Northern Blotting

Before you work with RNA

All experiments must be done extremely carefully to avoid contamination of RNase.
Be aware that you are the major source of RNase.
Always ware gloves during experiments, and change gloves frequently.
Always ware lab coat.
All water (MilliQ) for RNA experiments must be treated with DEPC (0.1% DEPC 1 h at RT, followed by autoclaving)
DO NOT talk during experiments, and let other people know you are working on RNA so that they will not talk to you when you are handling RNA. (This applies even when you are preparing buffers/reagents.)
Clean your bench with RNaseZap.
Wipe your Pipettes with RNaseZap.
Use tips/microfuge tubes dedicated for RNA work.
All equipments must be RNase-free. Soak buffer bottles, glass containers, gel boxes, gel plates, and combs in 3% H_2O_2. (For 30 min in the dark place, cover by foil.) Rinse these with small amounts of DEPC Water twice before use. You can also wipe glass container, gel plates, and combs with RNasZap.

1) RNA isolation form S. pombe
Follow the protocol for MastrPure Yeast RNA Purification Kit (EPICNTRE Biotechnologies)

2) Agarose electrophoresis
Prepare 1.2% agarose gel (without EtBr) in an RNase-free flask
Add 2.4 g agarose in 144 ml of MilliQ Water
Microwave to completely dissolve agarose, bring it to a fume hood, cool it down to 80˚C
after this step, prepare agarose gel in a fume hood.
Add 20 ml of 10 x MOPS buffer.
Add 36 ml of 37% formaldehyde.
Swirl to mix (Do not make bubbles).
Pour into a RNase-free, leveled gel plate, and place a comb.
Allow more than 30 min to solidify.
Place the gel with the comb in a gel box filled with 1 x MOPS buffer.
Remove the comb.

3) Sample prep for gel run (do this when gel is being solidified)

Precipitate 5 ug of RNA as follows:

Get 5 ug of RNA in a microfuge tube

Add MilliQ water to make it 50 ul

Add 5 ul of 3 M NaAc pH 5.3~5.6, and mix well by tapping

125 ul of 100% EtOH, and mix well by inversion

Incubate at -20˚C for 1 hour

Centrifuge at max speed for 30 min at 4˚C

Remove supernatant by pepetting (do not disturb pellet)

Add 200 ul of 75% EtOH slowly (do not disturb pellet)

Centrifuge at max speed for 5 min at 4˚C

Remove supernatant by pepetting (do not disturb pellet)

Air dry pellet

Dissolve the pellet in 20 ul of Sample prep buffer

**Make master mix to avoid pipetting error

for one sample, Sample prep buffer contains:

10 ul of formamide

2 ul of 10 x MOPS buffer

4 ul of formaldehyde

1 ul of 200 ug/ml EtBr

3 ul of MilliQ Water

Incubate samples for 20 min at 75˚C

Chill on ice for 10 min

Centrifuge briefly to collect all the content at the bottom of the tube

Add 2 ul of formaldehyde gel-loading buffer and keep samples on ice until gel run

4) Electrophoresis

Pre-run: 5 V/cm for 5 min

Stop running and load samples (do not use the first and the last lanes).

Run gel: 5 V/cm constant voltage (4h~, depending of the agarose percentage, voltage and gel size).

Run gel until the lower dye approaches to 3/4 position from the bottom.

Remove the gel place and put it on Saran wrap on the UV transilluminator, and take a image with a UV ruler.

Two distinct bands (26S and 18S rRNAs) must be clearly visible without smearing (if smeared, RNA is degraded).

5) Transfer

Soak the gel in 10 mM NaOH for 20 min with gently shaking

Rinse the gel with MilliQ water

Incubate the gel with 20 x SSC for 5 min with gently shaking

Incubate the gel with 10 x SSC for 40 min with gently shaking

During this 40 min, prepare:

1 sheet of Hybond-XL

5 sheets of 3MM paper

A lot of towel paper

*Important: Cut these membrane and paper to the exact size of gel.

Set up transfer sandwich. (See picture below.)

Place a plate over a glass container

(like a bridge: the plate must be longer but narrower than the glass container).

Lay a sheet of 3MM paper over the plate

(both ends of the 3MM sheet must be touching the bottom of the glass container).

Add 10 x SSC in the glass container so that the 3MM sheet gets wet.

Remove bubbles between the plate and the 3MM paper by rolling a glass test tube.

At this point, the two ends of the 3MM sheet must be under the buffer surface.

Carefully place the gel “up side down”.

(By adding some 10 x SSC, remove all bubbles between the gel and 3MM paper)

Soak Hybond-XL membrane in, MilliQ first, then in 10 x SSC buffer.

(the membrane must be the exact size of the gel)

Then place it on top of the gel. (remove all bubbles between the gel and the membrane)

If you put some 10 x SSC buffer between them, it is easy to remove all the bubbles.

It is important that the top edge of the gel and the top edge of the membrane are aligned perfectly.

Soak 3MM paper in 10 x SSC buffer, then place it on top, one by one. (3MM sheet must be the gel size)

Remove all the bubbles between the 3MM paper by rolling glass test tube again.

Then place a stuck of towel paper.

“BUT” before that, place Parafilm (or saran wrap) at all four sides of the gel to cover the surface of the bridge.

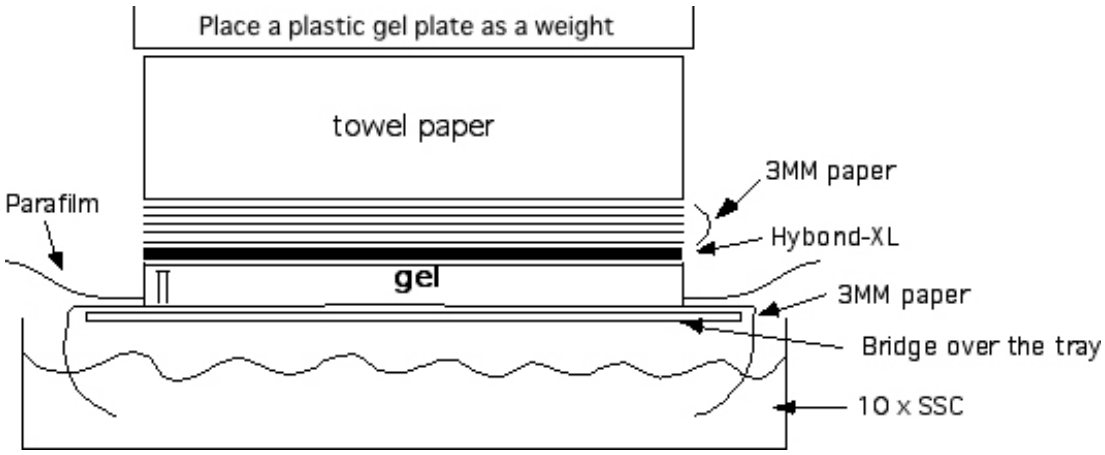
You don’t want the towel paper to touch the sponge.

Place something on top of the towel paper as a weight.

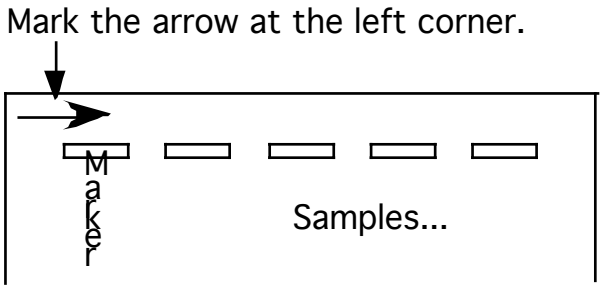
Don’t put a heavy thing or you’ll squish the gel, and the transfer does not work efficiently.

Plastic gel plate works well. Check the level by a plastic round level vial.

Leave the transfer set O/N in a safe place.



6) UV cross-link



After overnight transfer, take the membrane out with forceps. DO NOT touch the side that RNA is attached. Flip the membrane and soak it in 2 x SSC 0.1% SDS buffer (RNA side up).

Pick up the membrane using forceps and drain off the 2 x SSC 0.1% SDS buffer. Place the membrane on 3MM paper to remove excessive liquid. DO NOT dry the membrane.

Mark an arrow at the top left corner of the membrane with a ballpoint pen (or a water-resistant marker). Place the membrane on a new piece of 3MM paper, and place it in UV linker.

Expose the membrane to UV (Auto, 1200 J/m^2 but stop at 500).
(After this UV link, dry the membrane completely on 3MM paper, and store the membrane between 2 sheets of 3MM paper, in a safe place, such as in your drawer. But do not touch the surface that RNA is attached.)

7) Pre-hybridization
Warm up Northern Church buffer at 65˚C.
Soak the membrane in 2 x SSC 0.1% SDS, agitate a little, and discard the buffer.
Roll the membrane (RNA side in) and insert it in a hybridization bottle.

Add 20 ml of warmed Northern Church buffer to the bottle and incubate it at 65˚C rotor oven for 2 hour ~ O/N (Pre-hybridization). *Important: Make a balance.

8) DNA probe labeling (Roche, Random Primed DNA Labeling Kit, modified protocol)
After 1 hour of pre-hybridization, put the entire [α-^{32}P]dCTP blue container on the bench and allow 30 min to

thaw the content (***do not remove the actual vial from the blue container)

Start DNA labeling, after 1.5 hour you started the pre-hybridization (by this time [α-^{32}P]dCTP is thawed).

Prepare a screw-top 1.5ml tube with probe DNA: 10 μl (20~100 ng)

Boil for 5 min.

On ice for 5 min.

Centrifuge the tube briefly to collect all DNA at the bottom of the tube

Add the following reagents in the following order

1 ul of dATP (vial 2)

1 ul of dGTP (vial 4)

1 ul of dTTP (vial 5)

2 ul of reaction mixture (vial 6)

4 ul of [α-^{32}P]dCTP (40 uCi)

1 ul of Klenow enzyme (vial 7)

Mix gently but thoroughly by tapping

Centrifuge the tube briefly to collect the content at the bottom of the tube

Incubate at 37˚C for 30 min.

Centrifuge the tube briefly again to collect the content at the bottom of the tube

On ice to stop the reaction.

Add 30 ul of STE (see the instruction for Probe Quant G-50 Micro Column) to make it 50 ul

9) Purification of labeled probe DNA (Probe Quant G-50 Micro Column)

Mix the content of the G-50 column by vortex

Loosen the top cap, and snap off the bottom cap

Put the column in a screw-top 1.5ml tube, and centrifuge for 1 min at 3000 rpm, toss the tube

Put the column in a new screw-top 1.5 ml tube

Load the 50 ul of labeled DNA at the center of the G-50 resin, do not disturb the resin

Centrifuge for 2 min at 3000 rpm, purified probe will be collected in the 1.5 ml tube.

Keep the tube on ice.

10) Hybridization

Get the hybridization bottle from the 65˚C oven, discard the Northern Church buffer that you used for pre-hybridization.

Add 5~10 ml of fresh Northern Church buffer (pre-warmed at 65˚C) to the bottle, put it back to the oven.

Then, boil the purified probe for 5 min, on ice for 5 min

Centrifuge the tube briefly to collect the content at the bottom of the tube

Add the purified probe (25 ul) to the hybridization bottle, store the rest of the probe at -20˚C

Rotate it in the 65˚C oven for O/N.

11) Washing

Warm WashN1 and N2 at 50˚C

Rinse membrane with WashN1 once

Wash membrane with WashN1 at 50˚C for 10min twice in the bottle

If the background is high, wash membrane with WashN2 at 50˚C for 10min once of twice in the bottle

**When washing, it is important to get buffer between the bottle and membrane, remove bubble by rotating the bottle, then put the bottle back to the oven

Take the membrane out of the bottle, and wrap the membrane well

**If you plan to strip the probe for another hybridization, do not dry the membrane

12) Imaging

Expose membrane to phosphoimager screen for 2 hr - O/N

Analyze the blot with Storm 840/ImageQuant

13) Stripping the probe

Stripping buffer

0.1% SDS/2mM EDTA

Put the membrane in the glass container

Pour boiling Stripping buffer to the membrane, agitate for a min, and pour off the buffer to the sink (radioactive!)

Repeat this wash more as necessary

Every wash, check radioactivity

The membrane is now ready for re-hybridization

Reagent:
DEPC-autoclaved MilliQ
DEPC-autoclaved 0.5M EDTA pH 8.0
3M NaAc made with DEPC water, filter sterilized
20% SDS made with DEPC water, filter sterilized
*1 M Tris-HCl, pH 8.0 made with DEPC water, filter sterilized (***Tris cannot be autoclaved)*

Prepare all reagents with DEPC-treated MilliQ

10 X MOPS Running Buffer **(to 1L)**
200 mM MOPS (3-(N-morpholino)-propanesulfonic acid) **41.8 g**
To 700ml with ddH_2O, adjust to **pH 7.0 with NaOH**
80 mM NaAc **6.6 g**
20 mM EDTA **25 ml** (500mM stock)
0.2µ filter sterilize. Store RT protected from light.
Will yellow as it ages: cannot be stored indefinitely.

Formaldehyde Gel-loading buffer
50% (v/v) glycerol
10 mM EDTA (pH8.0)
0.1% Bromophenol Blue, 0.1% Xylene cyanol FF

20 X SSC **(to 1L)**
175.3 g NaCl
88.2 g sodium citrate
To 800 ml of water, adjust the pH 7.0 with a NaOH.
To 1L final. Sterilize by DEPC-autoclaving.

Northern Church buffer
0.5 M Phosphate buffer pH 7.
1 mM EDTA
7% SDS

Prepare Northern Church Buffer in the following order:
Add **67g** of Na2HPO4.7H2O to 400 ml of MilliQ, and stir well
Then add **2 ml** of 85% H3PO4 (caution, concentrated phosphoric acid!!), and stir well
Add **1 ml** of 0.5 M EDTA pH 8.0
Add **35g** of SDS
Add Milli Q to make it to 500 mL

Roche, Random Primed DNA Labeling Kit

[α-^{32}P]dCTP

STE (see the instruction for Probe Quant G-50 Micro Column)

Probe Quant G-50 Micro Column

Wash N1 (0.5L) (2 x SSC, 0.1% SDS), at 50˚C
20 x SSC 50mL
20% SDS 2.5mL
H2O 447.5mL

Wash N2 (0.5L) (0.5 x SSC, 0.1% SDS), at 50˚C
20 x SSC 12.5mL
20% SDS 2.5mL
H2O 485m