Two step TAP-Tag purification

NOTE: This is the full two step protocol which gives very clean samples (as used for the proteomic papers when you want to Mass Spec and ID complex components). However the yield sucks (most of it remains on the calmodulin beads) so protocol is not recommended for making active enzyme complexes for functional studies (**Use the 1 step protocol**).

Section I. Cell growth and lysis

1. Grow 1.5L cells in YPD to OD_{600} of 1 - 1.5
2. Collect cells by centrifugation (4000 rpm, 10', 4°C)
3. Wash in cold ddH_2O and transfer to 50 ml Falcon tubes
4. Spin cells 4000 rpm, 4', 4°C
5. Weigh cell pellet and wash with 20ml YEB + protease inhibitors

6. Spin cells 4000 rpm, 4', 4°C. Snap freeze the pellet in liquid N_2 and store at –80°C. (long term storage (couple of months) is possible).

Need ready: Liquid N_2
Dry ice pellets
Cleaned coffee grinder

7. When ready, take the tube from –80°C and drop it into liquid N_2 to make it nice and brittle. Wrap in some paper towels and smash it with a hammer. The pellet has to be smashed into relatively small pieces - one huge piece won't grind down efficiently.

SAFETY: Plastic and yeastie bits will fly everywhere – wear lab glasses.

8. Transfer the yeast fragments (avoiding the plastic) to the prechilled coffee grinder (grind some dry ice pellets to cool it down). Add 25% dry ice per pellet volume - pulse approx 90sec, scrape down powder with spatula and pulse again. **Don't let powder thaw.** Scrape the lysed powder into a 50ml Falcon on ice (leave lid off to allow the remaining CO_2 to sublime). (**NB.** this is important: if you add the buffer too early it will freeze and you'll have to wait until it thaws before you can move on)

NOTE: If using grinder for multiple samples **DO NOT CLEAN WITH WATER**, (it will freeze) - wipe clean with paper towels.

II. Protein A module purification (all @ 4°C - cold room)

9. Add 0.8 volume of YEB + protease inhibitors and thaw at RT°. Transfer sample to ultra tube and balance to 2 decimal places. Spin down extract at 4°C, 35000rpm, 60'. and collect supernatant. Dialyze down to 100 mM salt (2 x 1hr x 1L dialyses @ 4°C)

10. Spin down extract after dialysis to remove any precipitates: 35000rpm, 30', 4°C.

11. Transfer supernatant to a 15 or 50 ml falcon tube containing 250µL of IgG Sepharose mix that has been washed with 2 X 5 mL of IPP buffer. Rotate for 3 hours at 4°C.

12. Spin down the IgG sepharose (1500rpm, 5', 4°C) and discard supernatant.

13. Transfer the beads to a 2mL column (BIO-RAD cat. # 732-6008) using P1000 tips that have been clipped at the ends. Make sure the resin packs without air bubbles.

14. Wash 5 x 100µL IPP buffer

15. Wash 2 x 100µL TEV cleavage buffer

16. Close the bottom of the column and add 200mL TEV cleavage buffer and 100U TEV protease. Close top and rotate O/N at 4°C (or 16°C for 1-2 hours). **Re.** Protease inhibitors, add Aprotinin, Leupeptin, Benzamidine HCL, Pepstatin A and Antipain. Do not use PMSF.

NOTE. Unless you buy the super-expensive TEV from Invitrogen (or whatever they're called these days) you will have to determine the home-made TEV amount empirically (see MCK lab notebook pH13.56 and TEV protocol). The enzyme is usually so active that four hours at 4°C should go to completion. It is possible to determine this by αPAP (Sigma) reactivity in westerns – you're looking for the disappearance of the TAP-band. You can also use the αCBP antibody (although I'm not a huge fan of the latter).

III. Calmodulin column (all @ 4°C - cold room)

17. Remove the top and bottom plugs and collect eluate into an ependorff tube by gravity flow. Wash the column with 200µl TEV cleavage buffer and collect this too.

18. To the eluate add 1 vol of Calmodulin Binding Buffer (CBB) and 3µL 1M $CaCl_2$ / mL of IgG eluate

19. Transfer this to a 2mL column containing 200µL of **Calmodulin beads** washed with 2 X 5 mL of CBB and rotate at 4°C for 2-3 hours

20. Wash with 500µL CBB

21. Wash with 200µl Calmodulin Wash Buffer (CWB)

22. Elute with 5 or 6 x 100µl fractions Calmodulin Elution Buffer (CEB)
NB. do not use detergent if going to analyze by electro-spray mass spectrometry

NB. This seems to be the major stumbling block in the protocol - specifically releasing the complexes from the resin. Two options may improve your yield - increase the salt or increase the EGTA concentration. Try the following elutions (2 x 100µl fractions for each elution buffer).

1. 100mM KAc, 5mM EGTA
2. 100mM KAc, 10mM EGTA
3. 100mM KAc, 20mM EGTA
4. 150mM KAc, 20mM EGTA
5. 200mM KAc, 20mM EGTA

However this is probably a waste of time – if you're planning a quick enzymatic screen then the **one step TAP protocol** is good enough. If you're planning an MS-ID of the complex then purity is your prime concern, yield is secondary.

23. SDS-PAGE and Western analyse directly (10µl per fraction) or TCA precipitate 50µl protein and silver stain to visualise.

IV. TCA precipitation

24. To the eluate, add sufficient cold 20% TCA to bring to 8 % and let sit on ice for 30 minutes

25. Spin microfuge 14000rpm, 30' 4°C

26. Resuspend in 80µl 2X sample buffer and then 40µl of 1M Tris base (or half into sample buffer and half into Emily's trypsin buffer). Usually load 60µl onto a big 10% gel. Or alternatively, dry the sample down using a lyopholizer or speed-vac and then bring up dried sample in sample buffer and load on gel.

V. Buffers (NB. All contain protease inhibitors before use as standard; except see #16)

YEB Buffer
245 mM KCl
1 mM EDTA
5 mM EGTA-KOH (pH 7.9)
100 mM Hepes-KOH (pH 7.9)
2.5 mM DTT

Dialysis Buffer
100 mM NaCl
10 mM Tris-HCl pH 8.0
0.2 mM EDTA
20 % glycerol
0.5 mM DTT

IPP100	For 100 ml:
10 mM Tris-Cl ph 7.9	1ml 1 M Tris-Cl pH 7.9
100 mM NaCl	2ml 5 M NaCl
0.1 % Triton-X100	1ml of 10% stock

TEV Cleavage Buffer:	For 10 mL:
50 mM Tris-Cl pH7.9	500µl 1 M Tris-Cl pH7.9
0.2 mM EDTA	4µl 0.5 M EDTA
1 mM DTT	10µl of 1 M DTT
0.1% triton-X100	100µl of 10% stock

Calmodulin Binding Buffer (CBB)	For 100 mL:
10 mM Tris-Cl pH7.9	1ml 1 M Tris-Cl pH7.9
100 mM NaCl	2ml 5 M NaCl
2 mM $CaCl_2$	1ml 200mM $CaCl_2$
10 mM β-mercaptoethanol	70µl of 14.3M stock solution
0.1% Triton-X100	1ml 10% stock

Calmodulin Wash Buffer (CWB)	For 10 mL:
10 mM Tris-Cl pH7.9	100µl 1 M Tris-Cl pH 7.9
100 mM NaCl	200µl 5 M NaCl
0.1 mM $CaCl_2$	5µl 200mM $CaCl_2$
10 mM β-mercaptoethanol	7µl of 14.3M stock solution
0.1% Triton-X100	100µl of 10% stock

Calmodulin Elution Buffer (CEB)	For 10 mL:
10 mM Tris-Cl pH7.9	100µl 1 M Tris-Cl pH 7.9
10 mM β-mercaptoethanol	7µl of 14.3M stock solution
0.1% Triton-X100	100µl of 10% stock
Modify -	
100 - 200 mM KaC	?? of 4 M KAc (ph 7.8)
5 - 20 mM EGTA	? of 0.5 M EGTA Stock

2 x Reducing SDS-PAGE Loading buffer

60mM Tris pH6.8 (Stock 1M)
2% SDS (Stock 10%)
10% Glycerol (Stock 100%)
0.2% bromophenol blue
100mM DTT 1M (aliquots at -20ºC)

I usually make 50mls of a 2.2x stock (**no DTT**) and store at RTº. Then make 1ml of working stock at a time by adding DTT