TMM,4-06

Northern Blotting (acc. to Maniatis)

For work with RNA: Be aware of RNases! Use gloves, wash gloved hands with water, dry. Always use freshly autoclaved not yet opened pipet tips / Eppis! Treat pipets with RNase Erase spray!

Preparation of gel and buffers:

DEPC-Water (inhibits RNase activity): 0,01% DEPC in Millipore water. Let stand ON, autoclave. Instead autoclaved water may be used.

10 x running buffer: 0,2 M MOPS pH 7,
20 mM sodium acetate,
10 mM EDTA (pH 8).

Dissolve 41,2 g MOPS and 1,64 g sodium acetetate in 800 ml sterile water. Adjust pH to 7. Add 20 ml of sterile 0.5 M EDTA pH 8. Add sterile water to 1 l. Autoclave, buffer turns yellow. Store at RT in alufoil.

Gel: Special RNA gel chambers! Use 12 well/1,5 mm comb. Treat comb and chamber with *RNase Erase* (or other reagent for inactivation of RNAses) as requested (spray, then wipe). Medium-size gel requires 100 ml of gel. Melt appropriate amount of agarose (1g for 1 %) in 72 ml of autoclaved water. For RNAs from 1,5 to 6 kb 1 % agarose is ok. Cool to ~60°C. Add 10 ml of 10x running buffer, 18 ml of 37 % of formaldehyde and 5 µl of 10 mg/ml ethidium bromide (in the hood!!!). If the gel gets to cold, it might get solid. Do not reheat it! Allow gel to set for at least 30 min.
The formaldehyde is used to denature the RNA, so that no secondary RNA-structures can form and that the RNA runs according to its actual length.

Preparation of sample and running the gel:

Precipitate appropriate amount of RNA (up to 100 µg of total RNA/lane). The RNA has to be stored as a precipitate, so just spin it (14.000, 10 min, 4°C). Precipitate also the marker and treat it the same way. Wash once with 70 % EtOH, completely dissolve pellet to get rid of all salt. Otherwise the bands might run uneven (“smilies”). Dry the pellet.

Typically, use either 30-40 µg of total or 1-2 µg of polyA+-RNA.

Resuspend RNA in 7 µl sterile water, when dissolved, add
3 µl 10 x running buffer,
6 µl formaldehyde and
15 µl formamide.

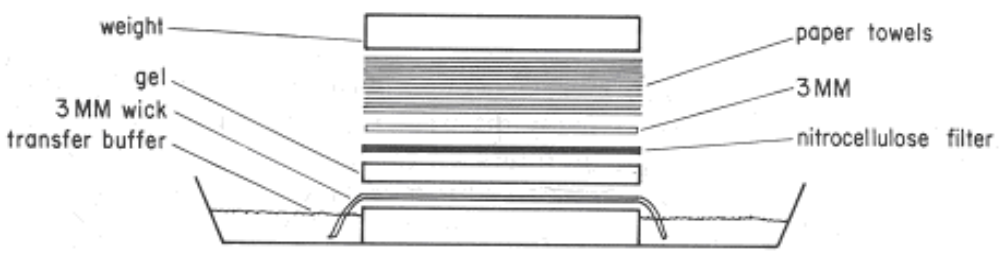
Heat 10 min at 65°C, snap-cool on ice, add 5 µl of RNA sample buffer (from RNA ladder), mix and spin briefly.

As size markers use 2 µg of 16/23S and 18/28S rRNAs or RNA ladders (treat as described above, otherwise the ladder runs very different).

Add the samples and wait 5-10 min before turning it on, to remove the last salt. Start gel run with 60 V (1h), then increase to 80 V. It might take up to 8 hrs, the time depends on the size range of RNA you want to detect. If necessary, run test gel.
Photograph gel on clean UV plate (clean first with 10 N NaOH, then with sterile water) with ruler next to marker lane and expose to UV light for the shortest possible time.

Transfer arrangement:

Before transfer wash gel 2 x 5 min in sterile water and 2 x 5 min in 10 x SSC. Use only RNA dedicated dishes for gel rinses. Set up transfer to Hybond (Hybond N) or other nylon membrane (12x14 cm) according to Maniatis using 10 x SSC as transfer buffer. Place Parafilm around the gel, eliminate all airbubbles between gel and membrane. Transfer ON.



After transfer mark the position of the pockets with a pencil and the orientation of the membrane (The marking is on the back side of the membrane!). Cut a corner of the membrane, this will be visible in the phosphoimager.
Rinse membrane briefly in 2 x SSPE, place on 3 MM paper and air-dry completely. Fix RNA by baking at 80°C for 1 h as described by manufacturer. Measure the distance pockets to upper side of membrane in cm. The membrane can be stored at RT.

Prehybridization/hybridization/wash

Carefully check all waterbaths and incubators for correct temperature!!!

Soak membrane briefly in 5 x SSPE, drain off excess liquid. Place in clean hybridization bottle and add 20-30 ml of hybridization solution 1 or 2. **Solution 1 is strongly recommended!!!** The Formamide prevents the probe from annealing.

Hybridization solution 1:

50 % Formamide ultrapure
5 x SSPE
5 x Denhardt
100 µg/ml of denatured herring-sperm DNA
1 % SDS
10 % Dextran sulfate (can be omitted if background problems exist)

Hybridization solution 2:

6 x SSPE
5 x Denhardt
100 µg/ml of den.herring-sperm DNA
1 % SDS
10 % Dextran sulfate (can be omitted if background problems exist)

The herring-sperm DNA needs to be boiled for 10 min, snap-cool on ice (denaturing), then add to the prepared hybridization solution (200µl / 20 ml).

If using solution 1, prehybridization is carried out at **42 °C** (stringent conditions, calculation see ‘notes’) for 30-60 min or ON. (With solution 2, prehybridize at 68°C (stringent conditions) for 30-60 min or longer.) This blocks unspecific binding of the probe to the membrane.

Probe (Fermentas DecaLabel Kit, K0622)

Typically, labelling is done with dCTP. For very hot probes, you can combine dATP and dCTP.

For labelling the probe, use 25 ng DNA.

- add 10µl buffer, fill to 40µl (sigma water)
- Vortex, spin down.
- 10 min in boiling water bath (heating block is not enough), snap cool on ice. The DNA is denatured now.
- Go to the isolab immediately (sample on ice), spin down there.
- Add 3µl MixC (dNTP without dCTP), 5µl radioactive labelled dCTP, 1µl Klenow enzyme.
- Mix, spin down, 5 min 37°C heating block. Now the labelled probe is created, the correct temperature is very important.
- Add 4µl dNTP, another 10 min 37°C. Check temperature!
- Stop reaction with 1µl EDTA (0,5M).
- Add TE to 200 µl, mix carefully.
- Precipitate with 400µl EtOH abs, 40 µl NaAcetate, 1µl glycogen. 15 min(!) 14.000 rpm RT.
- Collect supernatant for Szintillation counter.
- Wash pellet twice with 400 µl of 70% EtOH, spin 2 min. each time at 14.000 rpm, RT, air-dry.
- Resuspend in 50µl Sigma water
- 1µl into counter. A good incorporation rate is above 50%, you need at least 10 Mio counts in probe total.

Calculation: counts supernatant + counts probe = counts total (~ 50-60 Mio in 5µl fresh dCTP). Incorporation rate = counts probe / counts total.

Add the probe completely into 20 ml new hybridization buffer (don’t forget the denatured herring-sperm DNA). Discard prehybridization solution, add hybridization solution with probe to membrane. Hybridize at **42 °C** in sol. 1 (68 °C in sol. 2) for at least 20-24 hrs. Save probe if required (can be kept for 2-3 days for another hybridization).

Wash membrane 3 x 20-30 min at 66 °C in 0.1 x SSPE/0,1 % SDS (high stringency) under slight agitation. Use 200-300 ml of buffer per wash. This assumes the use of DNA probes. The unbound probe is removed by that step.

With riboprobes, temperature may be increased up to 75 °C. An additional washing step with 100 µg/ml RNAse A in 2 x SSPE for 2 x 15 min at 37 °C should be included, followed by 3 x 5 min in 2 x SSPE at 37 °C.

For different hybridization and washing conditions check Maniatis or other manuals.

Seal membrane in plastic bag and put phosphoimager on top. Expose at –80°C, first ON. Depending on signal strength use then other times.

If non-radioactive detection systems are used blocking and hybridization conditions might have to change.

Stripping and re-hybridization

Wash membrane for 30 min to 3 h in strip solution at 75 - 85 °C until no radioactivity can be detected on the membrane. Membrane can now be air dried and stored at RT for re-hybridization (up to 10 times) follow the hybridization protocol.

Stripping solution:

5 mM Tris pH 8; 0.2 mM EDTA; 0.05 % Na-pyrophosphate; 0.1 x Denhardt's solution

[500 ml: 2.5 ml 1 M Tris, 200 µl 0.5 M EDTA, 5 ml 5 % NaPP, 1 ml 50 x Denhardt's]

Incubate in roller derby, at 65°C for 1 hr, in 20 - 50 ml of:

75% Formamide

0.2X SSC

0.5% SDS

-Rinse blot thoroughly in tap distilled water.

-Rinse 5 min in neutralizing solution.

-Wrap in Saran Wrap (Frischhaltefolie) and store at –20°C.

Alternative protocol for stripping DNA probes:

Boil membrane for 30 min in a solution of 10 mM Tris-HCl, pH7.5-8.0, 1 mM EDTA, 1% SDS.

<u>Notes</u>

<u>Determination of melting point for perfect DNA-DNA hybrid</u>:

$T_M = 81.5 + 16.6 \log\ ]Na^+] + 0.41(G+ C) – \underline{500} – 0.62$ (% Formamide)

(L = length of double strand). Hybridization should be carried out at –25°C below T_M. T_M decreases 1°C / 1% mismatch for hybrid >150 bp.

Determination of hybridization time:

T_H = 1:X x Y:5 x Z:10 x 2

X = amount of probe in µg, Y = complexity of probe in kb, Z = hybridization vol in ml.

Multiply x 3 to achieve ~75 % hybridization efficiency.

Determination on melting temperature during washing:

(conditions are ~0.1 x SSPE) : $T_M = 69.3 + 0.41(G+C)$ %

For stringent washes, temperature should be ~5°C below T_M.