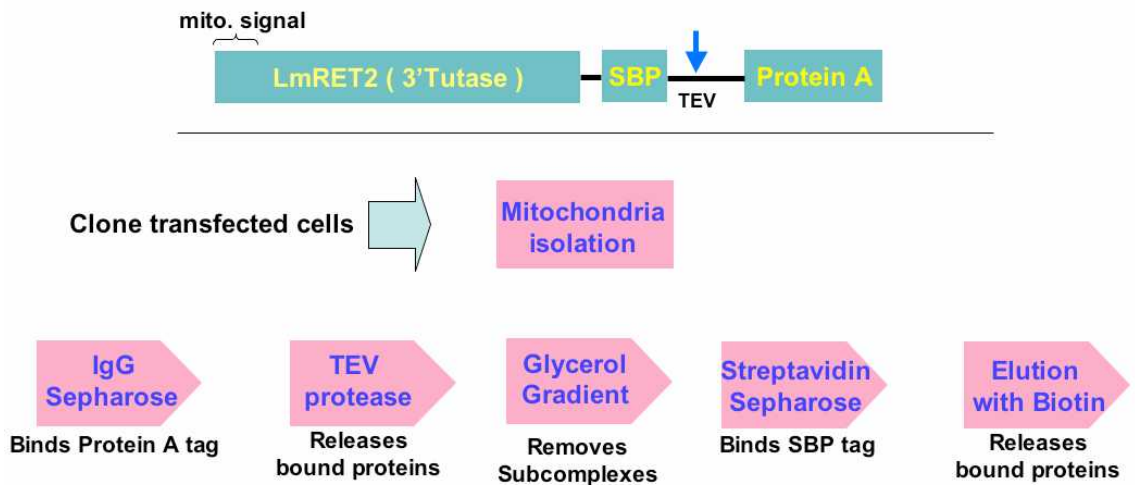


You can download a .gb type sequence file with annotation at <http://dna.kdna.ucla.edu/simpsonlab/vectors/vectors.htm#10>

Below is a protocol for the tandem affinity purification of the L-complex from *L. tarentolae* expressing this C-terminally tagged *L. major* RET2. This protein is a 3'-tutase required for RNA editing in mitochondria. Of course, if your studies involve protein or protein complexes expressed in other subcellular compartments, you can see that the protocol would be easily modified. Since we have sent you our vector with RET2 as the "stuffer", you need pop it out with BamH1/Xba1 double digest and slip in your ORF with BamH1 as your 5' end with an ATG immediately following and Xba1 as your 3' end that immediately follows your last sense codon. Coding is simple: the Xba1 site represents two complete codons, in frame, with the SBP sequence just downstream.

## THE PROTOCOL

### Transfection of *Leishmania tarentolae* with tagged fusion protein



All steps should be at 4°C and/or on ice.

Suspend 2 g of renografin-purified mitochondria in 8 ml of Buffer A with the aid of a hand-manipulated glass and teflon homogenizer. Add 0.5 ml 10% NP40 and gently mix.

Incubate on ice for 15 minutes. Sonicate with a micro probe 3x for 5 seconds, with 2 minute intervals on ice between sonication. After 15 additional minutes on ice, repeat sonications.

Centrifuge for 10 minutes at 90,000 rpm in Beckman TLA 100.4 rotor.

Save the supernatants on ice. Resuspend the pellets as before in 8 ml Buffer A, repeat sonications and centrifugation.

Pool both supernatants, and incubate with 0.6 ml (slurry volume ) IgG Sepharose 6 Fast Flow ( Amersham Biosciences ). In advance, the sepharose should be washed 3x in 20 ml Buffer B.

After 3 hours of gentle agitation on a ClayAdams Nutator, transfer the sepharose-extract mixture into two disposable plastic columns ( Pierce, cat #29920 ) and collect the extracts. Re-load the columns with the extracts and drain, 2x, without disturbing the gel beds. Fill the columns with Buffer B, cover with parafilm, and mix by hand, and drain. Repeat the Buffer B wash 5x. Fill the columns with Buffer C, cover with parafilm, and mixed by hand, and drain. Repeat the Buffer C wash 2x. Cap off the column tips, add 2 ml Buffer C and 20  $\mu$ l acTEV ( Invitrogen, cat #12575-015 ). Seal the columns with parafilm and agitate on the Nutator for six hours. You may want to extend this to overnight and get some sleep if your protein complex is quite stable.

Collect the TEV-released material by draining the columns and saving this "flow-thru". Agitate the gel bed with an additional 2 ml of Buffer B, drain, and save the flow-thru. Combine all flow-thru ( ca 8 ml ) and concentrate to 200  $\mu$ l using a Millipore 30 kD centrifugal filter device at 2000xg. In advance, the filter device should be washed 2x in Buffer B.

Fractionate the concentrated sample by velocity sedimentation through a 10 –30% glycerol gradient that, in advance, is prepared in Buffer D. For these small preps, we have been using the SW55 at 50,000 rpm for 5 hrs. By collecting twenty 250  $\mu$ l fractions, we pool only fractions that would be surrounding the 20s sedimentation position, based on a marker that we added to an identical gradient used to balance the rotor.

The pooled fractions, ca 2 ml and averaging 20% glycerol, are diluted 1:1 with Buffer D and mercaptoethanol added to 5 mM. In advance, 0.6 ml of Streptavidin Sepharose High Performance slurry ( Amersham, cat #17-5113-01 ) should be washed 3x in 20 ml Buffer E. The pooled and diluted glycerol gradient fractions are gently agitated with the sepharose for 30 minutes on a Nutator.

The mixture of gel beads and pooled samples is transferred to one disposable plastic column ( again, Pierce, cat #29920 ) and the flow-thru re-loaded 2x without disturbing the gel bed. The column is then filled with Buffer E, covered with parafilm, mixed by hand, and drained. This is repeated 4x.

The final purified "capture" is eluted as follows: successively, 250  $\mu$ l volumes of Buffer F are added to the gel bed, mixed by hand for 5 minutes, and the column drained and the fraction saved for analysis. Normally, the "capture" is eluted in the first three fractions, with fraction two containing most of the L-complex. Obviously, an advantage of the SA/biotin step is the small volume in which your final purification is quickly acquired without further concentration steps ( dialysis or filtration ) and avoids the losses associated when dealing with dilute solutions of purified proteins.

#### Buffers:

- A. 20 mM Tris, pH 7.8 at 4°C, 60 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, and one tablet protease inhibitors ( Roche, Cat #1873580 ) per 20 ml.
- B. 20 mM Tris, pH 7.8 at 4°C, 60 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, and 0.1% NP40.
- C. 20 mM Tris, pH 7.8 at 4°C, 60 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 0.1% NP40, and 1 mM DTT.
- D. 20 mM Tris, pH 7.8 at 4°C, 60 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, and 2 mM CHAPS.
- E. 20 mM Tris, pH 7.8 at 4°C, 60 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 2 mM CHAPS, and 5 mM mercaptoethanol.
- F. 40 mM Tris, pH 7.8 at 4°C, 60 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 2 mM CHAPS, 5 mM mercaptoethanol, and 2 mM biotin.

Note: Some of the composition of these buffers is "historical" ( changing from NP40 to CHAPS to avoid aggregation ) or in some sense illogical (10 mM MgCl<sub>2</sub>, in the same buffer with 1 mM EDTA ). Really important non-variables are 1) protease inhibitors at the extraction step only so that, later, added TEV will be active on the material bound to the IgG-Sepharose; 2) DTT at low concentration during release by TEV; 3) utilizing the long-life version of TEV, this is, acTEV , so that digestion really is active for at least six hours; 4) 40 mM Tris in Buffer F, so that the biotin will be in solution when it is at 2 mM at 4°C.