Yeast Nuclear Extract (Small Scale)

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Cell Growth

3 liters of cells are grown in YPD (3% glucose) to A600 of 3-5. Antifoam A (two drops from a P200 pipetman) is added to media before autoclaving.

For wild type cells, ~2.5 ml of YPD overnight inoculated per liter at 5:30 pm gives A600 of ~3-4 at 9:00 am if cells are grown at 30o. For wild-type cells grown at 25o, 10-12 ml inoculated per liter works best. Slow growing mutants need anywhere between 10-120 ml/liter inoculated depending on the strain.

Extract preparation

Day 1

Harvest Cells in 1 liter bottles (4 Krpm for 10 min). Drain excess media as well as possible and weigh cells. Expected yield is 20-35 g cells. Anything less than 18g will give poor extracts. If cells are overgrown, lyticase will work poorly in spheroplasting cells.

Resuspend cell pellets in 35 ml 50 mM Tris 7.5, 30 mM DTT. Usually this can be done by gently shaking the centrifuge bottles. Leave cells in 1 liter bottles. Incubate at 30o for 15 min.

Pellet cells (4K rpm for 8 min.) and resuspend in 20 ml YPD/S. Add 15 ml 2M sorbitol. Add 15 ml recombinant lyticase. Incubate at 30o with occasional gentle mixing.

Alternative: Instead of recombinant lyticase, can also use Zymolyase 100T from ICN. Use 12-18 mg per prep. The amount required can vary from ~12-18 mg depending on the yeast strain (often, wild-type takes the higher amount). If using zymolyase, add in one extra YPD/S washing step at 4 degrees. *(we typically use zymolyase)*

Check progress of spheroplasting every 15 min. To check, mix 4 microliters of cells with 4 microliters 1% SDS on a glass slide. Observe the number of cell ghosts under microscope. Incubate cells until about 80% spheroplasts are obtained. This can take anywhere from 30 min.

to 2 1/2 hours. If cells are spheroplasting slowly after 1 hr, an extra 5-10 ml of lyticase can be added. However, if cells were overgrown (A600 >5), the cells may never spheroplast. Spheroplasting is also somewhat strain dependent.

After spheroplasting has reached about 80%, add 100 ml YPD/S (room temp) and pellet cells (4K rpm for 12 min).

Resuspend cells in 250 ml YPD/S (room temp) and incubate at 30 degrees for 30 min. to allow cells to recover. The resuspension of spheroplasts works best if a small volume (~50 ml) of YPD/S is first added and cells are resuspended using a baking spatula. Then add the remaining YPD/S.

Pellet cells (4 K rpm for 12 min.) and resuspend in 200 ml cold YPD/S (4 degrees). Resuspend as in the previous step. Keep everything cold from this point on. Cells can be kept on ice for an hour or so if other cells are still spheroplasting.

(add an extra YPD/S wash if using zymolyase instead of lyticase)

Pellet cells (4K rpm for 12 min) and resuspend in 250 ml cold 1M sorbitol.

Pellet cells (4K rpm for 12 min) and and drain sorbitol media as well as possible (careful- sometimes the spheroplast pellet is not very tight). Resuspend in 100 ml Buffer A at 4° containing DTT and protease inhibitors. Transfer to 250 ml beaker.

Pass cell suspension 1X through Yamamoto LH1 homogenizer at 500 rpm in cold room. Transfer homogenized cells to GSA bottles.

Note: We have also had success douncing the spheroplasts three times in a B dounce if the homogenizer is not available.

Spin 5K rpm for 8 min. Transfer supernatant to new GSA bottles. Do not worry about the slimy loose pellet that also transfers. Repeat.

Spin supernatant 5K rpm for 5 min. Transfer supernatant to new GSA bottle. Repeat. By the last (fourth) spin, the slimy non pelleted material should be nearly gone and the pellets firm.

Optional: If the supernatant contains a very large amount of slimy material after this last spin, one more spin at 5K for 5 min can be done. However, do not do any additional low speed spins after this.

Transfer supernatant to 50 ml centrifuge tubes and pellet crude nuclei. Spin 13 K rpm for 30 min. in SS34 rotor. Remove supernatant by dumping and discard. Drain pellets.

Resuspend crude nuclear pellets with a small spatula in 10 ml Buffer B and transfer to 50 ml screwcap tubes. The prep can be stopped at this point. Quick freeze and store resuspended nuclear pellets at -70 degrees.

Day 2

Thaw nuclei on ice and measure volume. Add 3M ammonium sulfate (pH 7.5) to 0.5 M final concentration (1/5 original volume of nuclei) and immediately mix and incubate on roller in cold room for 30 min. After 10 min, break up any lumps with a glass rod. This step lyses nuclei.

Transfer to SW28 thick walled ultracentrifuge tubes and spin at 28K rpm for 90 min. at 4°.

Carefully remove supernatant with 5 ml pipette (and pasteur pipette if necessary) being careful to avoid the pellet. Do not worry about the white floating material. Transfer to 50 ml screwcap tube.

Add 0.35g solid ammonium sulfate/ml supernatant and immediately incubate on cold room roller for 30 min. The ammonium sulfate can be added all at once if a number of preps are being done. However, it is best if ammonium sulfate is added slowly while stirring supernatant in a beaker. The pH should remain above 7 (it almost always does) but should be checked. Adjust pH with 1M NaOH if necessary.

Transfer to thick walled ultracentrifuge tubes and spin in SW28 at 10K rpm for 20 min at 4°. Remove supernatant by dumping and re spin pellets at 10K rpm for 4 min. Carefully remove all remaining supernatant with a pasteur pipette.

Resuspend pellets in Buffer C (+ 0 salt) containing DTT and protease inhibitors. Depending on protein pellet size, resuspend in 1.5 - 0.4 ml buffer. This can be done with a small dounce homogenizer or a blue pipette tip depending on the amount of protein.

Dialyze nuclear extract vs Buffer C + 75 mM ammonium sulfate at 4°. Dialyze vs 500ml buffer with 3 changes of buffer over 4.5 hours total.

Aliquot extract and store at -70 degrees. Extracts should be 25-50 mg/ml in protein.

BioRad Protein Assay of extracts.

Extracts are sometimes difficult to get a reproducible measurement of protein concentration using the BioRad assay. This modified method works well.

Dilute extract 1/4 in 0.1% SDS. Add 1-2 microliters of diluted extract to 0.8 ml H20 in a 13x100 mm disposable test tube. Add 1 microliter 0.1% SDS to protein standards. Add 0.2 ml dye reagent. After 10 min, read absorbance at A595.

Buffers and solutions for nuclear extracts
(volumes given are for 6 2l extracts)

50 mM Tris, 7.5 250 ml

1.5g Tris in 250 ml H2O
pH to 7.5 with HCl
Before use, add 4.6 mg DTT/ml

YPD/S 2.5 liters at room temp. (YPD with 1M sorbitol)

25g Yeast extract
50g Bactopeptone
50g Dextrose (glucose)
455g Sorbitol
H2O to 2.5 liters total

YPD/S 1.5 liters (4 degrees)

15g Yeast extract
30g Bactopeptone
30g Dextrose
273g Sorbitol
Add H2O to 1.5 liters

1M Sorbitol (4 degrees)

273g Sorbitol
H_2O to 1.5 liters

Buffer A	**(650 ml)**
18% Ficoll 400 (or polysucrose 400)	117g Ficoll
10 mM Tris 7.5	6.5 ml 1M Tris 7.5
20 mM K Acetate	13 ml 1M K Acetate
5 mM Mg Acetate	3.25 ml 1M Mg Acetate
1 mM EDTA	2.6 ml 0.25M EDTA
0.5 mM Spermidine	82 mg Spermidine
0.15 mM Spermine	61 microliters 1.6M Spermine

3 mM DTT and protease inhibitors are added just prior to use

The Ficoll takes many hours to dissolve and frequently is stirred overnight.

Buffer B	**(250 ml)**
100 mM Tris acetate pH 7.9	3g Tris
50 mM potassium acetate	12.5 ml 1M K Acetate
10 mM $MgSO_4$	2.5 ml 1M $MgSO_4$
20% glycerol	50 ml glycerol
2 mM EDTA	2 ml 0.25M EDTA

3 mM DTT and protease inhibitors added just prior to use

Buffer C	**(250 ml)**
20 mM HEPES 7.6	1.19g HEPES
10 mM $MgSO_4$	2.5 ml 1M $MgSO_4$
1 mM EGTA	1 ml 0.25 M EGTA
20% glycerol	50 ml glycerol

adjust pH with KOH
3 mM DTT and protease inhibitors are added just before use

Buffer C + 75 mM Ammonium sulfate	**(1.5 liters)**
20 mM HEPES 7.6	7.14g HEPES
10 mM $MgSO_4$	15 ml 1M $MgSO_4$
1 mM EGTA	6 ml 0.25 M EGTA
20% glycerol	300 ml glycerol
75 mM Ammonium Sulfate	14.8g $AmSO_4$

adjust pH to 7.6 with KOH

Protease Inhibitors and DTT are added just before use (0.46g Benzamidine, 0.69g DTT, 15 ml PMSF, 3 ml 500X Leupeptin, 7.5 ml 200X pepstatin, 0.6 ml 2,500X chymostatin).

Zymolyase 100T (ICN)

This is reportedly contaminated with proteases, so extra care is needed to wash spheroplasts. Dissolve at 6 mg/ml in 50 mM Tris with 2X concentrated protease inhibitors. Incubate 10 min on ice before using. This material does not disolve well, so keep in suspension as well as possible.

Protease inhibitors and DTT

0.1 M PMSF (100x)
16 mg/ml Ethanol

Store at -20 degrees

0.2M DTT
32 mg/ml H2O
Store frozen at -20 degrees

Benzamidine (100X)
31 mg/ml H2O.
Store frozen at -20 degrees

Leupeptin (500X)
0.15 mg/ml Ethanol.
Store at -70 degrees for less than 6 months

Pepstatin (200X)
0.28 mg/ml methanol
Store at -20 degrees.

Chymostatin (2,500X)
5mg/ml DMSO

Store frozen at -20 degrees