platform, mounted on four guide posts; 8, power cables; 9, base.

(Example from biorad)
<table>
<thead>
<tr>
<th>Method</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary Transfer</td>
<td>- High sensitivity</td>
<td>- Consumes a relatively large amount of time (up to 12 hrs), buffer and blotting paper</td>
</tr>
<tr>
<td></td>
<td>- Economical</td>
<td>- Cannot transfer polyacrylamide gel</td>
</tr>
<tr>
<td>Vacuum Blotting</td>
<td>- Less time and fewer solutions are required as compared to the capillary method for blotting.</td>
<td>- Cannot transfer polyacrylamide gel</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Extensive prudence has to be taken to avoid vacuum leak</td>
</tr>
<tr>
<td>Semidry Blotting / Electroblotting</td>
<td>- Fastest to complete</td>
<td>- Requires special care to prevent crushing or melting of the agarose gel</td>
</tr>
<tr>
<td></td>
<td>- Efficient method for polyacrylamide gel transfer</td>
<td>- Less sensitive</td>
</tr>
</tbody>
</table>
Chemiluminescence Southern Blot

**Specimen Preparation**

- Salt Extraction

**Restriction Endonuclease Digestion of genomic DNA**

**Agarose Gel Electrophoresis**

- Gel Denaturation, Neutralization, and Transfer

**DIG Labeling of the Probe**

**Assessment Gel**

- Direct dot Blot Assay

**Probing Membranes with DIG-Labeled Probe**

**Posthybridization Washes**

**Band Visualization**

- Optimize the probe-generation protocol
- Hybridize using DIG Easy Hyb
- Serial dilution of matrix were spotted on membrane and incubate in Color Substrate Solution in dark. Assay development to optimize probe sensitivity
- Membrane treated with anti-DIG-Fab conjugated with alkaline phosphatase. Hydrolysis of CDP-Star. Results are recorded by exposing the membrane to XAR Film.

5µg DNA restrict with restriction enzyme for overnight at 37°C

Gel treated with HCl, blotted to Nylon membrane and baked at 80°C
Agarose Gel Electrophoresis

Agarose gel electrophoresis shows the separation of the DNA sample after incubated with restriction enzymes. Because of the high molecular weight, the gel look smear.
**Finishing Electrophoresis**

**Depurination**
– done by soaking the gel in acid to cleavage the DNA (will improve the transfer of large pieces of DNA)

**Denaturation**
- Done by soaking the gel in base solution to denature the DNA rendering it single stranded and in a form suitable to hybridize

**Neutralization**
- Done by soaking in high salt solution or tris pH 7. Its function to raise the pH to enable the DNA to bind to the membrane.
Duration of transfer

- Depends on methods, DNA length and transfer buffer.

- With a high-salt buffer, it takes about 18 hr to obtain acceptable transfer of a 15-kb molecule from a 5-mm thick 0.7% agarose gel; with the same gel 90% of the 1-kb molecules will be transferred in 2 hr.

- After transfer is complete the DNA is permanently immobilized on the membrane support by drying at ~80°C or exposing to UV irradiation.
Radioactive probe

The Random Hexamer Labeling Process produces a radioactive single-stranded DNA copy of both strands of the template for use as a probe.

Nonradioactive probe – DIG probe

This probe is easier to achieve because it just need double PCR to synthesis it with the usage of DIG-11-dUTP
How probe works

1. Mixture of molecules
2. Separate and immobilize target
3. Add probe
4. Remove unbound probe
5. Detect probe
Assessment gel

Direct dot blot assay

<table>
<thead>
<tr>
<th>DIG DNA</th>
<th>7µL</th>
<th>6µL</th>
<th>5µL</th>
<th>4µL</th>
<th>3µL</th>
<th>2µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>3:7</td>
<td></td>
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<td>1:10</td>
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<td>1:8</td>
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<td>1:6</td>
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<td>1:4</td>
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<td>1:2</td>
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Hybridization

– The labeled probe is added to the blocked membrane in buffer and incubated for several hours to allow the probe molecules to find their targets.
Washing

- Excess probe will have bound nonspecifically to the membrane despite the blocking reagents.
- Blot is incubated with wash buffers containing NaCl and detergent to wash away excess probe and reduce background.
Blocking

- Buffer binds to areas on the blot not occupied by patient DNA.
- Blocks the empty sites from being bound during hybridization.
Detection

- labeled probes enable detection on film.

Chemiluminescent detection – three step process

**First step**
membranes are treated with Blocking reagent to prevent nonspecific attraction of antibody to the membrane.

**Second step**
membranes are incubated with a dilution of anti-digoxigenin Fab fragments, which are conjugated to alkaline phosphatase.

**Third step**
The membrane carrying the hybridized probe and bound antibody conjugate is reacted with CDP Star and exposed to X-ray film to record the chemiluminescent signal.

detection of digoxigenin labeled compounds
Precaution on handling the exposure time

• Make sure the dark room is free from any light source.

• Make sure the working place is always dry in condition.
Analysis
**Striping membrane**

<table>
<thead>
<tr>
<th>For This Type Blot</th>
<th>To Remove</th>
<th>Use This Procedure¹</th>
</tr>
</thead>
</table>
| Southern           | Chemiluminescent product and probe             | • Rinse in H₂O, 1 min.  
|                    |                                                 | • Wash²,³ with 0.2 M NaOH/0.1% SDS, 2 x 15 min, 37°C.  
|                    |                                                 | • Rinse in 2x SSC, 5 min. Store in 2x SSC.               |
|                    | Colored product (from NBT/BCIP reaction) and probe | • Incubate in dimethylformamide at 50°–60°C for 1 h or more, until color has been removed. (Solution may need to be changed several times.)  
|                    |                                                 | • Rinse in H₂O, 1 min.  
|                    |                                                 | • Wash²,³ with 0.2 M NaOH/0.1% SDS, 2 x 20 min, 37°C.  
|                    |                                                 | • Rinse in 2x SSC, 5 min. Store in 2x SSC.               |

**Important precaution**: Membranes should never be allowed to dry before stripping. Once dried, the membrane cannot be stripped and reprobed.
Troubleshooting

**Poor Signal**
- Probe specific activity too low
- Not enough target DNA
- Probe concentration too low
- Hybridization time too short

**Spotty background**

**Patchy or generally high background**
- Insufficient blocking agents
- Part of the membrane allowed to dry out during hybridization or washing
- Not enough wash solution
- Probe concentration too high
- Probe not denatured

**Extra bands**

**Nonspecific background in one or more tracks**

**Cannot remove probe after hybridization**
- Membrane dried out after hybridization
"Before PCR and cheap fast sequencing changed our view of the universe that is genetics, the Southern Blot was a universal workhorse. It is still a useful tool today and you need to know about it so that you can interpret historical data."
Thank You...