

## THYMOCYTE ISOLATION AND IMMUNOPRECIPITATION

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### I. Make thymocyte suspensions.

1. Dissect mouse under sterile conditions.
2. Place thymus into approx. 2 mls of (DMEM, without methionine, without cystine) while cleaning up the mouse and preparing for the next step.
3. Place a sterilized piece (approx. 1 in X 1 in) of regular window screen with folded edges in one-half of a petri dish.
4. Dump thymus (and the 2 mls Media - Met) on top of the screen.
5. Pull apart a 5 ml syringe; and with the sterile rubber-tipped plunger portion, squash the thymus through the screen to disperse thymocytes into the media - met. Some connective-type tissue will remain on the screen.
6. Wash screen with approx. 4 - 5 mls of Media - Met. Remove screen.
7. Suck up thymocytes using a sterile 10 ml syringe (no needle).
8. Release thymocytes into a sterile 15 ml blue-capped conical tube through a sterile guaze filter (it removes the misc. tissue gunk).
9. Spin down the thymocytes....in Sorvall table-top centrifuge. Spin at room temperature for 10 min. at 1 - 1.5 X 1000 rpm.
10. Aspirate off the Media - Met.
11. Store in 5% CO<sub>2</sub> incubator if you have to, but better to use them immediately.

### II. Labeling thymocytes.

12. Resuspend thymocytes in 2 - 3 mls of DMEM, without methionine, without cystine, + 10% dialyzed fetal calf serum + antibiotics; ICN inc. (same media - met as above)
13. Pipet up and down to resuspend pellet.
14. Transfer thymocytes to a 3 cm tissue culture dish.
15. Starve cells for 1/2 hour in 5% CO<sub>2</sub> at 37°C.
16. Add 0.5 - 1 mCi <sup>35</sup>S-methionine to the dishes. (Yue keeps his <sup>35</sup>S-Met in 0.5 mCi aliquots.
17. Keep dishes in radioactive plexiglass bin in 5% CO<sub>2</sub> incubator (37°C).
18. Shake dishes now and then while labeling.

### III. Lysis of thymocytes.

19. When the labeling is complete, take the plate to the fume hood in the equipment room. Use the radioactive pipettors to resuspend the thymocytes (pipet up and down) and to add the thymocyte suspension to two 1.75 ml sterile Eppendorf tubes.
20. Spin down the cells in the RADIOACTIVE centrifuge (5 min, RT, 2 X 1000 rpm).

21. When pellet obtained, aspirate off the labeling media (IT HAS <sup>35</sup>S !!) into the radioactive flask. Rinse the pellets with 0.5 ml cold PBS, and add the resuspended pellet from one of the Eppendorfs to the other.
22. Spin as in step 20.
23. Aspirate as in step 21. Add 1 ml Lysis Buffer per pellet of thymocytes.

Yue's Lysis Buffer:

50 mM Tris-Cl (pH 7.5)  
150 mM NaCl  
0.5 % NP-40  
50 mM NaF

before use, add:

1 mM NaVO<sub>3</sub>  
1 mM DTT  
1 mM PMSF  
1 X Protease inhibitor

24. Let the tubes rotate in the cold room for 30 mins.
25. Spin in the Eppendorf centrifuge in the fume hood at max speed for 5 to 10 min. to remove nuclei.

#### IV. Pre-clearing the Extract.

26. Add 50 to 100 ml protein A beads (pre-washed in PBS) or activated Staph Protein A heat-killed bugs (BMB) to a new 1.5 ml Eppendorf centrifuge tube and transfer the supernatant into the tube. Rotate the tube in the cold room for 20 - 30 mins.
27. Centrifuge for 5 min. at low speed (4000rpm). The sup is now ready for the IP.

#### V. Immunoprecipitation.

28. Add desired antibody to a 1.5 ml Eppendorf tube and then add labeled cell lysates. Incubate the antibody with cell lysates for 2 hrs. to overnight at 4°C with rocking.
29. Add pre-washed protein A beads (approx. 5 ml beads) or Staph A bugs..depending on what you are using... to the IP tubes. Rock the tubes for 1 hour in the cold room.

#### VI. Washing the Immunocomplex.

30. Wash 3 times with cold lysis buffer (without protease inhibitors). Use 1 ml for each tube. If you are using Staph A bugs, you can spin at max. speed and vortex between washes. If you are using Prot. A beads, USE A VERY GENTLE SPIN...2000 rpm/3 mins. and DON'T VORTEX!!!!!! Abusive vortexing will smash the beads. Oh, and another thing, when aspirating off the wash media, use a very narrow guage tip (like the one used to load sequencing gels) or else you'll suck up the beads.

31. Add 10  $\mu$ l 1.5 X DTT-SDS (or b-Mercapto.-SDS) sample buffer, boil for 3 mins, spin down (GENTLY) in the Eppendorf centrifuge for 30 secs., and load the supernatant onto an SDS-PAGE.