Buffer Preparation for IP

Lysis Buffer (Non denaturing)
1M Tris-HCl, pH8: 0.5ml
10% NP-40: 1ml
5M NaCl: 0.3ml
ddH2O: 8.2ml

RIPA Buffer
1M Tris-HCl, pH8: 0.5ml
10% NP-40: 1ml
5M NaCl: 0.3ml
10% SDS: 0.1ml
10% Sodium Deoxycholate: 0.5ml
ddH2O: 7.6ml

IP General Protocols

Cell Lysate Preparation
1. Wash cells gently with ice-cold PBS.
2. Harvest cells (10^7) and transfer to conical tube. Wash cells with 10ml ice-cold PBS and centrifuge at 400xg for 10 mins at 4° C. (The number of cells used per IP should be optimized specifically for each protein and antibody)
3. Discard supernatant and repeat wash once.
4. Remove supernatant completely and resuspend cell pellet in 1ml ice-cold Lysis buffer (supplemented with protease inhibitors and phosphatase inhibitors).
5. Gently vortex and transfer to 1.5ml tube.
6. Place tube on ice for 30 mins with occasional mixing.
7. Centrifuge cell lysate at 10,000xg for 15-30 mins at 4°C.
8. Carefully collect supernatant into clean tube.
9. The protein concentration can be determined by Bradford assay. Samples can be diluted to 1µg/ul for further experiments.
10. Cell lysates can be frozen at -80°C or used immediately for IP procedures.

Preclearing
1. Add 50ul of prepared Protein A or G slurry to 500µl cell lysates.
2. Incubate on a rotator for 30 to 60 minutes at 4°C .
3. Centrifuge at 2500xg for 2-3 mins at 4 ° C . Transfer supernatant to clean tube.

Immunoprecipitation

1. Add 1-10µg of antibody to the pre-cleared lysates. (The concentration of antibody should be optimized)
2. Incubate at 4°C overnight on a rotator.
3. Add 50µl Protein A or G slurry to capture complexes. (Protein A or G should be equilibrated in the corresponding lysis buffer used).
4. Incubate for 1-2 hour at 4°C on a rotator.
5. Centrifuge the tube at 2500xg for 30s at 4°C.
6. Carefully discard the supernatant. Wash beads 3-5 times with 500µl ice-cold lysis buffer.
7. After last wash, carefully remove supernatant and add 50µl SDS Sample buffer to bead pellet.
8. Vortex and heat at 90-100°C for 10 minutes.
9. Centrifuge at 10,000xg for 5 mins. Collect the supernatant carefully and load onto SDS-PAGE for further analysis.

How Not to Fail a IP Experiment?

1. Lysis buffers

The denaturing ability of lysis buffer used during cell lysis can be critical. The ideal lysis buffer should protect proteins in their most native conformation while allowing adequate amounts of protein released for further analysis. Non-ionic detergents such as NP-40 or Triton X-100 are milder than ionic detergents such as SDS or Sodium Deoxycholate. Salt concentration or pH can also affect the binding capability of the antibodies to the protein of interest.

2. Elution method

There are various ways to perform elution step in IP experiments. The harshest method would be boiling of beads in a reducing SDS sample loading buffer. This method also elute non-covalently bound antibody fragments along with the protein of interest. Other methods such as using glycine buffer or applying pH shift are also applied to avoid disruption of antibody in order to obtain lower background.

3. Choice of Protein A or Protein G

Protein G is often considered a more universal IgG Binding Protein than Protein A, but different species, and subtypes of species, do vary in their binding to these proteins. Refer to the table at page 3 to find the best material that suit your antibody subclasses.

4. Secondary antibodies optimized for IP Western Blotting

Heavy chains or light chains contributed by the denatured primary antibodies during IP often cause problems for the Western Blot detection of protein of interest especially if it should be found to migrate around 50kDa or 35kDa. To overcome this issue, some secondary antibodies which only recognizes native form of IgG have been developed. These antibodies significantly eliminates the detection of denatured heavy or light chains during Western Blotting.

Species	Subclass	Protein A	Protein G	Protein L
Human	Total IgG	+++	+++	+++
	IgG1	+++	+++	+++
	IgG2	+++	+++	+++
	IgG3	-	+++	+++
	IgG4	+++	+++	+++
	IgA,M,D	-	-	+++
Mouse	Total IgG	+++	+++	+++
	IgG1	+++	+++	+++
	IgG2	+++	+++	+++
	IgG3	-	+++	+++
	IgG4	+++	+++	+++
	IgA,M,D	-	-	+++
Rat	Total IgG	-	++	+++
	IgG1	-	+	+++
	IgG2a	-	+++	+++
	IgG2b	-	+	+++
	IgG2c	+	+	+++
Chicken	IgY	-	-	-
Goat	Total IgG	-	++	-
Rabbit	Total IgG	+++	+++	-

+++ Strong binding ++ Medium binding + Weak binding - No binding

Protein A

Protein A is a cell wall component of Staphylococcus aureus which binds to the Fc region of immunoglobulins, especially IgG.

Protein G

Protein G is a cell wall component isolated from Group B streptococci which binds to most mammalian immunoglobulins through their Fc region.

Protein L

Protein L is a surface component of Peptostreptococcus magnus which binds immunoglobulins through their light chains. This protein is thus able to bind to antibody classes including IgA, IgD, IgE or IgM