RNA Gel Blot (Northern blot) Hybridization and Washing

A: Day 1, Crosslinking and Hybridization (Steps 1-4 done before class by TA)

1. After the blotting is finished, mark the gel wells on the nitrocellulose filter by stabbing though the gel and filter with a needle or scalpel blade.

2. Dry the nitrocellulose filter in a vacuum oven at 65 ˚C (to prevent explosions!) or UV crosslink to permanently fix the RNA onto the nitrocellose filter.

3. Soak the filter in 50 ml of DEPC H2O, then pour off the water and add 10 ml of prehybridzation buffer. Incubate the nitrocellulose filter in a hybridization oven at 42 C for one hour.

4. Pour off the pre-hybridization buffer and add 10ml of hybridization buffer containing 50 ng/ml non-radioactive Gene X ribo-probe. Hybridize the probe to the RNA on the nitrocellulose filter overnight at 42 C.

B: Day 2, Washing of non-specifically bound probe (Start here at Step 5)

5. Pour off the hybridization solution. Wash the nitrocellulose filter with 15 ml of 1 x SSC/ 0.1% SDS at 65C for 15 minutes (this is the high salt wash).

6. Pour off the previous solution and wash the nitrocellulose filter with 15 ml of 0.1 x SSC / 0.1% SDS at 65C for 15 minutes (this is the low salt wash).

C: Detection of Hybridized Non-Radioactive Probe

7. Pour off the previous wash solution and incubate the nitrocellulose filter with 10 ml of Buffer 1 at room temperature for 15 minutes (this step will saturate the filter with proteins and will prevent the antibody from nonspecifically sticking to the filter).

8. Pour off the previous solution and incubate the nitrocellulose filter with 5 ml of antibody solution (Anti-Dig antibody diluted 1:2500 in Buffer 1) at room temperature for 20 minutes. Rock the tube to make sure the membrane is covered at all times. The anti-Dig antibody (which costs $1,000 per ml) will bind to the probe in the hybrids.

9. Pour off the previous solution and incubate the nitrocellulose filter with 10 ml of Buffer 2 at room temperature for 5 minutes (this step will wash off unbound anti-Dig antibodies). Repeat this wash two more times.

10. Pour off the previous solution and incubate the nitrocellulose filter with 10 ml of Buffer 3 at room temperature for 1 minute (this step will equilibrate the filter to the proper pH for hybrid detection).
11. Pour off Buffer 3 and add 5 ml of the yellow color detection reagent (D.R.). Let the color develop under a sheet of foil for 10 minutes to overnight. (The length of incubation time depends on the abundance of the hybrids on the blot. The hybridization signal is a dark blue or black precipitate).

Prehybridization and Hybridization solutions:

Final Concentrations	Stocks	10ml final volume
50% Formamide	100%	5 ml
6X SSC	20X	3 ml
5X Denhardts	50X	1 ml
0.5% SDS	10%	0.5 ml
100μg/ml ssDNA*	20mg/ml	0.05 ml
tRNA (10 μg/ml)	100 mg/ml	1 μl
ddH2O		0.45 ml

The prehybridization and hybridization solutions are the same except we added the probe (50ng /ml) to the hybridization solution. * sheared single-stranded DNA.

Wash Solutions:

20X SSC:
Dissolve the following in 800ml of distilled H_2O.

175.3g of NaCl
88.2g of sodium citrate

Adjust the pH to 7.0 with a few drops of 1M HCl.
Adjust the volume to 1L with additional distilled H_2O.

Buffer I: (Antibody binding and washing buffer)

Reagents	Final Concentration
Maleic acid	0.1M
NaCl	0.15M
BSA	1%
Triton X-100	0.3%
pH 7.5	

Buffer II: Blocking buffer
Same as buffer 1, but no blocking reagent

Buffer III: Alkaline phosphatase detection buffer

Reagents	Final Concentration
Tris –HCL	0.1M
NaCl	0.1M
$MgCl_2$	0.05M
pH 9.5	