One step TAP-Tag purification

NOTE 1: This is the one step protocol which gives great yields and has been used to purify enzyme complexes for *in vitro* functional analyses. If you want super-pure samples for MS-studies uses the two step protocol (which has a much lower yield, but higher purity). I've used this protocol to purify kinases, phosphatases and HATs for *in vitro* analyses: all is the same until the dialysis buffer of choice.

Modification of protocol used in: Keogh *et al* (2006) *Genes Dev* 20:660-5.

Making extracts

1. Grow 1L cells in appropriate medium to $OD_{\lambda 600}$ <1.0 (exponential phase, etc). All steps below are with pre-chilled solutions on ice unless stated otherwise.

2. Collect cells by centrifugation. Successively wash with 100mls ddH_2O and 50mls Lysis buffer (LB^{o}, + inhibitors; LB^{o+}). Transfer cells to a 50ml Falcon and collect by centrifugation. Estimate the wet weight of the pellet (≈ 5g/L for 1L culture $OD_{\lambda 600}$≈1.0). Store pellets @ –80ºC (stable for months).

3. Thaw pellet on the bench (RT^{o}) and then place on ice. Add 5mls LB^{o+} and resuspend completely. Add an equal volume of glass beads and place on ice for 5-10mins to chill. Lyse with 5 x vortex pulses (1 min vortex, 1 min on ice). Centrifuge 10K, 10m, 4ºC. This is done in the preparative centrifuge with the Sorvall SS-34 rotor. You can spin the 50ml Falcon in the SS-34 rotor if you do not use the lid.

4. Collect the straw-colored supernatant to a Beckman untracentrifuge tube (26.3ml tube) on ice. Add another 5mls LB^{o+} to the lysate tube and resuspend completely with another 1 min vortex. Centrifuge 10K, 10m, 4ºC and pool the supernatant in the ultra tube. Balance the ultra tubes and centrifuge 45K, 30m, 4ºC. Collect the supernatant to a 15ml Falcon avoiding the crap at the bottom and the lipid payer at the top.

5. Using the Biorad Dc assay, estimate the concentration of the extract (usually ≈10mgs/ml). Given that you can collect ≈8mls of extract this gives you ≈80mgs(ish). Note the concentration, make 1ml aliquots and store @ -80ºC.

Purification

6. Thaw ≈12mgs WCE on ice. Make to 4mg/ml with LB^{o+} and dispense into 3 x 1.5ml ependorff tubes. Add 25µl IgG Agarose (Sigma, equilibrated in LB^{o}) per tube and incubate overnight / 4ºC with rotation.

7. Collect beads by centrifugation (1.5K, 2min, benchtop). Wash with 3 x 1ml LB^{o+} (NO PMSF) and pool all beads from the three ependorff tubes to one tube with 1 ml TEV buffer (TEV b^{o}). Collect by centrifugation and remove as much supernatant as possible. Make to

final volume ≈200µl with TEV b^{o}. Remove ≈5µl of the slurry to a new tube to confirm efficient digestion (before).

8. Add 8µl Jess-made rTEV (see **NOTE 2**). **NB.** This volume of rTEV is accurate for the batch available @ 5/28/05; see pH13.56. The activity of new batches will have to be determined empirically. Digest 4ºC, 4h on rotator. Remove ≈5µl of the slurry to a new tube to confirm efficient digestion (after). Collect the supernatant through a Promega Wizard column. Wash beads with 40µl TEV b^{o} and pool with supernatant.

NOTE 2: TEV, Tobacco Etch Virus NIa protease. Super expensive and available from Invitrogen (Cat# 10127-017; 1000U; 10U/µl). Protocol for making rTEV NIa protease available at http://mckeogh.googlepages.com/protocols. Each batch of rTEV should be titrated for activity. Below % substrate hydolysis data from Invitrogen.

Time	4ºC	16ºC	21ºC	30ºC
0.5h	34	58	56	77
1h	58	80	78	90
2h	71	99	99	99
3h	84	99	99	99

9. Transfer the supernatant to 2 x Slide-a-Lyzer mini dialysis units (3500MW cut-off, Pierce #69550: see **NOTE 3**). Dialyze for 90m, 4ºC against ≈ 400ml pre-chilled buffer of choice (sample buffers for PPAses, HATs and HDACs are given below). Collect dialysate and store in aliquots at –80ºC.

NOTE 3: Be careful to avoid piercing the dialysis membrane with the pipette. Ensure there's nothing suspicious about the volume in the dialysis unit (I've seen a significant number leak). To check for leaks add 100µl buffer to the unit and place it in a microfuge tube. Spin 5sec 1000rpm and discard those that allow buffer through.

Enzymatic reactions

In each case I've tested 1, 3 and 5µl of purified complexes and shown dose-dependence. Be sure to use an untagged strain (usually YF336 / BY4741) purified in the same manner as a control. The form the template takes is obviously up to you.

HATs: (example from [1], see MCK lab notebook pH13.57). *In vitro* HAT reactions were performed for one hour at 30ºC (25µl reactions containing 100ng histone substrates and 2µM Acetyl CoA (Na salt, A2056 (mw 809.6); 1mg ($15) to 1125µl = 1mM); conditions derived from [2]), resolved by 15% SDS-PAGE, and specific acetylation of Htz1-K14^{Ac} determined by immunoreactivity with a specific antibody.

PPAses: (example from [3]). Phosphatase reactions with γH2AX/H2B substrate were performed for one hour at 30ºC (conditions derived from [4]), resolved by 15% SDS-PAGE, and relative phosphatase activity determined by immunoreactivity with anti-γH2AX.

HDACs: (#1, example from [5]; Sin3/Rpd3). *In vitro* HAT reactions were performed for one to three hours at 30ºC. 25µl reactions contain 100ng acetylated histone substrates and HDAC under test. Samples are resolved by 15% SDS-PAGE, and specific acetylation of Htz1-K14^{Ac} determined by immunoreactivity with a specific antibody.

HDACs: (#2, example from [6]; Sin3/Rpd3). *In vitro* HAT reactions were performed for up to one hour at 37ºC (do a kinetic time course: 5, 10, 20, 40, 60m). 25µl reactions contain 100ng acetylated histone substrates and HDAC under test. Samples are resolved by 15% SDS-PAGE, and specific acetylation of Htz1-K14^{Ac} determined by immunoreactivity with a specific antibody.

Buffers

LB^o 20mM Tris.Cl pH 7.6
10% Glycerol
1mM EDTA
200mM KoAc
1mM DTT
Protease inhibitors as STD
Phosphatase inhibitors if desired (1M NaF = 1000x; 0.5M Na_3VO_4 = 1000x, 1M β-Glycerophosphate = 200X)

TEV b^o 50mM Tris.HCL pH 8.0
1mM DTT
0.5mM EDTA
Protease inhibitors as STD (but DO NOT ADD PMSF)

HAT b^o 50mM Tris.HCL pH 8.0
10% Glycerol
10mM butyric acid
1mM DTT
1mM PMSF (+ other protease inhibitors)
HAT b^o derived from [2], see MCK notebook pH13.57.

PPase b^o 20mM Tris.HCL pH 7.0
0.1mM EDTA
1mM DTT
0.01% Brij-35
1mM PMSF (+ other protease inhibitors)
PB1 derived from [4].

HDAC b^o 1 20mM Tris.HCL pH 7.65
100mM NaCl
10% Glycerol
1mM PMSF (+ other protease inhibitors)
Derived from [5] against Sin3/Rpd3.

HDAC b^o 2 10mM HEPES-KOH pH 7.8
75mM Kcl
0.1% NP40
5% Glycerol
2mM $MgCl_2$
5mm DTT
0.25mg/ml BSA
1mM PMSF (+ other protease inhibitors)
Derived from [6] against Sin3/Rpd3 (I prefer the look of this one).

2xSDS-PAGE b^{o}	120mM Tris pH 6.8 2% SDS 20% Glycerol 0.2% bromophenol blue 200mM DTT

References

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3. Keogh, M.-C., Kim, J.-A., Downey, M., Fillingham, J. *et al*. DNA damage checkpoint recovery requires a phosphatase complex acting on γH2AX. *Nature* **439:**497-501 (2006).
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6. Vermeulen, M. & Stunnenberg, H. G. An *in vitro* assay to study the recruitment and substrate specificity of chromatin modifying enzymes. *Biol Proced Online* **6**, 157-62 (2004).

Modification of protocol used in:

Keogh *et al* (2006) *Genes Dev* **20:**660-5.

In vitro HAT assays

Whole cell extracts (WCEs; untagged, Esa1.TAP, Gcn5.TAP or Elp3.TAP) were prepared as described (Keogh et al. 2003). Each HAT complex was affinity purified from 10mg WCE with IgG agarose (Sigma) (5µl resin / mg WCE). After overnight incubation at 4ºC the bead complexes were extensively washed with Lysis Buffer (LB: 20mM Tris.Cl pH 7.6, 10% Glycerol, 200mM KoAc, 1mM EDTA, 1mM DTT + protease inhibitors), resuspended to 200µl final in TEV buffer (TB: 50mM Tris.HCL pH 8.0, 1mM DTT, 0.5mM EDTA) and complexes cleaved from the beads with recombinant TEV protease (4 hours, 4ºC). The supernatant was collected, dialyzed for one hour against HAT buffer (HB: 50mM Tris.HCL pH 8.0, 10% Glycerol, 10mM butyric acid, 1mM DTT, 1mM PMSF) and stored in aliquots at –80ºC. *In vitro* HAT reactions were performed for one hour at 30ºC (25µl reactions containing 100ng histone substrates and 2µM Acetyl CoA; conditions derived from (Mizzen et al. 1999)), resolved by 15% SDS-PAGE, and specific acetylation of Htz1-K14 determined by immunoreactivity with anti-Htz1 K14^{Ac}.