Immunoprecipitation protocol

(Adapted from Sisson et al. (2000) JCB 151(4):905-917)

Prepare Beads

BioRad AffiGel Protein A gel is prewashed 7 x ~5 volumes in Buffer A + 150 mM KCl (no DTT or inhibitors) or 1xPBS

Transfer 30 μl of a 50% slurry (15 μl packed beads) to 1.5 ml tubes for each coupling reaction.

Coupling Antibody to Beads

Per reaction use 15 μl washed beads
5 μl antibody
20μl buffer
40 μl

Nutate 4°C overnight

Remove and save Ab

Wash 7 x 800 μl in buffer

Transfer beads for each IP to a fresh tube (to leave any Ab bound to walls of tube behind)

Protein Binding

Per reaction use 15μl Ab-coupled beads
250-750 μl S100 extract at 1mg/ml
Note: 750 μl can be depleted of Lva under these conditions

Nutate > 2.5 hours 4°C (3.5-4 h if possible for non-Lva antigens)

Remove sup and make gel sample from 75μl sup + 25μl 1xESB

Wash beads 5 x 750 μl buffer (transfer to a fresh tube for last spin to leave non-specific proteins on walls of tube behind)

Make gel sample of pellet by adding 100μl 1xESB.

For Western blotting load: 7.5 μl sup = 1/133 total
15 μl pellet = 1/6.67 total

NB: Pellet samples in ESB may degrade rapidly (< week) even if stored –80°C

Preparation of S100 for IP

1. Homogenize dechorionated embryos in 10 volumes of E-buffer + inhibitors. Add PMSF to 1 mM just before homogenizing. Use a motor-driven Teflon-tipped pestle and homogenize with 5 full strokes (or 2 strokes past when it becomes easier). (OP has done 7 strokes)

2. Spin the homogenate at 10,000 x g for 10 min at 4ºC.

3. Remove white lipid “cap.”

4. Place a funnel into a graduated cylinder and line the funnel with sterile cheesecloth. Decant the supernatant onto the cheesecloth and record the volume of the filtered supernatant.

5. Adjust the final concentration of Hepes to 50 mM, gently mix the supernatant, then spin it at 100,000 x g for 1 hour at 4ºC.

6. Finally, collect the supernatant (S100) with a pipette avoiding the lipid layer. Final concentration by Bradford assay should be approximately 2-3 mg/ml.

<u>E-buffer</u>
5 mM HEPES-KOH pH 7.5
0.5 mM EDTA
0.5 mM EGTA
0.05% NP-40
Protease Inhibitor Cocktail (1:100)

<u>Stock Protease Inhibitor Cocktail</u>
0.04 g Benzamidine HCl (10 µM)
0.003 g Phenanthroline (1.2 µg/ml)
0.025 g Aprotinin (10 µg/ml)
0.025 g Leupeptin (10 µg/ml)
0.025 g Pepstatin A (10 µg/ml)

Resuspend the indicated amounts of each of the protease inhibitors in 95% EtOH and store at –20ºC. Dissolve the inhibitors in the EtOH at 37ºC for a short time prior to use. Note that there will always be some undissolved material. Obtain inhibitors from Sigma. Final concentrations, after a 1:100 dilution in E-buffer, are indicated in parentheses above.

For IP wash beads and IP pellets in modified Buffer A:

<u>Buffer A/150 mM KCl (no DTT)</u>:
50 mM HEPES-KOH pH 7.5
0.5 mM EDTA
0.5 mM EGTA
0.05% NP-40
150 mM KCl
+ inhibitors