Thymocyte stimulation and co-immunoprecipitation

1. Harvest thymuses and make a single cell suspension in Medium 199-2 by grinding thymic lobes on a 40um cell strainer using the end of a 1 ml syringe
2. Strain cells into a 50ml conical tube
3. Count cells
4. Rest cells by incubating conical in 37C waterbath for 15-30 min. Afterwards, immediately place conical on ice for 10 min.
5. spin at 1500 for 5 min resuspend at 2 x 10^7 cells/ ml in cold PBS
6. Aliquot 1 ml to an Eppendorf tube on ice
7. spin at 1500 for 2 min and aspirate off all fluid
8. resuspend in 400ul cold PBS with no stimulating antibodies or 2 ul anti-CD3-biotin +/- 2 ul anti-CD4-biotin. Incubate on ice for 15-30 min.
9. Add 1 ml cold PBS to eppendorfs and spin at 1500 for 2 min and aspirate off fluid
10. Repeat wash and stimulate immediately following aspiration of wash buffer
11. Resuspend in 200 ul warm PBS containing 1 ug/ml streptavidin for desired time
12. Add 70 ul 4x lysis buffer containing protease and phosphatase inhibitors, ensuring that the volume is precise and that no air bubbles occur
13. Incubate on ice for 15min
14. Fast cool the centrifuge to 4C
15. Spin down samples for 15min at top speed
16. Take off supernatant and add to a separate tube
17. Distribute 200ul of supernatant into labeled tubes for precipitation and 45ul of supernatant into separate tubes for WCL. (For WCL tubes only: add 15ul 4x NuPAGE sample buffer + 3.75ul 0.8M DTT per tube)
18. Wash protein G beads (no need to wash PIP beads)
    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
19. From this 50% bead/buffer slurry, add 18 ul PIP or protein G beads to tubes containing the 200ul supernatant. For immunoprecipitation, add antibody (typically 0.5 ul – 2 ul).
20. In between each addition, vortex the beads and take beads from the middle of the tube using a wide mouth pipette tip
21. For PIP ppts: rotate in the fridge for 1hr 30min. Longer incubations will result in more protein binding and will alter the enhancement/displacement ratios
22. For Immunoprecipitations: rotate in the fridge 2 hr to overnight.
23. 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.

24. Remove lysate by pipetting.
25. Add 500 ul lysis buffer, vortex to wash. Spin at 1000rpm for 1 min, turn tubes and spin again for 30sec.
26. Repeat wash twice
27. after the third wash aspirate the remaining lysis buffer carefully but completely
28. Make sample buffer: mix ¾ volume lysis buffer, ¼ volume loading dye and x vol DTT (0.8M = 16X)
29. add 45ul of loading buffer to samples
30. store in -20C freezer until ready to run western