QUICKER RNA DOT BLOTS BY A DNase I-HEPARIN ISOLATION METHOD

We describe a very fast, one-step procedure for preparation of total RNA samples from cultured cells for dot blot analysis. Pelleted cells are resuspended in hypotonic solution containing heparin and vanadyl-ribo-nucleoside complex (VRC) and disrupted by freezing and thawing. DNase I is then added to the suspension in order to digest chromosomal DNA to mono- and oligonucleotides. This procedure liberates cellular RNA in non-degraded form (assayed on Northern blots after phenol extraction). The preparation can be directly used for dot blotting analysis without further purification. The detection of hsp70 RNA sequences in total Drosophila cellular RNA prepared by this method is as sensitive as that prepared by conventional organic extraction. Heparin was shown not to interfere with RNA binding to the nitrocellulose, nor did the presence of the remaining cellular protein in the lysate. Signals per dot obtained with various gene probes increased linearly up to 4 x 10^6 Drosophila Schneider line 2 cells/dot and to 2-4 x 10^5 HeLa or rat GH3 cells/dot. We are currently testing the lower limit of RNA detection by this method, which we think may be generally applicable for cultured cells and with some modification, to nuclear RNA, cytoplasmic RNA, and to tissue preparations.

Heparin has three main effects:

1. It lyses nuclei and dissociates chromatin proteins (mainly histones and HMG proteins). In the presence of heparin, nuclear DNA is digested as rapidly as protein-free DNA.
2. It has a partial inhibitory effect against the action of RNases.
3. It releases mRNA from the rough endoplasmic reticulum.

Solutions:

1. DNase I (Worthington DPRF)
   Dissolve DNase I in buffer containing 100 mM NaCl, 10mM Tris-Cl (pH 7.4), 100 mg/ml of BSA to make a final concentration of 200 u/µl.
   Freeze and store at -70°C in aliquots of 10 µl.
2. Heparin (sodium salt from porcine intestinal mucosa. 168 USP units/mg Sigma.)
   Make a stock solution of 50 mg/ml in water and store at 4°C up to one month.
3. Vanadyl ribonucleoside complex, VRC, (supplied by BRL at 200 mM).
4. Lysing buffer. Contains: 10 mM Tris Cl (pH 7.5), 1.5 mM $MgCl_2$, 1.5 mg/ml of heparin and 10 mM VRN. Prepare fresh.
5. 1.2X and 1X NaPF solution. 1X NaPF contains: 1M NaCl, 40 mM phosphate buffer (pH 7.0) and 6% formaldehyde.

Preparation of samples for dot blotting:

Drosophila Schneider line 2 cells are grown in the Schneider's medium supplemented 15% of calf serum and 0.42% bactopeptone. Cells are harvested at a density of 0.8 - 1.0 x 10^7 cells/ml.

1. Pipet aliquots of the cell culture containing 1 x 10^7 cells per aliquot into

Eppendorf (1.5 ml) centrifuge tubes.

2. Pellet cells by centrifugation for 10 sec in the Eppendorf microfuge and remove the supernatant.
3. Centrifuge again for 1 sec and pipette off the residual (SN) supernatant. The volume of the pellet shouldn't be greater than 10 µl.
4. Suspend the pellet in 50 µl of the cold lysing buffer. Vortex vigorously to disperse the cells.
5. Freeze the suspension at -70°C on powdered dry ice.
6. Thaw the suspension to room temperature, vortex vigorously for 2 - 5 sec.
7. Add 1.8 µl of DNase I (200u/µl) and incubate at room temperature for 15 min with occasional shaking.
8. Add 200 µl of 1.2xNaPF solution, mix and centrifuge for 2 min at room temperature.
9. Discard the pellet. Supernatant containing the RNA can be stored at -20°C for at least a week or longer at -70°C or directly used for dot blotting.

Dot blotting and hybridization:

1. Take aliquots of the supernatant (up to 100 µl, equivalent to 0.4 x 10^7 cells) and dilute with 1 x NaPF solution, making a final volume of 200 µl.
2. Heat the samples for 5 min at 65°C, and cool to room temperature.
3. Filter the samples through the nitrocellulose filter using eg.a Schleicher and Schuell minifold apparatus (before filtration soak the nitrocellulose in 20x SSC).
4. Wash filter once with 0.5 ml of 1 M phosphate buffer (pH 7.0), dry and bake at 80°C for 1.5 hr under vacuum.
5. Freeze the suspension at -70˚C on powdered dry ice.
6. Thaw the supspension to room temperature, vortex vigorously for 2-5 sec.
7. Add 1.8 µl of DNase I (200µ/µl) and incubate at room temperature for 15 min with occasional shaking.
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4. Wash filter once with 0.5 ml of 1 M phospate buffer , dry and bake at 80˚C for 1.5 h under vacuum.

Reference: *Zdzislaw Krawczyk and Carl Wu, NIH (manuscript in prep.).*