

Immunoprecipitation and Western

1. Determine how many samples you will need and make up the appropriate amount of lysis buffer. I usually plan for 1.5 - 2.0 mls of lysis buffer per tissue sample (Note: if you're using brain tissue, plan for a bit more Ly.Buf. than the others b/c there will be alot of cells in that extract, and you'll be centrifuging it more than the other extracts).

Lysis Buffer

150mM NaCl..... 1.5 mls 5M NaCl
50 mM Tris-HCl. pH8.... 2.5 mls 1M Tris
5 mM EDTA0.5 mls EDTA
0.5% NP40.....2.5 mls of 10% NP-40

.....up to 50 mls

**Right before use (when total amount is measured out),
add protease inhibitors: I use PMSF, Leupeptin, and Pepstatin
amount of inhibitors...see BMB protocols

2. Use razor blade to piece tissues. Then used about 0.2 g of tissue (comparable to 1/2 a mouse brain). I found that this amount was rather high for some the protein levels...for example: dl1135 mice..only take a 1/2 cm square piece of thymus for T-ag expression b/c anything more will give you boatloads of protein. I guess the bottom line is...trial and error will tell you exactly how much tissue to start with per line of mice. Note: for choroid plexus (which is only 0.5% of the entire brain, take at least 1/2 a brain...sometimes it seems if I only have half a brain). Put the tissue pieces separately into disposable 15 ml centrifuge tubes filled with the 1.5 - 2.0 mls of protease inhibitor-treated Ly. Buff.

3. Homogenize tissues on ice. Clean homogenizer with SDS, ethanol, and dH₂O between samples.

4. Sonicate tissues on ice. Clean the machine the same way.

5. The sonicated samples in the 15ml tubes can be centrifuged directly at about 3000rpm for about 10-15 mins. While they're spinning, label 1.75 ml Eppendorf tubes with sample name. Transfer sups to tubes. Brain samples and other ones that are cloudy will have to be spun again. Save about 10uls for Bradford protein analysis.

6. Preincubate with activated *Staph. aureus* cells....(follow the TVD protocol for Immunoprecipitation for preparation of the *S. a.* cells). Use 30-50 uls *S.a.* for clearing depending upon how much extract you have. Preincubate for at least 15 mins. on ice.

7. Spin at high speed in Eppen. centrifuge.

8. Add antibody: monoclonals = 300uls... or more depending on how sure you want to be in clearing the stuff. shake the tubes to mix now and then.
9. Incubate on ice: at least 1 hour. (Supposedly, the longer; the better, but I think one hour should be sufficient.).
10. Add activated *Staph. a.* to bring down the immunocomplex. About 25 - 40uls should be sufficient to be in *S.a* excess.
11. Incubate of ice for 15 - 30 mins. Spin for 4 mins in Eppendorf.
12. Wash immunocomplex; 2x with SNTE, 1x with NTE....see Immunoprecipitation protocol for full details.
13. Resuspend pellet in 30 uls protein sample buffer.
14. Run 11 uls on 12% polyacrylamide gel.
15. Transfer to nitrocellulose o/nite...I use Costar's Protoblot nitrocellulose. Transfer at 25 volts with a stir-bar.

Electroblotting buffer:

20mM Tris/150mM glycine

(ie. add 7.25g of Tris to 34g glycine and stir in 2 liters of dist. aqua. pH to 8.0 with HCl, add 600mls of methanol, and bring up to volume to 3 liters).

16. The next morning...

Air dry the filter, then rinse it in TBS....

	<u>10x TBS</u>
2M NaCl	(200mls 5M)
500mM Tris-Cl	(250mls 1M Tris)
pH 7.4	
	up to 500 mls

17. Block the filter with BSA (some people use dry milk, but I find that BSA is rather clean

block for 1 hour, shaking at 37 degrees C.

NOTE: for this step, #19, and #21, I use the heat-seal (seal-a-meal) bags with the liquids and filters sealed inside. For the washes, I just use tupperware.

18. Wash filter 3x with TBS and 0.1% Tween-20, and once with TBS (no tween-20)
19. Incubate 1 hour at rm. temp. with blot Aby...

Pab 412: use 1:20 in TBS
Pab 421: use 1:5 in TBS
Pab 2C2: use 1:15 in TBS
20. Rinse filter as in step 18.

21. Incubate with $2\mu\text{Ci } ^{125}\text{I}$ in 20 mls of TBS. at rm temp. for 30-45 mins.
22. Wash as in step 18.. Careful!, the rinses will be hot and should be discarded appropriately.
23. Air dry filters totally, and expose o/n against film (with a screen).