TAP-tag PROTOCOL

1. Grow 100mL of yeast hyrbid in YPD. (Do this 6 first steps twice because we are growing 6L).

Prepare Washing buffer and keep in 4ºC (Add 1 EDTA free tablet before use).

2. Add 33mL of pre-culture to each 1L of YPD (approx OD_{595}=0.2) to obtain an OD_{595}=0.5-07 (3h but check each 1h). If you want grow until 0.8 (for membrane is a must).
3. Centrifugate yeast at 4000rpm 4ºC 10min.
4. Wash with 12mL mqH_2O.
5. Wash with 10mL WB (Centrifuge) and the second until final V of 5mL.
6. Extrude into the liquid N_2 to form noodles, harvest cells with colander. Keep at -80ºC.

Prepare 100ml NP-40 Buffer (different to 10% NP-40) and keep in 4ºC.
Prepare 1ml PMSF 0.1M (17,419mg + 1ml EtOH 100%)
Clean everything with ethanol and change gloves to avoid any keratine residue.
Spatula: with spoon (colect the pounder) & normal (remove ice and cells of the ball)
Beaker 250ml

7. Griding (Retsch – mortar grinder).
   a. 10min cooling (N2 liquid – Second and a half line – refill constantly) 5-10min
   b. 10min 0 level.
   c. 5min 2 level.
   d. 5min 4 level
   e. 10min 8 level

Centrifuge at 4ºC
Surround with tissues the mortar grinder to dry

8. **(In the cold room)**Dissolve in 70mL NP-40 buffer (althoug we are adding 35ml, depends on your pellet) + 2 EDTA free tablets + 1ml 0.1M PMSF. Leave one hour with magnetic stirrer.
9. Transfer the lysate to 50ml falcon tubes. Centrifuge for 5min.

Clean Beckman tubes with EtOH
Ultracentrifuge tubes in -20C for 2-3min

10. Transfer supernatant to 35ml Beckman tubes. Weight the tube and equilibrate each other (use a beaker to help in the process).
11. Put resin to the tap.
12. Clarify in LC-90 Beckmann Ultracentrifuge (SW28 rotor) for 25000rpm 50min 4ºC (max accel & max Decel). To take the samples out press vacuum bottom.
13. Retrieve the supernatant to two 50ml falcon tubes (if you work with 35ml NP-40, use only one). Avoid to collect the bottom pellet.

Prepare 1h before IPP & TEV buffer (keep in 4ºC)
15min before finish the centrifuge Prepare Beads

14. Prepare the IgG Sepharose beads <4ºC fridge>:
    a. Take 600ul sepharose beads. Spin down to remove the supernatant (with pippete).
    b. Wash 3 times with 1ml of NP-40 Buffer w/o inhibitors.
    c. Resuspend them with 1ml NP-40 Buffer w inhibitors.
15. Add 500ul of IgG Sepharose beads and incubate for 2h in rotating platform (in cold room).
16. Spin the sample down to pellet down the beads (2000 rpm, 4ºC, 4min).

17. Remove supernatant with 25ml pipette.
18. Add 10ml of IPP150 buffer (*inject the buffer with gentle force so the beads are more effectively washed).* Stir gently. Centrifuge at 2000rpm 1min 4ºC. **Wash 2 times**.
19. Wash the beads with 10ml TEV cleavage buffer, stir gently. Spin down 2000rpm 1min 4ºC. Leave approx 1ml of buffer and remove supernatant.
20. Load the beads onto the column (BioRad, 732-6008, small columns). **Add more TEV buffer until the top if it's necessary.**
21. Add 10ul AcTEV (100U), close the column with the stopper supplied with the column. Seal the top with parafilm. Incubate on rotating platfor in RT 2h.
22. Prepare **CB resin (30min before end of incubation)**:
    a. Put 300ul CB resin into the eppendorf. Spin down and remove supernatant.
    b. Add 1ml CB binding buffer (w/o b-Mercapthoethanol) and wash. Spin down. **3 times.**
    c. Wash with 1ml CBB + b-mercapthoethanol
23. (On ice) Drain the eluate (in the case that you're working with two columns eluate in the same) into a new column sealed at the bottom (Poly-Prep CHr. Column, BioRad, 731-1550). **Careful open it with the bottom inside the top of the new column.**
24. Was out the old column (small one) with 300ul CB buffer + b-mer. Pour the eluate from both TEV cleavage columns into the same new column.
25. Add 3 volumes (7.8ml) of CB Buffer + b-MeOH to the IgG eluate. Add 7.8ul $CaCl_2$ 1M and 300ul of Calmodulin resin (using eluate to resupend the resin washed) and incubate on rotating platform for 1h at 4ºC.

<In cold room>

26. Brake the end of the column and wash the beads 2x10ml CB Buffer + 0.1% NP-40. Eluate is not important. (Use a beaker). **Pipette the buffer strongly to agitate the beads (no up and down).**
27. Wash the beads with 10ml CB Buffer + 0.02% NP-40. Eluate is not important. **Pipette the buffer strongly to agitate the beads (no up and down).**
28. Eluate with 1ml Calmodulin Elution buffer (move by inversion before) into a 50ml falcon tube (special one) on ice.
29. Take supernatant and put it on 1.5ml eppendorf with cap.

Switch on centrifuge at 4ºC

30. TCA precipitation (final concentration 25%). With 1ml add 250ul TCA (Trichloroacetic acid 10mM). On ice 30min with periodic vortexing.
31. Spin max speed at 4ºC for 30 min.
32. Wash with 1ml ice-cold acetone (-20ºC) Very carefully, fall down using the wall of the eppendorf.
33. Spin 5min at max speed 4ºC.
34. Remove supernatant and repeat 32 and 33 steps.
35. Remove supernatant, dry on air (5min).

<Running a gel>

Switch on bath at 98ºC.

36. Pellet at RT 1min.
37. Dissolve in 22ul of Loading Buffer SDS. By pipetting, avoid to form bubbles.
38. 7min at 98ºC.
39. Load in a polyacrylamide gel. 120v 1h and 10min.

Digest bands: In gel digestion protocol.

2. BUFFERS

** All working buffers must be made fresh on the day of experiment. Add DTT and B-Mercaptoethanol just before use. Stock solutions made in advance last for 3-5 months.*

Washing Buffer

		[Stock]
10 ml	Tris-HCl pH 8.0	1 M
3 ml	NaCl	5 M
1 ml	EDTA	0.5 M
2 ml	NP-40	10%

* add 1 complete EDTA-free tablet (Roche)
volume to 100 ml

1. IPP 150 (100 ml) KEEP IN 4ºC.

[Final]			**[Stock]**
10 mM	Tris-HCl pH 8.0	1ml	1 M
150 mM	NaCl	3 ml	5 M
0.1%	NP-40	1 ml	10%
	volume to 100 ml		

2. TEV Cleavage Buffer (TEV CB) (50 ml) KEEP IN 4ºC

[Final]			**[Stock]**
10 mM	Tris-HCl pH 8.0	0.5 ml	1 M
150 mM	NaCl	1.5 ml	5 M
0.1%	NP-40	0.5 ml	10%
0.5 mM	EDTA	50 µl	0.5 M
1.0 mM	DTT	25 µl	1 M *
	volume to 50 ml		

* Add DTT (Stock 500ul with 77mg DTT).

3. Calmodulin Binding Buffer (CBB) (100 ml)

[Final]			**[Stock]**
10 mM	Tris-HCl pH 8.0	1 ml	1 M
150 mM	NaCl	3 ml	5 M
1 mM	Mg^{2+} Acetate	100 µl	1 M
1 mM	Imidazole	100 µl	1 M
2 mM	$CaCl_2$	200 µl	1 M

<u>Add in Step 25.</u>
10 mM B-MeOH 69.9 µl 14.3 M * add before use
volume to 100 ml
<u>Do in step 26.</u>
divide into two 50 ml aliquots
adjust one 50 ml aliquots to 0.1% NP-40 by adding 500 µl 10% NP-40 (+35ul b-mercapthoethanol)
adjust one 50 ml aliquots to 0.02% NP-40 by adding 100 µl 10% NP-40 (+35ul b-mercapthoethanol)

4. Calmodulin Elution Buffer (CEB) (10 ml)

[Final]		[Stock]
10 mM	Tris-HCl pH 8.0	0.1 ml 1 M
150 mM	NaCl	0.3 ml 5 M
0.02%	NP-40	20 µl 10%
1 mM	Mg^{2+}Acetate	10 µl 0.5 M
1 mM	Imidazole	10 µl 1 M
20 mM	EGTA	400 µl 0.5 M
10 mM	B-MeOH	7 µl 14.3 M * add before use
	volume to 10 ml	

5. NP-40 Buffer (3L)

** NP-40 buffer must be made in advance and stored in room temperature.*

[Final]		[Stock]
6 mM Na_2HPO_4	2.56 g	
4 mM NaH_2PO_4 x H_2O	1.66 g	
1% NONIDET P-40	30 ml	100%
150 mM NaCl	26.3 g	
2 mM EDTA	12 ml	0.5 M
50 mM NaF	6.3 g	
4 ug/ml leupeptin	12 mg	
0.1 mM Na_3VO_4	0.6 ml	0.5 M

Add the following protease inhibitors per 50 ml NP-40 buffer:
1 complete tablet EDTA-free (Roche)
500 µl 0.1 M PMSF prepared fresh in 100% ethanol (17.41mg in 1ml 100% EtOH).
500 µl 1 mg/ml leupeptin prepared fresh in H_2O

EDTA-free protease inhibitor tablets 20 tablets, Roche
AcTEV protease (Invitrogen, Cat no: 12575-015)
IgG Sepharose 6 Fast Flow 10 ml (GE Healthcare, Cat no: 17-0969-01)
Bio Rad, 732-6008, small columns
Poly-Prep Chr. Column, Bio Rad, 731-1550
Calmodulin Affinity Resin 10 ml, Stratagene Cat no: 214303

TCA (Trichloroacetic acid) 100%: 10mM.

3. References

Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. 1999. A generic protein purification method for protein complex characterization and proteome exploration. *Nature Biotechnology* **17**: 1030-1032.