Mammalian TAP-tagging technique –

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1) Overview of the process

Derived from the system described by the Seraphin's laboratory, the Tandem Affinity purification system (TAP) is a two-step affinity purification scheme which generates protein complexes sufficiently clean to be analyzed by mass spectrometry.

2) Expression

a. Cell lines

I have been using both 293T and 293 cells for the transfections – each cell line being used for a different purpose, but they are grown and transfected in the same manner. You may want to use a cell line which is more physiological for your purposes, but I would suggest that you also use the HEK293 or 293T cells in transient transfections to detect whether your construct can be expressed prior to establishing stable cell lines. In all cases, you should start with a known source of cells – this is especially important when making stable lines: I try using a similar (and low) passage after reception of the cells from the ATCC for all my experiments.

i. 293T (ATCC CRL-11268):

I only use the 293T cells for transient transfections and mostly for testing expression and/or purification on small scale. The yield in protein is significantly higher in 293Ts, so you need less DNA/cells to obtain the same amount of proteins for your experiments. I would recommend to start the small scale tests using 293Ts, especially if you don't know how well your own (test) protein will behave (see below for suggested amounts for transfection).

ii. HEK293 (CRL-1573):

I use the 293 for making all my stable cell lines. When possible to generate, stable cell lines are my preferred choice for the real purification experiments. They express more moderate amount of protein, and are less likely to produce artifacts due to overexpression, such as mislocalization, and association with unnatural partners. They also are less likely to be misfolded and coated with chaperones. When stable cell lines cannot be obtained (for example when the protein is toxic), I still use 293 (and NOT 293T) in transient transfections to generate sample for large scale purification and mass spectrometry. In that case, I transfect 2 – 4 x 150 mm plates and split them to obtain 8 – 16 x 150 mm plates. I usually harvest at 48-60 hours, but this can be modulated depending of your protein of interest.

iii. General growth of the cells:

The cells are kept in DMEM high glucose supplemented with 5% FBS and 5% serum supreme (Biowhittaker) and Penicillin/Streptomycin. Alternatively, they can also be grown in DMEM high glucose supplemented with 10% FBS and Pen/Strep. The Serum Supreme is simply fortified calf serum and is much cheaper than FBS (40$ versus 200$ for a 500ml bottle, so the costs are significantly reduced in the first recipe). The cells can be trypsinized – they detach quite quickly and should not be incubated in trypsin for too long. For routine growth, I split the cells 1 → 4 to 1 → 8 (they do not like being too sparse).

b. Transfection - stable

Once the DNA has been sequenced and has showed expression in transient transfection, cells of interest are stably transfected. You need to decide whether you will want to obtain a pool of stably transfected cells, or individual clones of transfected cells. If you are working with only a few targets, you should consider establishing individual clones because the expression has the tendency to be more stable in a clone than in a pool (where often cells who do not express or express little of the protein of interest grow faster than the high expressers and tend to take over the culture after some time). In addition, you can know the expression of your protein of interest in all the cells from a clone, whether you can only detect the average from a pool. If working with clones, however, you may want to combine 3 – 4 decent* expressers for each experiment to prevent artifacts due to clonal variation. (* I never choose the clones which express HUGE amounts of proteins, by fear that artifact might arise. Instead, I'm trying to obtain clones which express in the same range as the endogenous protein). More recently I have derived vectors that have the selection marker under the control of an IRES promoter downstream of the TAP-Tag cassette, this tends to give a very homogeneous expression level in a polyclonal cell line and keep the level of expression low.

Use only low-passage cells for this procedure. For 293 cells, cells are transfected (10 cm plate) when they are ~60-70% confluent using CaP04 protocol. 48 hours post-transfection, they are split into 6 x 10cm plates, using serial 2- or 3-fold dilutions (so the final dilution will be ~1:6, 1:12, 1:24, and so forth). If you are only trying to obtain pools, perform only 2 serial dilutions in 150mm plates. The cells are returned at 37°C for 24 hours. The next day, the medium is changed for DMEM containing the appropriate selction marler (the IRES vectors use puromycin which is my favorite antibiotic for HEK293 cells). The cells are incubated in this medium for about 12-15 days, with occasional re-feeding. At the end of this period, the plates on which colonies are well defined/separated from their neighbors will be selected for picking clones. The other plates will be trypsinized and transferred into 150mm plates for use as "pools". For picking the clones, the position of the clones is first marked at the bottom of the plate. The plate is then rinsed with 5ml PBS. Sterile 3mm cloning disks (Sigma) are soaked in trypsin-EDTA, and carefully placed on each colony, using sterile forceps. HEK293 detach really fast (2-5 minutes), so proceed quickly. The disks are then transferred into the wells of a 24 or 48-well plate already containing medium. Clones and pools are grown in selection media and passaged as necessary. As soon as possible, they are screened for expression, and frozen away. Often, pools lose their expression quite fast, so clones are better for a more permanent line.

e. Reagents needed

DMEM high glucose, Fetal bovine serum, trypsin-EDTA, Sterile PBS, G418 sulfate, Penicillin-streptomycin can be purchased through Mediatech Cellgro, Biowhittaker, Invitrogen, etc.

All the TC plastics are purchased from TPP (plates), BD-Falcon (plates, pipettes), or Fisher (pipettes) – equivalent material can also be used.

Chemiluminescence detection reagents and films can be obtained from Amersham, and similar products can also be obtained through other suppliers.

Product description	Supplier	catalog number	unit size/price

Serum Supreme	Biowhittaker	14-492F	42$/500ml
Opti-MEM no phenol	Invitrogen	11058021	24$/500ml
Lipofectamine	Invitrogen	18324012	210$/1ml
PLUS reagent	Invitrogen	11514015	110$/0.85ml
Sterile 3mm cloning disks	Bel-Art / Fisher	37847 0001	18$/100
Rabbit normal serum	ICN	2941149	24.85$/100ml
Donkey anti-rabbit HRP	Amersham	NA934	158$/1ml
HEK293	ATCC	CRL-1573	175$/EA
293T	ATCC	CRL-11268	283$/EA

Purification of TAP-tagged (streptavidin/calmodulin) proteins for direct analysis by LC-MS

This procedure has been adapted from the protocol of Dr. Séraphin. I have optimized it for the purification of CYTOSOLIC MAMMALIAN proteins. The lysis and purifications conditions will need to be optimized for the purification of nuclear or membrane-associated proteins. The final washes + elutions are performed in a buffer allowing for the direct analysis of trypsin-digested proteins by LC-MS.

Troubleshooting notes. It is really important, 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. Steps which are not yielding an efficient recovery (see section II for expected recoveries) should be troubleshooted individually (see troubleshooting notes, section III).

The following protocol is aimed at small-scale purification and at purification of larger amounts for mass spectrometry, and approximation for the quantities to used are indicated (yellow highlight for the large scale; green highlight for the small scale).

PART I: PURIFICATION OF TAP-TAGGED PROTEINS

1. Extract preparation:

Wash cells 1x with PBS. Harvest cells with PBS-EDTA (2mm EDTA, 2mm EGTA). Spin down cells low spin for 5 mins. Lyse cells in 10 ml lysis buffer for 30 minutes (see appended recipe. Perform 2 freeze-thaw cycles to improve protein recovery (I alternate between liquid nitrogen and 37 degrees water bath – nerver let the temperature go over 4 degrees in the tubes). Spin down the debris for 10 minutes at maximum speed in a microfuge. (If larger volumes are used, the spins can be performed in a SS34 rotor at 10000rpms for 15 minutes, or in a clinical centrifuge at 2650g for at least 30 minutes.) Recover the cleared supernatant and monitor the protein concentration (I usually recover 5-10mg protein / 150mm plate of 90% confluent 293 cells). KEEP AN ALIQUOT FOR ANALYSIS (A – read section II for suggested quantities).

2. Binding to streptavidin-sepharose:

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.

Wash the appropriate amount of streptavidin beads 3x with lysis buffer. Remove excess liquid. Add the cleared lysate to the washed beads. Incubate with gentle rocking (or end-over-end rocking) at 4°C for overnight (make sure the slurry is well resuspended). The quantity of beads used is ~1-5 µl packed beads / mg extract, with the following exceptions: 1) When working with smaller quantities of lysate, I never use less than 15µl packed beads, to make it easier to see at the bottom of the tube; 2) Even when working with large amounts of cells (10-20 plates, or 50-200mg extract), I never use more than 75 - 100 µl packed beads (the capacity of the beads is >2mg streptavidin/ml packed gel, i.e. >200 µg streptavidin/100 µl packed beads.

3. Elution and cleavage with tobacco etch virus protease (TEV):

Spin down the streptavidin beads. Recover supernatant (USE FOR ANALYSIS - B). Wash the beads 3x with 1ml lysis buffer, and 2x with calmodulin binding buffer (see appended recipe). KEEP AN ALIQUOT OF THE BEADS PRE-CLEAVAGE FOR ANALYSIS (C). Drain liquid after the last wash. Resuspend beads in 800ul streptavidin elution buffer containing TEV protease. Add DTT to a 1mM final concentration. Incubate the slurry (again with gentle agitation) for at least 3 hours at 4°C (In most case, more is NOT detrimental – if the cleavage is inefficient, try incubating overnight). Make sure there is enough liquid so that the slurry stays resuspended.

4. Binding to calmodulin-sepharose:

Use about the same amount of calmodulin-sepharose beads as used for the streptavidin sepharose (you can usually get away with half as much). Wash the calmodulin sepharose beads 3x with calmodulin binding buffer (see recipe below).

Spin down the streptavidin beads post-cleavage. KEEP AN ALIQUOT OF SUPERNATANT FOR ANALYSIS (D). ALSO, WASH THE BEADS 3XWITH calmodulin binding BUFFER, AND USE FOR ANALYSIS (E). Transfer the supernatant to a fresh tube. Add (~5 ul) 1M CaCl2, mix by inversion. Spin again to remove traces of streptavidin sepharose and transfer the supernatant to the tube containing the calmodulin-sepharose. Incubate 90 minutes with gentle agitation at 4°C.

5. Elution from calmodulin-sepharose:

Spin down the calmodulin beads. Remove the supernatant (KEEP FOR ANALYSIS – F). At this point (and especially with the large scale), I transfer the beads (using calmodulin binding buffer) to a clean Bio-spin column to perform the subsequent washes and elution steps. These steps can also all be performed in eppendorf tubes, with spinning (especially for small scale). Wash the beads 3x with 10 – 20 volumes calmodulin-***binding*** buffer. (If using the Bio-spin, 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 10 – 20 volumes 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 (e.g. 100µl for 100µl packed beads), 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. 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. NOTE: If performing a small scale (test) experiment, Bio-spin columns are not suggested. Simply spin down beads and recover the supernatant for each elution.

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 also be detected through the presence of the streptag (using streptavidin-HRP) and calmodulin tags (using biotinylated calmodulin, followed by HRP-coupled avidin – I have never performed this latter detection myself). 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 strep beads	25µg = 1/20
B	lysate post streptavidin beads	efficiency of binding to strep beads	25µg = 1/20
C	Streptavidin beads pre-elution	efficiency of binding to strep beads; TEV cleavage efficiency	1/20
D	1st elution	TEV cleavage efficiency	1/10
E	Streptavidin beads post-elution	TEV cleavage efficiency; efficiency of binding to calmodulin beads	1/10
F	supernatant post-calmodulin (unbound)	efficiency of binding to calmodulin beads	1/10
G	calmodulin beads pre-elution	efficiency of binding to calmodulin beads efficiency of elution from calm. beads	1/10
HI	elutions 1, 2	efficiency of elution from calm. beads	1/5
J	calm. beads post elution	efficiency of elution from calm. beads	1/5

PART III: TROUBLESHOOTING NOTES

You should seriously consider troubleshooting individual steps is the recovery is significantly less than the values indicated below:
1) binding to streptavidin sepharose > 80%
2) biotin elution and TEV cleavage > 75%
3) binding to the calmodulin sepharose > 80%
4) elution with EGTA > 50%
5) total recovery > 30%

Things to try…

1) binding to streptavidin sepharose
   a. Make sure you wash you cells well prior to lysis
   b. Try different batch / brand streptavidin sepharose

2) TEV cleavage
   a. If cleavage is incomplete: Increase amount of TEV, time of incubation, temperature of incubation; try a different lot or source of TEV
   b. If cleavage occurs, but cleaved protein is not recovered: maybe your protein sticks to the beads; try reducing strep amount, and/or adding more detergent; determine whether your protein sticks to sepharose
3) Binding to calmodulin sepharose
   a. Make sure you have added calcium and that there is no excess of EDTA
   b. Try a different brand / lot of the calmodulin-sepharose
4) Elution with EGTA
   a. Decrease the amount of calmodulin-sepharose you are using – your protein might stick to it

b. Incubate for longer periods, at room temp.; incubate with higher concentrations of EGTA; perform repeated elutions
c. Try adding some Rapigest (detergent which is compatible with mass spectrometry after cleavage – sold by Waters)

PART IV: REAGENTS AND RECIPES

Product description	Supplier	catalog number	unit size/price
streptavidin sepharose 6 fast flow	Amersham	17-5113-01	10ml / 243$
calmodulin-sepharose 4B	Amersham	17-0529-01	10ml / 212$
recombinant TEV	Invitrogen	10127017	1000U / 86$
Micro Bio-spin columns	Bio-rad	732-6204	100 / 80$
Protease inhibitor cocktail	Sigma	P8340	5ml / 126$

*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
2 mM	DTT	1 M	0.2 ml
1 x	Sigma prot inhibitor	500 x	0.2 ml
10 mM	NaF	1 M	1 ml
0.25 mM	NaOVO3	100 mM	0.25 ml
5 nM	okadaic acid	10µM	25µl
5 nM	calyculin A	10µM	25µl
50 mM	ß-glycerolphosphate	1 M	5 ml
	H2O		to 100ml

Aliquot and freeze.

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. Phosphatase inhibitors okadaic acid, calyculin A, ß-glycerolphosphate and NaOVO3 can also be used to prevent phosphatase activity

Streptavidin Elution Buffer

Calmodulin-binding buffer containing 10mM D+Biotin

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. After the tryptic digest, dry the sample by speed-vac. Resuspend in 200 ul water, and speed-vac to remove traces of Ammonium bicarbonate. Resuspend in buffer A and load onto the C18 reverse-phase column used for MS. Wash extensively offline, with 5% acetonitrile in buffer A. Analyse by LC-MS. If necessary, load onto a large capacity C18 reverse-phase column first, then elute in acetonitrile, and lyophilize prior to running on the LC-MS. Alternatively, a strong cation-exchange step can be performed. Samples eluted in NH4OH:MeOH can be lyophilized and loaded on the LC-MS reverse-phase column.

Buffer A

0.1% Trifluoroacetic acid (HPLC grade), 2% acetonitrile