

ULTIMATE MAMMALIAN NON-DENATURING IP

Where possible, we prefer to use denaturing IPs to recover labeled proteins from pulse-chase experiments. This protocol, however, describes a non-denaturing IP. The non-denaturing protocol is useful when you wish to separate soluble from insoluble proteins, and if your antibody recognizes a native epitope. The protocol here is described for four sets of pulse-chase experiments (16 samples).

A. EXTRACTING PROTEINS.

Prepare: (i) 1 set of labeled snap-cap tubes
(ii) 18 ml of RIPA (add inhibitors fresh)

1. Resuspend each frozen cell pellet in 800 µl of RIPA by pipeting.

2. Rotate in cold room for one hour.

B. IMMUNOPRECIPITATION.

Prepare: (i) 2 sets of labeled snap-cap tubes
(ii) 450 µl of Protein A Sepharose combined with 450 µl of Protein G Sepharose. Washed 2 times in RIPA and resuspended in 450 µl of RIPA (with inhibitors)

1. Spin lysates in microfuge, 5 minutes at top speed (cold room). Transfer supernatant to a fresh tube.

2. To each sample, add 50 µl of Protein A/G Sepharose mix. Rotate in cold room for one hour.

3. Spin samples in microfuge for 20 seconds, top speed (cold room). Transfer supernatant to a fresh tube.

4. Add antibody to each sample (e.g., 0.8 µl 12CA5 ascites). Rotate in cold room for 1-2 hours.

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

5. Spin tubes briefly. Add 50 µl of Protein A/G Sepharose mix. Rotate in cold room for one hour.

Prepare: (i) 50 ml RIPA (with 2 Complete Inhibitor tablets)
(ii) 2.0 ml 2x Laemmli Buffer (with 50 mM DTT)

6. Spin samples in microfuge—2 minutes, setting #3—in cold room.

7. Aspirate supernatant. Add 1 ml RIPA, and rock back and forth to wash.

8. Spin samples in microfuge—2 minutes, setting #3—in cold room. Aspirate supernatant. Repeat washing two more times.

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

C. SOLUTIONS.

RIPA	**For 500 ml**
150 mM NaCl	15 ml 5 M NaCl
1% NP-40	50 ml 10 % NP-40
0.5% DOC	25 ml 10 % DOC
0.1% SDS	5 ml 10 % SDS
50 mM Tris (pH 7.5)	25 ml 1M Tris (pH 7.5)
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).