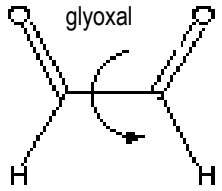
Howard Judelson, Nov. 1998

Northern Blotting

A. PREPARATION OF SAMPLE

i. mix together and then heat for 1 hr at 50°C:

RNA plus (DEPC-treated) water	5.5 µl
Dimethylsulfoxide (DMSO)	11.25 µl
0.1 M sodium phosphate pH 7.0	4.25 µl
Deionized glyoxal (6 M, 40%)	3.3 µl
Total:	25.3 µl



Basic principles of glyoxal gels: RNA can be completely denatured by glyoxylating the bases in the presence of DMSO, thus prohibiting base pairing. Samples treated in this way should be relatively clean, so that the glyoxal is not depleted by contaminanting junk.

Hint: Prepare 0.1 M sodium phosphate pH 7.0: for 0.4 l, add 2.1 g NaH_2HPO_4, 3.46 g Na_2HPO_4.

Note: Glyoxal is purchased as a 40% (6M) solution. Glyoxal readily oxidizes in air; the pH will drop as carboxylic acids accumulate. These can be removed by passage through a mixed-bed resin (e.g. Bio-Rad AG 501-X8) until the pH is about 5.0. This must be done quickly to minimize exposure of the glyoxal to air. Freeze small aliquots and discard tubes after each use.

ii. prepare sample for loading on gel:
Place on ice (spin down condensation if necessary).
Add 2.5 µl loading buffer. (50% v/v glycerol, 10 mM $NaP0_4$ pH 7.0, 0.25% bromophenol blue).

B. GEL ELECTROPHORESIS

i. If necessary, prepare an RNAse free gel unit by soaking in 1% SDS overnight (or commercial product likeRNAse-Off from CPG)
ii. Place a stir plate under the cathode and anode buffer chambers (stirring is required to prevent a pH gradient from building up).
iii. Pour a 1 to 1.4% agarose gel (depending on desired size range) in 10 mM $NaP0_4$ pH 7.0. After gel is solidified, add running buffer to 2 mm above gel.
iv. Load samples and run at 5 V/cm maximum.
v. After samples have entered the wells (or after 30 min) start the stir bars (a modest speed, ca. 60 rpm, is adequate).
vi. Electrophorese until the running dye has run about 10 cm (2/3 to 3/4 of the way down the gel).
vii. It is normal for the gel to become warm during the run.

C. STAINING STANDARDS WITH SYBR GREEN II

i. Normally, molecular weight standards are run in a side lane which is cut off and stained while the rest of the gel is blotted (stained lanes transfer poorly). About 2 µg of the Promega markers are commonly used.
ii. Note: SYBR dyes have several unusual features compared to traditional dyes: staining must be done in a buffered solution at pH 7.5 to 8.3, solutions must be kept in the dark (thedye bleaches readily),

and glass staining dishes should be avoided (use plastic with recycling number "5"). Safety note: these dyes come in a DMSO solution.

iii. Dilute dye stock 1:10,000 in 10 mM Tris 8.0 or other suitable buffer (see ii). The dye is expensive, so use a minimal volume.

iv. Agitate at room temperature 40 min or longer. Overnight at 4°C, in the dark, works very nicely.

v. For optimal results, photograph with the yellow filter (Wrattan #15).

D. BLOTTING RNA TO NYLON FILTER (HYBOND N+)

Electroblotting or capillary blotting are both acceptable. Electroblotting is reported give better results for high molecular weight species but is more difficult; disasters can occur if bubbles are not removed from the apparatus. Nevertheless, in HJ's quantitative tests using the following protocols, the signals for capillary-blotted 0.7 k, 1.3, and 4.2 kb bands were about the same (±30%) as electroblotted RNAs; no reproducible trend towards enhanced transfer of the larger bands by electroblotting was noted.

i. capillary transfer

a. RNA gels require no denaturation step prior to blotting, unlike DNA gels. If large RNAs are to be examined, gels can be treated in 50 mM NaOH for 20 minutes (to cause nicking and enhance transfer of large molecules). Then quickly move to step b, and repeat step b again.

b. Soak the gel in 10X SSPE at room temperature for 20 minutes.

c. Prepare a wicking apparatus for the gel (as with Southerns) in 10X SSPE

d. Blot 12-16 hrs. Disassemble apparatus.

e. Fix the RNA to the membrane by UV crosslinking at 1200 µJ (place RNA-side up in UV oven).
alternate fixation procedure: treat membrane in 50 mM NaOH, 15 min, then wash twice in 3X SSPE

ii. electroblotting

a. Prepare transfer buffer and cool to 4°C. 50X transfer buffer is 0.4 M Tris, 0.2 M NaAcetate, 30 mM Na_2EDTA pH 7.3 (adjust pH with acetic acid). The Pharmacia unit requires about 5 liters. *If you are reusing the buffer, beware of shifts in pH; after a single 14 hr run, the pH dropped from 7.3 to 6.2.*

b. Cut 3 pieces of Whatman paper and one piece of Hybond to the same size as the gel. Don't use nitrocellulose for electroblotting!

c. Saturate the blotting paper in transfer buffer. This and following steps are best performed in a deep tray containing transfer buffer, since this will help eliminate air bubbles that confound transfer.

d. Assemble in order: negative side of gel cassette>WET sponge>2 sheets of WET blotting paper>gel>Hybond>1 sheet of WET blotting paper>WET sponge>positive side of gel cassette. Remove any bubbles (roll with a glass tube, etc.)

e. Insert the blot assembly into the chamber (filter side towards "+").

f. Transfer at 15V overnight at 4°C with stirring (about 0.21 amp). Alternatively, go 30V for 6 hr)

g. Disassemble the cassette and fix the RNA to the membrane.

E. REMOVAL OF GLYOXAL (*This step increases hybridization signals. In several tests by HJ, deglyoxalation resulting in signals 1.3 to 2.8X that of non-deglyoxylated samples).*

After fixing the RNA to the membrane, wash the membrane in 20 mM Tris 8.0 for 20 min at 65°C.
(or) boil a beaker of 10mM Tris-Cl 8.0 and then turn off the heat. Drop in filter and wait 10 min.