Small RNA Northern by Attila Molnár

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A. RNA isolation with TRI-Reagent (Sigma, Cat. No. T9424) **or TRIZOL** (Invitrogen, Cat. No. 15596-026)

1. Harvest *Chlamydomonas* cells (50ml cells in midlog phase: ~2 x 10^6 cells/ml) using a 50 ml Corning tube (Cat. No. 430290) by centrifugation for 5 min at 4000 rpm.
2. Pour off the supernatant and freeze the cells in liquid N_2. Store the sample at -80 ^oC or proceed with step 3.
3. Transfer the tube on ice and re-suspend the pellet in 6 ml of TRI-Reagent with a sterile glass pipette. Pass the sample several times through the pipette to form a homogenous lysate.
4. Pellet polysaccharides by centrifugation for 15 min at 4000 rpm, 4^{o}C (Sigma 4K15C, refrigerated laboratory centrifuge with swing out rotors).
5. Transfer the upper phase to a fresh 15 ml Corning tube (Cat. No. 430790) and incubate at room temperature for 5 min to ensure the complete dissociation of nucleoprotein complexes.
6. Add 1.2 ml of chloroform (no isoamyl alcohol!), vortex vigorously for 15 sec and allow to stand for 5 min at room temperature.
7. Centrifuge the resulting mixture for 15 min at 4000 rpm, 4^{o}C.
8. Transfer the upper phase containing the RNA to a fresh 15 ml tube kept on ice (avoid the interphase which contains the DNA as well as the protein-containing red organic phase).
9. Precipitate the RNA by adding 1 volume of isopropanol. Mix by shaking and allow the sample to stand for 5-10 min on ice (or store the precipitated RNA at -20 ^{o}C, overnight).
10. Recover the RNA by centrifugation for 30 min at 4000 rpm, 4^{o}C. Remove the supernatant by aspiration.
11. To remove residual salts rinse the pellet with 8 ml of 80% ethanol and centrifuge immediately 5 min at 4000 rpm, 4^{o}C. Do not re-suspend the RNA pellet in 80% ethanol, because short RNAs are soluble to some extent in 80% ethanol in the absence of salt.

12. Remove the ethanol and repeat step 11.
13. Remove the ethanol by aspiration without disturbing the pellet and collect the residual ethanol at the bottom of the tube by an additional 10 sec centrifugation. Remove the residual liquid completely using a small pipette tip and dry the tube at room temperature for 3-5 minutes.
14. Place the tube on ice, and add 100 µl of RNase-free water. Allow the RNA to re-hydrate for 15 min on ice then vortex vigorously for 15 sec. Dilute 1 µl into 200 µl water to estimate the concentration of the total RNA by measuring the absorbance (A_{260}) in a quartz/plastic disposable cuvette. Store the remaining RNA extract at -70^{o}C. The quality of the RNA can be checked by denaturing 1-2 µg of sample in 5 µl final volume with one volume of 2X gel-loading solution at 65°C for 5 minutes and analyzing on a sterile 1.2% agarose gel (Fig. 1).

B. Separation of total RNA on 15% denaturing polyacrylamide gel using Bio-Rad Mini-PROTEAN Tetra system (Cat. No. 165-8000).

15. Prepare RNA size marker (Decade Marker, Ambion, Cat. No: AM7778) according to the manufacturer's instruction. Separate the labelled RNA from unincorporated nucleotides on a Microspin G-25 column (GE Healthcare, Cat. No. 27-5325-01).
16. Clean the electrophoresis equipment with washing up liquid then rinse with water and subsequently with sterile distilled water. Wipe the glass plates with 70% ethanol.
17. Prepare a 15% denaturing polyacrylamide gel (7M Urea, see protocol below).
18. Assemble the electrophoresis apparatus. Rinse the wells with running buffer (0.5X TBE) using a syringe and pre-run the gel at 100 V for 30 minutes.
19. Mix the RNA samples (15-20 µg) with an equal volume of 2X loading buffer. Use equal volumes to ensure similar running behaviour. Denature the RNA as well as the radio-labelled size marker (~20-50cps) at 65°C for 5-10 minutes and then place the tubes on ice.
20. Wash the wells of the gel and load the RNA samples leaving one well empty between the sample and the size marker to avoid contamination.

21. Separate the small RNAs by running the gel at 50 V until both the dyes enter the gel (20-30 minutes). The small RNAs run between the bromophenol blue and xylene cyanol in a 15% denaturing polyacrylamide gel.
22. Wash the wells to remove the high molecular weight RNA and continue to run at 100-150 V until the bromophenol blue (bottom dye) reaches the bottom of the gel.

C. Small RNA transfer by capillary blotting

23. Dismantle the gel-running apparatus and soak the gel in 50-100 ml of 20X SSC for 10 minutes.
24. Cut a membrane (Hybond-N, NX, N+ or ZetaProbe GT) to the size of the gel and equilibrate it in distilled water. Then soak in 20X SSC for 5 minutes.
25. Set up the capillary blot according to Fig. 2, and transfer the RNA overnight.
26. Dismantle the capillary blot and dry the membrane with the RNA side up for a few minutes on Whatman 3MM paper. Crosslink the RNA to the membrane with UV at 120000 µJOULES (Stratagene, UV Stratalinker 2400).
27. Cut the membrane slightly above the xylene xyanol dye. Hybridize the upper part with a *Chlamydomonas* U6 probe (5' CTCGATTTATGCGTGTCATCC 3'; loading control) and the bottom part with a small RNA-specific probe.

D. Hybridization with an oligoprobe

28. Pre-hybridize the membrane in 5-10ml of hybridization solution at 40°C for at least 30 minutes.
29. Label the single-stranded DNA (ss DNA) oligo (reverse complement to the small RNA you would like to detect) as described below. Denature the oligo at 90°C for 5 minutes and then place the tube on ice before adding buffer, ATP and enzyme.

    2 µl 10 µM ssDNA oligo (21-24nt)
    10 µl sterile distilled water
    2 µl 10X Polynucleotide kinase buffer
    5 µl γ32P-ATP
    1 µl T4 Polynucleotide kinase 10 (µ/µl)

30. Incubate the reaction mixture at 37°C for 30-60 minutes.

31. Separate the labelled ssDNA from unincorporated nucleotides on a Microspin G-25 column according to the manufacturer's instruction (GE Healthcare, Cat. No. 27-5325-01). 1 µl of separated probe should count 500-2000 cps.
32. Add 2 µl of 0.5M EDTA to the radioactive probe and denature at 90°C for 5 minutes, then place the tube on ice.
33. Pour off the pre-hybridization solution. Mix the denatured ssDNA probe with 5 ml of fresh hybridization solution and add to the filter.
34. Hybridize at 40°C overnight.
35. Wash the membrane with gentle shaking using 2X SSC, 0.1% SDS at 40°C for 10 minutes. Repeat the washing step.
36. Wrap the membrane with Saran wrap and expose to Phosphor Image plates. If necessary, increase the stringency of the washes by lowering the salt content of the washing buffer (i.e. 1X SSC) or increasing the temperature during the wash.

Reagents and Solutions

2X gel-loading buffer
10 ml deionized formamide
200 µl 0.5 M EDTA, pH 8.0
1 mg xylene cyanol FF
1 mg bromophenol blue
Store up to 1 year at 4^oC

15% denaturing polyacrylamide gel (10ml for two 0.75mm thick gels)
4.2 g Urea
0.5 ml 10X TBE
3.75 ml 40% (w/v) 19:1 acrylamide:bis-acrylamide
2.5 ml water
Stir at room temperature to dissolve Urea.
Add 70 µl 10% (w/v) ammonium persulphate (APS) and 3.5 µl TEMED
Pour immediately. Polymerization time is 30 minutes.

Hybridization Solution
0.25M sodium phosphate buffer pH 7.2 (1M buffer is made up from 1M Na_2HPO_4 and 1M of NaH_2PO_4, see Maniatis)
7% SDS

Additional information

- This protocol can also be used to detect small RNAs extracted from plant tissue. Grind 0.5-1g of fresh plant tissue in a sterile mortar with liquid N_2. Add 6 ml of

TRI-Reagent and pass the sample several times through a sterile glass pipette to form a homogenous lysate. Transfer the lysate to a fresh 15 ml Corning tube and proceed with step 4.

- Avoid nuclease contamination throughout the procedure. Use sterile solutions and disposable plasticware, keep the tube containing the RNA solution on ice.

- Be sure that your RNA is intact! This is one of the most crucial parts for good detection.

- Other phenol-chloroform based nucleic acid extraction protocols may also be used. The relatively small amount of genomic DNA in total nucleic extract will not interfere with further processes. Avoid commercial RNA isolation kits with silica-gel–membrane purification because the small RNA fraction does not bind to the column. LiCl precipitation should also be avoided because small RNAs do not co-precipitate with long RNA transcripts.

- Alternative hybridization solutions may be used (PerfectHyb Plus, Sigma, Cat. No. H7033 or ULTRAHyb, Ambion, Cat. No. AM8670).

Fig 1. *Chlamydomonas* RNA separated on a 1.2% native agarose gel.

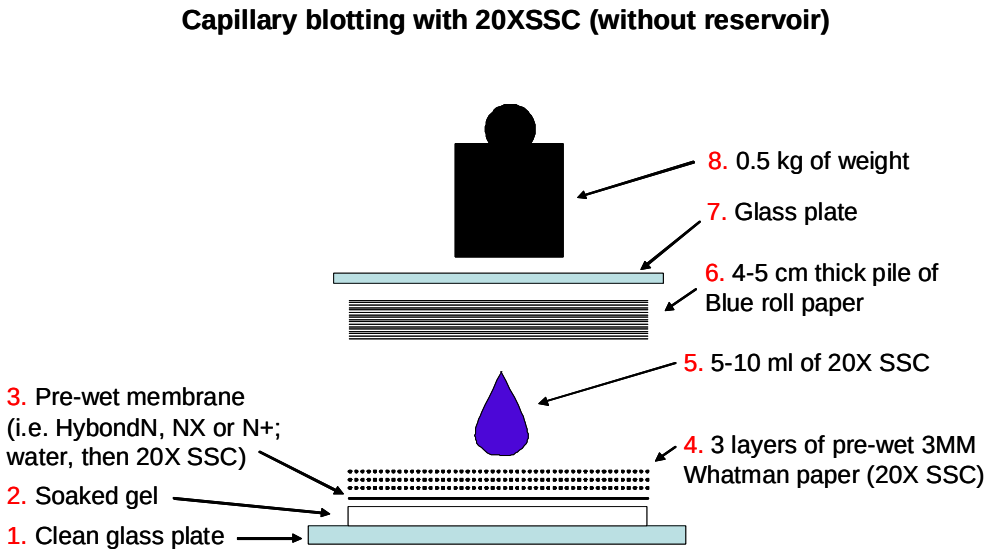

Fig 2. Capillary blotting by Attila Molnár. Set up the system following the numbers. Transfer the RNA overnight.