Standard Prep for ZZ tagged proteins from *S.cerevisiae*

Buffers

- 5x Lysis Buffer (Make 500ml stock)

		500ml	10 ml
150mM	Hepes pH 7.4	75.0 ml 1M	-
250mM	K-Acetate	62.5 ml 2M	-
10mM	Mg-Acetate	5.0 ml 1M	-
1mM	EGTA	2.5 ml 0.2M	-
5 mM	DTT	-	50 µl 1M
2 mM	PMSF	-	100 µl 0.2M
0.5mM	Mg.ATP	-	50 µl 0.1M

I usually take 10ml of stock (without DTT, PMSF or ATP) and add the appropriate volumes (50, 500 & 50 µl).

- Wash Buffer (Lysis Buffer with 300mM KCl)
  10 ml 5x Lysis Buffer stock
  10 ml 50% Glycerol
  7.5 ml 2M KCl
  H_2O to 50ml
  50 µl DTT, 100 µl PMSF, 50 µl Mg.ATP

- TEV Buffer

		500ml	50ml
10mM	Tris pH 8	5.0 ml 1M	-
150mM	KCl	37.5 ml 2M	-
10%	Glycerol	62.5 g	
1mM	DTT	0.5 ml 1M	50 µl
0.1 mM	Mg.ATP	0.5 ml 0.1M	50 µl

Growth and Harvest

1) Day1 afternoon: Innoculate a 10ml YPD (YP + 2% Glucose) starter culture with a yeast colony.
2) Day2 morning: Transfer 10ml culture to 200ml of YP+2% raffinose (or YPD, either is fine)
3) Day2 evening: Transfer 200ml culture to 2 litres of YP+2% galactose (in a 6 litre shake flask).
4) Day3 afternoon: Harvest when cells are dense (OD of 2-5)
   - Spin at 5000g for 5-10 minutes (1 litre bottles)
   - Wash in H_2O
   - Resuspend in a small volume of water.
   - Freeze cell pellets – drop into a plastic beaker of liquid nitrogen (inside an ice bucket)
   - Lyse cells – mash cell pellets to a fine power in a pestle and mortar or a small Warring blender/Coffee grinder. The grinding vessel needs to be pre-cooled with liquid nitrogen, the pellets can then be ground to a fine powder whilst remaining frozen (do not allow yeast to thaw). After grinding store the yeast powder in a 50ml falcon tube in the -80 until ready to use.

Protein Prep

1) Prepare 5x lysis buffer: add fresh DTT, PMSF and ATP
2) Estimate volume of 5x lysis buffer needed and add it to the frozen yeast powder.
   - 30ml of yeast powder will typically have a volume of ~18ml on thawing. I therefore usually estimate the volume of 5x lysis to add as follows:
   - 2 litre growth: ~30 ml yeast powder: Assume 18ml thawed volume: Add 4.5ml 5x Buffer)
3) Thaw yeast powder rapidly in a 37^{o}C water bath then return to ice
4) High speed spin: eg Rotor **Ti 70**: 70K / 30min
   - I typically use the Ti70 rotor. Tubes hold 26ml and must be filled completely or otherwise they collapse. Make up the difference with 1x lysis buffer if your volume is less than this.
5) Prepare IgG beads (Use 0.2ml of packed beads for a typical 2 litre prep) by washing twice in 1x lysis buffer.
6) Decant lysate from the high speed spin into a 50ml falcon (avoiding the gunge on top of the pellet).
7) Add NP40 or Triton X100 to a final concentration of 0.2%.
8) Add IgG beads to lysate and put on a rotary wheel or agitator for 1 hour (4^{o}C)
9) Prepare Wash Buffer and Tev Buffer with fresh DTT etc.
10) Wash the beads 3-4 times in Wash Buffer
    - We usually decant the lysate bead mix into a BioRad disposable column and do all wash steps in there. You can also wash by pelleting the beads gently (3000rpm in a desktop centrifuge) resuspending in buffer.
11) Wash twice in Tev buffer.
12) Resuspend beads in Tev buffer and transfer to a 2ml eppendorf tube.
    - For 200ul (packed volume) of beads, resuspend the bead slurry to a final volume of 400ul. We use a 2ml eppendorf to allow the beads to be agitated during the cleavage step.
13) Add Tev protease to the bead slurry.
    - (2ul of 4mg/ml Tev protease for 400ul of bead slurry)
14) 1 hour at 16^{o}C with agitation (eg rotary wheel)
15) Collect supernatant and freeze in 50ul aliquots.
    - We typically transfer the bead slurry to a small 0.22u spin column (#UFC40VV25, Millipore), spin and collect the flow through containing the dynein.
    - Alternatively you can also spin in a microfuge and take the supernatant, trying to avoid taking any beads.