

ULTIMATE MAMMALIAN DENATURING IP

Where possible, we prefer to use denaturing IPs to recover labeled proteins from pulse-chase experiments. Use of denaturing IPs reduces background considerably. If, however, you prefer to use non-denaturing conditions, see the 'Ultimate Mammalian Non-Denaturing IP' protocol. The protocol here is described for four sets of pulse-chase experiments (16 samples).

A. EXTRACTING AND DENATURING PROTEINS.

Prepare: (i) 2.0 ml TSD
(ii) Labeled snap-cap microfuge tubes

1. Do not thaw cells before making extract. To frozen cell pellet, add 100 µl of TSD. Mix up and down by pipetting. Boil 10 minutes.

2. Spin lysates in microfuge for 5 minutes at room temperature. Transfer supernatant to a fresh tube.

B. IMMUNOPRECIPITATION.

Prepare: (i) 25 ml ice-cold TNN.
(ii) Labeled snap-cap microfuge tubes
(iii) 450 µl of Protein A Sepharose combined with 450 µl of Protein G sepharose. Washed 2 times in TNN and resuspended in 450 µl TNN.

1. To each sample, add 1.2 ml of TNN and mix by pipetting. Add 50 µl of Protein A/G sepharose mix. Rotate in the cold for 1 hr.

2. Spin lysates in microfuge at top speed for 20 seconds. Transfer supernatant to a fresh tube.

3. Add antibody (*eg.*, 0.5 µl 12CA5 ascites)

4. Rotate in cold room for 1 hour.

Prepare: (i) 450 µl of Protein A Sepharose combined with 450 µl of Protein G sepharose. Washed 2 times in TNN and resuspended in 450 µl TNN.

5. Add 50 µl of Protein A/G Sepharose mix. Rotate in cold room for 1 hr.

Prepare: (i) 50 ml ice-cold TNN (with Complete Inhibitor)
(ii) 3 ml 2x Laemmli buffer (with 50 mM DTT)

6. Spin samples 10 seconds in the cold (or 2 minutes on setting number 3). Remove supernatant.

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

8. Spin samples 10 seconds in the cold (or 2 minutes on setting number 3). Remove supernatant. Repeat wash two more times.

9. Aspirate all liquid from the beads using a 26G needle. Resuspend in 100 µl of 2 x Laemmli (50 mM DTT). Boil and load 50 µl on SDS-PAGE.

Note that the usual concentration in 2 x Laemmli is 200 mM. For these experiments, the concentration is dropped to 50 mM.

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).

TSD	**For 200 ml**
50 mM Tris (pH 7.5)	10 ml 1M Tris (pH 7.5)
1% SDS	10 ml 20 % SDS
5 mM DTT	**ADD 1:200 of 1M DTT FRESH**