Cell Fractionation Assay (Lipid raft flotation assay)

1. Day1 : Transfect 293 cells with LMP1 use 25ul lipofectamine
   5-6ug 1990 (Or 1281 ; Ngan says 1990 is better for wtLMP1) or 2492 SubLZ1LMP1
   2ug GFP (2134 or 2145)
   1ug Gαi-RFP (2513)
2. Day2 : Check the transfection efficiency by GFP or RFP
   Put the rotor and tubes at 4ºC for o/n
3. Day3 : Harvest 3×10^7 cells from 15cm dishes (It is O.K. to harvest at day4)
4. Lysis the cell pellet in 1ml ice-cold TritonX-100 + TNE buffer
   150mM NaCl
   25mMTrisHCl pH7.5
   5mM EDTA
   1%TritonX-100
   Protease Inhibitor Cocktail –stock is usually 100X
   Incubate on ice for 30'
5. Dilute 1:1 with 70% Nycodenz (Histodenz from Sigma) in TNE buffer (use the same conc. as 4) so final conc. would be 35%
6. Load sample carefully on the bottom of 5ml ultracentrifuge tube (Beckman SWi50)
   Let sample follow the wall of the tube.
7. Overlay with 400ul 25%, 22.5%, 20%, 18%, 15%, 12%, 8% Histodenz in TNE from bottom to top. (Histodenz gets dissolved easily in TNE buffer)
8. Fill the tube with 8% Histodenz in TNE to the neck of the tube (leave 2mm)
9. Apply grease inside the rotor tube and on the cap of the rotor tube.
10. Carefully put your sample tube in the rotor. Make sure the number on the cap matches the number of the rotor.
11. Spin at 45,000rpm (or 200,000g on average) in the ultracentrifuge at 4ºC for 4hrs
    (As long as you rotor was in the frig for o/n, it is O.K. if the temp. of centri. gets higher for an hour.)
12. Carefully collect fractions #1-13 , 400ul each from the top.
    13^{th} fraction would include the pellet, you can sonicate, if necessary.
13. Use 10ul of each fraction for SDS-PAGE
    Use 9-10% SDS-PAGE gel for LMP1, 12% gel for GFP and Gαi-RFP(30kD)

1º	2º
Anti-GFP 1:100 Clontech	rabbit-AP
Anti-RFP 1:16,000 Clontech	rabbit-AP
Anti-LMP1 1:500 DAKO	mouse-AP

* Concentrating protein using TCA method.
1. add 1ml 10%TCA
2. 0ºC on ice 10'
3. vortex, spin at max speed 10' remove supernatant
4. wash with 0.5ml acetone kept in -20ºC
5. vortex , spin at max 5' remove supernatant
6. wash with 0.1ml acetone kept in -20ºC
7. vortex, spin at max 5' remove supernatant
8. add SB to pellet and boil to use for SDS-PAGE