NUCLEAR RUN-ON ANALYSIS OF TRANSCRIPTION

Isolation of nuclei is basically the procedure of Wright and Bishop, PNAS 86:505-509, 1989. Purification of the ^{32}P-labeled RNA is basically according to O'Conner and Wade, BioTechniques 12:238-243, 1992.

1. Pellet 5 x 10^7 cells (per rx) for 8 min @ 1.2k RPM @ 4°.

2. Wash 1X with ice-cold PBS (12ml) and re-pellet in a 15-ml cent. tube.

3. Resuspend cells in ice-cold RSB (2ml) on ice and then add an equal volume of RSB containing 0.2% NP-40, pipet up and down 2-3X to mix, leave on ice for 3 min, vortex briefly.

4. Pellet nuclei @ 2.5k RPM for 10 min at 4° in a desk-top centrifuge (swinging-bucket).

5. Aspirate SPNT (this can be used for extraction of cytoplasmic RNA).

6. Resuspend nuclei in 162ul of rx buffer and transfer to a 1.5-ml microfuge tube on ice. Final volume will be about 200ul. The rx buffer consists of:

100ul 2X buffer
10ul 20X NTPs
50ul (500uCi) ^{32}P-alpha-UTP (800 Ci/mmol)
2ul RNasin

7. Incubate @ 37° for 5-10 min.

8. Add $CaCl_2$ to 1mM (2.2ul of 100mM stock) and 20 units of RQ1 DNase. Incubate @ 37° for 15 min.

9. Add 0.1 vol 11X STE (22ul), mix and add 2.5ul 10mg/ml Proteinase K. Mix well and incubate @ 42° for 30 min.

10. Add 1ml of RNazol B and vortex well to dissolve nuclear debris.

11. Add 0.1 vol chloroform (125ul), vortex for several seconds and then place on ice for 20 min.

12. Centrifuge @ 12.5k RPM @ 4° for 15 min in a microfuge.

13. Re-extract aqueous phase with phenol:chloroform, divide into two tubes if necessary.

14. Precipitate with an equal volume of isopropanol @ -20° for 1 hr and pellet.

15. Rinse RNA pellet with 70% EtOH, dry briefly and hydrate with 10ul of water. Resuspend pellet in 1M NH_4OAc, vortex briefly and ppt with 250ul EtOH.

16. Repeat step 15. NOTE: It is very difficult to dissolve the RNA pellet in NH_4OAc; If you have some sterile RNase-free G-50 push columns, such as those from Stratagene, these should work well to remove unincorporated nucleotides after step 14.

17. There is still a significant amount of DNA present and it is necessary to digest the RNA at this point with RNase-free DNase (RQ1 DNase). This is an absolute requirement if the cells contained transfected DNA (short-term or long-term) containing your gene of interest.

Dissolve RNA in 165ul water
2.5ul RNasin (about 80 units)
20ul 10X buffer (see manufacturer's instructions)
10 U/ul RQ1 DNase

Incubate @ 37° for 15-20 min

18. Extract 1X with phenol:chloroform (more if an interphase is apparent) and 1X with chloroform. Add 0.1 vol 3M NaOAc, mix and ppt with 2.5 vol EtOH.

19. Rinse with 70% EtOH and dissolve in 200ul $T_{10}E_1S_{.1}$ (S=SDS). You can determine your CPM at this point by counting 1ul; you should get about 1-4 CPM per nuclei. Add enough yeast tRNA so that its final concentration is 100ug/ml in the hybridization buffer (do not add it to the pre-hyb soln. and <u>do not</u> use salmon sperm DNA, your background will be very high).

The filter should be pre-hybridized for 2-4 hr @ 42° before adding the probe. I use GeneScreen Plus membrane and pre-hyb and hybridize in 50% Formamide/10% Dextran Sulphate (from a 50% stock)/1 M NaCl/1% SDS. Hybridize for 48 hr and wash as you normally would for a northern blot (you will have to use 1% SDS in your wash solns. if using a nylon membrane). After your last wash, incubate the filter(s) for 15 min in a 37° solution of 2X SSC containing 10ug/ml of RNase A. Rinse the filter and then dry and expose.

<u>Stock Solutions</u>:

<u>RSB</u>
10mM Tris (pH 7.6)
10mM NaCl
5mM $MgCl_2$
$\pm$ 0.2% NP-40

<u>2X Buffer</u>
20mM Tris (pH 7.6)
180mM KCl

10mM $MgCl_2$
50mM DTT
50% glycerol

<u>20X NTPs</u>
20mM ATP
20mM CTP
20mM GTP

<u>11X SET</u>
110mM Tris (pH 7.4)
55mM EDTA
5.5% SDS

Treat and prepare all reagents as you normally would for RNA to prevent or eliminate RNase contamination.