

Experiment 12

Southern Blot Analysis of Plasmids pRIT4501 and pRIT4502

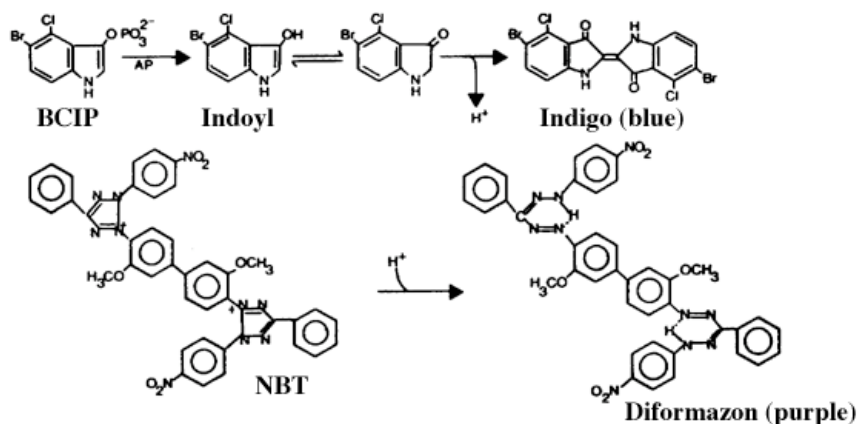
It is often desirable to screen nucleic acids to determine whether or not they carry specific sequences. Typically, this is done by making the nucleic acid in question single-stranded, fixing it to a solid support, and challenging it with a small, labeled, single-stranded DNA (probe) under conditions that promote hybridization. If the probe sequence is found within the target DNA, the probe will bind to its complementary sequence on the support. All that is left is to detect probe binding. Probe is usually a cloned gene or other DNA fragment that is labeled with either ^{32}P or biotin. Upon autoradiography or development with a chromogenic substrate, it is possible to visualize bound probe.

Both DNA and RNA can be bound to a membrane and there are a variety of ways in which this can be done. For example, whole bacterial colonies can be screened by transferring them to a solid support, lysing them *in situ*, denaturing the released DNA, and binding it irreversibly to the support. DNA can also be separated by size on a gel and transferred to a support by capillary action (blotting) or by electrophoretic transfer. The blotting method was invented by E. M. Southern in 1975 and has come to be known as Southern Blotting. Subsequently, Southern's technique was applied to RNA and came to be known as "Northern Blotting." The technique has also been adapted to proteins. Proteins are separated by SDS-PAGE electrophoresis, blotted to a solid support, and challenged not by nucleic acid probes, but by labeled antibodies. The jargon has also been adapted to proteins, and this technique is known as "Western Blotting."

After transfer of the DNA's, the membrane is treated with salmon sperm DNA. Salmon sperm DNA is heterologous, i.e. it has no relationship to any of the DNA sequences that you are using, but it serves to block non-specific hybridization of the probe. Typically, there is always some low level of non-specific hybridization, and the intention is that any such non-specific hybridization be carried out by the unlabeled salmon sperm DNA and not by the labeled probe. Finally, the probe is added and only specific hybridization sites are available. The conditions under which probe is permitted to hybridize can be varied to demand greater or lesser degrees of complementarity. This is known as *stringency*.

Stringency can be defined as the degree of complementarity that you demand for a specific hybridization. As stringency decreases, the probe can bind to an increasing number of sequences that are similar but not identical. The lower the stringency, the less the complementary must the sequences be. Sometimes, low stringency is desirable. For example, genes in eukaryotes form families and a low stringency hybridization may permit probe to bind to related, but not identical genes in the same family. Another example would be a case in which you have cloned a gene from one species and want to find a similar gene in another species. Depending on evolutionary distance, the sequences in the two species may have diverged quite a bit. Stringency during hybridization can be controlled by varying the temperature, or by adjusting the salt concentration and/or the amounts of urea or formamide in the hybridization solution. Urea and formamide are denaturing agents that lower the melting temperature of the hybrid. In a typical Southern blot, hybridization is performed at low stringency and then the membrane is put through a series of washes in which the stringency is progressively increased by progressively decreasing salt concentration. The strategy is to promote the maximum hybridization and then wash away any probe that is only loosely bound.

The final stage in a Southern Blot is to visualize the probe. If the probe is radioactively labeled, then one merely performs an autoradiography. GE lab is not equipped to use radioisotope probes, so you will use a biotinylated probe. Biotin is incorporated into the probe via a nick translation reaction in which normal dATP is replaced by biotin-7-dATP. In biotin-7-dATP, the biotin is attached to the dATP by a 7-carbon linker. Detection of the probe is based on the strong affinity between biotin and avidin. Avidin is conjugated to an enzyme such as alkaline phosphatase, which can subsequently be used with a chromogenic substrate to produce a color change. Generally, avidin, which comes from egg white, is replaced with streptavidin, which comes from *Streptococcus*. Both compounds have similar affinities for biotin but in practice, streptavidin produces a lower background. In a standard protocol, the membrane is blocked to prevent streptavidin•alkaline phosphatase conjugate from binding non-specifically to the membrane. Blocking is accomplished by coating the membrane with a layer of bovine serum albumin. Streptavidin•alkaline is added to the membrane and finally, a chromogenic substrate is added which the alkaline phosphatase converts to a purple colored end product. The chromogenic substrate is a combination of nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The reaction is shown in the diagram below.



In the final experiment of this course, you will use Southern Blotting to examine plasmids pRIT4501 and pRIT4502. First, you will cut pRIT4501 and pRIT4502 as well as their parent plasmids pUC9 and pPL608 and blot them to a nitrocellulose membrane. In addition, you will also be given a sample of chromosomal DNA. Then, you will use the two parent plasmids to prepare probes. At the end of the experiment, you will be able to correlate parent plasmid DNA with the particular regions of the restriction maps of the recombinants.

**First Day
Preparation
of Probe**

1. Probe will be made by the nick-translation reaction using either pPL608 or pUC9. You should team up with another lab pair so that one pair does one plasmid and one does the other.
2. Prepare the following reaction mix:

| | |
|--------------|-----------------------|
| 5.0 μ l | dTTP + dCTP + dGTP |
| 2.5 μ l | biotin-7-dATP |
| X μ l | DNA (2 μ g total) |
| Y μ l | water |
| <hr/> | |
| 45.0 μ l | total volume |

+ 5.0 μ l DNA polymerase I + endonuclease A

 - X depends on the concentration of your DNA
 - Y is the volume of water necessary to bring the reaction up to 45 μ l
3. Prepare a 14^o water bath by adding ice to 4 liters of tap water until it cools to the proper temperature (we will do this as a class). Place your nick-translation reaction in the water bath and allow it to incubate for 90 minutes.
4. Adjust the reaction mix to 0.1 M NaCl. Add 100 μ l ice-cold ethanol and place in freezer for 30 minutes.
5. Centrifuge for 20 minutes at 17,000 rpm.
6. Resuspend in 50 μ l TE buffer.

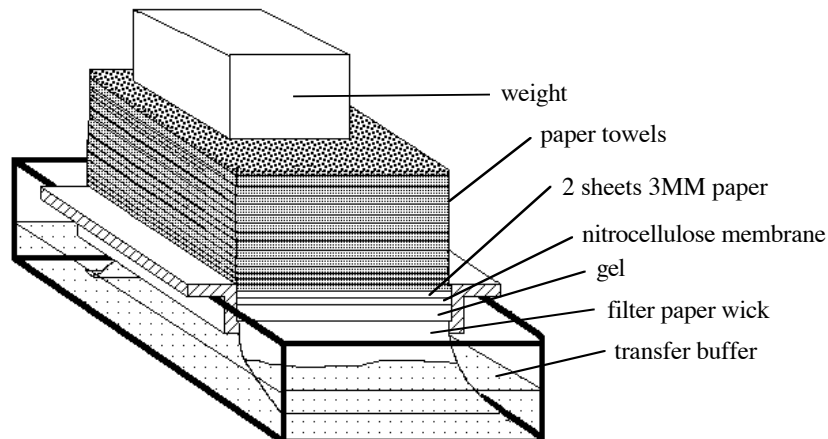
**First Day
Electrophoresis
and Blotting**

1. Cut pUC9, pPL608, pRIT4501, pRIT4502, and chromosomal DNA with *Eco*RI. You will need two samples of each, so scale your reactions up accordingly.
2. Prepare 2 samples each of 1 kb ladder and λ *Hind*III molecular weight standards.
3. Pour a 1% “piggy-back” gel.
4. Load your gels so that the samples are in the middle wells according to the table:

| lane | top | bottom |
|------|-----------|-----------|
| 1 | | |
| 2 | | |
| 3 | | |
| 4 | λ | λ |
| 5 | 1kb | 1kb |
| 6 | pUC9 | pUC9 |
| 7 | pPL608 | pPL608 |
| 8 | pRIT4501 | pRIT4501 |
| 9 | pRIT4502 | pRIT4502 |
| 10 | Chrom | Chrom |
| 11 | | |
| 12 | | |
| 13 | | |
| 14 | | |

4. At the end of the electrophoresis, stain the gel and take *two* photographs.
5. Transfer the gel to a plastic dish and add several volumes of 1.5 M NaCl + 0.5 M NaOH. Shake gently at room temperature for one hour. This will denature the DNA so that single-stranded DNA is transferred to the membrane.
6. Neutralize the gel by washing in 1 M Tris, pH 8 + 1.5 M NaCl for 1 hour. If the gel is not neutralized, then the NaOH will degrade the membrane and cause it to turn brittle and shatter when you try to unload the blot.

7. Set up the transfer according to the instructions below and the following diagram:
 - a. Cut a piece of nitrocellulose paper to the same size as the gel. Float it on the surface of 2X SSC until it is completely wet. Submerge the filter for 1 - 3 minutes.
 - b. Suspend the gel tray in a plastic dish and cover it with a piece of 3MM paper, draping down into the dish.
 - c. Fill the dish partially with 10X SSC. Remove all bubbles from the filter paper with a glass rod.
 - d. Lay the gel face down on the filter paper. Smooth out all bubbles.
 - e. Retrieve the nitrocellulose from step "a" and place it on top of the gel. Smooth out all bubbles.
 - f. Cut strips of saran wrap and lay them on each edge of the membrane and drape them over the side of the tray. Be careful to cover as little of the wells as possible.
 - g. cut two sheets of 3MM paper to the same size as the gel, soak in 2X SSC and lay them over the nitrocellulose.
 - h. Place a stack of paper towels 10 - 12 cm thick on top of the 3MM paper and place a weight on top. The dry towels will draw the 10X SSC by capillary action up through the gel, eluting the DNA onto the nitrocellulose where it is trapped.



**Second Day
Blotting**

1. Remove the towels and discard the gel. Make a nick in one corner of the nitrocellulose membrane so that when the side touching the gel is laying face up, the nick will be in the upper left-hand corner. This will allow you to orient the blot later on.
2. Allow the membrane to dry and then loosely sandwich it between two sheets of 3MM paper and bake it for 2 hours at 80° in a vacuum oven. This will irreversibly bind the DNA to the membrane.

**Third Day
Hybridization**

1. Lay the membrane in a tray and soak with 6X SSC for 5 minutes. Remove SSC and add pre-wash solution. Cover the tray and
2. incubate for 90 minutes at 42°.
3. At the end of the 90 minutes, put 15 ml of hybridization solution into a large disposable plastic centrifuge tube. Obtain a microfuge tube containing 150 µl of salmon sperm DNA and boil it for 10 minutes to denature it, then chill quickly in ice. Transfer the salmon sperm DNA to the hybridization solution in the centrifuge tube and vortex vigorously to mix, as the DNA is quite viscous.
4. Remove the pre-wash solution from the membrane and replace it with the hybridization solution + DNA. Incubate for 4 - 6 hours at 42°.
5. Remove about half of the hybridization solution so that the filter is covered with the minimum amount of liquid as possible. Boil your probe for 10 minutes, chill and add to the membrane. Do not pipette the probe directly on top of the membrane. Rather, dip the tray so that all of the liquid pools at one end. Add the probe to the pooled liquid, mix, and then rock the tray to cover the membrane. Incubate overnight at 42°.

**Fourth Day
Stringency
Washes**

1. Remove the hybridization solution.

There is still usable probe in the solution. If you wanted to probe another blot, you would use it at step 5 of Day Three. Instead of removing half of the hybridization solution and adding fresh probe, you would remove all of the solution and replace it with your re-cycled probe. Before adding the recycled probe, you would have to first boil and chill it to denature the DNA. Biotinylated probe can be stored in the freezer for up to a year, and can be reused several times.
2. Wash the membrane twice, 3 minutes per wash, in 2X SSC with 0.1% SDS.
3. Wash twice in 0.2X SSC with 0.1% SDS. Each wash should be 3 minutes.
4. Wash twice in 0.16X SSC with 0.1% SDS at 50°. Each wash should last 15 minutes.
5. Briefly rinse in 2X SSC with 0.1% SDS at room temperature.

**Fourth Day
Probe Detection**

1. Wash for 1 minute in Triton X-100 buffer.
2. Incubate filter for 20 minutes in blocking buffer at 65°. The blocking buffer should be pre-warmed.
3. If you wish to pause, this is a convenient braking point. You could dry in a vacuum oven for 20 min. at 80° and come back to it later.

We will not stop here.
3. Obtain streptavidin•alkaline phosphatase (1 mg/ml) and dilute it to 1 µg/ml (i.e. 1:1000) in Triton X-100 buffer. It takes 13.6 ml to cover the face of the membrane, so you should add 13.6 µl of streptavidin*alkaline phosphatase.

This should be done in a polypropylene tube. Add the streptavidin•alkaline phosphatase and incubate at room temperature for 10 minutes with gentle agitation.
4. Transfer the membrane to a fresh tray and wash the previous one. This transfer will help reduce background.

5. Wash the membrane, with gentle agitation, 3 times with Triton X-100 buffer. Use 20-40 times as much buffer as you used streptavidin•alkaline phosphatase solution. Each wash should last three or four minutes.
6. Wash twice with TSM buffer.
7. Transfer the membrane to a heat-seal bag. The reaction works best if not exposed to air.
8. Add 10 ml of the chromogenic substrate (NBT + BCIP) to the bag and seal. Incubate the membrane in dark or low light. Color will appear quickly, maximum development usually takes 4 hours.
9. Terminate the color reaction by rinsing in 20 mM Tris, pH 7.6) and 5 mM EDTA, pH8. Allow to dry and then take a photograph. The membrane will eventually fade.
10. Cut your membrane, your photograph, and the photograph of the original gel into four quadrants. Keep one of each to paste into your lab notebook and give a second to your partner. Exchange the remaining pieces with another pair who did the probe that you did not do.

20X SSC

| | |
|---------------------|---------|
| NaCl..... | 175.3 g |
| sodium citrate..... | 88.2 g |
| water..... | 800 ml |

Adjust to pH 7
Bring volume up to 1 liter
Dilute as needed

20X SSPE

| | |
|--|---------|
| NaCl..... | 174.0 g |
| NaH ₂ PO ₄ | 27.6 g |
| EDTA..... | 7.4 g |

Per 800 ml
Adjust to pH 7.4
Bring volume up to 1 liter
Dilute as needed

Pre-Wash Solution

| | |
|-----------------------|---------|
| 1 M Tris, pH 8..... | 12.5 ml |
| 0.5 M EDTA, pH 8..... | 1.0 ml |
| 10% SDS..... | 2.5 ml |
| NaCl..... | 14.61 g |

per 250 ml

Hybridization Solution

| | |
|------------------------------|---------|
| formamide..... | 67.6 ml |
| 50X Denhardt's solution..... | 1.0 ml |
| 20X SSPE..... | 37.5 ml |
| 10% SDS..... | 1.0 ml |

50X Denhardt's Solution

| | |
|---------------------------|---------|
| ficoll..... | 175.3 g |
| polyvinylpyrrolidone..... | 88.2 g |
| bovine serum albumin..... | 800 ml |

per 500 ml

Triton X-100 Buffer

0.1 M Tris, pH 7.5
0.1 M NaCl
2 mM MgCl₂
0.05% Triton X-100

Blocking Solution

3% bovine serum albumin in Triton X-100 buffer

TSM Buffer

0.1 M Tris, pH 9.5
0.1 M NaCl
50 mM MgCl₂