

Sucrose gradient analysis of polysomes

(As in Monzo et al. (2006) PNAS 103(48):18160-18165)

Extracts:

Embryos (fresh or flash frozen and stored < 3 days optimally) or ovaries can be successfully homogenized as below. Frozen embryos require additional homogenization as specified, and will yield poorer polysome profiles. Ovaries can be dissected in PBS and immediately transferred to cycloheximide-containing buffer on ice over a period of ~ 1 hr. Collect ~50 pairs for one ~12 ml gradient.

Cellularizing embryos were obtained by collecting 1 h, age 2 h 20 min (OR). For *fmr-* embryos aging was 2 h 30 min to achieve the comparable stage of development. Embryos were dechorionated and either used immediately or frozen in liquid nitrogen. Mutant embryos were collected from bottles on grapejuice agar caps with each collection (~10-30 μ g) dechorionated and frozen in a minimum volume of EWB immediately. Multiple collections over a 3-day period were necessary to stockpile the 200 μ l embryos required for enough material to load 2 gradients. Speed during the thawing, pooling, and homogenization was beneficial.

PGB (Polysome Gradient Buffer)

15 mM Tris pH 7.5 @ 4°C

100 mM KCl

10 mM MgCl₂

add fresh

2 mM DTT (from 1 M stock in water, stored -20°C)

200 μ g/ml cycloheximide (from 100 mg/ml stock in 96% ethanol, -20°C)

50 U/ml rRNAsin (Promega)

protease inhibitors 1:100 including PMSF (stocks in ethanol, -20°C)

Procedure:

Prepare:

--Chill dounce, PGB, and gradient buffers

--Thaw protease inhibitors, DTT, cycloheximide

- 1.) Pour 5/20/35/50% sucrose layered gradients made in PGB and let sit (tilted horizontally) 4°C during extract preparation 1.5-2 h. Prechill rotor and centrifuge. Estimated pouring time ~ 45 min.
- 2.) Homogenize embryos using 5 vols PGB (15 x loose pestle followed by 5 x tight for fresh embryos or 7-10 times tight pestle for frozen embryos).
- 3.) Generate post-nuclear supe (3,000 x g, 10 min., 4°C).
- 4.) Centrifuge S3 at 14,000 x g, 10 min., 4°C.
- 5.) Transfer supe (S14) and save pellets on ice (P14). Estimated time from thaw to S14 1 h.
- 6.) Quantitate yields and calculate loads. S14 is typically about 6 mg/ml (range 4-10 mg/ml). Aim for <200 μ l per gradient with ~10 OD₂₆₀ (estimated time 30-45 min).
- 7.) Load on 5-50% sucrose gradients (estimated time 30 min). [Note that no more than a few millimeters of air space are permissible at the top or tube will collapse. Likewise any jostling of the tube will cause your sample to be lost prior to centrifugation.] Centrifuge 2.5 h 40,000 rpm in SW40 4°C, break to 800 rpm. [Label collection tubes during spin as necessary.]

8.) During last 30 minutes of spin prepare and zero fraction collector (using 35% sucrose in PGB or an extra blank gradient). Collect 22 x 0.6 ml fractions (I do this manually by premarking the 0.6 ml level on each tube). Estimated time from end of spin to departure post-cleanup = 1.5-2 h.

Checklists:

Pouring Gradients

Sucrose solutions
5 ml pipet and pipetting device
marker
parafilm
prong rack for gradient tubes
gloves

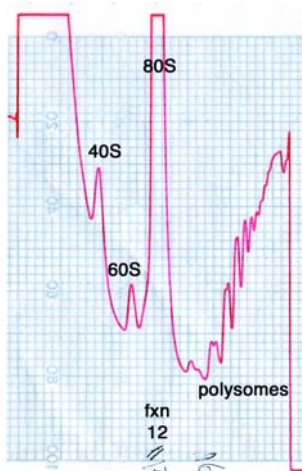
Loading Gradients

protein samples
P1000 tips and p1000 pipettor
notepad and pen
gloves

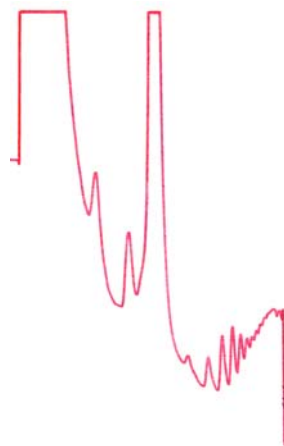
Harvesting Gradients

ice bucket
numbered collection tubes (and glass tube holders if needed)
65% sucrose in water
blanking solution (35% sucrose in PGB)
cleaning solutions for gradient collector (eg. Liquinox, 70% ethanol)
beakers
sheet protector for charts
gloves
electronic data transport medium
notepad and pen

Sample gradient collection traces (A₅₅₄)



fresh embryos



frozen embryos