Immunoprecipitation Protocol

PRINCIPLE: The antigen is extracted from the cell in an appropriate lysis buffer, and antibodies are added to the lysate to allow formation of the immune complex. A solid phase matrix containing Protein A or G is added, and the immune complexes are allowed to bind by adsorption of the antibody to Protein A or G. After the Protein A (orG)-antibody interaction occurs, the unbound proteins are removed by washing the solid phase, leaving the purified antibody-antigen complexes bound to the matrix.

REHYDRATE PROTEIN A OR G AGAROSE/SEPHAROSE:

1. Weigh out ~ 100mg of Protein A into a microfuge tube (enough for 10 reactions). (If Protein G is used, start with step 4). See Protein A/G affinity tables 2+3.
2. Rehydrate the 100mg of Protein A with ~1ml PBS.
3. Mix and incubate at 4˚C for 1 hour.
4. Wash Protein A or G three times in 1ml PBS, micro-centrifuging at 14000rpm for ~10 seconds and aspirating supernatant in between washes.

TUBE	PROTEIN A OR G 50% SLURRY	IRRELEVANT ANTIBODY		TEST ANTIBODY		PRE-CLEARED LYSATE	COMPLETE RIPA BUFFER
		DILUTION	µL AB	DILUTION	µL AB	2MG/ML	
1	60µl	2.5 µg				400 µg:200µl	
2	60µl			2.5µg		400 µg:200µl	
3	60µl			2.5 µg			200 µl
4	60µl					400 µg:200µl	
5	60µ						200 µl

BLOCK PROTEIN A OR G:

1. Resuspend Protein A or G with an equal volume of 5%BSA/PBS to make a 50% Protein A or G slurry.
2. Incubate Protein A or G at 4˚C on a rocker for 2 hours or overnight. (Blocking prevents binding of non-specific proteins, which form covalent bonds with Protein A or G beads.)

PRE-CLEAR CELL LYSATE:

1. Prepare complete RIPA buffer by adding protease inhibitor tablet into RIPA buffer.
2. Thaw appropriate amount of lysate and dilute to 2mg/ml with complete RIPA buffer.
3. Add 50ul of 50% Protein A or G to lysate.
4. Rotate mixture at 4˚C for 1hour.
5. Micro-centrifuge pre-cleared lysate at 14000rpm for 20 seconds to pellet Protein A or G.
6. Carefully transfer pre-cleared lysate to a clean tube and then transfer ~20 ul of pre-cleared lysate to a labeled tube as the lysate positive control.

FORM AND PURIFY THE IMMUNE COMPLEX:

Label 5 microfuge tubes according to the following reactions:

1. Add 200µl of pre-cleared lysate to tube #1, #2, and #4 and 200µl Complete RIPA buffer to tube #3 and #5 according to the table.
2. Add 2.5µg of irrelevant antibody to tube #1 and 2.5µg of test antibody to tube #2 and #3 according to the table.
3. Rotate reaction mixture of antigen and antibody at 4˚C overnight.
4. Next day, add 60 µl of Protein A or G to each tube and rotate at 4˚C for 2 hours.
5. Collect IP complex by micro-centrifuging mixture for 30 seconds at 14000rpm, aspirate off supernatant.
6. Wash all reactions five times with 1ml complete RIPA buffer. To wash, resuspend the Protein A or G with the buffer, vortex briefly, centrifuge at 14000rpm for 30 seconds, and aspirate supernatant (make sure to aspirate all the supernatant at the last wash).

IP/WESTERN:

1. Resuspend Protein A or G with 50 µl of 2X reducing sample buffer. Prepare lysate positive control by mixing 20µl of pre-cleared lysate with 5µl of 5X reducing sample buffer.
2. Boil samples for 5 minutes. Micro-centrifuge briefly to pellet the Protein A or G.
3. Load ~15µl of the supernatants, pre-cleared lysate and non-precleared lysate on SDS-PAGE gels. Samples can be stored at -70˚C if the gel will be run later.

➜ **For gel transfer and Western blot analysis see Western blot protocol**

PRINCIPLE: When immunoprecipitations are coupled with SDS-PAGE, a number of important characteristics of the antigen can be determined readily. These assays can determine:

- The presence and quantity of the antigen.
- Relative molecular weight of the polypeptide chain.
- Rate of synthesis or degradation.
- Presence of certain post-translational modifications.
- Interactions with proteins, nucleic acids, or other ligands

TABLE 1 Required Immunoprecipitation buffer

RIPA BUFFER	50mM Tris, pH8.0 150 mM NaCl 0.1% SDS 1.0% NP-40 0.5% Sodium Deoxycholate Complete with Protease Inhibitor Cocktail tablets

TABLE 2 Protein A/G Affinities for Monoclonal Antibodies

ANTIBODY ISOTYPE	AFFINITY
Human IgG1	Protein A or Protein G
Human IgG2	Protein A or Protein G
Human IgG3	Protein G
Human IgG4	Protein A or Protein G
Rat IgG1	Protein G (weakly)
Rat IgG2a	Protein G
Rat IgG2b	Protein G (weakly)
Rat IgG2c	Protein G (weakly)
Mouse IgG1	Protein G
Mouse IgG2a	Protein A or Protein G
Mouse IgG2b	Protein A or Protein G
Mouse IgG3	Protein G
Rat IgM	neither - use bridging antibody

TABLE 3 Protein A/G Affinities for Polyclonal Sera

ANTIBODY ISOTYPE	AFFINITY
Human	Protein A or Protein G
Horse	Protein G
Cow	Protein G
Pig	Protein A or Protein G
Sheep	Protein G (weakly)
Goat	Protein G (weakly)
Rabbit	Protein A or Protein G
Chicken	Protein G (weakly)
Hamster	Protein G (weakly)
Guinea Pig	Protein A
Rat	Protein G (weakly)
Mouse	Protein A or Protein G (both weakly)

→ **Also available: Immunoprecipitation Troubleshooting Guide**