



## Protocol #2

### Northern Blot

#### Introduction

Northern blot analysis allows the detection and quantification of specific RNA species from a particular cell type. Isolated RNA is electrophoresed through an agarose/formaldehyde gel which separates the RNA species by size. The faster migrating RNA fragments are the smallest, however, the distance of migration is not linear, rather it is inversely proportional to the size RNA molecule. When RNA has separated following electrophoresis, it is stained with ethidium bromide and visualised using ultra violet light. For gels of total RNA the 28S and 18S ribosomal subunits are visible and act as convenient size markers (approx 4.8 and 1.9kb, respectively).

To probe for a specific mRNA species by northern blot, it is first necessary to transfer the RNA from the agarose/formaldehyde gel to a nylon membrane. RNA is detected by hybridisation using a labelled probe. The probe is a DNA or RNA molecule which is chemically or radioactively labelled. We will use a [<sup>32</sup>P]-dUTP-labelled probe.

#### Timetable

- 1<sup>st</sup> Day* Prepare gel and electrophorese  
Transfer o/n
- 2<sup>nd</sup> Day* Synthesize and purify probe  
Pre-hybridize membrane & Hybridize with probe o/n
- 3<sup>rd</sup> Day* Wash membranes and expose to film o/n
- 4<sup>th</sup> Day* Develop film – interpret data

**1<sup>st</sup> Day (AM) – prepare and load gel***Materials required*

RNase free water (i.e. DEPC treated)	Agarose
10 x running buffer (0.2 M MOPS pH 7.0,	Formaldehyde
10 mM EDTA)	UV transilluminator
Formamide	Ethidium bromide
0.5 M iodoacetamide	Microfuge

**Formaldehyde, Ethidium bromide, UV, High Voltage – Caution**

Formaldehyde is toxic and a potential carcinogen; use the fume hood – do not inhale!

Formamide, ethidium bromide and iodoacetamide are also toxic and should be handled with gloves

UV light can damage your eyes if not protected – use wear facemask or goggles.

Molten agarose can cause nasty burns – handle with care.

*Preparation of the samples*

1. Keeping all samples on ice, add 10-20 µg of RNA from the control cells and/or treated cells to a sterile Eppendorf tube. The volume of RNA should be increased to 15 µl by the addition of DEPC treated water.
2. To this add 2.5 µl deionised formamide, 2.5 µl formaldehyde and 2.5 µl of running buffer (10 x stock), and 2.5 µl loading buffer
3. Denature samples by heating at 60°C for 20 min, then snap cool on ice.

*Gel Preparation*

1. You will be provided with 130 ml molten agarose at 60 °C (1.5 g agarose/130 ml H<sub>2</sub>O).
2. Carefully add:

15.0	ml	10 x running buffer
3.0	ml	0.5 M iodoacetamide
2.25	ml	40% formaldehyde
10.0	µl	Ethidium bromide (10 mg/ml stock)
3. Mix by swirling and pour into sealed gel tray (don't forget the comb to form the wells).

*Load and run the gel*

1. Load samples into the wells and run at 100 volts for 2 h (e.g. over lunch)

**1<sup>st</sup> Day (PM) – stop gel and transfer o/n***Materials required*

Hybond N (Nylon) membrane	20 x SSC (0.3M Na <sub>3</sub> citrate, 3M NaCl)
Saran wrap (DOW chemical company)	Glass plate
Whatmann 3MM paper	Bottle of water as weight for blot
Sponge soaked in 20 x SSC	

*Visualise integrity of RNA by UV illumination/EtBr staining*

1. Wearing gloves, remove gel from the tank, carefully place gel on transilluminator and take a photograph. It is often useful to place a fluorescent ruler on the gel prior to the photograph. This aids later in matching the position of the ribosomal markers on the photograph (which may not be actual size) with the final X-ray film of the hybridized mRNA.
2. Use extra blue roll when transporting gel from tank to UV transilluminator. Take care not to spill the running buffer because it may contain ethidium bromide (EtBr).

*Transfer to nylon membrane*

1. Set up transfer as shown and leave to transfer by capillary action overnight.

## 2nd Day(AM) Synthesize and purify probe

The Northern blot can be probed with any labelled RNA or DNA. *Here* we will probe the blot with DNA which is radiolabelled. See appendix for details of other probes.

### Materials required

2 x SSC buffer	50 $\mu\text{Ci}[\alpha^{32}\text{P}]\text{dCTP}$ , 3000Ci/mM
High Prime solution	Screw cap vials

### Radioactivity – Caution

$\beta$ -particles are emitted by  $^{32}\text{P}$  radionucleotides need to be handled with care and disposed of in a designated radioactive waste bin – wear two pairs of gloves

### *Transfer disassembly*

1. Carefully disassemble the transfer apparatus and remove the nylon membrane.
2. Fix the RNA to nylon membranes using UV crosslinker.
3. Label your hybridization chamber
4. Place blot in hybridization chamber (RNA 'side' facing upwards).

### *Probe synthesis*

High Prime is a reaction mixture that contains random oligonucleotides, Klenow polymerase, labelling grade dATP, dGTP, dTTP, and an optimized reaction buffer concentration in 50% glycerol for rapid and efficient labeling of DNA with  $^{32}\text{P}$ - or  $^{35}\text{S}$ -labeled dCTP. The plasmid we will use contains the P2X<sub>2</sub> cDNA. We use this plasmid as the template DNA with High Prime and  $[\alpha^{32}\text{P}]\text{dCTP}$ .

1. Place 25 ng template DNA (final volume 11  $\mu\text{l}$ ) in a screw cap vial.
2. Denature DNA by heating in boiling water bath for 10 min.
3. Chill quickly on ice.
4. Pulse spin tube in microcentrifuge.
5. Mix on ice:

25 ng	denatured DNA
4 $\mu\text{l}$	High Prime solution
5 $\mu\text{l}$	50 $\mu\text{Ci}[\alpha^{32}\text{P}]\text{dCTP}$ , 3000 Ci/mM
6. Incubate for 10 min at 37°C.
7. Stop the reaction by adding 2  $\mu\text{l}$  0.2 M EDTA and heat to 65°C for 10 min.

**2<sup>nd</sup> Day(PM)      Pre-hybridize membrane/hybridize with probe o/n***Materials required:*

Radiolabelled probe

Hybridisation buffer 50% formamide, 5X SSPE, 5X Denhardt's reagent, 1% SDS)

Sonicated salmon sperm DNA (10mg/ml).

**Radioactivity– Caution**

Radioactivity should not be used without a demonstrator present.

The blot is probed in Northern hybridisation buffer which comprises SDS together with a variety of reagents to block non-specific background. The probe is double stranded DNA, so it must be boiled (in order to separate the two strands) *before* being added to the blot. The probe is applied overnight in the smallest volume necessary to wet the blot in a shaking water bath or hybridisation oven.

*Prehybridise/hybridise*

1. Boil sonicated salmon sperm DNA for 5-10min and snap cool on ice. Add to hybridisation buffer to a final concentration of 200µg/ml. (e.g. 0.3ml of salmon sperm DNA (10mg/ml) to 15ml hybridisation fluid.
2. Add the hybridisation fluid (with added SS DNA) to the hybridisation chamber containing the membrane. Prehybridise the membrane at 42°C for at least 1h (no upper time limit).
3. Boil probe for 10 mins and snap cool on ice.
4. Open the hybridisation chamber, and without disturbing the membrane, pour the hybridisation solution into a 15-20ml tube (you will not recover the full 15ml). Add denatured (boiled) probe to the hybridisation buffer and mix gently, then return it to the hybridisation chamber and incubate overnight at 42°C.

**3<sup>rd</sup> Day(AM) Wash membranes and expose to film o/n**

*Materials required:*

2 x SSC with 0.1% SDS

Saran wrap

X-ray film

0.1 X SSC with 0.1% SDS

**Radioactivity– Caution**

The small fraction of the RNA that comprises the P2X<sub>2</sub> mRNA on the membrane should have hybridised to the radioactive probe. However, the membrane will have high background radioactivity which needs to be washed in order to yield a clean film and clear results.

1. Discard the radioactive hybridisation solution in the designated container/sink. Leave the membrane in the hybridisation chamber for subsequent washes.
2. Add about 50ml 2 x SSC with 0.1% SDS (room temperature) to the hybridisation chamber and wash the membrane for 10 min
3. Repeat the above washing step.
4. Discard the second wash solution. Add to the hybridisation chamber, 50ml 0.1 X SSC with 0.1% SDS, preheated to 42°C. Wash for 20 min.
5. Discard the final wash solution. Remove the membrane and air dry of a piece of Whatman filter paper. Wrap in Saran wrap and expose overnight to X-ray film.

**4<sup>th</sup> Day (AM) Develop autoradiograph – interpret data**

Gels may be developed after 24 hours, or can be left for longer periods if necessary.

**Appendix**

*Saran wrap and gels*

Saran wrap is NOT the same as cling film. Saran wrap can be purchased from Sigma. Standard protocols for these types of gels have high concentrations of formaldehyde that will not allow ethidium bromide addition so the gel has to be stained afterwards. In this course, we utilise a lower formaldehyde concentration which means that that ethidium bromide can be included in the gel. Iodoacetamide is included in the gel as an RNase inhibitor and is hazardous.