Polysome Gradients of Mammalian Cells

Pour Gradients
1. Wash chambers with dH₂O → 0.5% SDS → EtOH → dH₂O before pouring
2. Add 5.25 mL of 15% sucrose in left chamber with 15% sucrose and 50% into right chamber.
3. Tubing should be wrapped around outside of wheel pump and clamp should be engaged. Since tubing has a tendency to pull to left, place most of tubing to the left of wheel pump.
4. Turn the valve on the chambers to “Up”
5. Magnetic stirrer in right chamber should be started during sucrose flow
6. Turn probe adjustment to “Up”, this retracts the probe while the gradient is being poured.
7. Adjust the tip of the probe so that it is touching the bottom of the tube.
8. Set speed to 3.5
9. Turn switch to “Deposit”
10. Once poured, keep cold and undisturbed.
11. Use 20 million cells per 11 mL gradient and 50 million cells per 30 mL gradient

Collect Cells
1. Aspirate media and add complete media containing 200 µM cycloheximide
2. Incubate for 15 minutes at 37°C
3. Aspirate media, wash with cold PBS containing 200 µM cycloheximide.
4. Scrape cells into 10 mLs cold PBS with 200 µM cycloheximide
5. Pellet cells at 1500 rpm for 5 min at 4°C
6. Aspirate supernatant
7. Add ~0.25 mL cells per 10 million cells
8. Incubate on ice for 10 minutes
9. Spin for 10 min at 10,000 rpm at 4°C to pellet nuclei and unbroken cells
10. Transfer supernatant to new eppendorf (can be snap frozen and stored at -80°C)

Run and collect gradient
1. Very Gently, Load onto gradient
2. Spin large gradients for 6 hours at 27,000 rpm at 4°C in an SW28 rotor or small gradients for 3 hours at 35,000 rpm at 4°C in a SW41 rotor. (Make sure you know how to use the ultra-centrifuge).

<table>
<thead>
<tr>
<th>Ultra-Centrifuge control panel settings for polysome gradients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temp</strong></td>
</tr>
<tr>
<td>Run</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

3. Allow 30 minutes for the centrifuge to get to temperature
4. Check weights of gradients and make sure they don’t diverge by much.
5. Make sure to wipe off steel sleeves to prevent the tubes from sticking when finished
6. Insert gradients into rotor sleeves and attach ALL sleeves even if you’re not using all of them.
7. Open centrifuge by switching vacuum to “auto” in order to break seal and, simultaneously, depress lid lever and vacuum release button.
8. Close lid and re-engage vacuum by switching it to “manual”
9. Once vacuum gauge reached 500, start the run.
10. When centrifuge reaches 10,000 rpm, switch vacuum back to “auto” or centrifuge will not get to speed.
11. Collect fraction with Brandel gradient collect or continue with hand collection
12. If collecting by hand, pierce bottom of tube with 16 gauge needle and collect 300 – 400 µL fractions for small gradients or 1 mL fractions for large gradients
13. Spec fractions at 260 nm to track ribosomal RNA sedimentation and graph
14. Collect either RNA or Protein from fractions. If you need both, use every other fraction. If you only need either protein or RNA, it is usually best to combine fractions for easier loading on a gel.
15. If you’re using the Brandel gradient collector, make sure you read that manual.

**RNA isolation from Gradient Samples**

1. Bring each sample up to 750 µL with GT buffer + 0.1M β-mercaptoethanol and mix
2. Add 250 µL of acidic phenol (pH = 4.5) and mix
3. Rotate samples for 15 minutes at room temperature
4. Add 200 µL chloroform to each and mix for 1 – 2 minutes
5. Spin samples at 7000 rpm for 10 minutes at 4°C
6. Transfer aqueous layer to new ependorf tube
7. Add 1/10 volume of 3M NaOAc (pH 5.2) to each and 1 volume of isopropanol
8. Mix well and precipitate O/N at -20°C
9. Pellet by spinning at full speed for 15 min at 4°C
10. Wash with 0.5 mL cold 70% Ethanol
11. Air dry and resuspend in 5 µL of dH₂O

**Protein Isolation from Gradient Samples**

1. Thaw samples and bring each up to 1mL with dH₂O
2. Add 100 µL of 100% TCA to each sample. Mix and incubate at -20°C at least 1hr
3. Spin 15 minutes at 4°C at full speed
4. Carefully pipet off supernatant
5. Add 1 mL cold 5% TCA to each sample
6. Vortex and spin samples again at 4°C for 10 minutes at full speed
7. Carefully remove supernatant and add 1 mL of ice cold acetone
8. Spin samples at 4°C for 10 minutes at full speed
9. Remove supernatant carefully
10. Allow to air dry thoroughly on bench top and add 35 µL of sample buffer
11. Mix and vortex. Heat samples at 65°C for 15 minutes
12. If samples turn yellow, add 1 – 2 µL of unbuffered 2M Tris to correct pH and reheat at 65°C. This will be because of residual TCA.

**Reagents:**

<table>
<thead>
<tr>
<th><strong>Cycloheximide:</strong></th>
<th><strong>Lysis buffer</strong></th>
<th><strong>GT Buffer [pH 7.0]</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM stock</td>
<td>400 mM KOAc</td>
<td>4 M Guanadinium Thiocyanate</td>
</tr>
<tr>
<td>15% sucrose or 50% sucrose</td>
<td>25 mM HEPES</td>
<td>25 mM Na-citrate [pH 7.0]</td>
</tr>
<tr>
<td>400 mM KOAc</td>
<td>15 mM MgOAc₂</td>
<td>0.5% N-lauryl-sarcosine</td>
</tr>
<tr>
<td>25 mM HEPES</td>
<td>1 mM DTT</td>
<td>5 mM EDTA [pH 8.0]</td>
</tr>
<tr>
<td>15 mM MgOAc₂</td>
<td>200 µM cycloheximide</td>
<td>Adjust pH to 7.0 with NaOH</td>
</tr>
<tr>
<td>200 µM cycloheximide</td>
<td>1% NP-40</td>
<td>Always add DTT, PMSF, RNase inhibitor and cyclohexamidine fresh.</td>
</tr>
<tr>
<td>10U/mL Rnase inhibiton</td>
<td>0.5% deoxycholate</td>
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<tr>
<td></td>
<td>1 mM PMSF</td>
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<tr>
<td></td>
<td>50U/mL RNase inhibitor</td>
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