Polysome fractionation with RNA purification

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Material:

Sucrose
Beckman Centrifuge Tubes, 11 x 60 mm (328874)
Cyclohexemide (CHX)
1 x PBS (Phsophate buffered saline)
Ultracentrifuge
SW60 rotor
Fractionator (Teledyne ISCO UA-6)
Phenol : Chloroform : Isamylalcohol (PCI) 25 : 24 : 1 (AppliChem, A0944)
Isopropanol
GlycoBlue (Ambion, AM9515)
Ethanol
RNAse-free water

2 x Polysome buffer
30mM Tris-HCl pH7.4 30mM MgCl2 600mM NaCl

Polysome lysis buffer (for 10 ml)	
2 x Polysome buffer	5 ml
Water	4.8 ml
Triton-X-100	100 □l
CHX (10 mg/ml)	100 □l
complete protease inhibitor (Roche cOmplete Mini, EDTA-free)	1 tablet
RNAsin (Promega)	50 □l
□-mercaptoethanol	10 □l

Solution II (for 100 ml)	
1 M Tris-HCl pH 7.5	1 ml
5 M NaCl	7 ml
0.5 M EDTA	2 ml
10% SDS	10 ml
Urea	42 g
water	ad 100 ml

Preparation of gradients:

> dissolve sucrose in 1 x Polysome buffer: 17.5%, 25.6%, 33.8%, 41.9% and 50%
> start with 790 □l of 50% sucrose per tube
> freeze at -80°C
> continue with 41.9% sucrose, freeze etc.
> store gradients at -80°C

Protocol:

1. On the day before:

> seed cells: they should not be confluent, otherwise they might reduce translation;
for adherent cells, a 10 cm dish per gradient is a good starting point.

> thaw gradients in the cold room overnight (4°C)

> prepare 60 % sucrose

2. Polysome fractionation

> add 100 µg/ml cyclohexemide (CHX) to all dishes

> incubate for 5 min at RT

> optional: wash once with ice-cold 1 x PBS containing 100 µg/ml CHX

> depending on the cell type:

scrape cells off in ice-cold 1 x PBS + 100 µg/ml CHX and pellet by centrifugation at 4°C

or: lyse directly in the dish

> lyse in 350 µl ice-cold polysome lysis buffer per sample

> tumble for 10 min in the cold room

> centrifuge at 9'300 g, 4°C for 10 min (to remove the nuclei)

> take the supernatant into a fresh eppendorf tube without touching the nuclear pellet

> take 30 µl for isolation of total cytoplasmic RNA (into 270 µl polysome lysis buffer + 300 µl Solution II)

> load about 300 µl lysate per gradient (fill up with lysis buffer if necessary; work in the cold room)

> centrifuge for 2.5 hrs, 35'000 rpm at 4°C in a SW60 rotor (Acceleration: 7, Deceleration: 1)

> collect 16 fractions of about 250 - 300 µl into eppendorf tubes that already contain 300 µl of Sol II.

We elute the gradients using an ISCO UA-6 gradient fractionator, which monitors UV absorbtion at 254 nm. Typical settings for fractionation are 14 sec per fraction at 50% pump speed.

3. RNA purification

> add 300 µl Phenol-Chloroform-Isoamylalcohol (PCI, 25:24:1) to each fraction (under the fume hood!) and vortex

> heat the samples for 10 min at 65°C (heating block)

> open and close the eppendorf tubes (under the fume hood) to release the pressure

> centrifuge for 20 min at 17'000 g at RT

> transfer the aqueous (upper) phase (about 500 µl) to a fresh tube and add 1 µl GlycoBlue to each sample. GlycoBlue contains glycogen (to assist RNA precipitation) and a blue dye (so that you can see the RNA pellet after precipitation).

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> add 600 µl Isopropanol

> vortex

> precipitate over night at -20°C

> vortex briefly

> centrifuge for 20 min at full speed and 4°C

> take off and discard the supernatant (carefully with a pipet, do not disturb the pellet)

> wash once with 800 µl cold 70% ethanol

> centrifuge for 10 min at full speed and 4°C

> take off the supernatant again with equal care

> add 20 µl RNase-free water

> dissolve the pellet by heating at 65°C for 10 min and vortexing

4. mRNA quantification

> measure mRNA of interest in the different fractions using qPCR or Northern blot analysis. You can also pool fractions to reduce the number of samples.

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