Immunoprecipitation

BETHYL LABORATORIES, INC

Immunoprecipitation protocol
Reagents Needed:
Immunoprecipitation (IP) lysis buffer
Protease Inhibitors (Calbiochem Cat#539131)
Primary Antibodies made in Rabbit
Normal IgG, negative control (Rabbit IgG- Bethyl Cat. No. P120-101)
Protein A Sepharose Beads (Amersham Cat# 17-0780-01)
Cell Lysate
Sample Buffer

IP lysis Buffer

- 12.5 ml 1M NaCl (250mM)
- 2.5 ml 1M Tris (50mM)
- 500ul 0.5M EDTA (5mM)
- 2.5ml 10% NP-40
- 32 ml dH20

Protein A Beads
Resuspend 400 mg of Protein A beads in 10 ml of distilled H2O. Mix well to resuspend. Spin at 250 rpm for 5 minutes. Wash 3X in 10 ml IP Lysis buffer. Resuspend to 10 ml with IP lysis buffer for a 20% solution. Use 100 mcl per IP reaction.

4X Sample Buffer

- Glycerol 4.0 g
- Tris Base 0.68 g
- Tris HCL 0.67 g
- LDS 0.80 g
- EDTA 6 mg
- Brilliant Blue G250 2.5 mg
- Phenol red 2.5 mg
- Adjust volume to 10 ml with ultra pure water.

Store at 4 C.
4X sample buffer is available from Invitrogen (Cat# NP0007)

1X Sample Buffer

- 4X sample buffer 150 mcl
- 1M DTT 60 mcl
- Distilled water 390 mcl

Make fresh for each use.

Procedure:

1. Prepare cell lysate according to protocol. Place 500 mcl of the prepared cell lysate (1-3 mg/ml) into a 1.5 ml micro-centrifuge tube.
2. To this tube add 2 to 10 mcg of the primary antibody (If using neat sera or an IgG fraction such as Protein-A purified antibody, larger amounts are likely to be required. For best results, optimal amounts of antibody should be empirically defined.)
3. To a negative control reaction, add an equivalent amount of normal rabbit IgG.

4. Add 100 mcl of a 20% Protein A suspension.(Amersham Biosciences, Cat# 17-0780-01) to the mixture of antibody and cell lysate. Rotate the immunoprecipitation reactions (end-to-end) for 3 hours at room temperature or overnight at 4 C.
5. Centrifuge (200 x g; 5 minutes) to pellet the complex.
6. Remove the supernatant and add 500 mcl cold cell lysis buffer. Centrifuge (200 x g; 5 minutes).
7. Repeat wash step 6 twice more. After each centrifugation remove as much of the supernatant as possible.
8. After removing the supernatant from the third wash, add 40 mcl of freshly prepared 1X sample buffer to each tube and heat at 90 C for 5 minutes.
9. Continue with electrophoresis and immunoblotting as described under western blotting protocol. Load 8 to 16 mcl (20 to 40% of the IP reaction) to a polyacrylamide gel.

Note: For optimal results, complete reduction of the sample is required. We recommend the use of 0.1 M DTT in SDS-PAGE sample buffer and immediately heating samples, loading and running gels.

Immunoprecipitation with Agarose-immobilized Antibody

Reagents Needed:

Agarose-immobilized Antibody

Cell Lysis Buffer

NaCl	7.31g
1M Tris, pH 8	25ml
0.5M EDTA, pH8	5ml
10%NP-40	25ml
Distilled H2O	445ml

Store at 40C.

4X Sample Buffer

Glycerol	4.0 g
Tris Base	0.68 g
Tris HCL	0.67 g
LDS	0.80 g
EDTA	6 mg
Brilliant Blue G250	2.5 mg
Phenol red	2.5 mg

Adjust volume to 10 ml with ultra pure water.
Store at 4 C.
4X sample buffer is available from Invitrogen (Cat# NP0007)

1X Sample Buffer

4X sample buffer	150 mcl
1M DTT	60 mcl
Distilled water	390 mcl

Make fresh for each use.

Procedure:

1. Place 500 mcl of the pooled cell lysate (1-3 mg/ml) into a 1.5 ml micro-centrifuge tube.
2. To this tube add 15-25 mcl of gel slurry of the Agarose-immobilized Antibody. (For best results, optimal amounts of lysate and slurry should be empirically defined.)
3. Rotate the immunoprecipitation reactions (end-to-end) for 3 hours at room temperature or overnight at 4 C.
4. Centrifuge (200 x g; 5 minutes) to pellet the complex.
5. Remove the supernatant and add 500 mcl cold cell lysis buffer. Centrifuge (200 x g; 5 minutes).
6. Repeat wash step 6 twice more. After each centrifugation remove as much of the supernatant as possible.
7. After removing the supernatant from the third wash, add 40 mcl of freshly prepared 1X sample buffer to each tube and heat at 90 C for 5 minutes.
8. Load 8 to 16 mcl (20 to 40% of the IP reaction) to a polyacrylamide gel.

Note: For optimal results, complete reduction of the sample is required. We recommend the use of 0.1 M DTT in SDS-PAGE sample buffer and immediately heating samples, loading and running gels. We use 4X sample buffer from Invitrogen (cat#NP0007) to which DTT is added.

Immunoprecipitation FAQs

My protein of interest migrates in the vicinity of IgG heavy chain, how can I detect my immunoprecipitated protein without interference from the IgG signal?

Bethyl offers the ReliaBlot IP/Western Kit (Cat. No. WB120) that eliminates the interfering IgG signal when westerns are performed on samples immunoprecipitated with antibodies made in rabbit.

When I perform a western on immunoprecipitated samples, do I need to use more or less primary antibody when probing the western blot?

When probing immunoprecipitated samples on a western blot, the concentration of primary antibody can be increased resulting in an increase in sensitivity. However, for best results, the optimal dilution of antibody should be empirically determined.

Why does my IP reaction still appear as a slurry and not a pellet after spinning?

The IP reaction should only be spun at a speed of 200 x g to 500 x g (maximum 2400 rpm in a microfuge). Spinning at higher speeds will results in rupture of the protein A/G beads and a pellet will not form.

Do I have to use protease inhibitors in the lysis buffer used in the wash steps of the IP reaction?

Inhibitors are not necessary for the wash the steps.

How do I know how much antibody to use in the immunoprecipitation reaction?

For best results, the optimal amounts of antibody should be empirically determined. But a general rule is to add 2 to 10 micrograms of antibody per 500 micrograms of lysate. If you are using neat antisera, or an IgG fraction (such as protein-A purified antibody), greater amounts of antibody are likely to be required.