Purification of TAP-tagged (protein A/calmodulin) proteins for direct analysis by LC-MS: Adaptation for yeast
ACG 2004/09/24

This procedure has been adapted from the protocol of Dr. Séraphin, and I initially have optimized it originally for the purification of CYTOSOLIC MAMMALIAN proteins for direct LC-MS analysis.

This protocol is a simple adaptation of my protocol to work with S. cerevisiae extracts. Please also refer to my main protocol at www.proteomecenter.org.

Troubleshooting notes. It is really helpful, especially when performing the TAP-tagging expression/purification for the first time, or with a new protein, to take aliquots for analysis of the purification procedures at all the steps indicated in red.

PART I: PURIFICATION OF TAP-TAGGED PROTEINS

1. Extract preparation:
Grow 1L S. cerevisiae strains to OD 0.8 – 1.0. Collect the cells by centrifugation. Transfer to 15 ml tube and wash with ice-cold 20mM Hepes pH 7.5, 10mM EDTA. Measure the volume of the pellet, and add an equal volume of TAP-lysis buffer containing a protease inhibitor cocktail. Aliquot 1ml per tube in 2ml snap-cap tubes, and freeze until needed. To lyse, thaw partially, add ~500ul glass beads, and transfer to a vortex in the cold room. Vortex at maximum speed 4 x 5 minutes, with 5 minutes rest period on ice between each pulse. Spin down beads and debris at max speed in a 4C microfuge for 10 minutes. Recover the supernatant in a fresh tube. If desired, add 400ul lysis buffer to the pellet, vortex for 2 minutes and centrifuge again. Combine both supernatants. Repeat the centrifugation steps to get rid of last debris. You should recover 50-200mg extract.
KEEP AN ALIQUOT FOR ANALYSIS (A – read section II for suggested quantities).

2. Pre-clear
NOTES: All spin steps involving beads are performed at very low speeds (e.g. 1500 rpm in a microfuge for 1-2 minutes). Removal of the liquid is performed slowly, preferably with a transfer pipette, or a pipetman. All spins and incubations are performed at 4°C, and all the buffers are pre-chilled on ice.
Transfer the cleared supernatant to a fresh 2ml tube containing 100 – 200 ul pre-washed packed sepharose beads (I have been using older batches of glutathione sepharose, but any non specific or non-conjugated sepharose will do). Incubate 1 hour at 4C on an end-over-end or similar device. Spin down beads.

2. Binding to IgG sepharose:
Wash IgG beads 3x with lysis buffer (75-100ul packed beads per pull-down). Remove excess liquid. Add the pre-cleared lysate to the washed beads. Incubate with gentle

rocking (or end-over-end rocking) at 4°C for > 3 hours (make sure the slurry is well resuspended).

3. Cleavage with tobacco etch virus protease (TEV):
Spin down the IgG beads. Recover supernatant (USE FOR ANALYSIS - B). Wash the beads 1x with 1ml lysis buffer without protease inhibitors, and 2x with 1ml TEV buffer (see appended recipe). KEEP AN ALIQUOT OF THE BEADS PRE-CLEAVAGE FOR ANALYSIS (C). Drain liquid after the last wash. Resuspend beads in TEV buffer containing TEV protease (~100 units TEV in 300 µl TEV buffer). Incubate the slurry (again with gentle agitation) overnight (or at least 4 hours) at 4°C.

4. Binding to calmodulin-sepharose:
Wash the calmodulin sepharose beads (50-75ul per pull-down) 3x with calmodulin binding buffer (see recipe below). Distribute in tubes, remove excess liquid, and set aside.

Spin down the IgG beads post-cleavage. KEEP AN ALIQUOT OF SUPERNATANT FOR ANALYSIS (D). Transfer the supernatant to a fresh tube (not the calmodulin-containing tube). Add 300ul of calmodulin binding buffer. Incubate for 5 minutes end-over-end at room temp. Spin down the IgG beads again. Combine with the first wash. Repeat twice, so that you will get a combined TEV eluate of 1200ul. KEEP AN ALIQUOT OF THE BEADS FOR ANALYSIS (E). To the eluate, add 1/250 volume (~5 ul) 1M CaCl2, mix by inversion. Spin again to remove traces of IgG sepharose and transfer the supernatant to the tube containing the calmodulin-sepharose. Incubate > 2 hours with gentle agitation at 4°C.

5. Elution from calmodulin-sepharose:
Transfer the slurry to a clean Bio-spin column; recover flow through in a new eppendorf tube (KEEP FOR ANALYSIS – F). Wash the beads 1x with 1ml calmodulin-***binding*** buffer. (resuspend the slurry in buffer, let drip the buffer through, pushing the liquid out gently with a rubber bulb if the buffer does not drip by itself). Wash 2x with 1 ml calmodulin ***rinsing*** buffer. (KEEP AN ALIQUOT OF THE BEADS PRE-ELUTION FOR ANALYSIS – G). Drain the beads well (push the remaining droplets with a rubber bulb). Transfer the Bio-spin column + beads into clean tubes (2ml screw-cap tubes work well). Resuspend beads in one volume of calmodulin elution buffer (100µl), and incubate a few minutes. Lift the Bio-spin to let drip, and push the remaining droplets out with a rubber bulb. Add another volume (100µl) calmodulin elution buffer to the Bio-spin column and let drip in the same eppendorf tube (push the remaining droplets out with a rubber bulb). Transfer the Bio-spin column to a new tube and repeat these steps 3 more times. *If you know that your protein is sticky, you might want to include another set of elutions with a detergent such as Rapigest. ANALYSE A FRACTION OF EACH OF THESE ELUTIONS (H, I). ALSO ANALYSE THE BEADS POST-ELUTION (J). Freeze the final eluates until the analysis is performed

PART II: ANALYSIS OF THE PURIFICATION PROCEDURE.

The expressed protein is detected by Western blot using antibodies specific to the expressed protein. If antigen-specific antibodies are not available, expression can be detected through the presence of the protein A (using whole rabbit sera as a primary antibody 1:2500, and HRP-coupled anti-rabbit as a secondary antibody) and/or the Open Biosystems new anti-TAP antibody (1:5000). In all cases, chemiluminescent detection is performed and the amounts of protein in each fraction are approximated. By loading each fraction, it is really easy to see which steps require further optimization. The following table is an indication of how much sample I have loaded for each of these fractions in test experiments (starting material = 500µg). The proportions are decreased when starting with more material. The gels are loaded in the order A to J. If an assessment of the complexity of the samples is needed, the final eluates can also be analyzed by silver staining of the SDS-PAGE gel.

ID	fraction name	function	amount loaded
A	total lysate	efficiency of binding to IgG beads	25µg
B	lysate post IgG beads	efficiency of binding to IgG beads	25µg
C	IgG beads pre-TEV	efficiency of binding to IgG beads; TEV cleavage efficiency	1/100
D	TEV eluate	TEV cleavage efficiency	1/100
E	IgG beads post-TEV	TEV cleavage efficiency; efficiency of binding to calmodulin beads	1/100
F	supernatant post-calmodulin (unbound)	efficiency of binding to calmodulin beads	1/100
G	calmodulin beads pre-elution	efficiency of binding to calmodulin beads efficiency of elution from calm. beads	1/100
HI	elutions 1, 2	efficiency of elution from calm. beads	1/25
J	calm. beads post elution	efficiency of elution from calm. beads	1/25

PART III: REAGENTS AND RECIPES

Product description	Supplier	catalog number	unit size/price
IgG sepharose 6 fast flow	Amersham	17-0969-01	10ml / 243$
calmodulin-sepharose 4B	Amersham	17-0529-01	10ml / 212$
Calmodulin-sepharose	Stratagene	214303	10ml/227$
recombinant TEV	Invitrogen	10127017	1000U / 86$
Micro Bio-spin columns	Bio-rad	732-6204	100 / 80$
Protease inhibitor cocktail	Sigma	P8340	5ml / 126$
Glass beads 0.5mm	Biospec	11079105	454g/26$

*all other chemicals are molecular biology grade (or equivalent) from Sigma

Lysis buffer:

final concentration	product	stock concentration	vol. for 100ml 1x
10 %	glycerol	100 %	10 ml
50 mM	Hepes-KOH pH 8.0	1 M	5 ml
100 mM	KCl	2 M	5 ml
2 mM	EDTA	0.5 M	0.4 ml
0.1 %	NP-40	10 %	1 ml
10 mM	NaF	1 M	1 ml
0.25 mM	NaOVO3	100 mM	0.25 ml
50 mM	ß-glycerolphosphate	1 M	5 ml
	H2O		to 100ml
2 mM	DTT	1 M	0.2 ml
1 x	Sigma prot inhibitor	500 x	0.2 ml

Keep at 4C. Add DTT and protease inhibitors just before use.

TEV buffer:

final concentration	product	stock concentration	vol. for 100ml 1x
10 mM	Hepes-KOH pH 8.0	1M	1 ml
150 mM	NaCl	5 M	3 ml
0.1 %	NP-40	10 %	1 ml
0.5 mM	EDTA	0.5 M	100 µl
1 mM	DTT	1 M	100µl
	H2O		to 100µl

Aprotinin, leupeptin and PMSF do not inhibit TEV, and can therefore be used in this buffer if endogenous protease activity is suspected.

Calmodulin-binding buffer:

final concentration	product	stock concentration	vol. for 100ml 1x
10 mM	ß-mercaptoethanol	14 M	69.7 µl
10 mM	Hepes-KOH pH 8.0	1 M	1 ml
150 mM	NaCl	5 M	3 ml
1 mM	MgOAc	1 M	100µl
1 mM	imidazole	1 M	100µl
0.1 %	NP-40	10 %	1 ml
2 mM	CaCl2	1 M	100 µl
	H2O		100 ml

Protease inhibitors can be used in this buffer if endogenous protease activity is suspected.

Calmodulin-rinsing buffer for direct analysis by LC-MS:

final concentration	product	stock concentration	vol. for 100ml 1x
50 mM	Amm.bicarb. pH 8.0	1 M	5 ml
75 mM	NaCl	5 M	1.5 ml
1 mM	MgOAc	1 M	100µl
1 mM	imidazole	1 M	100µl
2 mM	CaCl2	1 M	100 µl
	H2O		100 ml

Calmodulin-elution buffer for direct analysis by LC-MS:

final concentration	product	stock concentration	vol. for 100ml 1x
50 mM	Amm.bicarb. pH 8.0	1 M	5 ml
25 mM	EGTA	0.5 M	5 ml
	H2O		100 ml

The ammonium bicarbonate is volatile, and will be evaporated in the speed vac, following the tryptic digest. The EGTA will not be evaporated, and there will also be traces of NaCl, MgOAc, imidazole and CaCl2.