Northern blot protocol for the detection of RNA in Neurospora

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Proceedure

a. Extract RNA from tissue powder

1. Harvest and grind the tissue with a mortar and pestle in liquid nitrogen.
2. Transfer powder (~200 mg) into a 1.5 ml eppendorf tube containing a mixture of 0.45 ml lysis buffer and 0.45 ml of phenol*: chloroform: IAA (25:24:1). Vertex briefly and mix on a rotator until all the samples are ready.
   (* phenol saturated with H2O, pH4~5)
3. Centrifuge at 10,000 g for 10 min at 4˚C.
4. Transfer the upper phase to a new tube containing 0.45 ml of phenol: chloroform: IAA (25:24:1), vertex well and spin again.
5. Precipitate RNA with one-tenth volume of NaOAc and 2.5* volumes of cold 100% ethanol. Mix well and then spin again.
6. Wash the pellet with 70% EtOH and air dry the pellets.
7. Dissolve the pellets in RNase-free water and measure the RNA concentration by UV spectroscopy. One A_{260} units of RNA is 40μg/ml.
   (Usually we take 2 μl of RNA+498μl H_20 for measuring.)
   [RNA]= $A_{260} \times 40$ (μg/ml) × 250 (dilution fold) × 10^{-3} (ml/μl) = X × 10 μg/μl

b. Electropheresis and transfer RNA

1. Prepare a 1.3% agarose gel (100 ml) by mixing 1.3 g agarose, 10 ml 10× MOPS/EDTA/NaOAc buffer and 85 ml H_20.
2. Microwave the mixture to boiling and cool to 60˚C in water bath.
3. Add 1μl EtBr (10mg/ml stock) and 5ml formaldehyde (37% stock solution). Mix and pour the gel. Let the gel sit for 1 hour in hood before use.
4. Prepare the loading samples by suspending 2~40 μg RNA in 10 μl H_20 plus 26 μl of loading buffer mix and 4 μl of loading dye. Heat samples at 65˚C for 15 min to denature RNA. Place in ice water immediately.
5. Spin the sample briefly and load the sample on the gel, run at 80mA for 1 to 1.5 hours.
6. Soak the gel in 10×SSC once for 30 min with gentle shaking, and carefully pre-wet the membrane in 10×SSC for 5 min.
7. Transfer the RNA from the gel to a membrane (what membrane and from

where) using capillary action with 10×SSC buffer overnight. For the exact procedure for transfer, please check Molecular Cloning.

c. Preparing the membrane for probing and preparation of a riboprob.

* Riboprobe is more sensitive than DNA probe, but it is difficult for stripping and reprobing. For highly expressed genes, DNA probe is fine.

1. Crosslink the RNA to the membrane by UV crosslinking (Please check the manual of your crosslinker for the time needed for this).
2. Put the membrane into a hybridization tube and fill the tube with Millipore H_2O so that the membrane will stick to the tube without forming bubbles in-between the membrane and the tube. Pour the water out, and leave the tube upside down on a piece of paper to absorb the residual water. Then add 5 ml of prehybridization buffer to the bottom of hybridization tube.
3. Incubate in a hybridization oven at 65°C for at least 1 hour.
4. Make a riboprobe by *in vitro* transcription (Maxscript kit, Ambion)

I. prepare the template for riboprobe:

-- Using plasmid as template: linearize (~1kb) with a restriction enzyme downstream of the insert to be transcribed. Gel purify the linearized DNA (avoid using enzymes leaving 3' overhang ends, such as Kpn1, Pst1 etc.).

-- Using PCR products as template: A phage promoter sequence, such as T7 or T3, can be added to one or both of PCR primers to be incorporated into PCR products.

* We add TAATACGACTCACTATAGGG to the 5' end of the antisense strand of the gene of interest.

II. Synthesize the RNA probe by mixing together the following: (Maxscript kit, Ambion)

10 × buffer	2 μl
10 mM ATP	1 μl
10 mM GTP	1 μl
10 mM CTP	1 μl
0.1mM UTP	0.5 μl
RNA template (1μg) in 7.5μl H_2O	
T7 Polymerase	2 μl
α^{32}P-UTP	5 μl

III. Incubate the reaction at 37 °C for 1 hour and then dilute with 60 μl H_2O.

IV. Purify labeled riboprobe by applying the diluted sample to a Micro

Bio-Spin P-30 Column (Bio Rad), centrifuge at 1000g (3020 rpm) for 4 min. The labeled riboprobe is in the column flow-through.

d. Hybridization:

1. Add the purified riboprobe to the hybridization tube for incubation overnight at 55°C (the actual temperature for each probe needs to be determined experimentally. 55C is a good starting point).
2. Pour the hybridization buffer out and rinse the membrane with wash buffer (0.1× SSC, 0.1% SDS) once. Then wash the membrane 3 times with 100ml of prewarmed wash buffer for 20min each in the hybridization oven at 68 °C.
3. Wrap the membrane in Clingwrap and expose the membrane to X-ray film or phosphoimager screen.

Lysis buffer

- 0.6 M NaCl
- 10 mM EDTA
- 100 mM Tris pH8.0
- 4% SDS

10 × MOPS(for 500ml)

- 46.2g MOPS
- 6.8g NaOAc
- 3.8g EDTA

Adjust pH to 7 with NaOH and autoclave (add around 8 g NaOH to 1L buffer)

20 × SSC

- 3M NaCl
- 0.3M Na_3Citrate

(For 1L, dissolve 175.3g of NaCl and 88.2g sodium citrate in 800 ml deionized water. Adjust pH to 7 with a few drops of 10N NaOH and then add water to 1L)

Loading buffer mix (26 μl for each sample)

- 10 × MOPS buffer 150 ul
- De-ionized formamide 450ul
- 0.66M formaldehyde 45ul

Hybridization solution:	For 500 ml
- 10 × Denhardt's	100 ml 50×
- 50% formamide	250 ml
- 50 mM Tris pH7.5	25 ml 1M
- 1 M NaCl	29.22 g
- 0.1% Napyrophosphate	0.5g
-100 mg denatured salmon sperm DNA	5 ml 10mg/ml
water to 500 ml	

**make up in fume hood, mix and store at -20°C

Denhardt's reagent 50× stock:
- 5g Ficoll (Type 400, Pharmacia)
- 5g polyvvynlpyrrolidine
- 5g bovine serum albumin (Fraction V; Sigma)

water to 500 ml

**mix, filter and store at -20°C. It takes a while to get everything into solution, and a long time to filter.

Denatured salmon sperm DNA:

Salmon sperm DNA (Sigma type III sodium salt) is dissolved in water at a concentration of 10 mg/ml. Stir for 2-4 hours to get into solution (it's very sticky). Shear the DNA by passing the solution through a 17-gauge needle 12-20 times (until it goes through easier)

References: Molecular Cloning