Extraction of protein complexes by Tandem Affinity Purification

Testing tagged protein expression

Grow 20 NGM plates of integrated worm strain. Proceed o a denaturing total protein extraction of mixed stage population. Then follow the anti-TAP antibody western blot protocol.

Extract preparation

Grow worms in liquid culture.
Wash them several times with M9 and let them incubate for 30 min during the last wash.
Wash 2x with H_2O.
Resuspend in a minimal volume of WFB.
Quickly put the worms in a mortar with liquid N_2 .
Grind and add liquid N_2 to scrape down worms to middle of mortar.
Repeat till it seems a powder.
Put ground worms in a pre-chilled Falcon50 and let them thaw on ice.
From here work always on ice with precooled buffers and equipments.
Add and adjust PEB.
Homogenize 3x at 30000 rpm 15 sec with the 12mm Polytron stem, then put on ice 1 min.
Load the 40K French pressure cell and operate it at 10000 psi. Repeat once if necessary.
Split in Sepcor tubes and centrifuge 10 min at 16000 r m at 2°C.
Collect the soluble protein supernatant fraction and l t it on ice at 4°C.
Add minimal volume of PEB to resuspend, transfer to a lcon and put on an ice-water bath.
Set the sonicator on medium power and the amplitude on 2. Sonicate 30 sec and wait 3 min.
Repeat 2-3X.
Set the amplitude on 3. Sonicate 30 sec.
Centrifuge 10 min at 16000 rpm at 2°C.
Collect the chromatin fraction supernatant and pool th supernatants together.
Centrifuge at 38000 rpm during 20 min at 2°C. During t ime wash IgG/Sepharose beads.
Collect the interphase.

Washing IgG/Sepharose beads

Resuspend beads and wash 200 µl of suspension with cold PEB (without Complete mini).
Centrifuge 30 sec at 2000 rpm, put 30 sec on ice and remove supernatant.
Repeat the wash 3x, resuspend in 1:1 slurry with PEB and keep on ice.

Washing Calmodulin/Sepharose beads

Resuspend beads and wash 200 µl of suspension with CBB (0.1% NP-40).
Centrifuge 30 sec at 2000 rpm, put 30 sec on ice and remove supernatant.
Repeat the wash 3x, resuspend in 1:1 slurry with CBB (0.1% NP-40) and keep on ice.

TAP

Mix lysates and IgG/Sepharose beads in a Falcon and incubate on a rotating platform between 2h and o/n at 4°C.

Open the bottom of a Poly-Prep Chromatography Column (Biorad).
Pour lysates and beads and allow to pack by gravity.
Wash 3x the beads with 10ml IWB.
Wash beads with 10 ml TCB
Close bottom of the column.
Add 1ml of TCB and 100U TEV.
Plug the top of the column and incubate on rotating platform o/n at 4°C.

Wash Calmodulin/Sepharose beads.
Recover the TEV eluate in a new sealed column.
Wash the old column with 1 ml TCB and recover in the new column.
Add 3 volumes (~ 6ml) of CBB (0.1% NP-40) to the TEV eluate plus 3 µl 1M $CaCl_2$ per ml of TEV eluate (~6µl).
Add Calmodulin/Sepharose beads and incubate on rotating platform at 4°C for 1h.

Open the bottom of the column.
Wash beads twice with 1 ml CBB (0.1% NP-40).
Wash beads twice with 1 ml CBB (0.02% NP-40).
Close the bottom of the column.
Elute 10 min one ice with 1ml CEB (0.02% NP-40).
Recover the eluate in a microcentrifuge tube.
Close bottom of the column.
Elute with additional 1ml CEB (0.02% NP-40).
Recover the second eluate in another microcentrifuge tube.
Add 333µl of TCA 100% to both eluates to obtain a 25% TCA volume.
Let 30 min on ice with periodic vortexing.
Centrifuge at maximum speed at 4°C for 30 min.
Remove supernatant and wash once with –20°C acetone containing 0.05M HCl.
Centrifuge at maximum speed at 4°C for 5 min.
Remove supernatant and wash once with –20°C acetone.
Centrifuge at maximum speed at 4°C for 5 min.
Remove supernatant and dry in a speed vacuum for maximum 10 min.
Resuspend the pellet of the 2nd eluate in sample buffer for 1D gel analysis or re-hydration buffer for IEF.
Then combine both eluates by suspending the pellet of the 1st eluate with the same buffer.

SOLUTIONS

Pre-cool all solutions on ice before use. All volumes are for one purification.

WFB (Worm freezing buffer)		
25mM HEPES pH7.2	1.0M	250µl
150mM KCl	2.5M	600µl
10% Glycerol (v/v)	100%	1ml
H_2O		
		to 10ml

PEB (Protein extraction buffer)		
50mM HEPES pH7.2	1.0M	1ml
150mM KCl	2.5M	1.2ml
20% Glycerol (v/v)	100%	4ml
H_2O		
		to 20ml

Add once the worm volume of PEB, then add these compon ding to the total volume :

1mM DTT	0.5M
2mM EDTA	0.5M
0,1% NP-40	10%
1mM PMSF	0.5M
mini complete (EDTA free)	1/10ml

IWB (IgG/Seph beads washing buffer)		
25mM Tris-HCl pH 7.4	1.0M	1.25ml
150mM KCl	2.5M	3ml
0.1% NP-40	10%	500µl
H_2O		
		to 50 ml

TCB (TEV cleavage buffer)

25mM Tris-HCl pH 7.40	1.0M	500µl
150mM KCl	2.5M	1.2ml
0.1% NP-40	10%	200µl
0.5mM EDTA	0.5M	20µl
1mM DTT	0.5M	40µl
H_2O		
		to 20 ml

CBB (Calmodulin binding buffer)

25mM Tris-HCl pH 7.40	1.0M	1ml
150mM KCl	2.5M	2.4ml
1mM MgAcetate	1.0M	40µl
1mM Imidazole	1.0M	40µl
2mM $CaCl_2$	1.0M	80µl
10mM βME	14.3M	28µl
H_2O		
		to 40 ml

Divide into two 20ml aliquots, adjust one to **0.1%** NP-40 by adding 200µl of 10% NP-40, adjust the other to **0.02%** by adding 40µl.

CEB (Calmodulin elution buffer)

25mM Tris-HCl pH 7.40	1.0M	250µl
150mM KCl	2.5M	600µl
0.02% NP-40	10%	20µl
1mM Mg Acetate	1.0M	10µl
1mM Imidazole	1.0M	10µl
20mM EGTA	0.5M	400µl
10mM βME	14.3M	7µl
H_2O		
		to 10 ml

General considerations

PEB

The pH of HEPES will rise slightly ($\Delta pK_a/°C$ = -0.014) when temperature drop. The pK_a of HEPES (pK_a=7.55 @ 20°C,) is the closest to physiological pH, however PIPES (pK_a=6.8, $\Delta pK_a/°C$ = -0.009) or Tris-HCl (pK_a=8.3, $\Delta pK_a/°C$ = -0.031) can be used depending on the proteins that will be extracted. Moreover PIPES or Tris-HCl have a negligible metal ion binding and none fore HEPES.

To avoid unspecific binding of proteins and thus to reduce background, till 300 mM of salt can be used. However, for most cases 150 mM is a good startin point. NaCl can also be used; it is somewhat less physiological, but both salts work.

The lysate can also be pre-cleared with 500 ml uncoupled Sepharose beads prepared in a 1:1 slurry with PEB and incubated 30 min at 4°C on a rotating pla form.

NP-40 and Triton X-100 are quite similar but the second one is a little bit harsher and is more appropriated to solubilize membrane proteins. 0.1% is good starting concentration for most proteins however it could be raised till 1% or as little as 0.05% for highly soluble proteins. But with such lower concentration it will be better to pass the extract at least two times in the French press to ensure cell membrane breakage.

PMSF permanently inactivate some serine proteases and omplete tablet has a half-life of 30 minutes. They must be added directly before use. Phosphatase inhibitors are recommended however they can be avoided.

Worm extract

Avoid maximum as possible temperatures above 4°C since it will result in degradation of proteins.

At least 5g of worms must be used.10g is suitable in most of the cases unless your complex is not abundant or difficult to solubilize then you can use till 120g of worms (ex: receptors, enzymes,…). 1g of ground or living worms pellet correspond to about 1ml.

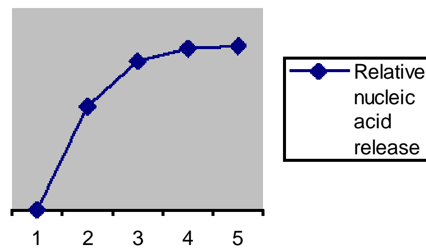
Before freezing worms pre-chill the mortar and pestle for at least 5 minutes with liquid N_2. Worm beads or ground worms powder can be stored at –80°C in a Falcon indefinitely. To store worms the Falcon has to be prechilled in an EtOH/carbonic ice bath. To thaw let them 1 hour on ice.

Homogenization should be done more times with bigger volumes.

All centrifugations are performed in an OTD combi ultracentrifuge with a Beckman Type 50 Ti rotor. Centrifugation at 16000 rpm corresponds to an average of 16945 RCF and 38000 rpm to an average of 95581 RCF. Supernatant have to be clear. An additional centrifugation must be done if needed. After centrifugation of the lysate, lipids and ribosomes are found on top whereas membrane and DNA on the bottom. Thus it is better to take the interphas n visible.

The power of sonication is set up depending of the viscosity of the solution. The number of sonication step needed can be established by checking A shearing by UV adsorption at 260 nm that monitor nucleic acid release.

Sonication test



TAP

1u of TEV cleave about 3 µg of control at 30°C. TEV cleavage is performed o/n at 4°C to prevent proteins degradation, however it is more active at higher temperature. Thus to increase yield it is better to increase TEV concentration till 500u if needed than increasing incubation temperature. TEV has an half-life of about 4 days at 4°C. To avoid autocatalysis use Invitrogen AcTEV.

The elution can also be performed with urea or by addi on of sample buffer directly to Calmodulin/Sepharose beads. However EGTA elution followed by TCA/Acetone precipitation will allow to obtain the best concentration/volume ratio for further gel separation and MS analysis. Moreover TCA/Acetone precipitation will remove salts, esidual buffers and other charged small molecules that can interfere with IEF.

Reference:

Puig, O. and al. " The tandem affinity purification (TAP) method: A general procedure of protein complex purification". *Methods* 2001, 24, 218-229.

Polanowska, J and al. "Tandem immunoaffinity purificat of protein complexes from "*Caenorhabditis elegans*". *BioTechniques* 2004, 36, 778-782.

Gould, K.L. and al. "Tandem affinity purification and ntification of protein complex components". *Methods* 2004, 33, 239-244.

Cheeseman, I.M. and al. " A combined approach for the m affinity purification of protein complexes from metazoans". Science's stke 2005.