Southern Blotting

(Technical Review)

Lim Wei Chun

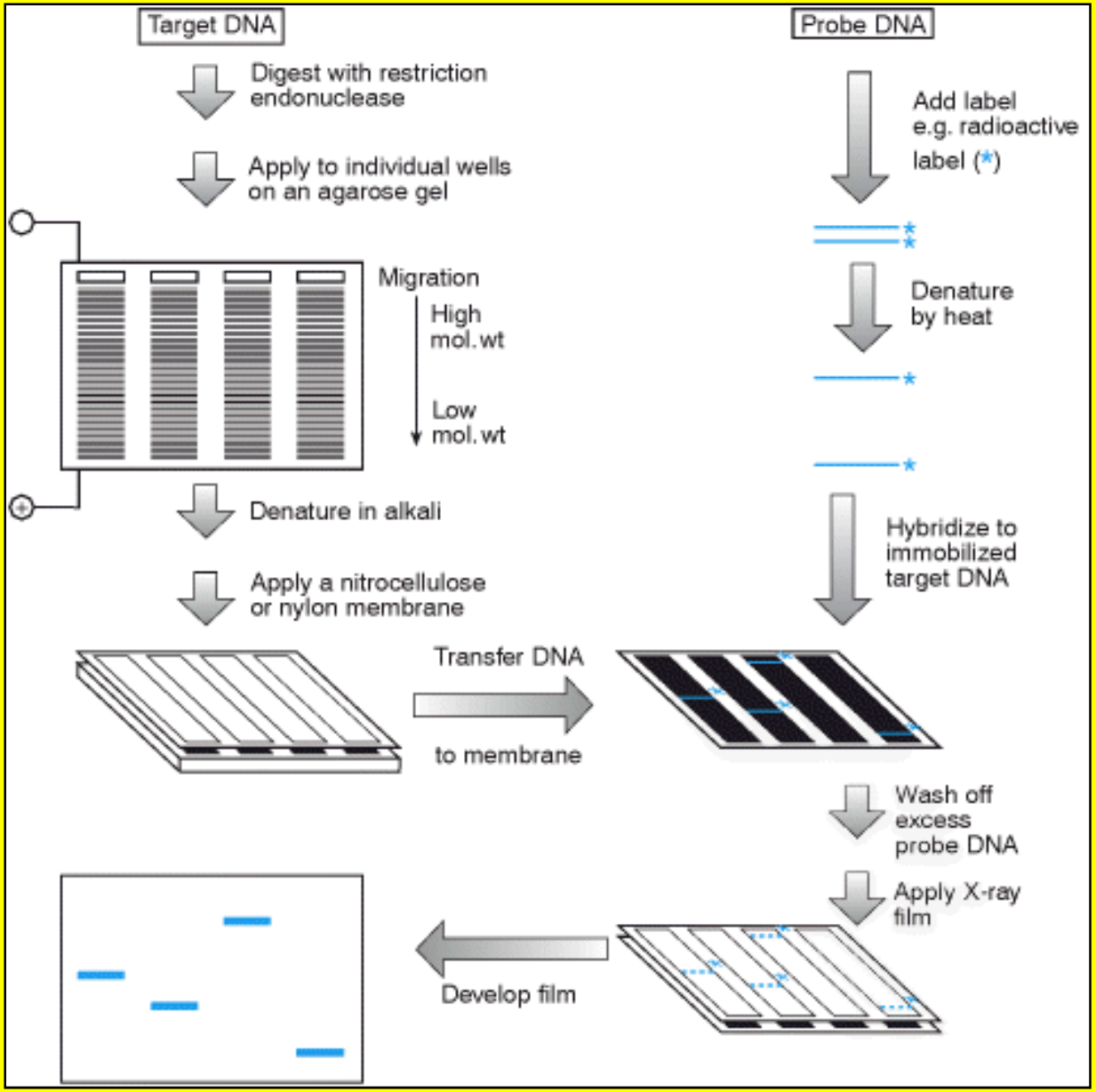
Southern Blot

- **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



Type of Membrane

	Nitrocellulose	Nitrocellulose with Reinforcing Fleece	Nylon Membrane	Highly Positive charged Nylon Membrane
FEATURE:	Superior for chemiluminescence detection using horseradish peroxidase.	Suitable for repeated probing.	For nucleic acid applications. Easier to block than highly positively charge membrane	For nucleic acid applications. Highest binding capacity available.
MEMBRANE TYPE:	Nitrocellulose, 100% pure	Nitrocellulose, reinforced	Nylon, moderately positively charged	Nylon, highly positively charged
APPLICATIONS:	Western, Southern, Northern blotting	Western, Southern, Northern blotting	Southern, Northern blotting	Southern, Northern blotting
BINDING:	75-110 µg/cm^2	75-90 µg/cm^2	>400 µg/cm^2	>600 µg/cm^2
PORE SIZES:	0.45 µm 0.2 µm 0.1 µm	0.45 µm 0.2 µm -	0.45 µm 0.2 µm -	0.45 µm - -
TRANSFER METHODS				
Semi-dry Blotting	++	++	++	++
Tank Blotting	++	++	++	++
Vacuum Blotting	++	++	++	++
Capillary Blotting	++	++	++	++
Alkaline Method	not recommended	not recommended	++	++
IMMOBILIZATION				
UV-crosslinking, DNA, RNA	++	++	++	++
Baking (80 ℃), DNA, RNA	++	++	+	+
Drying, DNA, RNA	-	-	+	+
Drying, Protein	++	++	-	-
DETECTION METHODS				
Colorimetric	++	++	+	+
Chemiluminescent	++	++	++	++
Isotopic	++	++	++	++
Fluorescent	++	-	-	-
REPROBING	limited	++	++	++

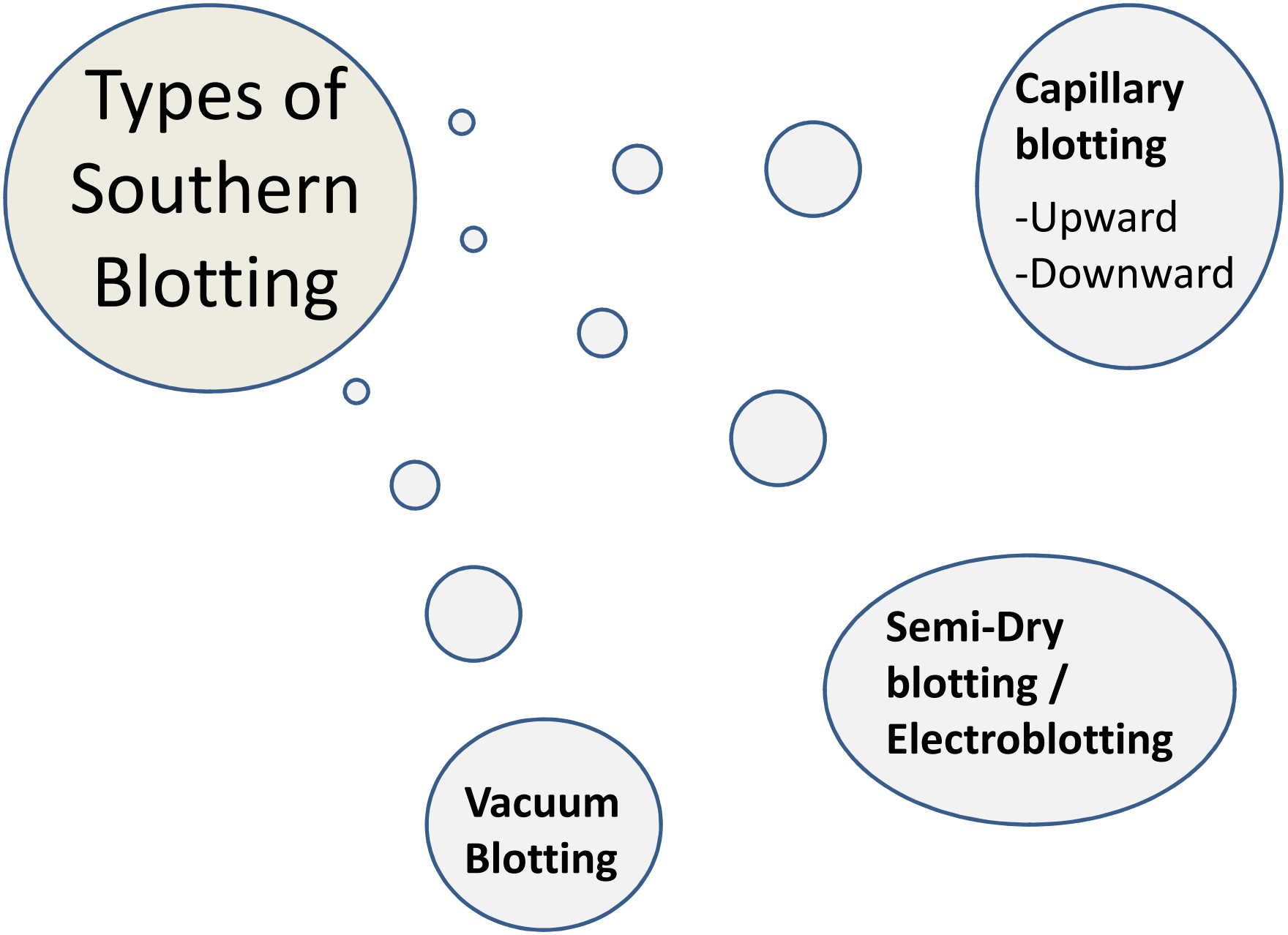
++ Recommended
+ Satisfactory

Comparison of membrane

- Nitrocellulose – high ionic strength – usually 10x SSC
- Nylon membrane - 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
- Positively charged nylon membrane – 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

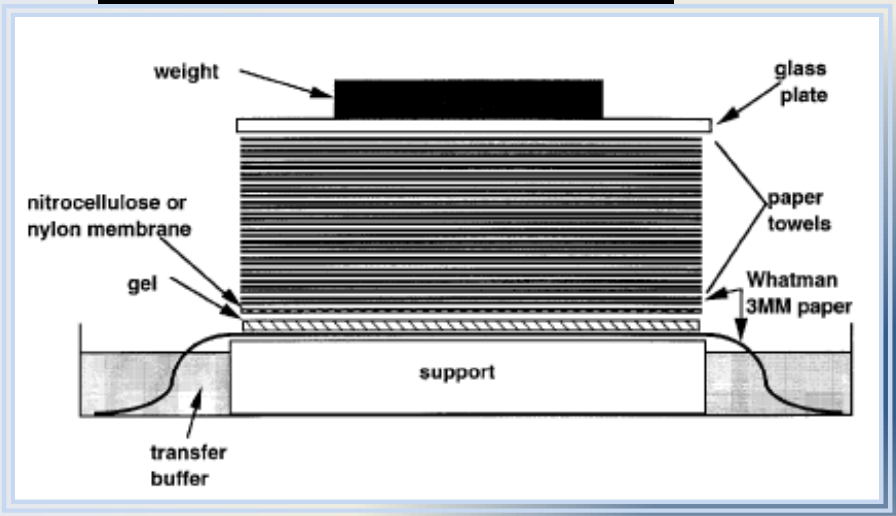
Handling of Membrane

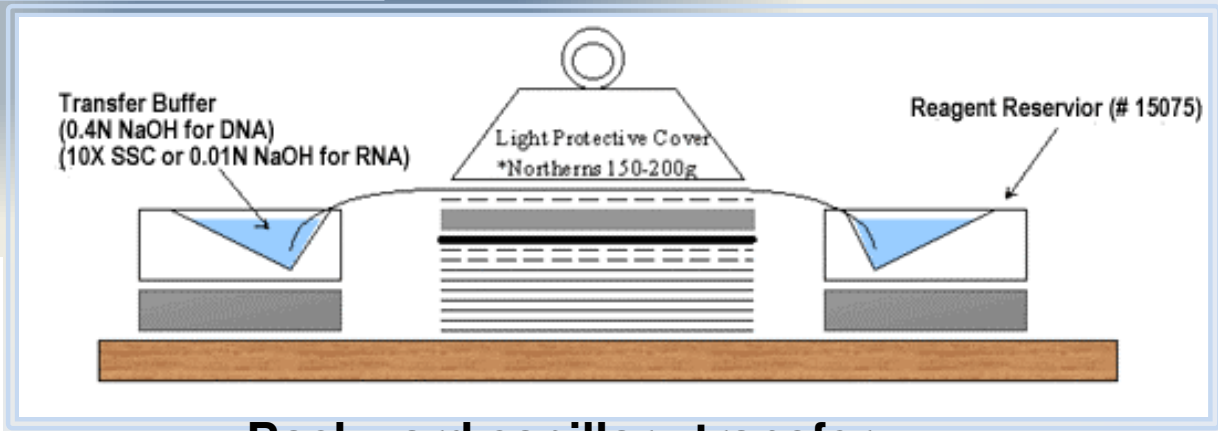
- Wear non-powdered gloved to avoid contamination
- Cut membranes only with clean blunt-ended forceps


Types of Southern Blotting
Capillary blotting
-Upward
-Downward
Semi-Dry blotting / Electroblotting
Vacuum Blotting

Capillary Blotting

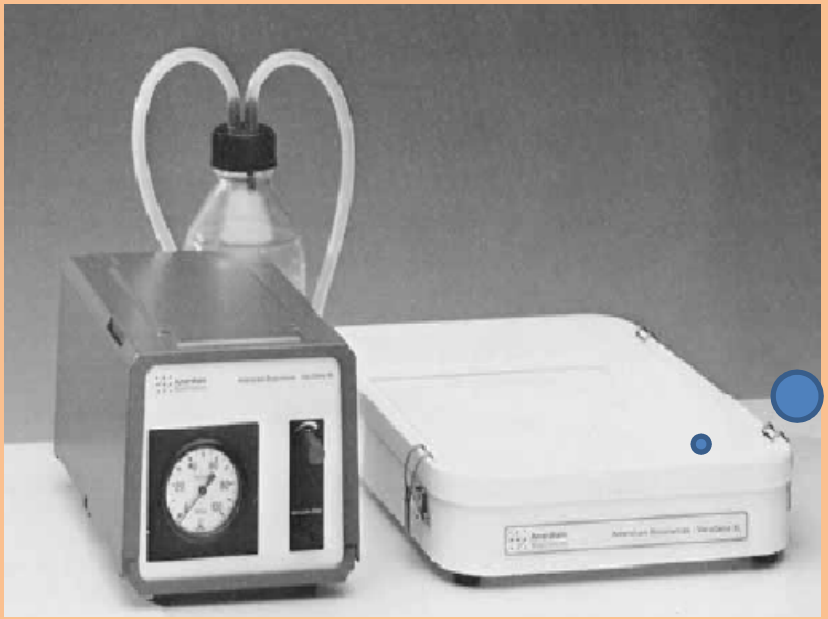
Upward capillary transfer

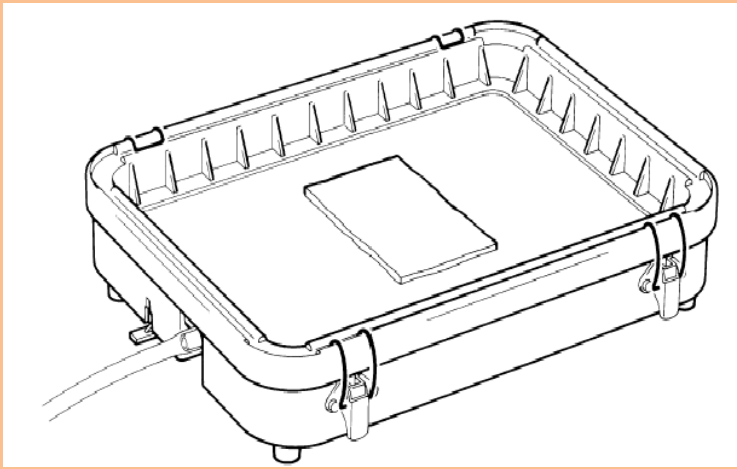




Backward capillary transfer

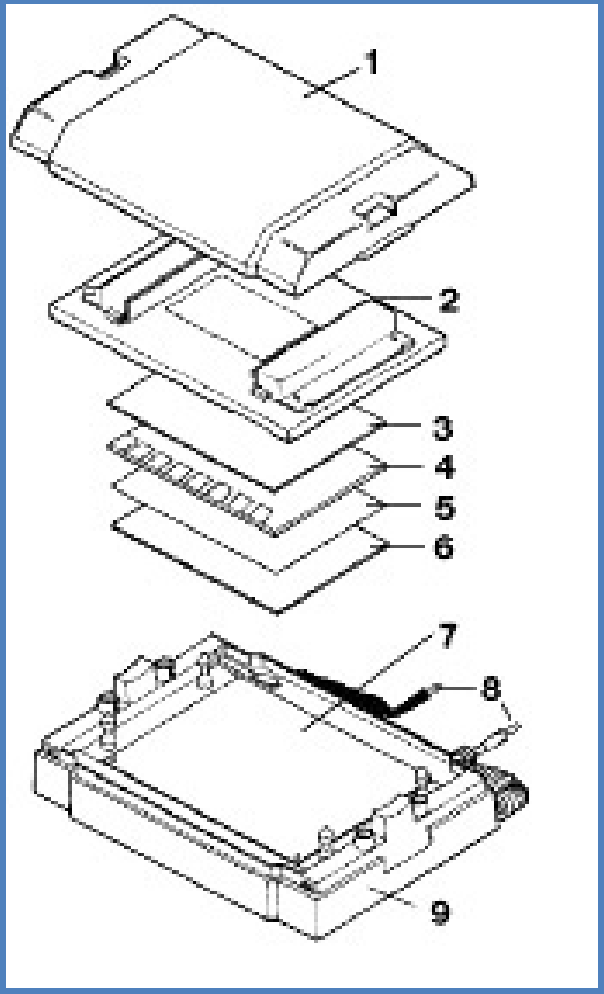
Vacuum Blotting





(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)

Comparison of Southern blot transfer method

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

Chemiluminescence Southern Blot

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UKM MEDICAL MOLECULAR BIOLOGY INSTITUTE

Specimen Preparation

Salt Extraction

Restriction Endonuclease Digestion of genomic DNA

5µg DNA restrict with restiction enzyme for overnight at 37^{0}C

Agarose Gel Electrophoresis

Gel Denaturation, Neutralization, and Transfer

Gel treated with HCl, blotted to Nylon membrane and baked at 80^{0}C

Probe synthesis by normal PCR and labeling of probe with DIG using PCR

DIG Labeling of the Probe

Assessment Gel

Direct dot Blot Assay

Optimize the probe-generation protocol

Serial dilution of matrix were spotted on membrane and incubate in Color Substrate Solution in dark. Assay development to optimize probe sensitivity

Probing Membranes with DIG-Labeled Probe

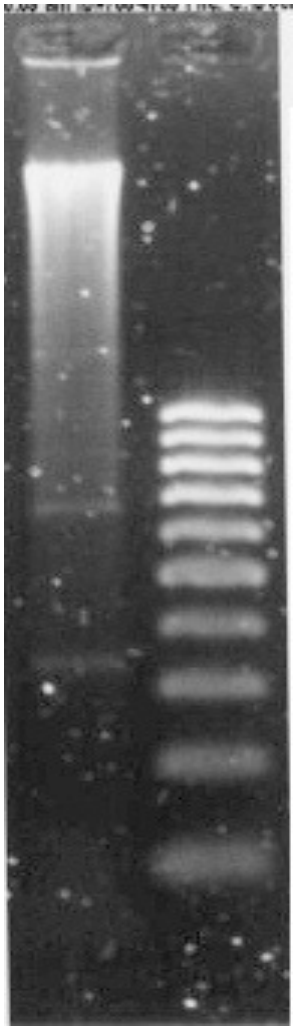
Hybridize using DIG Easy Hyb

Posthybridization Washes

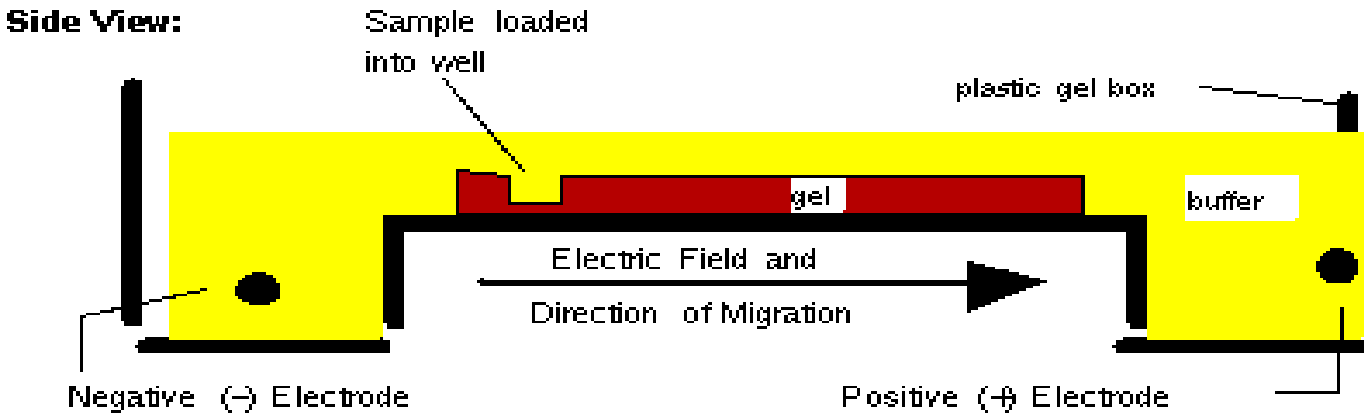
Band Visualization

Membrane treated with anti-DIG-Fab conjugated with alkaline phosphatase. Hydrolysis of CDP-Star. Results are recorded by exposing the membrane to XAR Film.

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)

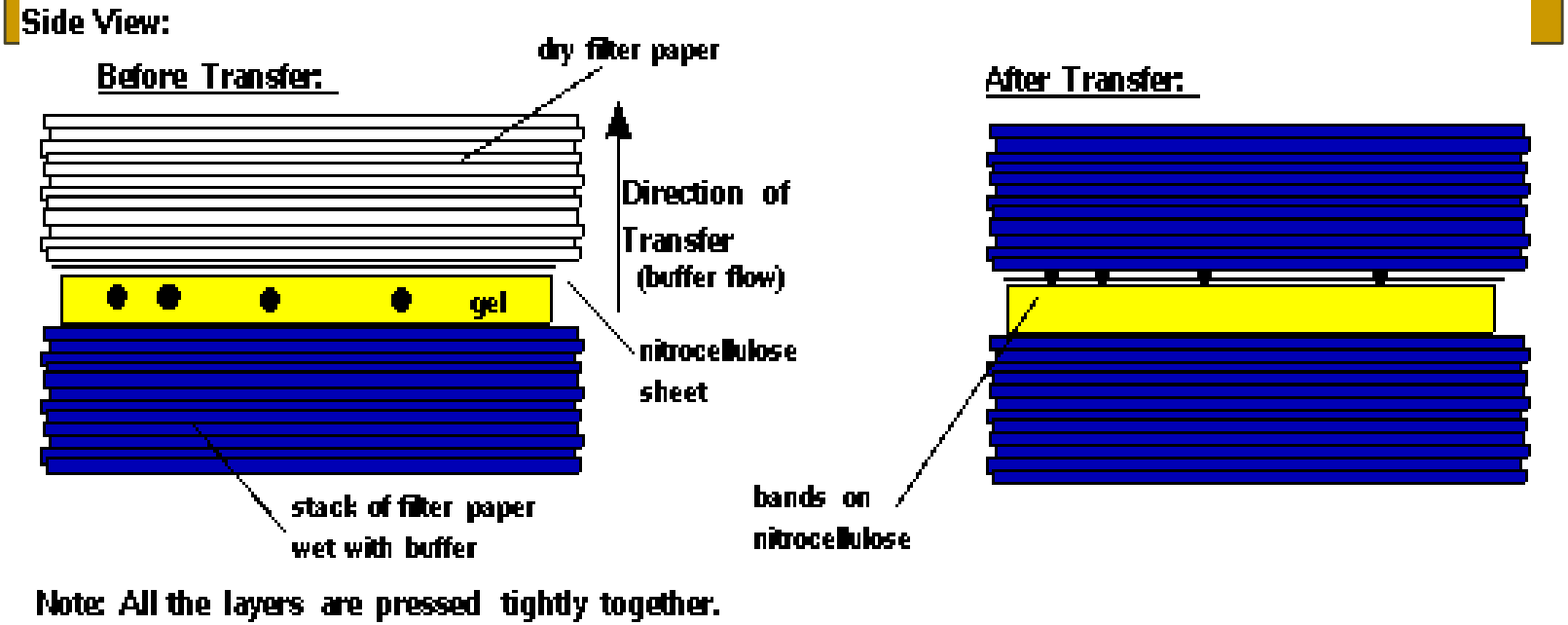
Denaturation
- Done by soaking the gel in base solution to denature the DNA rendering it single stranded and in a form suitable to hybridize

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

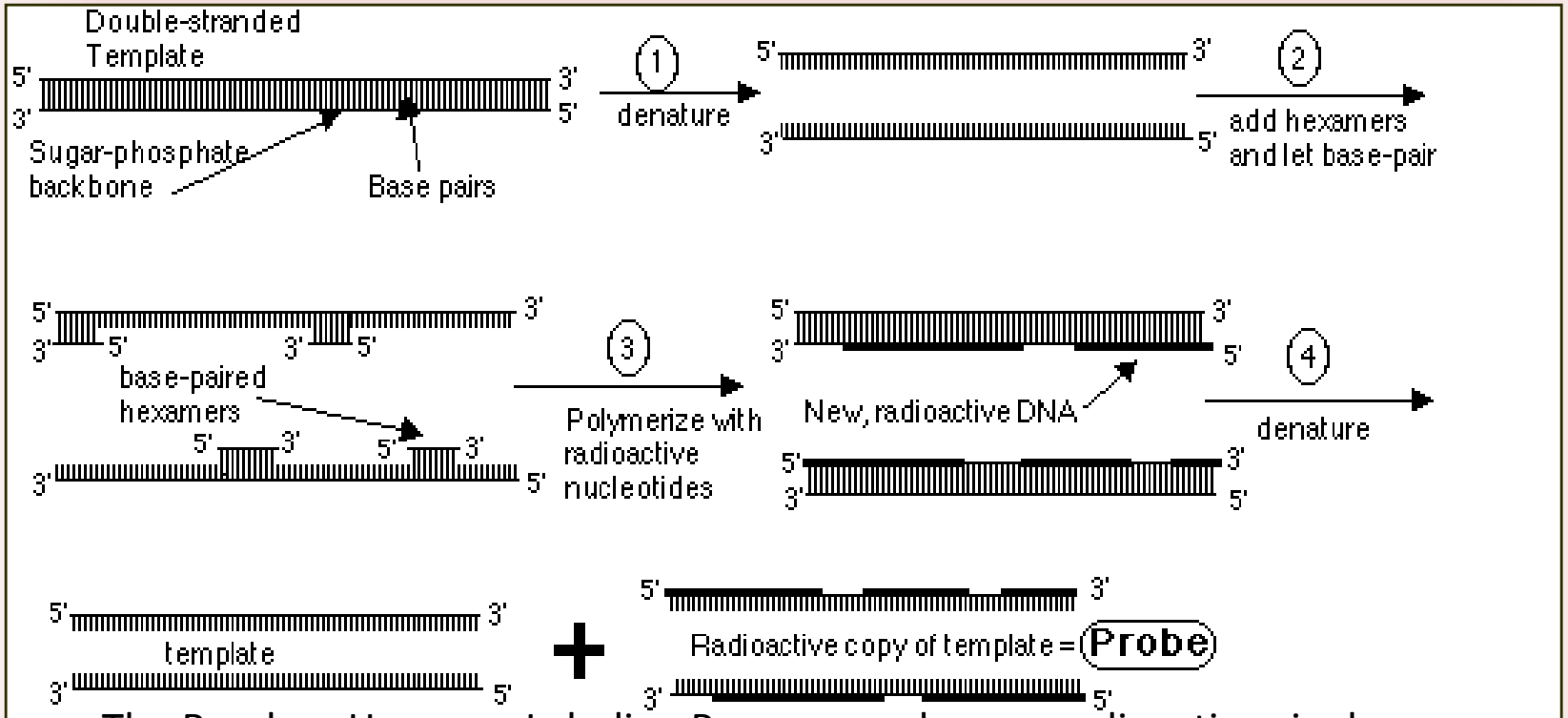
-Depends on methods, DNA length and transfer buffer.

-With a high-salt buffer, it takes about 18 hr to obtain acceptable transfer of a 15-kb molecule from a 5-mmthick 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℃ or exposing to UV irradiation

Radioactive probe

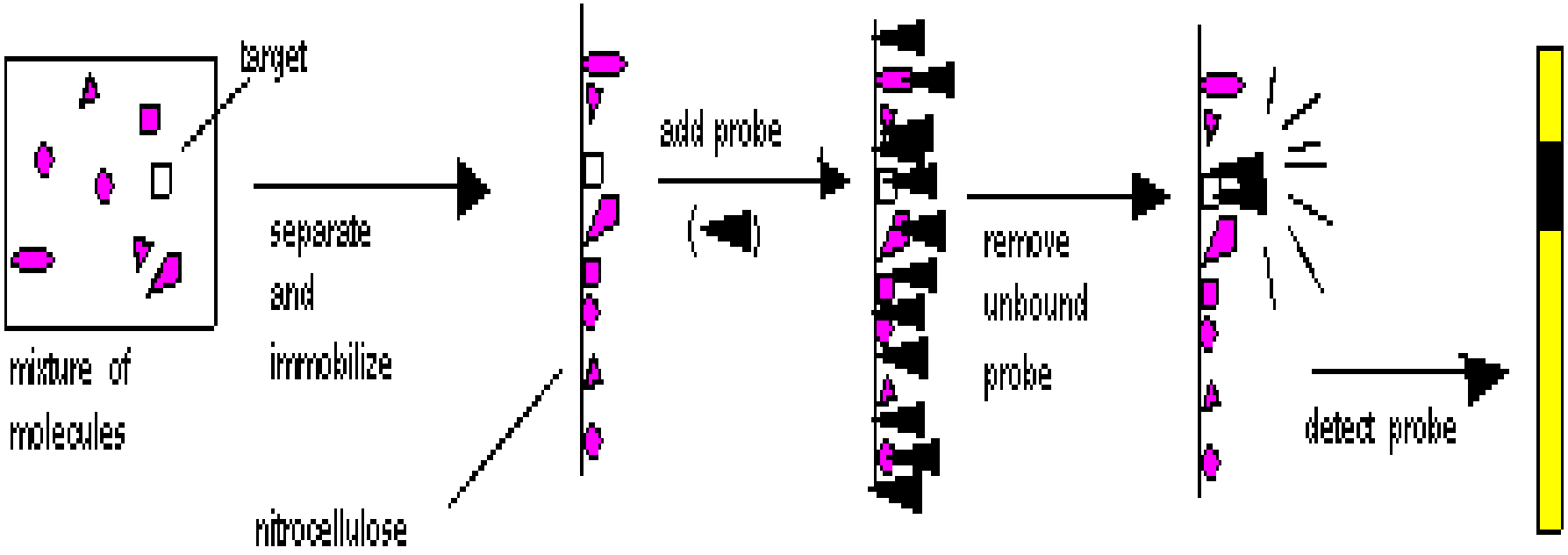


The Random Hexamer Labeling Process produces a radioactive single-stranded DNA copy of both strands of the template for use as a probe.

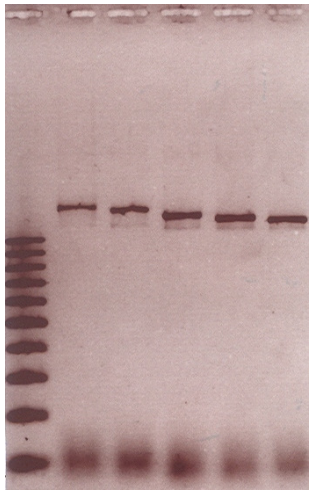
Nonradioactive probe – DIG probe

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

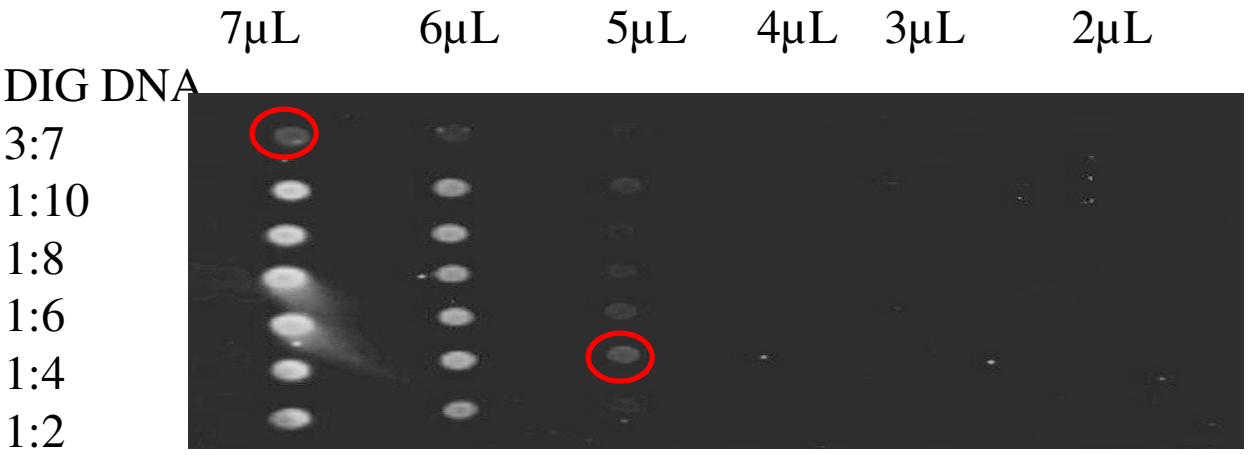
How probe works



Assessment gel



Direct dot blot assay



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.

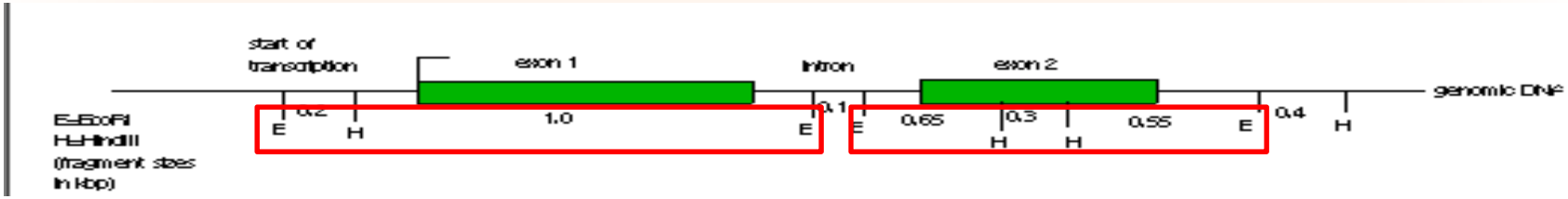
detection of digoxigenin labeled compounds

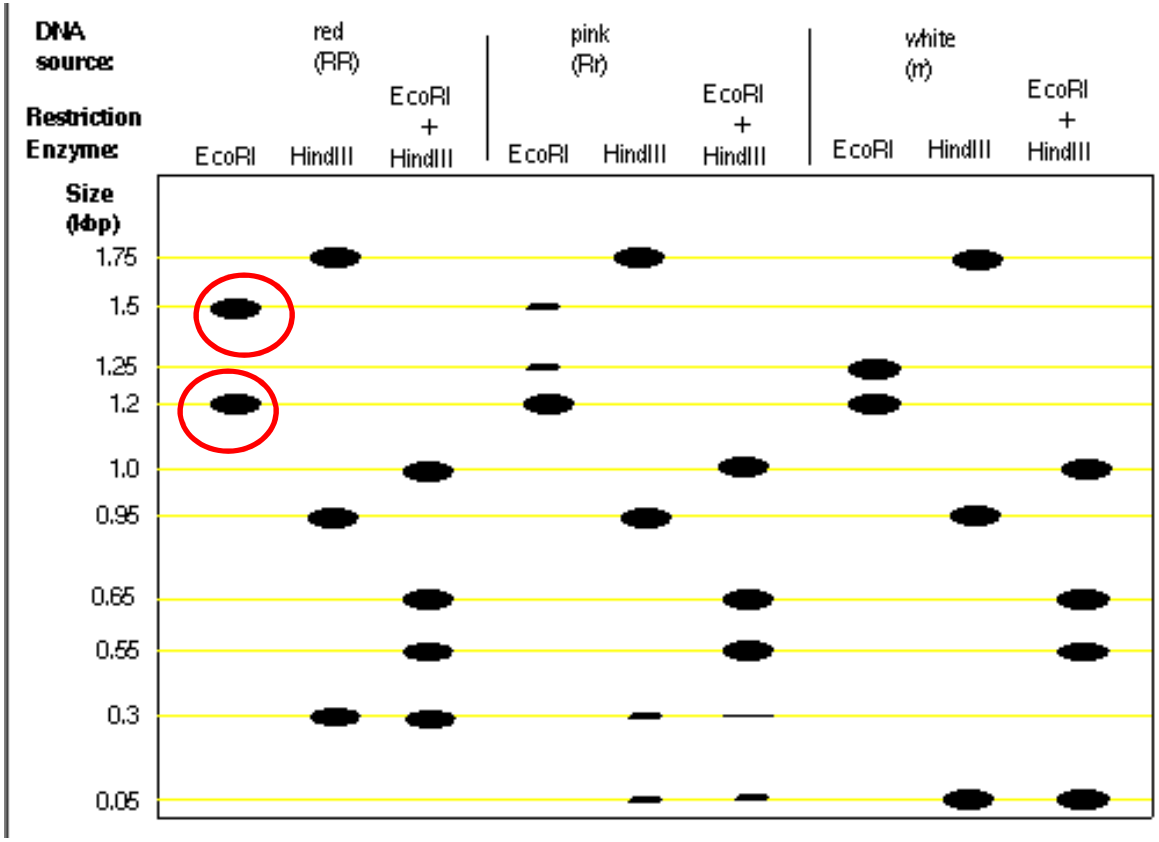
Third step
The membrane carrying the hybridized probe and bound antibody conjugate is reacted with CDP Star and exposed to X-ray film to record the chemiluminescent signal.

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


start of transcription
exon 1
intron
exon 2
genomic DNA
E=EcoRI
H=HindIII
(fragment sizes in kbp)
E
H
1.0
0.65
0.55
0.2
0.1
0.3
0.4


DNA source:
red (RR)
pink (Rr)
white (r)
Restriction Enzyme:
EcoRI
HindIII
EcoRI + HindIII
Size (kbp)
1.75
1.5
1.25
1.2
1.0
0.95
0.65
0.55
0.3
0.05

Striping membrane

For This Type Blot	To Remove	Use This Procedure[1]
Southern	Chemiluminescent product and probe	• Rinse in H_2O, 1 min. • Wash[2,3] 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 H_2O, 1 min. • Wash[2,3] 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

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...