EXTRACTION AND ANALYSIS OF MAMMALIAN POLYSOMES

Morris Lab Protocols

The rationale for interpretation of polysome analysis and the strategies for troubleshooting these procedures are given in (Ruan et al., 1997). This document updates the protocols given in the 1997 review, as of July, 2004. The compositions of the reagents are given in Section IV.

I. CELL HARVESTING

The procedure differs according to whether the cultures are grown in suspension or attached and, furthermore, whether the investigator prefers to harvest monolayer cultures by scraping or by digestion with trypsin.

A. Harvesting of suspension cultures

Harvest the cells by centrifugation at room temperature (the number of cells required will differ with cell size). Subsequently, all operations are carried out in the cold room, on ice. The cells are resuspended in PBS+CHX, centrifuged and then washed once in LB. The cells are finally resuspended in 750 μL LB and carried on to Section II.

B. Harvesting of monolayers by trypsinization

This is carried out exactly as described in (Ruan et al., 1997).

C. Harvesting of monolayers by scraping

CHX is added to the cultures to a final concentration of 100 μg per mL and the cells are incubated at 37^{o}C for 10-15 min. After washing the monolayer twice with PBS+CHX, the cells are scraping into the same solution. The cells are harvested by centrifugation, washed once in LB, resuspended in 750 μL LB and carried on to Section II.

II. CELL LYSIS

The procedure here will vary somewhat, depending on whether there is concern as to polysome association with either the cytoskeleton or the endoplasmic reticulum in a particular cell type. This situation is indicated by a significant appearance of rRNA and/or mRNA in the pellet after homogenization. Solutions to these situations are discussed below. In addition, we have had some indication recently that we get an improved yield of polysomes using M-PER® Mammalian Protein Extraction Reagent from Pierce. That procedure is discussed in Section B below.

A. "Standard" Lysis Procedure

1. As noted above, all operations are carried out on ice, in the cold room.

2. To the final cell pellet from Section I, suspended in 750 μL LB, add 250 μL DB. Homogenize with 8 strokes in glass homogenizer (RNase-free).

3. Spin 10 min at 14,000 rpm in microfuge to remove particulate matter, including nuclei. Transfer the supernatant solution into a tube with 100 μL HB and mix gently

4. **Note:** Samples should be frozen of the total lysate, the final supernatant and the pellet for checking RNA if trouble-shooting is required.

5. Dilute 3 μL of the supernatant into 600 μL H_2O and read A_{260}. Optimally, 25-50 A_{260} units are loaded onto the sucrose gradient.

B. M-PER® Lysis Procedure

In this procedure, attached tissue culture cells are lysed directly on the culture dish. We've shown this method to work well with HeLa cells. Alternatively, cells could be scraped into PBS+CHX, centrifuged to concentrate the cells, and lysed in the M-PER lysis reagent with additives.

1. To each P150 culture dish, add 400 μL of the CHX stock solution (Section IV), which has been diluted 1:10 in PBS. Incubate 10- 15 min at 37° C.

2. Wash cell monolayer twice with PBS+CHX (Section IV), and to each P150 add 500 μL M-PER lysis solution prepared as described below. Swirl dishes for ~ 5 min. Collect the cells by scraping and transfer to an Eppendorf tube.

3. Homogenize the cells with 8 strokes in glass homogenizer (RNase-free) and centrifuge at low speed (~3,000 g) and 4° C for 10 min. Remove supernatant to a new tube and snap freeze pellet for subsequent analysis, if trouble shooting is indicated.

4. Spin the supernatants from step 3 at ~14,000 rpm and 4° C for 30 min. Reserve pellet and snap freeze for trouble shooting.

5. Apply the supernatant to a sucrose gradient.

III. SUCROSE GRADIENT CENTRIFUGATION

The gradients are prepared and fractionated exactly as described in (Ruan et al., 1997), except that we are now using 7% to 47% gradients and spinning at 39,000 rpm for 90 min in the SW40 rotor. For preparation of reproducible gradients, it is important to standardize the length of time allowed for diffusion of the two layers. We now use 16 hours.

IV. REAGENTS

Lysis Buffer (LB):

10 mM HEPES, pH 7.5
10 mM KCl
15 mM $MgCl_2$
1 mM DTT
100 μM cycloheximide

Detergent Buffer (DB):

Lysis buffer + 1.2% Triton N-101 + 3.3% DOC

Heparin Buffer (HB):

Lysis buffer + 1.5 M NaCl + heparin (1 mg/mL)

Cycloheximide (CHX) Stock Solution

50 mg/mL in ethanol

Phosphate-buffered saline (PBS):

0.14 M NaCl
5 mM KCl
8 mM Na_2HPO_4
1.5 mM KH_2PO_4
pH 7.3

PBS+CHX:

PBS containing 1:1000 dilution of CHX solution (50 μg/mL final concentration)

M-PER® Lysis Solution:

Final concentration	**Stock***	**For 8 mL M-PER® reagent**:**
10 mM KCl	2M	40 μL
15 mM $MgCl_2$	1M	120 μL
RNAsin (Fermantas)	40 U/ μL	200 μL
1.0 mM DTT	1 M	8 μL

*RNAse free
**M-PER Mammalian Protein Extraction reagent Cat. #78501/78505

REFERENCES

1. Ruan, H.J., C.Y.Brown, and D.R.Morris. 1997. Analysis of ribosome loading onto mRNA species: implications for translational control. *In* Analysis of mRNA formation and function. J.D.Richter, editor. Academic Press, New York. 305-321.