293 N3S and MRC5 Phase Partitioning

The basic principle of the detergent phase partitioning method is to solubilize the membranes (P100) in the detergent Tx114. Cytoskeletal components (CSK) of the membranes are not soluble in the detergent and are pelleted out by centrifugation. Following phase separation at 24oC centrifugation can be used to separate hydrophobic membrane proteins contained in the detergent phase (DET) from the luminal contents and membrane skeleton components Finally a last centrifugation step at 150.000 x g separates the aqueous phase (Aq), which consists mainly of soluble hydrophilic proteins, and the membrane skeleton phase (MSK).

1. To 25ul P100 in Dog Buffer C add:
    - 1ul DTT (of 1M DTT)
    - 63ul 2x solubilisation buffer
    - 10ul PIN (of 200x PIN)
    - 10ul PMSF (of 100 mM PMSF)
    - incubate for 5 min on ice
    - add 37ul H20
    - spin 10 min in ufuge at 6000 - 8000 rpm @ 40C.
2. Keep the pellet this is the cytoskelal components, CSK.
3. To further fractionate the supernatent:
    - layer on 300ul sucrose cushion
    - incubate 3min at 300C (upper layer should turn cloudy)
    - centrifuge 3 min at 3000 rpm in ufuge (240C)
4. Remove supernant (100ul) and add to it:
    - 10ul PIN (of 200x)
    - 10ul of 10% Tx114 (Tx114 diluted in MQ water)
    - incubate 3 min on ice (should turn clear)
    - layer back on same sucrose cushion (see cushion of step 3)
    - incubate 3 min at 300C
    - spin 3 min at 3000rpm at room temperature (24oC)
5. Keep the bottom, detergent phase. See 8 for further processing.

    Remove supernatent (200ul) and add:
    - 10ul PIN
    - 20ul of 10% Tx114
    - incubate 3 min on ice
    - layer on new cushion (300ul)
    - spin out detergent 3 min at 3000rpm @ 240C
    - discard detergent phase (bottom) and keep supernatant (270ul)

6. Spin supernatant fractions 1hr at 30psi in the airfuge. Keep pellet, this is the membrane skeletal fraction: MSK Keep aqueous phase (sup)
7. Add 1/2 vol 50% TCA to supernatant and then:
    - incubate 15 min on ice
    - spin 15 min in the microfuge, full speed, 40C, discard supernatant
    - add 500ul of ethanol/ether (50:50 vol:vol) to the pellet (this step washes out the remains of the TCA but be careful with ether it is highly flammable
    - vortex
    - spin 15 min/40C in ufuge
    - discard sup
    - keep pellet this is the aqueous phase (Aq.)
8. To the bottom detergent phase from step 4
    - resuspend in 500ul wash buffer (+ 1x PIN, + 1 mM PMSF)
    - incubate 3 min on ice
    - layer on new cushion (600ul)
    - Spin 3 min/3000rpm in ufuge @ 240C, discard supernatant
    - resuspend pellet in 500ul wash buffer
    - add 1/2 vol. 50% TCA
    - incubate 15 min on ice
    - spin 15 min full speed in ufuge @ 40C
    - wash pellet with ethanol/ether (vol:vol/50:50) as in step 7 above.
    - vortex
    - spin 15 min/ufuge/full speed @ 40C, discard supernatent.
    - keep pellet this is the detergent phase: Det.
9. Solubilize each pellet in 80ul of tricine gel loading buffer and run 20ul/well on a Tricine SDS protein gel

Sucrose cushions	1X Solubilization Buffer	Wash Buffer
6% Sucrose	10mM Tris/HCl pH 7.4	10mM Tris/HCl pH 7.4
10mM Tris/HCl pH 7.4	150mM NaCl	150mM NaCl
150mM NaCl	5% Glycerol	
0.06% Triton X114	1% Triton X114	
1% Glycerol		