ISAKSON LAB

Caveolin Cellular Fractionation

Grow up cells on 10-cm round dishes to confluence. For HCoSMCs, serum starve for 48 hours at 80-90% confluence.

Stimulate as necessary.

Aspirate off media and add ice-cold detergent-free lysis buffer, moving the plate onto ice to scrape off and pipet cells + LB into a microcentrifuge tube. Suggested volume of LB: 750uL, moving cells + LB from plate to plate to harvest multiple plates per condition.

Dounce homogenize cells with 20 strokes, twisting and grinding.

Sonicate 20 x 1 second bursts, leaving cells on ice to keep them cold. Greater time between sonication bursts allows cells to remain cold throughout sonication, which is high energy.

BCA assay to determine protein concentrations of samples; dilute more concentrated sample down to the concentration of the less concentrated one.

Turn on ultracentrifuge and turn on vacuum for it to cool to 4 degrees C.

Add equal volumes of each to ultracentrifuge tubes in parallel (suggested 750uL), mixing 1:1 with 85% sucrose. This is the bottom 42.5% layer – 1.5mL total. Pipet very gently 1mL of 30% sucrose on top of this layer, mixing as little as possible. Pipet very gently 1mL of 5% sucrose on top of everything, mixing as little as possible. Total volume should be identical for each condition, total volume 3.5mL.

Turn off ultra's vacuum, open, and load rotor into ultracentrifuge, double checking that the rotor is loaded in a balanced manner. Turn back on vacuum and run overnight, 16 hours at 4 degrees C, at 240,000 rpm, with slow acceleration (e.g. setting 3) and no brake. This will add roughly 45 minutes to total run time.

Prepare microcentrifuge tubes for each of the 10 fractions to be produced from each sample, ahead of time.

(Day 2) Remove samples from ultra very gently, jostling them as little as possible. Pipet out 250uL at a time from the top of the gradient in each sample, numbering each fraction 1-10 from top/lightest (1) to bottom/heaviest (10). Take care to pipet off gently enough to avoid creating a convective current that mixes the fractions, to preserve their resolution.

Mix 20uL of each fraction with 5uL of 5x loading buffer. Boil 3 min at 95 degrees C. Run on western and blot for proteins of interest. Be sure also to blot for cell compartment markers, e.g. transferrin receptor for membrane or caveolin 1 for caveolar/lipid raft membranes. The fractions have no specific location marker on their own, only in comparison to the known markers that fractionate out alongside proteins of interest, so be sure to know what you need to indicate in order to draw conclusions on the question at hand.

Fractions can be co-IP’d to determine whether two proteins only interact in a certain context or cellular location. Be sure to use enough total protein – e.g., 4 plates per condition for SMCs looking for a caveolin-Panx1 interaction, to yield ~400ug of total protein from which to IP.

Fractions can also be useful for determining localization of PTMs such as phosphorylation. Compare phospho banding to total banding pattern for the protein of interest, using the total protein of interest as the control for phospho protein of interest across fractions.