NUCLEAR RUN-ON TRANSCRIPTION

Reagents:

1. 50X Denhardt's solution: 1% Ficoll, 1% polyvinylpyrrolidone, and 1% bovine serum albumin (BSA). Filter sterilize.
2. Dnase I
3. Hybridization buffer: 50% formamide, 6X SSC, 10X Denhardt's solution, 0.2% SDS
4. Lysis buffer: 10mM NaCl, 3mM $MgCl_2$, 10mM Tris-HCl, pH 7.4, 0.5% Nonidet P-40. Autoclave.
5. Nuclei storage buffer: 40% glycerol, 50mM Tris-Hcl, pH 8.5, 5mM $MgCl_2$, 0.1mM EDTA. Autoclave.
6. Proteinase K stock solution: 50mg/ml (store at -20°C)
7. 2X reaction cocktail: 50μl{^{32}P}UTP (500μCi), 250μl 4X reaction mix, 125μl 8X tri-phosphate mix, 75μl ddH_2O (enough for 13 reactions at 40μl per reaction)
8. Nuclei run on stop buffer: 2% SDS, 7M urea, 0.35M NaCl, 1mM EDTA, 10mM Tris-Hcl, pH 8.0
9. 4X reaction mix: 100mM HEPES, pH 7.5, 10mM $MgCl_2$, 10mM DTT, 300mM Kcl, 20% glycerol (store at 4°C)
10. 50% TCA (w/v)
11. 8X tri-phosphate mix: 14μl 25mM ATP, 14μl 25mM GTP, 14μl 25mM CTP, 0.4μl 1mM UTP, 83 μl ddH_2O (final concentrations of 2.8mM ATP, GTP, CTP, and 3.2mM UTP).
12. carrier tRNA

Procedure: perform all procedures on ice unless otherwise specified

I. Isolation of Nuclei

1. Wash 6 x 10^6 cells or more (i.e. 3-5 100x20mm plates of HeLa cells, or 10-15 ml of 5 x 10^5 cells/ml HeLa S3 culture) in 1X PBS and harvest cell pellet.
2. Lyse the cells by the addition of several volumes of lysis buffer (about 200μl for 6 x 10_6 cells) and repeated pipetting to disperse the pellet. Spin 3s (no longer) in cold-room microcentrifuge to pellet the nuclei. Remove the supernatant and save for RNA isolation if desired.
3. Estimate the nuclear pellet volume (usually around 50-200μl) and resuspend in an equal volume of nuclei storage buffer. Pipetting should be done with a wide-bore pipet tip (cut the end off a pipet tip with a clean razor blade) to avoid breaking them. Store at -80°C.

II. DNA Dot Blot Preparation

1. Sonicate 10μg DNA in 750μl ddH_2O for 15s to linearize DNA.
2. Add 30μl of 10 N NaOH to each sample, and chill on ice to denature DNA. Neutralize with one volume of 2M ammonium acetate (780μl).
3. Filter 1μg (150 μl) of the DNA solution on to a nitrocellulose membrane on top of one piece of 3MM Whatman paper using dot blot apparatus. Cut the nitrocellulose membrane into strips, each containing one set of DNA samples to be tested and bake in a vacuum oven for 2h at 80°C. The DNA blots can be stored at room temperature.

III. Transcription Reactions

1. Freshly prepare the 2X reaction cocktail that contains radiolabeled nucleotide. Thaw the nuclei on ice and add 50μl of nuclei (using wide-bore tip) to 50μl of the 2X reaction cocktail. Incubate at room temperature for 20 min. Stop the reaction by adding 2μl of DNase I and incubating at 37°C for 10 min.
2. Add 300μl stop buffer, 300μg proteinase K (final concentration 1mg/ml), and 100μg tRNA to each reaction. Homogenize by pipetting or by passage through a 23gauge needle and 1ml syringe if it is very viscous, and incubate at 40-50°C for 2h.
3. Precipitate by adding ice cold TCA to each reaction to a final concentration of 10% and incubating on ice for 20 min.
4. Spin 15 min to pellet the nucleic acids. Wash pellet with cold absolute ethanol to remove any trace of TCA. Air-dry the pellet and resuspend in 50μl of TE containing 0.5% SDS. Incubate at 65°C for 15-30 min to dissolve the RNA.

IV. Hybridization Reactions

1. Prepare the hybridization buffer. Prehybridize filter-bound DNA in 2.5ml hybridization buffer at 42°C for at least 6h. It is convenient to hybridize the nitrocellulose strip in 12x75mm culture tubes with caps.
2. Add 50μl radiolabeled RNA to the prehybridization buffer. Hybridize at 42°C for at least 72 hours.
3. Wash the DNA blots once with 6X SSC and 0.2% SDS at room temperature for 10 min, then twice with 2X SSC and 0.2% SDS, followed by two washes in 0.2X SSC and 0.2% SDS, all at 65°C for 10-30 min each wash. Place the DNA blot strips on an old X-ray film as backing, cover with plastic wrap, and analyze by autoradiography.

References:

Udvardy and Selfart (1976) Eur. J. Biochem. 652:353-363.
Marzluff et al. (1974) Biochem. 13:3689-3698.

Reeder and Roeder (1972) J. Mol. Biol. 67:433-441.
Groudine et al. (1981) Mol. Cell Biol. 1:282-288.
Fodor and Doty (1977) Biochem. Biophys. Res. Comm. 77:1478-1485.
Mathew et. al. Mammalian Heat-Shock Transcription Factors

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