Tandem Affinity Purification in Drosophila Heads and Ovaries

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[Abstract] Tandem affinity purification (TAP) (Pugi *et al*., 2001; Rigaut *et al*., 1999) is a method that uses a tagging approach of a target protein of interest for a two-step purification scheme in order to pull down protein complexes under native conditions and expression levels. The TAP tag consists of three components: a calmodulin-binding peptide, a *Tobacco etch virus* (TEV) protease cleavage site and Protein A which is an immunoglobulin G (IgG)-binding domain. This protocol was modified from the original methodology used in yeast cells (Pugi *et al*., 2001; Rigaut *et al*., 199) for isolation of protein complexes from Drosophila heads and ovaries expressing a TAP tagged protein of interest. To determine in vivo binding partners of the Drosophila fragile X protein (dFMR1), we developed a transgenic strain of flies expressing a recombinant form of dFMR1 with a carboxy-terminal TAP tag (Tsai and Carstens, 2006). To ensure that the construct was expressed at wild-type levels, we engineered this form of the tagged protein in the context of a genomic rescue construct that rescued a mutant sterility phenotype. The purification process was performed using mild conditions to maintain native protein interactions. For TAP methods in Drosophila S2 cell culture, we have successfully used a protocol previously published by Tsai and Carstens (Tsai and Carstens, 2006; Bhogal *et al*., 2011).

Materials and Reagents

1. 1x Phosphate buffered saline (PBS)
2. Fly sieves: 106, 355, 600 and 850 mm
3. IgG Sepharose Fast Flow Beads (GE Healthcare Dharmacon, catalog number: 17-0969-01)
4. AcTEV Protease (Life Technologies, Invitrogen™, catalog number: 12575-015)
5. Calmodulin Sepharose 4B (GE Healthcare Dharmacon, catalog number: 17-0529-01)
6. Micro Bio-Spin column (Bio-Rad Laboratories, catalog number: 732-6204)
7. NuPAGE Novex 4-12% Bis-Tris gel (Life Technologies, catalog number: NP0321)
8. Silverquest Silver Staining Kit (Life Technologies, catalog number: LC6070), Colloidal Blue Staining Kit (Life Technologies, catalog number: LC6025) or Coomassie Blue.
9. Beta-mercaptoethanol

10. 1 M $CaCl_2$
11. Hepes
12. $MgCl_2$
13. KCl
14. NP-40
15. DTT
16. Na_3VO_4
17. EDTA
18. EGTA
19. NaF
20. Glycerol
21. Mg-acetate
22. Imidazole
23. Bouwmeester's buffer (see Recipes)
24. Buffer A (see Recipes)
25. Buffer B (see Recipes)
26. Buffer C (see Recipes)
27. TEV Cleavage buffer (see Recipes)
28. Calmodulin binding buffer (see Recipes)
29. Calmodulin blution buffer (see Recipes)

Equipment

1. Benchtop centrifuge (that fits 1.5 ml microcentrifuge tubes)
2. 1.5 ml microcentrifuge tubes
3. Nutator
4. Vortexer
5. Ceramic mortar and pestle
6. Dounce homogenizer
7. Disposable pestles for 1.5 ml tubes
8. Beckman table top centrifuge (that fits 15 ml and 50 ml conical tubes and can be cooled to 4 °C)
9. Sorvall with a Beckman JA-20 rotor that is capable to cool to 4 °C
10. Mass Spectrometry Facility

Procedure

A. Isolation of protein complexes

1. For protein complex isolation from drosophila ovaries:
   a. Feed (1-3 day old) flies expressing your TAP-tagged protein for three days with yeast paste.
   b. Dissect ovaries in 1x PBS, collect in a 1.5 ml microcentrifuge tube, and freeze at -80 °C. Collect more than 200 pairs of ovaries. Ovaries can be collected following several independent rounds of dissection and frozen to amass an appropriate amount.
   c. Homogenize ovaries in 1 ml Bouwmeester's buffer using disposable pestles that fit 1.5 ml microcentrifuge tube.
   d. Incubate on ice for 20 min and transfer to Beckman centrifuge tubes (1/2 x 2 inch).
   e. Spin tubes at 4 °C, 20,000 *x g* for 15 min.
   f. Remove and save supernatant on ice. This is your cytoplasmic extract.
   g. Spin tubes at 4 °C, 100,000 *x g* for 45 min to remove remaining extract following additional spin.
   h. Remove and save supernatant on ice. This is your nuclear extract (can combine the extracts from step A6 and A8, if you want total extracts, or keep separate).
   i. Take out 20 µl from each of your isolated extracts as a lysate control (control A). Freeze in liquid nitrogen and store at -80 °C. Go to step #a in section 2: Pulldown of protein complexes.
2. For protein complex isolation from drosophila heads:
   j. Freeze flies expressing TAP-tagged protein of interest at -80 °C. You will need at least 50 ml of frozen flies to begin. Weigh starting materials on a standard scale and be sure to have at least 1 gram of heads.
   k. Chill sieves in liquid nitrogen in preparation for head popping procedure.
   l. Pop heads off using liquid nitrogen and the proper sized meshes (http://cmgm.stanford.edu/~vesicle/protocols/droshead.html). Keep purified head samples on dry ice or at -80 °C until you are ready for the next step.
   m. Pre-freeze mortar and pestle using liquid nitrogen. Add heads and liquid nitrogen to chilled mortar and pestle and grind sample on dry ice, continuing to add liquid nitrogen until you have a fine powder.
   n. When you have fine powder, add more liquid nitrogen to pour out powder into a 50 ml falcon tube.
   o. Let liquid nitrogen de-gas in uncovered tube for 5-10 min.
   p. Add 2 ml bouwmeester buffer to powder, transfer to a dounce homogenizer and dounce homogenize 10 times. Transfer to several 1.5 ml microcentrifuge tubes. Spin

15 min, 15,000 *x g* at 4 °C, remove supernatant and repeat spin to remove as much as supernatant as possible.

q. Take out 20 µl as a lysate control from each sample (control A). Snap freeze in liquid nitrogen and store at -80 °C.

B. Pulldown of protein complexes

3. Prepare 200 µl IgG Sepharose Fast Flow beads in a 15 ml falcon tube and wash twice with 500 µl buffer B (add 100 µl PIC and 5 µl DTT fresh to 10 ml buffer B). After each wash with buffer B, spin at 4 °C, 100 *x g* for 2 min in a Beckman table top centrifuge and remove supernatant.
4. Add lysate and rotate overnight at 4 °C on a nutator.
5. After overnight incubation, spin at 4 °C, 100 *x g* for 5 min in a Beckman table top centrifuge and remove supernatant. Save 20 µl of supernatant for post-incubation control (control B).
6. Wash beads 3 times in 1 ml buffer B. Spin 4 °C, 100 *x g* for 2 min after each wash in a Beckman table top centrifuge.
7. Wash beads 4 times in 1 ml buffer C. Spin 4 °C, 100 *x g* for 2 min after each wash in a Beckman table top centrifuge.
8. Wash twice in 1 ml TEV cleavage buffer. Spin 4 °C, 100 *x g* for 2 min after each wash in a Beckman table top centrifuge. Remove 10 µl beads as a control (control C).
9. Add 400 µl TEV cleavage buffer and 4 µl AcTEV protease to beads and transfer to a 1.5 ml microcentrifuge tube. Rotate overnight at 4 °C on a nutator.
10. Spin 300 *x g*, 1 min at 4 °C in a bench top centrifuge.
11. Take off supernatant and add 0.6 µl 1 M $CaCl_2$ per 200 µl supernatant (usually 1.2 µl for 400 µl total supernatant).
12. Prepare a 1.5 ml microcentrifuge tube with 100 µl Calmodulin sepharose resin and wash twice with 500 µl Calmodulin binding buffer. Spin 2 min, 1,500 rpm, 4 °C after each wash.
13. Add 3x volume Calmodulin binding buffer to supernatant and add this to prepared calmodulin resin. Rotate 3-4 h at 4 °C.
14. Spin 2 min, 1,500 rpm, 4 °C.
15. Wash three times with 500 µl Calmodulin binding buffer. Spin 2 min, 1,500 rpm, 4 °C after each wash.
16. Elute TAP-tagged protein with Calmodulin elution buffer using preferred strategy.
17. Run eluate on a 4-12% Bis-Tris 10 well gel.
18. Run controls and protein ladder on a separate gel from final eluate so there is no contamination.

19. Stain with either Coomassie Blue, Colloidal Blue Staining Kit, or Silverquest Kit. Cut out bands of interest and send to the Mass Spectrometry Facility.

Recipes

Note: Make buffers without Protease Inhibitor Cocktail without EDTA (PIC w/o EDTA), Na_3VO_4, $CaCl_2$ and/or DTT. These should be added fresh right before use.

1. Buffer A (20 ml)

   See note above

Stock and concentration	volume	Final concentration
1 M Hepes (pH 7.9)	200 ml	10 mM Hepes
1 M $MgCl_2$	30 ml	1.5 mM $MgCl_2$
2 M KCl	100 ml	10 mM KCl
1 M DTT	10 ml	0.5 mM DTT
PIC w/o EDTA	20 ml	1x
H_2O	to 20 ml	

2. Bouwmeester's buffer (20 ml)

   Filter sterilize and store at 4 °C.

   See note above

Stock and concentration	volume	Final concentration
1 M Tris-HCl (pH 7.5)	1 ml	50 mM Tris-HCl
5 M NaCl	2.5 ml	125 mM NaCl
100% glycerol	1 ml	5% glycerol
100% NP-40	80 ml	0.2% NP-40
1 M $MgCl_2$	30 ml	1.5 mM $MgCl_2$
1 M DTT	20 ml	1 mM DTT
1 M NaF	500 ml	25 mM NaF
200 mM Na_3VO_4	100 ml	1 mM Na_3VO_4
500 mM EDTA	40 ml	1 mM EDTA
2 mM EGTA	80 ml	500 mM EGTA
PIC w/o EDTA	20 ml	1x
H_2O	to 20 ml	

3. Buffer B (20 ml)

   Make fresh on the day of experiment.

   See note above

Stock and concentration	volume	Final concentration
1 M Hepes (pH 7.9)	400 ml	20 mM Hepes
87% glycerol	4.6 ml	20% glycerol
100% NP-40	200 ml	0.5% NP-40
2 M KCl	2.5 ml	200 mM KCl
1 M DTT	10 ml	0.5 mM DTT
500 mM EDTA	40 ml	1 mM EDTA
20 mM EGTA	80 ml	500 mM EGTA
PIC w/o EDTA	200 ml	1x
H_2O	to 20 ml	

4. Buffer C (20 ml)

   See note above

Stock and concentration	volume	Final concentration
1 M Hepes (pH 7.9)	400 ml	20 mM Hepes
87% glycerol	4.6 ml	20% glycerol
100% NP-40	200 ml	0.5% NP-40
2 M KCl	2.5 ml	200 mM KCl
1 M DTT	10 ml	0.5 mM DTT
H_2O	to 20 ml	

5. TEV cleavage buffer (100 ml)

   Filter sterilize

   See note above

Stock and concentration	volume	Final concentration
1 M Tris-HCl (pH 8.0)	1 ml	10 mM Tris-HCl
5 M NaCl	3 ml	150 mM NaCl
20% NP-40	500 ml	0.1% NP-40
1 M DTT	50 ml	0.5 mM EDTA
H_2O	to 100 ml	

6. Calmodulin binding buffer (100 ml)

Stock and concentration	Volume	Final concentration
1 M Tris-HCl (pH 8.0)	1 ml	10 mM Tris-HCl
Beta-mercaptoethanol	69.7 ml	10 mM Beta-mercaptoethanol
5 M NaCl	3 ml	150 mM NaCl
1 M Mg-acetate	100 ml	1 mM Mg-acetate
1 M Imidazole	100 ml	1 mM Imidazole
1 M $CaCl_2$	200 ml	2 mM $CaCl_2$
20% NP-40	500 ml	0.1% NP-40
H_2O	to 100 ml	

7. Calmodulin elution buffer (100 ml)

Stock and concentration	Volume	Final concentration
1 M Tris-HCl (pH 8.0)	1 ml	10 mM Tris-HCl
Beta-mercaptoethanol	69.7 ml	10 mM Beta-mercaptoethanol
5 M NaCl	3 ml	150 mM NaCl
1 M Mg-acetate	100 ml	1 mM Mg-acetate
1 M Imidazole	100 ml	1 mM Imidazole
20% NP-40	500 ml	0.1% NP-40
2 mm EGTA	400 ml	500 mM EGTA
H_2O	to 100 ml	

Acknowledgments

This protocol was modified from the original methodology used in yeast cells (Pugi *et al.*, 2001; Rigaut *et al.*, 1999).

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