**Cushion density gradient ultracentrifugation (CDGUC) purification of small and large EVs**

Written by Nan Hyung Hong (2016), last updated by Weaver lab (2024). This is essentially the procedure from the Raffai lab, published in Li et al., Methods Mol Biol, 1740:69-83, 2018, and modified for serum-free media collection.

Specialized materials required:

3mL ultracentrifuge tubes – Beckman Coulter Cat #362305

Ti45 ultracentrifuge tubes – Beckman Coulter Cat #355622

SW32 tubes – Beckman Coulter Cat #344058

SW40 tubes – Beckman Coulter Cat #344060

Concentrators – Millipore/Sigma Cat #UFC501024

Long metal syringe – Cadence Sci Ref 7942/Fisher Sci 14-825-16N

Prep/Day 1

1. Cells should be plated in their standard media.
   1. Plate with 20mL media for a T175, 30mL for a T225, etc.
   2. You will need *at least* 6, preferably 8 T175 flasks for each condition/cell line.
   3. Based on the cell line’s growth rate, plate the appropriate number of cells such that flasks will be ~80% confluent in 24 hours.

Day 2

1. Check that cells are ~80% confluent.
2. Remove media and wash 3x with PBS (10mL washes for a T175, 15mL for a T225, etc).
3. Add conditioning media - ideally serum-free, same volume as cells were plated in.
4. Incubate for 48 hours, typically. Conditioning time may vary depending on cell line, growth rate, experimental objectives, etc.

Day 4

1. Before starting, make sure ultracentrifuge is on, vacuum is locked, cooled to 4°C.
2. Collect the conditioned media from flasks into 50mL conical tubes, combining for each condition.
3. Centrifuge media at 300xg (0.3 RCF) for 5 minutes to remove cells.
4. Count cells while media is spinning.
   1. For every 4 flasks, count cell number from at least one.

From this point forward, keep tubes, samples, solutions and centrifuges at 4°C / ice cold

1. Transfer supernatant to new 50mL conical tubes and centrifuge at 2,000xg for 25 minutes at 4°C to remove cell debris.
2. Pipet supernatant from conical tubes into Ti45 ultracentrifuge tubes, and spin at 10,000xg in ultracentrifuge for 30 minutes at 4°C.
   1. Ti45 ultracentrifuge tubes hold 60mL, make sure they are at least ¾ of the way full of sample.
   2. *DO NOT* touch pellet when transferring supernatant from conical tubes.
3. After 10,000xg spin, transfer supernatant into 50mL conical tubes and set aside on ice – this supernatant contains the small EVs.
   1. The pellet contains large EVs. Resuspend in 3mL PBS and transfer to 3mL ultracentrifuge tubes.
   2. Centrifuge for 30 minutes to 1 hour at 10,000xg in tabletop ultracentrifuge at 4°C.
   3. Discard supernatant, resuspend pellet in 10-50uL PBS, and transfer to microcentrifuge tube. Store on ice and quantify large EVs by NTA asap.
4. During step 7 large EV spin, wash concentrators with 40-70mL ddH2O and spin at 2,000xg for 2 minutes. Discard wash.
5. Transfer supernatant containing small EVs (collected in steps 6/7) to concentrators and spin at 3,000xg for 5 minutes in tabletop centrifuge at 4°C. Continue spinning until the volume remaining in the top of the concentrator has reduced to ~30mL of supernatant. Collect supernatant and empty flow through.
6. Take off top of concentrator and flip it, place it on top of collector and spin at 3,000xg for 5 minutes at 4°C to collect any residual sample. Pool with supernatant from step 9.
7. Cushion:
   1. Transfer supernatant to SW32 tubes.
   2. Place 2mL of 60% Opti-prep (iodixanol) on bottom of the tubes underneath the supernatant, using a long metal syringe.
   3. Balance tubes and make sure they are nearly full to avoid collapse – should have at least 32mL solution total per tube (30mL supernatant + 2mL Opti-prep). Spin in SW32 swinging bucket rotor in ultracentrifuge at 100,000xg at 4°C for 4 hours (or O/N for proteomics).
8. Collect bottom 3mL from each tube with long metal syringe. Do not combine.
   1. The collection will contain 2mL of 60% Opti-prep and 1mL of small EVs.
      1. Small EVs will be resting on top of the 60% Opti-prep “cushion”.
   2. MIX 3mL LAYERS TOGETHER VERY WELL! When combined, the samples are at 40% final Opti-prep concentration.
9. Make Opti-prep dilutions for density gradients. The following table shows volumes to dilute in order to prepare two gradients:

|  |  |  |
| --- | --- | --- |
|  | Opti-prep/previous Dilution (mL) | Dilution Solution (mL) (0.25M sucrose, 10mM Tris pH 7.5) |
| 20% | 4.5 of Opti-prep | 9 |
| 10% | 6.5 of 20% | 6.5 |
| 5% | 6.5 of 10% | 6.5 |

1. Prepare the 40/20/10/5% iodixanol gradients:
   1. Place the 3mL sample (which is the 40% Opti-prep layer) onto the bottom of the SW40 ultracentrifuge tube, then layer on top 3mL 20% Opti-prep dilution, 3mL 10% dilution, and finally 3mL 5% dilution.
      1. Mix each dilution WELL *before* applying to gradient.
      2. LAYER VERY SLOWLY, DO NOT MIX DILUTIONS TOGETHER.
2. Spin gradients in SW40 swinging bucket rotor in ultracentrifuge at 100,000xg for 18 hours at 4°C.

Day 5

1. Mark on each gradient where the “haze” is (if you can see it).
   1. It is helpful to take pictures of the gradients after the spin against a dark background to document the haze location and appearance.
2. Collect 12 1mL fractions from top of gradient down to bottom into 3mL centrifuge tubes, changing pipette tips between fractions.
3. “Washing fractions”: add 2mL PBS to each 1mL fraction in the ultracentrifuge tubes, mix well and spin for 3 hours in the TLA-110 rotor at 100,000xg in tabletop ultracentrifuge at 4°C.
4. Remove supernatant and resuspend each fraction’s pellet in 10-50uL of PBS.
   1. DO NOT MIX FRACTIONS
   2. There will not be a visible pellet in every fraction so be mindful of which side of the tube is facing toward the outside. It can be helpful to draw a circle on the tube in advance so that you know where the pellet should be.
5. Keep fractions on ice and characterize asap by NTA, protein quantification, etc. Equal protein amounts of each fraction can be mixed with SDS-PAGE sample buffer and frozen at -80°C for western blotting later. Fractions can be stored at 4°C on ice for up to a week for functional assays.