

Cravatt-lab Cell Fractionation Protocol [M. M. Dix, 2008]

Prior to collecting conditioned media (CM), change cells to serum-free (SF) media at 80%-100% confluency. To change media, wash 3x with PBS to avoid contamination, then add SF media. The confluency at which cells are changed to SF media varies with each cell line. Ideally cells will be 100% confluent with out too many dead or lysed cells at the time of harvesting.

After ~48hours incubation in SF media, cells and the conditioned media (CM) are collected as follows.

1. Collect CM: pour off media from plates into 50mL conicals on ice.
2. Tilt plates and transfer the remaining CM with a pipette (for 10X 15cm dishes using 5 conicals, filling them to about 40mL each works well).
3. Without allowing the cells to dry out, rinse the dishes 3x with PBS, about 8-10 mL each wash.
4. Scrape cells using a cell-scraper/rubber policeman with about 8mL of cold PBS, transferring the scraped cell suspension to each new plate until all plates have been scraped and pooled.
5. Transfer the final cell suspension in to a 50mL conical on ice.
6. Pellet the cells at 1,400 x g (2,400 RPM) for 3 min at 4°C. Discard the supernatant and resuspend the pellet in appropriate volume of PBS.
7. This can be stored at -80 or you may continue with the prep (see cell prep section below).

Conditioned Media (CM) preparation:

8. Clarify the CM by centrifuging out any dead cells: spin the 50ml conicals containing the serum free media previously collected in step 1 at 2,800 x g (3500rpm) for 5 min at 4°C.
9. Take the supernatant from step 8 and spin in a high speed centrifuge at 100,000 x g for 45 minutes (24,000 RPM with SW-28 rotor)
10. Take the supernatant and precipitate CM proteins using **ammonium sulfate**: 0.561 g of ammonium sulfate/mL of CM (80% cut). We usually do this by measuring the total volume of clarified CM then add it to a beaker along with the ammonium sulfate.
11. Stir in the cold room until ammonium sulfate is completely dissolved, remove the stir bar and transfer the beaker into a ice bucket in the cold room over night (at least 6 hrs).
12. Centrifuge the CM for 30 min at 37,000 x g (16,500 RPM with JA-17 rotor) at 4°C. If volume is large, decant supernatant after spinning and add additional CM to the same tube and repeat.
13. After all the media is spun, discard supernatant and do a final quick spin to remove any residual supernatant.
14. Resuspend the pellets in ice-cold PBS to a final volume of 2.5mL (note: the pellet may be very small or invisible. Keep track of where the pellet *should* be by marking the tube).

15. Equilibrate a PD-10 column (Biorad) with 25mL of ice-cold PBS.
16. Apply the 2.5mL of CM to the column. Discard flow-through.
17. Elute with 3.5mL of PBS. Collect the eluate in a spin concentrator (10,000 MW cutoff, Amicon Ultra) and spin at high speed (3,200 x g) for ~15 minutes.

Particulate and cytosol preparation:

18. Move the cell suspension (from step 7) into a thick-walled microcentrifuge tube and do 3 x 10 second pulses with a probe sonicator on ice (in the cold room). Keep sample on ice between each 10 second pulse.
19. Spin at 145,000 x g (64,000 RPM with TLA 100.3 rotor) for 45 min at 4°C. The pellet is the particulate (membrane) fraction, the supernatant is the cytosol.
20. Move the cytosol to a new tube. Wash the pellet with 2x 1mL PBS. Resuspend the pellet in appropriate volume of PBS. Sonicate briefly to resuspend. This is the particulate fraction.

Optional: The particulate fraction can be **solublized** in 1% TX-100 by rotating for 1 hour at 4°C, followed by a 45 minute 100,000 x g spin). The supernatant from this spin is the solublized membrane fraction.

21. Determine protein concentration and use 200µg of each fraction to run on a gel for PROTOMAP analysis.

Optional: Deglycosylate the particulate and CM fractions to increase resolution:

PNGaseF treatment (using enzyme from NEB):

1. Place 50µL protein in a microcentrifuge tube.
2. Add 5.5µL of denature buffer to each tube and boil 8-10 minutes on 90°C heat-block.
3. Spin down and cool on ice
4. Add 5.5µL NP-40
5. Add 5.5µL G7 buffer and 0.2µL (per 50µg) of PNGaseF
6. Incubate at 37°C for 30-45 minutes
7. Add SDS loading buffer
8. Boil 8-10 minutes on 90°C heat-block, cool on ice
9. Load directly on gel or store at -20°C until ready to run gel.