

ULTIMATE YEAST DENATURING IP

Where possible, we prefer to use denaturing IPs to recover labeled proteins from pulse-chase experiments. Use of denaturing IPs not only reduces background, but also makes protein harvest easy—yeast are simply boiled in an SDS-containing solution to liberate total proteins. This is also much safer than bead beating radioactive yeast samples.

A. EXTRACTING YEAST PROTEINS.

Prepare: 250 µl SHD per set of four samples (ie., one pulse-chase).

1. Resuspend each frozen yeast pellet in 50 µl SHD by pipeting. Heat at 95°C for 10 minutes.

2. Spin briefly. Transfer supernatant to a fresh snap-lock tube.

B. IMMUNOPRECIPITATION.

Prepare: (i) 1.2 ml ice-cold TNN with inhibitors per sample (5 ml for 4 samples).
(ii) 2 sets of labeled tubes.
(iii) 25 µl of Protein A Sepharose combined with 25 µl of Protein G sepharose per sample (125 µl of each for 4 samples). Wash two times in TNN and resuspend in 25 µl of TNN (125 µl for 4 samples).

1. Add 200 µl of TNN to each sample, vortex, and spin for 2 minutes in the cold.

2. Transfer supernatant to a fresh tube containing 1 ml of TNN plus 50 µl of Protein A/G sepharose.

3. Rotate in cold room for 1 hr.

4. Spin lysates in microfuge for 20 seconds in the cold. Transfer supernatant to a fresh tube.

5. Add antibody as appropriate (we use 0.5 µl of 12CA5 ascites for HA-tagged proteins).

6. Rotate in cold room for 1 hour.

Prepare: (i) 25 µl of Protein A Sepharose combined with 25 µl of Protein G sepharose per sample (125 µl of each for 4 samples). Wash two times in TNN and resuspend in 25 µl of TNN (125 µl for 4 samples).

7. Spin samples briefly. Add 50 µl of Protein A/G Sepharose mix. Rotate in cold room for 1 hr.

Prepare: (i) 3 ml TNN with Complete Inhibitor per sample (15 ml for 4 samples).
(ii) 100 µl of 2xLaemmli buffer with 50 mM DTT (NB: this is one-quarter the usual amount of DTT).

8. Spin samples for 2 minutes on setting number 3 in microfuge. Remove supernatant.

9. Wash sepharose beads by adding 1 ml TNN and rocking back and forth 10 times.

10. Spin samples for 2 minutes on setting number 3. Remove supernatant. Repeat wash two more times.

11. Aspirate all liquid from the beads using a 26G needle. Resuspend in 100 µl 2 x Laemmli. Boil and load 50 µl on SDS-PAGE.

C. SOLUTIONS.

TNN	**For 500 ml**
50 mM Tris (pH 7.5)	25 ml 1M Tris (pH 7.5)
250 mM NaCl	25 ml 5 M NaCl
5 mM EDTA	5 ml 0.5 M EDTA
0.5% NP-40	25 ml 10 % NP-40
Add fresh each time:	
0.4 mg/ml Pefabloc	1:100 of 40 mg/ml stock
10 μg/ml Leupeptin	1:1000 of 10 mg/ml stock
10 μg/ml Pepstatin	1:500 of 2 mg/ml stock
5 μg/ml Aprotinin	1:2000 of 10 mg/ml stock

or....Add 1 COMPLETE INHIBITOR TABLET per 25-30 ml for IP washes.

Note: We use individual protease inhibitors when performing the extraction and initial IP steps. For the washes, we use Complete protease inhibitor tablets (Roche: 1 tablet per 25 ml TNN).

SHD	**For 1 ml**
2% SDS	200 μl 10% SDS
90 mM HEPES (pH 7.5)	90 μl 1M HEPES (pH7.5)
Add fresh each time:	
30 mM DTT	30 μl 1M DTT
0.4 mg/ml Pefabloc	1:100 of 40 mg/ml stock