Yeast large scale Whole Cell Extracts (WCEs)

NOTE 1: This is old school WCE preparation (original from: Sayre *et al* (1992) *JBC* **267:**23376), used for making large amounts of WCEs and downstream biochemical purifications (it is not really suitable for IPs – the salt concentration is too high).

NOTE 2: *S.cerevisiae* are extremely difficult to break open compared to other eukaryotes, so efficient mixing during the glass bead lysis step is essential. If the beads just sit there your protein extraction will suck. Also note that these extractions take place on ice with pre-chilled solutions or proteolysis will be a problem.

Variant of this protocol used in: Keogh *et al* (2002) *Mol Cell Biol* **22:**1288-97

1. Grow cells to late log phase in YPD (OD_{600} ≈6). Should get 10-20g cells /L. (Alternatively, **and preferable if at all possible**, use a fermenter: 12L of an overnight WT-like YPD culture will also give >10g cells /L).

2. Spin down and wash once with pre-chilled ddH_2O. Confirm the wet weight.

3. Resuspend pellet in 3 x lysis buffer (0.5ml per gram of cell wet weight)

4. Put suspension in Bead Beater (250-300 ml suspension for large chamber, 200ml 500µ glass beads; Sigma G8772). Ensure there is no air in the chamber – bubbles kill protein complexes. Pack an ice-salt mix around the chamber to keep it chilled.

5. Disrupt cells with 10-15 cycles: 30s on, 90s cooling. Check breakage under microscope. If necessary, do a few more cycles.

6. Pour off supernatant into GSA bottles (450ml preparative centrifuge flasks). Centrifuge 20m, 9K, 4°C in GSA. Pour off supernatant into a beaker.

7. Add a magnetic stir-bar and, while slowly stirring, add 1/7th volume of 4M KAc dropwise. Continue gentle stirring for 30m. (*This step increases the salt concentration to about 650mM, and helps to blast transcription factors out of nuclei*).

8. Pour extract into 45Ti centrifuge bottles (one 45Ti ultracentrifuge rotor holds 6 x 70ml). Spin 90m, 42K, 4°C. Carefully pipette the supernatant to a clean tube, avoiding the dark brown gunk near bottom.

9. Quantitate the total protein in each preparation with Bradford Dc assay. Expect 8-12 mg protein / ml lysate. Note the concentration and store extracts at -70°C until ready to move on.

Buffers:

3x Lysis buffer (per L, store at 4°C)	Stock	Final
450 mM Tris-Acetate pH 7.8	2M	225 ml
150 mM Potassium Acetate (KAc)	1M	150 ml
60% Glycerol	100%	600 ml
3 mM EDTA	500mM	6 ml
3 mM DTT	1M	3 ml
Protease Inhibitors (add fresh, just before use)		

From: Keogh *et al* (2002) *Mol Cell Biol* **22:**1288-97 (used a version of this protocol for the early steps)

Biochemical separation of Kin28 complexes. Extracts were made from yeast strains containing both 6.His tagged Tfb1 and HA-tagged Kin28 (WT, T162A or T162D). Purification of TFIIH by BioRex-70 and Ni-NTA column was modified from the procedure of Svejstrup et al. (69). 12 liters of each strain was grown overnight in a fermenter and disrupted as described (59). All manipulations from this point on were performed at 4°C and all buffers contained phosphatase and protease inhibitors. 1/7 Volume of 4M potassium acetate (pH 7.6) was added dropwise with stirring and after 30 minutes the mixture was centrifuged in a Beckman 45Ti rotor at 40000 rpm for 90 minutes. The supernatant (≈120 mls) was removed and diluted with buffer A-0 (20mM HEPES-KOH pH 7.6, 20% Glycerol, 1mM EDTA, 1mM DTT) until the conductivity matched buffer A-0.2 (Buffer A plus 0.2 M potassium acetate) and then chromatographed on a 150 ml Biorex-70 (Biorad) column equilibrated in buffer A-0.2. The column was washed with 200 mls buffer A-0.2 and 400 mls buffer A-0.4 before elution with 300 mls buffer A-0.65. The peak protein fraction (≈120 mls) was pooled and dialyzed against 2 x 2L of buffer A-0.2. The conductivity of the eluate was adjusted to match buffer A-0.2 before chromatography on a 50 ml phosphocellulose (Whatman) column equilibrated in buffer A-0.2. The column was washed with 50 mls buffer A-0.2 and eluted with a 300 ml gradient of 0.2 - 1.0 M KAcetate in buffer I. 3.5 ml fractions were collected from this gradient and subjected to immunoblotting analysis with antibodies against components of TFIIH (Kin28, Tfb3, Ccl1 and Tfb1). Two peaks were identified (see Figure 5).
Each peak was pooled and bound overnight to 2 ml Ni-NTA beads (Qiagen) equilibrated in buffer I (20mM HEPES-KOH pH 7.6, 20% Glycerol, 0.01% NP40, 0.2% Tween20, 10mM imidazole, 5mM β-mercaptoethanol) plus 0.5 M KAcetate and the beads collected by centrifugation. The supernatant was collected from the first peak (trimer) and stored in aliquots at -80°C while the beads from the TFIIH peak were washed in a column (0.7cm diameter) with 20 mls buffer J-1.0 and 20mls buffer J-0.4 (20mM Tris-Ac pH 7.6, 20% Glycerol, 5mM β-mercaptoethanol, 0.4 M potassium acetate) containing 20mM imidazole. TFIIH was then eluted with 10 mls buffer J-0.4 containing 100mM imidazole). 1ml fractions were collected and assayed by western analysis for Tfb1. Positive fractions were pooled and stored in aliquots at -80°C. Immunoprecipitations were performed from the Kin28-Ccl1-Tfb3 trimer and TFIIH fractions as described above.