$PIP_{2/3}$ Pulldown from 293 cell lysates
(Thymocytes: Lyse 2 x 10^7 cells in 200ul lysis buffer: begin at step 9)

1. Add 5 ml of PBS+EDTA to 293T TC plates and incubate for 5-10 min at 37C
2. Resuspend cells and add to a 15ml conical tube
3. Count cell in all samples
4. Spin at 1500 for 5 min
5. Resuspend at 2 - 4 x 10^6 cells/ ml in PBS
6. Aliquot 1 ml to an Eppendorf tube on ice
7. Spin at 1500 for 5min and aspirate off all fluid
8. Resuspend in 1ml lysis buffer ensuring that the volume is precise and that no air bubbles occur
9. Incubate on ice for 15min
10. Fast cool the centrifuge to 4C
11. Spin down samples for 15min at top speed
12. Take off supernatant and add to a separate tube
13. Distribute 180ul of supernatant into labeled tubes when pipetting up the liquid pipette back into the tube the first time and then start distributing
14. Wash PIP beads (Echelon)
    a. Vortex bottle of beads before pipetting up beads (always wash 50ul more beads than needed for the samples)
    b. Dispense beads with wide mouthed pipette tips into an eppendorf containing 500ul of lysis buffer and vortex.
    c. Spin down beads at 800 rpm for 1min (Do not spin at higher speed as this will collapse beads)
    d. Take of supernatant
    e. Check how many microliter of beads are in the tube and add the same volume of lysis buffer to the tube to make a 50% lysis buffer slurry
15. From this mixture, add 18 ul PIP_3-coated beads to tubes containing whole cell lysate
16. In between each addition, vortex the beads and take beads from the middle of the tube using a wide mouth pipette tip
17. Rotate in the fridge for 1hr 15min – 1hr 30min. Longer incubations will result in more protein binding and will alter the enhancement/displacement ratios
18. Spin down eppendorfs at 1000rpm for 1 min, then turn tubes 180 degrees in centrifuge and spin for another 30 secs to pellet beads evenly to the bottom of the eppendorf.
19. Remove lysate by pipetting.
20. Add 500 ul lysis buffer, vortex to wash. Spin at 1000rpm for 1 min, turn tubes and spin again for 30sec.
21. Repeat wash twice
22. After the third wash aspirate the remaining lysis buffer carefully but completely
23. Make sample buffer: mix ¾ volume lysis buffer, ¼ volume loading dye and x vol DTT (0.75M = 15X)
24. Add 45ul of loading buffer to samples
25. Store in -20C freezer until ready to run western