

RNA-Preparation and Northern Blotting

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Extraction of RNA

Incubation of cells under the appropriate conditions (usually on petri-dishes with 10 cm diameter)
 Complete removal of the media with a sterile pasteur pipette linked to the vacuum pump: after the first removal of the media lean the petri-dishes nearly vertically against a support (for a short time) so that the rest of the media can be collected from the bottom (in the laminar flow).

Add 1 ml of TriZol Reagent (Gibco: 15596-018) to the petri-dish and cover quickly the whole area (by shaking) - use sterile tip (if possible with a filter included).

This step should be done in the fume cupboard because of the phenol of the reagent. If you want to prevent any potential contamination with RNases, you can do it in the laminar flow, too.

Extract the cells by repeated pipetting of the solution over the whole area (the solution usually gets a little bit turbid) and transfer the extract to a sterile eppendorf tube.

Preparation of total RNA

according to the protocol provided by Gibco.

Quantification of the RNA

The final pellet is dried for 10 min in the laminar flow and dissolved in 22 µl of nuclease-free distilled water by heating to 56°C for 30 min. 2 µl of the solution are diluted with 500 µl of distilled water and the A₂₆₀ and A₂₈₀ values are determined using quartz cuvettes (switch on the reader at least 1 h before in order to warm up the lamp).

40 µg RNA/ml have an A₂₆₀ = 1

µg RNA/ml = measured A₂₆₀ x 250 (dilution factor) x 40; thus: **A₂₆₀ x 10 = µg RNA/µl**

The ratio A₂₆₀/A₂₈₀ should be 1.8 - 2.0 for clean RNA solutions.

Dot Blot (optional):

Calculate the amount of RNA solution to give 5 µg of RNA (for higher sensitivity: 10 µg).

The volume has to be 3 µl or less. Pipette the corresponding volume into a fresh sterile eppendorf tube and add nuclease-free water to give a total of 3 µl. Centrifuge the tubes to get a drop at the bottom of the tube. Then directly pipette 3 µl onto a dry Hybond-N⁺ membrane (you can draw a grid with 1 cm squares onto the membrane using a pencil and apply the drops in the middle of each square). Let the membrane dry for 10 min and then wet it a little bit from below using 5x SSC buffer. Immobilise the RNA on the membrane by UV-crosslinking (Stratagene Crosslinker - Dorian Bevec lab: Auto-crosslink with setting of 1200). Store the blot in Saran wrap at -20°C.

Electrophoresis in denaturing Agarose-Gels

5x MOPS: 41.2 g 3-(N-morpholino)-propanesulfonic acid
 800 ml 50 mM Na-Acetate solution (4.1 g/l in nuclease-free water)
 10 ml 0.5 M EDTA solution
 adjust the pH to 7.0 with 2 N NaOH
 adjust the volume to 1 l with nuclease-free water
 autoclave the buffer and store at R.T. protected from light (wrapped in foil)

Preparation of the gel (1% Agarose)

1.5 g Agarose (Pharmacia, NA-Agarose) are suspended in 93 g of nuclease-free water (fresh bottle!) and heated for 3 min at full power (850 W) in the microwave oven. Shake a little bit to dissolve completely; weigh the amount of water that is lost due to evaporation and fill up to the original weight (if necessary heat

again to dissolve completely and check the weight again).

Add 30 ml 5x MOPS buffer, 27 ml 37% formaldehyde (2.2 M) and 7.5 µl ethidiumbromide solution (10 mg/ml) - mix and try to prevent air bubbles.

The gel is poured into the gel-bed (this should be completely horizontal - check with bubble of spirit level) and the sample comb is put in (the sample comb is usually stored in ethanol to prevent RNase contamination and dried in the laminar flow before use). Polymerisation of the gel is allowed for 30 min and then the gel is transferred to the electrophoresis apparatus (filled with 1 l of 1xMOPS - diluted from 5x MOPS with nuclease-free water). The surface of the gel should be covered. The sample comb is carefully removed.

Preparation of RNA-samples for electrophoresis

10 µg total RNA (calculated volume)

2.5 µl 5x MOPS

3.5 µl 37% formaldehyde

10 µl formamide

are combined in an eppendorf tube, briefly centrifuged and the RNA is denatured by heating to 56°C for 15 min. After a brief cooling on ice, 2 µl of 10x loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenolblue, 0.4% xylencyanol) are added and the samples are again briefly centrifuged.

Electrophoresis

The samples are mixed with loading buffer (at the bottom of the tube after centrifugation) by repeated pipetting. Then they are carefully filled into the sample pockets of the gel. Electrophoresis is carried out at 20V over night or at 100V for 3 h (RNA migrates towards the plus-pole). The bromophenolblue should migrate about 8 cm, before ending the run. The gel is checked on the UV-monitor and a picture is taken with the Polaroid camera (put a ruler to the gel). Two bands should be visible (28S and 18S rRNA). Partly RNase-digested samples migrate faster.

Capillary Blot

The gel is marked on the right lower edge, removed from the electrophoresis container and submersed in 0.05 N NaOH for 20 min (with some shaking). This is important for the transfer of RNA larger than 2.5 kb. Wash the gel with nuclease-free water.

Shake the gel for 45 min in 20x SSC-buffer.

A tray is filled with 5x SSC, and a glass plate is put on the tray. Two Whatman 3MM filters (11 cm x ca. 40 cm) are wetted with the 5x SSC and laid over the plate so that both ends are in the buffer. Air bubbles between the plate and the filters are removed by rolling a sterile pipette on the filter. The gel is put on the

wet filters with the upper side down (transfer is more efficient in this way; besides, the orientation of the samples on the blot is then equal to the orientation on the gel). Air bubbles between gel and filters have to be removed with the pipette !!!! A pre-cut Hybond-N⁺ membrane (10 cm x 14 cm, dry) is put exactly on the gel (the membranes becomes wet when it touches the gel).

2 Whatman 3MM filters (10 cm x 14 cm) are wetted in the 5x SSC buffer and laid on the Hybond-membrane. Air bubbles are removed again. (If the pre-cut 10 cm x 13 cm Whatman filters are used, the gel and the Hybond membrane have to be cut to this size; in this case just 13 instead of 15 samples can be applied to the gel).

Parafilm is put exactly to the edges of the gel, in order to prevent contact between the wet filters below the gel with the filters above the gel.

A stack of dry Whatman filters (height: 5 - 8 cm) is laid on top, followed by a glass plate and a 500 g weight (bottle). The capillary transfer from the gel to the membrane should be carried out for 18 - 24 h. Afterwards, the transfer is checked under UV-light. There should be no bands visible on the gel, but only on the Hybond-membrane (a picture can be taken with the Polaroid camera)

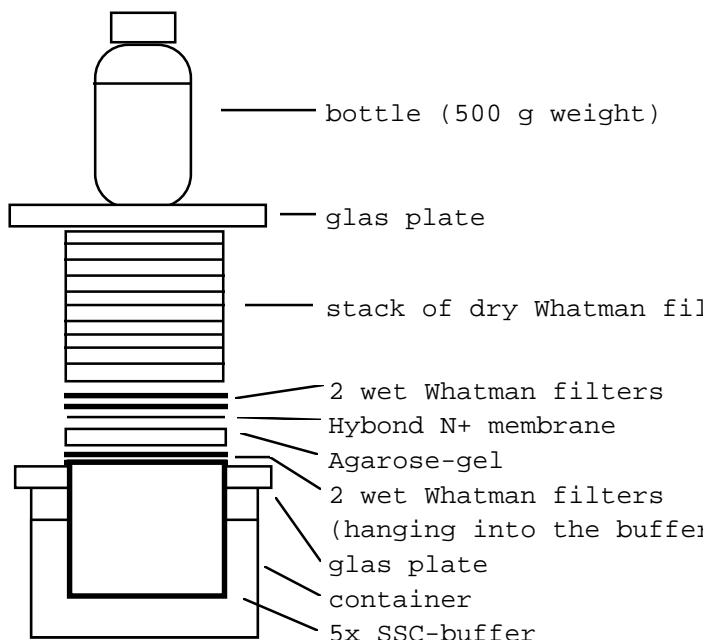


Fig. 1: Capillary Blot

The RNA is immobilised on the membrane by UV-crosslinking (Exposure to 120 mJ; Bevec's lab: Stratagene Auto-Crosslink set to 1200 units).

Methyleneblue staining of the Blot (optional)

Wash the membrane for 10 min in 3% HAc (under nuclease-free conditions)

Stain for 30sec - 1 min with 0.04% methyleneblue/0.5 M Na-acetate pH5.2

Destain with nuclease-free dist. water until the background is nearly white.

Northern Blot Hybridisation

Pre-hybridisation

Reagents:

Pre-hybridisation solution:

5x SSC	(25 ml 20x SSC)
5x Denhardt's	(10 ml 50x Denhardt's)
20 mM Na-phosphate	(20 ml 1 M Na-phosphate buffer pH7.0)
7% SDS	(35 ml 20% SDS)
	(10 ml nuclease-free water)
	(stored in aliquots at -20°C)

The pre-hybridisation solution is pre-warmed to 65°C before use.

Hybridisation solution

= pre-hybridisation solution containing 10% dextran sulfate (10 g/100 ml) (stored in aliquots at -20°C).

For the pre-hybridisation, the blots (wet) are put into the hybridisation tubes (not more than 2 blots per tube) with the RNA facing inside, and 10 ml of pre-warmed pre-hybridisation solution are added, as well as:

100 μ l Poly-A(10 mg/ml; final concentration 100 μ g/ml)

100 µl sonicated fish sperm DNA (10 mg/ml; final concentration: 100 µg/ml)
(for 10 ml = for one tube)

The blots are pre-hybridised for 4 h at 65°C (2.5 h are sufficient, too) under continuous rolling of the tube. 1 h before the end of the pre-hybridisation, the hybridisation solution is pre-warmed to 65°C and the radioactive labelling of the oligo is carried out.

32P-labelling of the oligonucleotide (with Terminal deoxynucleotidyl-Transferase)

- 1 µl Oligonucleotide solution (10 ng/µl) = 10 ng
- 2 µl 5x TdT reaction buffer
- 1 µl CoCl₂ solution (10 mM)
- 1 µl TdT enzyme (included in the TdT-Kit: Boehringer Nr. 220582)
- 5 µl (α -³²P) dATP (50 µCi) (Amersham, Redivue)

Mix by centrifugation and incubate for 1 h at 37°C

In the meantime prepare NICK spin column (Pharmacia) for the purification of the labelled oligo: put the column in vertical position, let the gel settle and remove the lids. Let the column drain and apply 1 ml TE-buffer, let it drain again and apply 2 ml TE-buffer. Let the column drain again, put into a centrifugation tube and centrifuge it at 500 g for 4 min using a counterbalance (2000 rpm in the Heraeus centrifuge). Remove the water that was spun off, and put a small cryotube (1.5 ml) into the centrifugation tube, followed by the column. (see Fig. 2). After the incubation of the oligonucleotide with (α -³²P) dATP and TdT, add 100 µl of TE-buffer containing bromophenolblue and mix shortly. Briefly centrifuge the sample and apply it to the prepared NICK spin column (the solution is taken up by the semi-dry gel). Centrifuge the column under exactly the same conditions as before (500 g, 4 min). Low molecular compounds like bromophenolblue or unbound (α -³²P) dATP remain in the gel, whereas high molecular compounds as the (α -³²P) dATP-labelled oligonucleotide are spun into the cryotube.

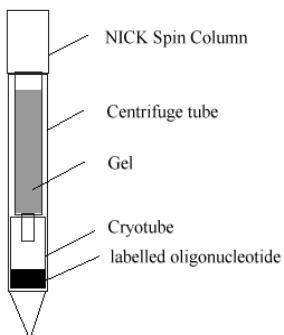


Fig. 2

Hybridisation

The pre-hybridisation solution is poured off and 10 ml pre-warmed hybridisation solution is added, as well as 100 µl Poly-A solution and

100 µl sonicated fish sperm DNA

Finally the labelled oligonucleotide is added, and the tube is transferred to the hybridisation oven.

Hybridisation is carried out over night at 65°C with continuous rolling of the tube.

Post-hybridisation washing

After the hybridisation, the radioactive solution is poured onto some paper towels on a bench coat and can be thrown to the solid radioactive waste. The membranes are removed from the tubes into a plastic container and washed with continuous shaking under appropriate conditions in a water bath. Two or three washing solutions are used, usually at 65°C for 20 min, each.

Oligo-Wash 1:

150 ml 20x SSC

100 ml Na-phosphate buffer pH7.0

100 ml 50x Denhardt's

400 ml nuclease-free water

250 ml 20% SDS (at last)

Oligo-Wash 2:

50 ml 20x SSC

900 ml nuclease-free water

50 ml 20% SDS (at last) = 1x SSC/1% SDS (Na^+ -concentration: 0.225 M)

Oligo-Wash 3 (optional):

10 ml 20X SSC

980 ml nuclease-free water

10 ml 20% SDS = 0.2x SSC/0.2% SDS (Na^+ -concentration: 0.045 M)

The washing solutions are pre-warmed to the appropriate temperature (usually 65°C)

For each oligonucleotide the melting temperature has to be calculated for the different washing solutions according to the formula:

$$T_m = 81.5 + 16.6 \log (\text{Na}^+ \text{-concentration}) + 0.41 (\%GC) - 600/N - 0.63 (\text{formamide}\%)$$

%GC.... percentage of G and C in the oligo

N length of the oligo (number of bases)

(in our procedure there is no formamide included)

The last washing step should be about 10°C below the calculated melting temperature.

For oligonucleotides in the range of 30 bases, oligo-wash 1 and 2 are usually sufficient, for oligos in the range of 60 bases a third wash is recommendable. Either the temperature or the sodium concentration has to be adjusted in a way that the applied temperature is 10°C below the melting point.

After the use, the first washing solution has to be poured into the container for liquid radioactive waste, the second and the third washing solution can be poured into the decontamination sink. The membranes are wrapped in Saran wrap and either measured on the InstantImager detector or exposed to film (Kodak X-OMAT or BioMax). The film cassette is stored at -70°C until development of the film.

Dehybridisation:

After evaluation of the northern blot, the membrane can be stripped and reprobed. The stripping is carried out by washing in 0.1% SDS at 80°C for 20 - 30 min. The next probing starts with the pre-hybridisation.