ANALYSIS OF POLYSOMES BY SUCROSE GRADIENT SEDIMENTATION

(I) Materials

1. Polysome buffer: 300 mM KCl, 5mM $MgCl_2$, 10mM HEPES, pH 7.4
   Potassium concentrations less than 300 mM can also be used.
2. 15% sucrose in polysome buffer
3. 45% sucrose in polysome buffer

We use Schartz/Mann Ultrapure sucrose (RNase-free). The above solutions can be treated with diethylpyrocarbonate, if so desired. All solutions should be filtered through a 0.45 µm filter before use. We find it convenient to make a large quantity of the sucrose solution and keep it frozen in 50 ml aliquots.

4. Lysis buffer: Polysome buffer containing 0.5% NP-40 and 100 µg/ml cycloheximide. Vanadyl adenosine (10 mM) and/or dithiothreitol (5 mM) can be included to inhibit RNases. It is generally not necessary to use vanadyl adenosine if one is working with HeLa cells.

(II) Preparation of the lysate

(a) For HeLa JW36 cells growing on 10 cm plates:

The media is aspirated off and 5-10 ml of ice-cold PBS containing 100 µg/ml cycloheximide is immediately added and the cells are put on ice. The buffer is then aspirated off; the plates are taken to the cold room held at an angle to allow the remaining buffer to drain into the edge of the plate. The remaining buffer is removed with a pipet and the cells are lysed by adding 200 µl of lysis buffer directly on the plate. The lysate is scraped to the edge of the plate with a policeman, passaged 2-3 times through a 27 gauge needle, and centrifuged in a microfuge in the cold for 3-5 min. to pellet the nuclei.

(b) For HeLa JW36 cells growing in spinner culture:

To a sample of 1-2 x 10^7 cells cycloheximide is added to 100 µg/ml from a 10 mg/ml stock. The cells are then quick-chilled by immersing them for 5-10 sec. in a dry-ice/ethanol bath with constant swirling and then in an ice-water bath for at least 30 sec. The cells are then pelleted by centrifugation, resuspended in 1 ml of ice-cold PBS containing 100 µg/ml cycloheximide, transferred to an Eppendorf tube and centrifuged again. The buffer is aspirated off; the cells can be frozen in a dry-ice bath and stored at -70°C or processed immediately. Frozen or fresh cell pellets are lysed with 500 ul of lysis buffer, passaged 2-3 times through a 27 gauge needle, and centrifuged to remove the nuclei.

(c) For chicken reticulocytes;

Cells (0.5-1.0 x 10^9 cells per aliquot) are incubated at a concentration of 10^9 cells/ml in 1.5 ml Eppendorf tubes. Cycloheximide is added to a concentration of 100 µg/ml and the cells are quick-chilled as above and centrifuged for 1-2 min. The supernatant is removed and the cells are then frozen in dry ice/ ethanol or processed immediately. Cell pellets are lysed in 500-600 µl of lysis buffer, dispersed by pipetting, and centrifuged for 3-5 min to pellet the nuclei.

Notes:

(1) It is important to chill the cells quickly and keep the cells or cell lysate cold after chilling to avoid polysome "run-off" during preparation. The use of cycloheximide is optional and can be omitted if so desired.

(ii) In order to release polysomes with puromycin, add puromycin to a final concentration of 100-500 µg/ml (from a stock concentration of 10 mg/ml in water) to the cells and incubate for 10-20 min. at 37°C before quick-chilling. It is necessary to use high salt polysome and lysis buffers (e.g., 300 mM salt) for puromycin release

(III) Sucrose gradient sedimentaiton.

We typically use 12 ml 15%-45% linear sucrose gradients, although other concentrations of sucrose should be suitable (e.g., 10%-50%). The gradients are poured 2-12 hours before use and allowed to equilibrate in the cold room. Centrifuge tubes are frequently "dusty" so I routinely clean them by soaking them in 0.1% DEPC for 10 minutes, followed by two rinses with sterile distilled water. Typically 200 ul of lysate corresponding to 2-6 x 10^6 HeLa cells or 1-2 x 10^8 reticulocytes are loaded on each gradient. The gradients are centrifuged for 60-90 min. at 3°C in a Beckman SW41 rotor at 38,000 rpm. The length of time of the spin will depend upon which size class of polysomes you wish to resolve. A 90 minute spin will place polysomes of about 4-6 ribsomes near the middle of the gradient, while a 60 min. spin will place polysomes of about 10-mers in this region.

The gradients are collected while monitoring the absorbance at 260 nm. The full scale absorbance on the chart recorder should be set to 0.2 (for reticulocytes) to 0.4 (for HeLa cells). We collect 24 fractions per gradient. I usually monitor the absorbance of a "blank" gradient passed through the flow cell first to get an idea what the background absorbance is.

(IV) Hybridization anaylsis of gradient fractions:

Buffers and solutions:

2 x NaPF: 2 M NaCl
80 mM sodium phosphate buffer, pH 7
12% formaldehyde
1 M sodium phosphate buffer, pH 7

Hybridization Buffer: 50% formamide,
6 x SSC
10 x Denhardt's
0.2 % SDS,
(optional) 50% µg/ml tRNA

6 x Proteinase K buffer: 1.2 M LiCl
60 mM Tris pH 7.6
60 mM EDTA
1.2% SDS

An aliquot of each gradient fraction (100-200 µl) is added to an equal volume of 2 x NaPF, heated to 65°C for 5 min., and cooled to room temperature. The sample is then centrifuged for a few minutes to pellet any insoluble material and then filtered through a nitrocellulose filter using a "dot blot" apparatus (e.g., Schleicher and Schuell Minifold). Before use, the nitrocellulose should be wet in water and then soaked for 5 min in 20 x SSC. After filtering the fractions, wash the wells of the dot-blotter with 0.5 ml of sodium phosphate buffer. The filter is then air-dried, baked at 80°C for 1.5-2 hours, prehybridized in hybridization buffer for 2 hours to overnight, and then hybridized overnight to a nick-translated probe. The filter is then washed at 65°C in several changes of 6 x SSC, 0.2% SDS; 2 x SSC, 0.2% SDS, and then 0.2 x SSC, 0.2% SDS. If the probe is not completely homologous to the RNAS, you may want to skip the 0.2 X SSC washes.

It may be necessary to deproteinize the topmost 4-5 fractions of the gradient if the protein there is interfering with the hybridization signal. This is done by adding one-fifth volume of 6x proteinase K buffer ans 10µl of 10 mg/ml proteinase K, incubating at 37°-50°C for 20-30 min, extracing one time with phenol:chloroform, and precipitating with one volume of isopropanol or 2.5 volumes of ethanol. Deproteinization does not improve the signal in fractions in the rest of the gradient.