Yeast WCEs for immunoprecipitations

NOTE 1: *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 are just sitting there your protein extraction will suck. Also note that these extractions take place on ice or proteolysis will be a problem.

NOTE 2: This is **NOT** my preferred method for quick screening *in vivo* tagged proteins or looking at chromatin modifications. For those I use the TCA extraction protocol – it's quicker, easier and MUCH more efficient at extracting chromatin.

NOTE 3: If you only intend one or two westerns then go with a 10ml culture (and I really suggest the TCA method in **Note 2**). If you want to do some immunoprecipitations (IPs) then you'll want to go up to about 50mls. Note that it's more hassle to deal with the larger volumes: you'll probably need a shaker rather than a rotator, flasks rather than tubes, etc, etc (both Option 1). If you're intending to do a lot of IPs, such as to prepare enzymes for downsteam analyses then you'll want to go to Option 2.

Variant of this protocol used in:

Keogh *et al* (2002) *Mol Cell Biol* **22:**1288-97
Keogh *et al* (2003) *Mol Cell Biol* **23:**7005-18.

SAFETY: Don't stab yourself with the red-hot 22G needle at **Step 1.4**

Option 1: **Small Scale**

1.1. Innoculate yeast from fresh plates into an 10ml overnight culture. The choice of medium depends on the yeast strain / plasmid incorporated. If knockout of an essential gene has been performed and loss of the covering plasmid would be lethal then YPD can be used. If an *in vivo* epitope tagging was performed, this too should be stable and YPD can be used. However if you have a non-essential plasmid with no-selective pressure (except the auxotrophy covered by the plasmid), then it will be required to grow in minimal dropout media. Place culture tube on rotator at 30°C.

1.2. Next morning allow the tubes to sit on the bench for about 20 minutes before sub-innoculating. This is a handy method of observing possible bacterial contamination. If the prep is yeast only they will settle quickly such that the top few mm of the culture tube will clarify. Innoculate 1/10 into 10 – 50 mls fresh medium ($OD_{600} \approx 0.15$) and grow to an $OD_{600} \approx 1.0$ (measure OD every 1 - 2 hours). The time it takes depends on the growth curve of the strain (WT in YPD, doubling time ≈ 90 minutes and you want to allow 2 - 3 divisions in log phase. For a WT strain this will take about 5 hours.

All buffers from now on pre-chilled on ice

1.3. Transfer culture to a centriguge tube and 3000rpm 5' to pellet (benchtop centrifuge)
Flick pellet to resuspend and resuspend in 1ml lysis b°
Under these conditions a 10ml culture should give a packed cell volume (pcv) ≈ 150µl
Transfer to ependorff tubes (no >300µl pcv per tube)
Flash spin (up to 10K in a benchtop microfuge) to pellet
Discard supernatant and place tubes on ice.

1.4. Resuspend in 1 volume lysis b° (+ inhibitors) / pcv
Add small acid washed glass beads (Sigma G8772) to meniscus and incubate on ice, 2'
Vortex 4 x 1' (with 1' on ice between mixes)
Invert tube and puncture bottom with red hot 22G needle (**CAREFUL**). Then puncture lid.
Place in a 2nd Epen (cut lid off this one) and spin microfuge 4000rpm, 2'
Wash beads with 1/2 pcv volume of buffer and also collect this by centrifugation.
Discard the top tube (but use the cap for the bottom) and spin 14000rpm, 10', 4°C.
Transfer S/N to fresh ependorff.

1.5. Quantitate total protein in each tube with Bradford Dc assay
Store proteins at -70°C until ready to use (expect 2-5 mg protein / ml lysate)

Option 2: **Large Scale**

2.1. In the AM innoculate a loopful of yeast from a fresh plate into a 10ml culture. The choice of medium depends on the yeast strain / plasmid incorporated. If knockout of an essential gene has been performed and loss of the covering plasmid would be lethal then YPD can be used. If an *in vivo* epitope tagging was performed, this too should be stable and YPD can be used. However if you have a non-essential plasmid with no-selective pressure (except the auxotrophy covered by the plasmid), then it will be required to grow in minimal dropout media. Place culture tube on rotator at 30°C.

2.2. After about 5 hours place the tubes to sit on your bench for about 20 minutes. This is a handy method of observing possible bacterial contamination. If the prep is yeast only they will settle quickly such that the top few mm of the culture tube will clarify. Innoculate 500µl into 1L culture medium (in a 2L flask). *I usually grow 1L of cells per strain for most downstream experiments using this protocol but scaling up to the 4L level requires no major changes*. Place flasks in a rotating incubator (250rpm, 30°C). By early the next AM, the $OD_{600} \approx 1.0$ for a WT strain (≈ 5g cells /L). This time is approximate, and will depend on the growth phenotype of the strain, the media and the incubator temperature. Your window is large: I've harvested strains from OD_{600} 0.8 – 1.3 and seen no difference.

All buffers from now on pre-chilled on ice (volumes are for 1L cells; $OD_{600} \approx 1.0$)

2.3. Transfer culture to preparative centriguge flasks and 3000rpm, 10', 4°C to pellet. Resuspend cells in 8ml lysis b° (+ all inhibitors). Transfer to a 50ml Falcon tube and add an equal volume of glass beads (Sigma G8772). Place tube on ice for 5' to cool everything down. Vortex high speed 5 x 1' (with 1' on ice between mixes).

2.4. Place 50ml Falcon into preparative centrifuge (Rotor SS-34 with lid removed, but screwed into place; maximum 4 samples / spin). Centrifuge 6000rpm (**no faster**), 10', 4°C to pellet. Carefully remove the supernatant to an ultra tube on ice. Resuspend the slurry with 5ml lysis b° (+ all inhibitors) (mix it up with a wooden applicator and then vortex to resuspend as much as possible). Place Falcon back in rotor and centrifuge 6000rpm, 10', 4°C to pellet. Collect the supernatant and pool with the first batch. Balance all ultra tubes to two decimal places and centrifuge 40000rpm, 30', 4°C. Carefully collect the supernatant avoiding any gunk and transfer to a clean falcon tube on ice.

2.5. Quantitate total protein in each sample with Bradford Dc assay (expect 6-10 mg protein / ml lysate). Make all samples to 4mg/ml with lysis b° (+ all inhibitors), and transfer 1ml aliquots to ependorff tubes. Store all at -70°C until ready to use.

Lysis buffer (store at 4°C)

	Stock	per 500ml
20mM Hepes pH7.6	1M	10 mls
10% Glycerol	100%	50 mls
200mM KoAc	5M	20 mls
1mM EDTA	500mM	1 ml

Take about 50mls at a time and use for a week or so. Add
1mM DTT (1M stock)
Protease inhibitors (leupeptin, pepstatin-A, aprotinin; all 1000x stocks at –20°C)

And add just before use (PMSF is unstable in aqueous):
1mM PMSF (Stock 100mM in MeOH)

A version of this protocol was used in:
Keogh *et al* (2002) *Mol Cell Biol* **22:**1288-97
Keogh *et al* (2003) *Mol Cell Biol* **23:**7005-18.

Protein analysis and immunoprecipitations. For the preparation of whole cell extracts, cells were grown to an optical density (600nm) of 1.0 and pelleted. Glass beads were used to disrupt cells in lysis buffer (20mM Hepes pH7.6, 200mM KOAc, 10% Glycerol, 1mM EDTA) supplemented with phosphatase inhibitors (1mM NaF and 0.5mM Na_3VO_4) and protease inhibitors (1mM PMSF, 1μg/ml aprotinin, 1μg/ml leupeptin and 1μg/ml pepstatin-A). Equivalent amounts of protein were then subjected to SDS-PAGE analysis. Immunoblotting was done using standard methods. Polyclonal rabbit α-Kin28 was from Covance. FLAG-M2 agarose beads, polyclonal goat anti-rabbit*HRP and goat anti-mouse*HRP were from Sigma. Rabbit polyclonal α-Tfb1 and α-Tfb3 were made against the appropriate his-tagged recombinant proteins by Covance. The B3 monoclonal antibody was a gift from Ben Blencowe and polyclonal α-Ccl1 was from Roger Kornberg.

HA-tagged Kin28 mutant proteins were immunoprecipitated from whole cell extracts or chromatographic fractions as indicated using 12CA5 bound to protein A beads. Complexes were prepared by mixing 10μl of protein A resin and 2μl of 12CA5 ascites per sample in TE (pH 8.0) followed by incubation with gentle rolling for 30 minutes at 4°C. Beads were washed twice with one ml TE (pH 8.0) and diluted 1:1 with TE (pH 8.0). 10μl of this mix was then added to each yeast protein sample. Reactions were incubated with gentle rolling overnight at 4°C. Finally, beads were pelleted by centrifugation and washed three times in one ml lysis buffer (20mM Hepes pH7.6, 200mM KOAc, 10% Glycerol, 1mM EDTA) prior to analysis by SDS-PAGE.