S.cerevisiae fractionation

NOTE 1. In addition to fractionating *S.cerevisiae*, this protocol should work for purifying chromatin / nucleosomes suitable for direct analysis or affinity purification of binding factors.

Variant of protocol used in: Keogh *et al* (2005) *Cell* **123:**593-605
Keogh *et al* (2006) *Genes Dev* **20:**660-5
Keogh *et al* (2006) *Nature* **439:**497-501

1. Grow 100mls – 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. Volumes given are for cells harvested from ≈50mls with OD_{600} <1.0. For this pellet size you can perform the sucrose gradient fractionation step in a 2ml ependorff tube (this is important – with a fixed angle microfuge, a 1.5ml ependorff is not recommended).

2. Collect cells by centrifugation. Successively wash with 10mls ddH_2O and 10mls SB in a 14ml U-bottom Falcon tube. Store pellets @ –80ºC.

3. Thaw pellets on ice. Successively wash with 1.5mls each PSB and SB in a 2ml ependorff tube (flash spin to 9K in a benchtop microfuge).

4. Resuspend completely in 1ml SB. Add 125µl Zymolase 20T (10mg/ml in SB). Remove 10µl and add to 1ml 1% SDS; determine $OD_{\lambda 600}$, blanking with 10µl SB in 1ml 1% SDS. Incubate RTº with rotation. Determine $OD_{\lambda 600}$ every 30min, continuing incubation until it drops > 80% (should take about one hour). NOTE: Some mutants may be more zymolase-resistant than WT: if digestion is suspiciously slow at one hour add another 75µl Zymolase 20T and continue incubation.

5. When digestion is complete add 1ml SB, collect by centrifugation (microfuge: 2K, 5m, 4ºC) and repeat SB wash (**NB.** Be careful – spheroblasts are more fragile than cell-walled yeast).

6. Resuspend spheroblasts gently with 500µl EBX (0.25% Triton X-100 + Phenol red). Accounting for the pellet volume, add Triton X-100 to 0.5% final to lyse outer cell membrane. Place on ice for 10min with gentle mixing every few minutes **(* aliquot / westerns; TOTAL)**. Layer over 1ml NIB. Centrifuge 12K, 15m, 4ºC.

7. Should now be three layers : (i) cloudy reddish (because of the phenol red) upper (cytoplasmic fraction - *** aliquot / westerns; CYTOPLASM)**, (ii) possibly a white lipid (?) interphase (it may be on the side of the tube), and (iii) nuclear pellet: glassy, white, and the stuff you want.

8. Remove the supernatant and gently resuspend nuclei in 500µl EBX (0.25% Triton X-100). Accounting for the pellet volume, add Triton X-100 to 1% final to lyse nuclear membrane. Place on ice for 10min with gentle mixing every few minutes **(* aliquot / westerns; NUCLEI)**.

9. Collect chromatin by centrifugation (15K, 10m, 4ºC). Remove S/N **(* aliquot / westerns; NUCLEOPLASM)**. There are numerous options at this point depending on what you want to do.

(i) Salt stability: Wash the chromatin from above (with EBX) and split into numerous aliquots in 1.5ml ependroff tubes. Wash individual preps twice with EBX –100 to –500 (all mM NaCl; EBX is usually 100mM; In my hands most histones are kicked out between –250 and –500, so choose steps accordingly). Resuspend pellet in 50µl Tris pH8, and add 100µl 2x SDS-PAGE loading bº. Incubate 95ºC for 5 minutes, microfuge (15K, 5m, RTºC) and collect supernatant to a new 1.5ml ependorff tube. Run 10-15µl samples on SDS-PAGE gels to see if your modification of interest is relatively sensitive.

(ii) Solubilize nucleosomes for immunoprecipitation: Wash the chromatin from above (with EBX) and resuspend to 500µl with EBX. Sonicate 3 x 20s as per ChIP output (12) with one min on ice between pulses (this was determined empirically: MCK notebook pH13.152). Collect insoluble by centrifugation (15K / 10m / 4ºC). PCI extract an aliquot of the supernatant and run on an EtBr gel to see how much DNA was released and average size. Also run samples on SDS-PAGE gel to see how many histones were released. Determine concentration by Biorad Dc to balance different samples and bind onto beads for further experiments.

Other useful reagents = Control Abs to confirm efficient fractionation

1. Rabbit α-Rpt1 (19S proteasome component), use 1/1000; mainly cytoplasmic
2. Rabbit a-Htz1 (Upstate 07-718), use 1/400, nuclear
3. Rabbit α-H3 (Abcam ab1791), use 1/750; Nuclear
3. Rabbit α-eIF-5a (gift from R.S. Zitomer; SUNY Albany), use 1/4000; cytoplasmic

Buffers

SB 1M Sorbitol
20mM Tris.Cl pH 7.4

PSB 20mM Tris.Cl pH 7.4
2mM EDTA
100mM NaCl
10mM β-ME

EBX 20mM Tris.Cl pH 7.4
100mM NaCl
0.25% Triton X-100
15mM β-ME
Protease inhibitors as STD
Phosphatase inhibitors if desired (1M NaF = 1000x; 0.5M Na_3VO_4 = 1000x, 1M β-Glycerophosphate = 200X)

0.005% Phenol red (from 0.5% stock (1000x) in PBS) as indicated. Note that in addition to marking the upper layer, this is a pH indicator (yellow 6.8 – 8.2 red).

NIB
20mM Tris.Cl pH 7.4
100mM NaCl
1.2M Sucrose
15mM β-ME
+/- 50mM Na-butyrate
Protease inhibitors as STD
Phosphatase inhibitors if desired

(If you decide to try MNase to release the nucleosomes rather than Sonication)

1x MNase bº
20mM Tris.Cl pH 8.0
100mM NaCl
0.1% Triton X-100
5mM $MgCl_2$
2mM CaCl
10mM β-ME
Protease inhibitors as STD
Micrococcal Nuclease @ 1 – 50U as determined empirically.

2xSDS-PAGE bº
120mM Tris pH 6.8
2% SDS
20% Glycerol
0.2% bromophenol blue
200mM DTT