TAP PURIFICATION

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Theory:

TAP tag is a quick, easy sequential affinity purification technique. The protein of interest is expressed in cells, fusion-tagged with a calmodulin-binding peptide, followed by a TEV protease cleavage site, followed by Protein A.

Brief Flowthrough:

Days Prior: Grow cells. Freeze them.

Day One: Lyse cells with a mortar and pestle. Thaw the cell powder, then make a crude lysate extract by ultracentrifugation. Then, incubate the lysate extract with IgG sepharose resin—this binds the Protein A epitope. After collecting and washing the resin, the protein of interest is cleaved off of the IgG resin by TEV protease, a sequence-specific protease uninhibited by most protease inhibitors (overnight cleavage incubation).

Day Two: The freed protein is then bound to calmodulin resin in the presence of calcium, which chelates the calmodulin-binding peptide in the protein tag to the calmodulin resin. After more collection and washing, EGTA is used to rip the calcium out of the resin-protein complex, freeing the protein of interest. The eluent is concentrated, the EGTA is dialyzed out (if not it would kill many biochemical reactions), and the enzyme is aliquotted and frozen.

Important Notes:

The buffer we use for chromatin remodeling TAP purifications is “E buffer.” E buffer is the base of all buffers made during the protocol. It is buffered at pH=7.3-7.5 by 20mM Hepes, has 350mM NaCl to raise the ionic strength enough to remove contaminant DNA and nonspecifically bound proteins that would otherwise be bound to chromatin remodeling enzymes, 0.1% tween detergent to stabilize proteins, and 10% glycerol, again to stabilize proteins. Make 1L of E buffer for the prep.

Always keep everything cold, either on ice or at 4^{o}C. Also, **avoid making bubbles** at all costs. Finally, be sure to **make sure all buffers are homogenous.**

To all buffers, always freshly add protease inhibitors (PIs): PMSF (phenylmethylsulfonyl fluoride; lab stocks are 10mg/ml in ethanol; 100X; @-20^{o}C), Leupeptin (lab stocks are 2 mg/mL in 20mM Tris8; 1000x; @-20^{o}C), Pepstatin (freezes at 4^{o}C; lab stocks are 2mg/mL in DMSO; 1000x; @-20^{o}C), Aprotinin (Sigma-ordered 1.7mg/mL in PBS; is 10000x; @4^{o}C), and benzamidine (lab stocks are 1M; 1000x; @-20^{o}C).

To most buffers, you will also add DTT (lab stocks are 1M; 1000x; @-20^{o}C).

Thaw all these stocks out to room temperature and make sure they are all fully redissolved and mixed before use (**no particulates**). In particular, Pepstatin A takes a long time to thaw and redissolve.

Make all buffers **right before** you need them.

Strains of Interest:

CY1552: SWI2-TAP wild-type, endogenous purification

CY1503: RSC2-TAP wild-type, endogenous purification

CY1497: SIR4-TAP, Sir2(HA)/Sir4p Galactose overexpression

Materials:

1M $ZnCl_2$, filter sterilized	IgG Sepharose 6 Fast Flow Resin (GE #17-0969-01; @4^oC)
1M $CaCl_2$, filter sterilized	Calmodulin Affinity Resin (Agilent #214303-52; @4^oC)
0.5M EGTA, filter sterilized	Polyprep columns w/ caps (Bio-Rad #731-1550; @RT)
Protease Inhibitors (see above)	TEV protease (we have lab-made stocks @-80^oC; or Invitrogen)
Dialysis apparatus; 10000 MWCO caps	Appropriate cell culture media (3x2L YEPD)

Vivaspin PES concentrators 6mL and 500uL (Sartorius Stedium #VS0601 and #VS0101)

E Buffer (make 1L for a prep): 20mM Hepes pH=7.4, 350mM NaCl, 0.1% Tween 20, 10% Glycerol. Filter Sterilize.

Specific Buffers:

"Lysis Buffer" – E Buffer with protease inhibitors; no DTT. If paranoid, 0.5mM DTT won't harm IgG.

"TEV cleavage buffer" – E buffer with protease inhibitors and 1mM DTT

"Calmodulin binding buffer" – E buffer with 2mM $CaCl_2$ and protease inhibitors and 1mM DTT

"Calmodulin elution buffer" – E buffer with 10mM EGTA and protease inhibitors and 1mM DTT

"Dialysis buffer" – E buffer with PMSF and 1mM DTT; also **FOR SWI/SNF PREPS ONLY** add 50μM $ZnCl_2$

Growing Cells, Lysis, and Ultracentrifugation:

From the corresponding glycerol stock, streak out a plate. When you get colonies, inoculate two colonies into overnight cultures. At the end of the next day, inoculate ~0.33mL of overnight saturated culture into each 2L flask of media. Typical prep scale is 3x2L of culture. The following day, collect cells following my "YEAST LYSIS WITH MORTAR AND PESTLE" protocol.

The following does not apply to wild-type yeast: For the slow-growing CY1497, it helps to patch colonies onto fresh plates and let grow before inoculating overnights with large yeast gobs, and to do an intermediate 50mL culture before inoculating the 2L flasks—try and keep the OD high.

The aforementioned mortar and pestle protocol ends with a clarified cell lysate, fresh from ultracentrifugation, in a 50mL Falcon tube.

IgG Chromatography and TEV Elution:

1. **Put 400µL of resuspended IgG resin slurry into a poly-prep column, then wash the resin with 10mL of Lysis Buffer.**

   Do this while the lysate is clarifying in the ultracentrifuge. To hold the poly-prep column, I fill a small ice bucket, embed a long-necked flask in it, lay a prep column holder across the mouth of the flask, and keep this apparatus in a 4^{o}C fridge. I keep a small loose sheet of folded foil as a protective cap over the column to stop particulates from falling into the open top of the column. When opening a poly-prep column, ensure the bottom hole is not obstructed by twisted plastic. Then, get the IgG resin and resuspend it in buffer by gently swirling. Never introduce bubbles or cake resin onto the cap/sides by shaking.

   Add wash buffer slowly, by dripping it down the side of the column so as to not disturb the resin bed. As the meniscus reaches the resin bed, cap the bottom of the polyprep column. Never let the resin dry out. Save a 20µL aliquot of the clarified lysate (frac. 1) for troubleshooting later if the prep fails.

2. **Transfer IgG resin into lysate. Nutate the resin + lysate @4^{o}C for 2 hours.**

   Transfer beads by resuspending the IgG beads from the column in a little bit of lysate, then transferring the bead suspension into the lysate tube. Cap and save the polyprep column.

3. **Spin down the resin in a prechilled J6B rotor for three minutes at 1k rpm and 4^{o}C.**

   Take a 20µL fraction of the supernatant (frac. 2). Carefully remove most of the supernatant without disturbing the resin at the bottom. Then, resuspend the resin and transfer it back to the polyprep column.

4. **Transfer resin to column. Uncap the bottom of the column and let the lysate drain. Wash resin with 10mL Lysis Buffer, then 10mL TEV cleavage buffer.**

   Collect a 10µL fraction of IgG-beads (frac. 3).

5. **Cap the bottom of the column, add 4mL TEV cleavage buffer and 300 units (20µL of our lab prep) TEV Protease. Cap the top of the column and nutate overnight at 4^{o}C.**

Ensure neither end of the column is leaking; if paranoid, apply parafilm.

Calmodulin Affinity Chromatography and Dialysis:

1. **Set column upright and collect eluent.**

   When uncapping the column, first set the column upright over a brand new 15mL conical to collect the eluent enzyme. Remove the top cap first, then remove the bottom cap while hovering the bottom of the column over the conical. Collect all 4mL, then wash the resin with an additional 1mL TEV cleavage buffer (try to rinse the sides of the column down), and collect that 1mL too. Save 20µL of beads (frac.4) and 20µL of elution (frac. 5).

2. **While eluting, prepare the second column by putting 400µL of Calmodulin affinity resin into a second polyprep column. Wash this column with 10mL Calmodulin binding buffer, then cap the bottom.**

3. **Bring the [$CaCl_2$] of the TEV elution up to 2mM with 1M $CaCl_2$ (10µL 1M per 5mL), then add the elution into the Cal column. Cap up the top of the column, and nutate 2h @4^oC.**

4. **Set the column upright and let the unbound flow out.**

   Save a 20µL fraction (frac. 6).

5. **Wash column with 20mL E Buffer + PIs + DTT.**

   Collect a 10µL aliquot of the beads (frac. 7).

6. **Cap the bottom of the column and add 2mL Cal elution buffer. Cap the top of the column and nutate for five minutes at 4^oC. Set the column back upright and collect the elution.**

   As before, rinse the column with an additional mL of Cal elution buffer and collect that as well. Save a 20µL fraction of the beads (frac 8) and 10µL of the elution (frac 9).

7. **Concentrate the pooled elution in a Vivaspin concentrator down to ~150µL.**

   Prewash all concentrators in appropriate buffer before use.

8. **Dialyze for at least three hours in 400mL dialysis buffer to remove excess EGTA.**

   Slide-a-lyzer dialysis caps are a convenient size. If desired, concentrate post-dialysis sample down to ~100µL (dialysis may cause volume expansion).

9. **Aliquot into appropriate volumes and flash-freeze in liquid nitrogen. Store @ -80C.**

If something went wrong, or if looking to optimize a prep, compare the fractions by western blot. Standardize active enzyme concentrations by ATPase assay, and verify enzyme purity by silver stain.