

Northern Blotting

Materials

⌘ DEPC-treat solutions

Add 0.1% DEPC to the solution in an autoclavable bottle. Mix the solution well and allow it to stand with the cap tightly closed overnight. Then loosen the cap and autoclave. This must be done in a fume hood as DEPC is very toxic.

⌘ 20X SSPE (1L)

175.2 g NaCl
27.6 g NaH₂PO₄*H₂O
7.4 g Na₂EDTA in 800 ml H₂O
Adjust pH to 7.4 with NaOH

⌘ 10 x Northern Running Buffer (containing MOPS) 500 ml

0.2 M MOPS, 10 mM EDTA, 50 mM Na Acetate

<u>Solution</u>	<u>Stock</u>	<u>Volume</u>
MOPS	0.2 M	20.93 g
EDTA	0.5 M	10 ml
Na Acetate	3 M	8.33 ml
Water (DEPC)		up to 500 ml

The buffer needs to be brought to a pH of 7.0 (for 500 ml, add approx 2 g of NaOH)

Note: Autoclaving of the buffer is not necessary

Store at 4°C

⌘ 1x RNA Sample Buffer (prepared fresh from frozen stocks) 500 µl

<u>Solution</u>	<u>Stock</u>	<u>Volume (µl)</u>
Running Buffer	10x	50
Ethidium Bromide	10 mg/ml	2
Deionised Formamide		250
Formaldehyde		90
Water (DEPC)		108

⌘ 10x RNA Loading Dye

<u>Solution</u>	<u>Stock</u>	<u>Volume</u>
EDTA	0.5 M	20 µl
Xylene cyanol	1.25%	80 µl
Glycerol		5 ml
Water (DEPC)		4.9 ml

⌘ **Pre-hybridization and Hybridization Solution**

1 mM EDTA, pH 8
0.5M Na₂HPO₄, pH 7.2
7% SDS
Autoclave 20 min then aliquot 10ml/tube and store at -20°C.

⌘ **Wash Buffer**

Wash 1: 1mM EDTA, pH 8; 40mM Na₂HPO₄, pH 7.2; 5% SDS.
Wash 2: 1mM EDTA, pH 8; 40mM Na₂HPO₄, pH 7.2; 1% SDS.

Methods

⌘ **Formaldehyde agarose gel electrophoresis**

Note: Formaldehyde vapours are toxic and casting this gel should be performed in a fume hood. The gel tank must be covered when in use.

1. Soak the gel tanks, combs etc in 0.2 M NaOH or 30% H₂O₂ for 15 minutes to destroy any contaminating RNases before rinsing in DEPC treated water.
2. Prepare 125 ml of a 1% RNA gel by adding 1.25 g agarose, 12.5 ml 10 x running buffer, 102.5 ml DEPC water. Dissolve the agarose in the microwave, let the solution cool to less than 60°C, then add 6 ml 35% formaldehyde and cast the gel.

Note: ethidium bromide is not added to the gel but rather to the sample buffer.

3. Prepare RNA samples by drying 20 µg of each sample in a Speed Vacuum Centrifuge. Resuspend each sample in 1x sample buffer. Shake 15 min at RT.
4. Heat samples at 65°C for 10 minutes then cool on ice. Add 3µl of 10x RNA loading dye and load the gel.
5. Run the gel at between 60-90 V for several hours, until the xylene cyanol dye front has migrated 3 to 4 cm into the gel and the bromophenol blue is about 2/3rds down the gel. Circulate the buffer from end to end every half an hour, especially if running the gel at high voltage. When the RNA has run an appropriate distance, photograph the gel including a ruler aligned with the wells.
6. Pre-soak the gel in 20x SSC while setting up the Northern blot transfer (about 15 minutes).

⌘ **Northern membrane blotting**

Northern membrane transfer is the same as described as in the Southern protocol with the following exceptions:

1. Use 20 x SSPE instead of 20 x SSC for transfer.
2. All of the vessels need to be treated for removing RNase.
3. Extreme cautions need to be paid for avoiding the RNA degradation.

⌘ **Probe labeling**

Follow the instruction of the labeling Kit (Prime-It II Random Primer labeling Kit, Stratagene)

1. Add the following components to the bottom of a clean microcentrifuge tube:
 - Sample: 25 ng (1-23 μ l) of DNA template to be labeled
 - 0-23 μ l of high quality H₂O
 - 10 μ l random oligonucleotide primers (total reaction volume should be 34 μ l)
2. Heat the reaction tubes in a boiling water bath for 5 minutes and then centrifuge briefly at room temperature to collect the liquid, which may have condensed on the cap of the tubes.
3. Add 10 μ l of 5 \times primer buffer: The 5 \times *dCTP primer buffer contains dATP, dGTP and dTTP, and it should be used when [α -32P]dCTP. The 5 \times *dATP primer buffer contains dCTP, dGTP and dTTP and it should be used when [α -32P]dATP or a dATP analog is to be incorporated.
4. Add 5 μ l of labeled nucleotide: Use either [α -32P]dCTP at 3000 Ci/mmol or [α -32P]dATP at 3000 Ci/mmol. Mix the contents of the tube thoroughly with your pipet tip.
5. Add 1 μ l Exo(-) Klenow enzyme (5 U/ μ l).
6. Mix the reaction components thoroughly with your pipet tip.
7. Incubate the reactions at 37–40°C for 20 minutes
8. Add 2 μ l of the stop mix to stop the labeling.

⌘ **Pre-hybridization**

1. Add 20ml of pre-hybridization solution per membrane to the hybridization bottle.
2. Place hybridization bottle in the rotating hybridizer (65°C) for 3 hr at speed set of 10.

⌘ **Hybridization**

1. Heat the labeled probe at 100°C for 5 min and chill it in ice if probe is DNA.
2. Add 10-50 μ l of the probe directly to the hybridization solution in the hybridization bottles.
3. Set up overnight hybridization in the hybridizer at 65°C at speed of 10.
4. Place the washing solutions in the 65°C oven for the next-day washing.

⌘ **Membrane wash**

1. Add 50 ml Washing 1 solution and wash membranes for 30 minutes at 65°C.
2. Add 50 ml Washing 2 solution and wash membranes for 30 minutes at 65°C.

⌘ **Detection**

1. Expose membranes to X-ray film in a darkroom with only the safe light on. Inside a film cassette, place the saran wrapped membranes at the bottom. Place a sheet of X-ray film on the top of the film, and place an intensifying screen face down on top of the film. Close the cassette.
2. Place the cassette at -80°C for 1-14 days, depending on the signal strength. The intensifying screen acts at low temperatures to enhance the efficiency of detection of radioactivity. This occurs when the photon emitting intensifying screen absorbs beta particles that pass through the film.

⌘ **Probe removal**

Shake membranes in 0.1 x SSC, 1%SDS and 40mM Tris-Cl (pH 7.5) for 30 minutes at 80°C.

References

1. Sambrook J, Fritsch EF, Maniatis, editors (1989) Molecular Cloning: A Laboratory Manual (2nd ed) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, Chapter 9-31 to 9-62