Immunoprecipitation protocol

General IP procedure including a list of reagents and a table to help you choose the correct protein beads

Immunoprecipitation is a method that enables the purification of a protein. An antibody for the protein of interest is incubated with a cell extract enabling the antibody to bind to the protein in solution. The antibody/antigen complex will then be pulled out of the sample using protein A/G-coupled agarose beads. This physically isolates the protein of interest from the rest of the sample. The sample can then be separated by SDS-PAGE for western blot analysis.

1. a. Lysis buffers

The ideal lysis buffer will leave proteins in their native conformation, minimizing denaturation of antibody binding sites while at the same time releasing adequate amounts of protein from the sample for subsequent analysis. Non-ionic detergents such as NP-40 and Triton X-100 are less harsh than ionic detergents such as SDS and sodium deoxycholate. Other variables that can affect the success of IP include salt concentration, divalent cation concentration, and pH. Therefore to optimize these variables they should be tested within the following ranges (From Harlow and Lane, page 231; see References):

Salts: 0-1 M

Detergent, non-ionic: 0.1-2%

Detergent, ionic: 0.01-0.5%

Divalent cations: 0-10 mM

EDTA: 0-5 mM

pH: 6 - 9

1. Non-denaturing lysis buffer

Use for antigens that are detergent soluble and can be recognised in native form by the antibody. Triton X-100 can be substituted for NP-40.

20 mM Tris HCl pH 8

137 mM NaCl

1% Nonidet P-40 (NP-40)

2 mM EDTA

Store up to 6 months at 4°C. Immediately before use add protease inhibitors

2. RIPA (RadioImmunoPrecipitation Assay) buffer

More denaturing than NP-40 or Triton X-100 lysis buffer, RIPA buffer contains the ionic detergents SDS and sodium deoxycholate as active constituents and is particularly useful for nuclear membrane disruption for nuclear extracts. RIPA buffer gives low background but can denature kinases.

50 mM Tris HCl pH 8

150 mM NaCl

1% NP-40

0.5% sodium deoxycholate

0.1% SDS

Store up to 6 months at 4°C. Immediately before use add protease inhibitors.

For convenience, a 10% sodium deoxycholate stock solution (5 g into 50 ml) may be prepared. It must be protected from light.

3. Detergent-free soluble protein lysis buffer

Some soluble proteins may not require use of detergents. Use this buffer with mechanical breakage of cells, e.g. repeated passage through a syringe or homogenization with a Dounce homogenizer.

PBS containing:

5 mM EDTA

0.02 % Sodium Azide

Store up to 6 months at 4°C. Immediately before use add protease inhibitors

4. Denaturing lysis buffer/buffer for non-detergent soluble antigens:

Epitopes of native proteins are not accessible to antibodies that only recognise denatured proteins. When harvesting and lysing the cells, heat the cells in denaturing lysis buffer. This method can also be used for antigens that cannot be extracted from the cell with non-ionic detergents. Use of DNase1 will aid extraction of proteins from chromatin.

1% SDS

5 mM EDTA

Store up to 1 week at room temperature

Immediately before use add:

10 mM dithiothreitol or beta-mercaptoethanol

Protease inhibitors

15 U/ml DNase1

5. Wash buffers

10mM Tris; adjust to pH 7.4

1mM EDTA

1mM EGTA; pH 8.0

150mM NaCl

1% Triton X-100

0.2mM sodium ortho-vanadate

Protease inhibitor cocktail

Store up to 6 months at 4°C. Immediately before use add protease inhibitors

1. b. Other reagents

Protease inhibitors

As soon as lysis occurs, proteolysis, dephosphorylation and denaturation begin. These events can be slowed down tremendously if samples are kept on ice or at 4°C at all times and appropriate inhibitors are added fresh to the lysis buffer. Mixtures (“cocktails”) of protease and phosphatase inhibitors are commercially available. If not using a cocktail, two of the most commonly used protease inhibitors for IP are PMSF (50 µg/ml) and aprotinin (1 µg/ml). For more details of protease and phosphatase inhibitors, please see our western blot guide.

Other reagents required:

Sterile PBS pH 7.4

Sterile PBS-BSA 1% w/v (filtered)

TBST buffer

Loading/sample buffer for western blotting

100 mM EDTA stock solution is made with 1.86 g EDTA dissolved into 40 ml H2O. Add NaOH to adjust the pH to 7.4. Finally, adjust the total volume to 50 ml.

2. Preparation of lysates

2.1. Lysates from cell culture

2.1.a. Non denaturing:

1. Place the cell culture dish on ice and wash the cells with ice cold PBS.
2. Drain the PBS, then add ice cold lysis buffer (1 ml per 107 cells/100 mm2 dish/150 cm2 flask; 0.5 ml per 5x106cells/60 mm2 dish or 75cm2 flask).

3. Scrape adherent cells off the dish using a cold plastic cell scraper then gently transfer the cell suspension into a pre-cooled micro centrifuge tube.
4. Maintain constant agitation for 30 min at 4°C.
5. Centrifuge in a micro centrifuge at 4°C.
   You may need to vary the centrifugation force and time depending on the cell type. A guideline is 20 min at 12,000 rpm but this must be determined by the end user (e.g. leukocytes need a very light centrifugation).
6. Gently remove the tubes from the centrifuge and place on ice. Aspirate the supernatant and place in a fresh tube kept on ice, and discard the pellet.

2.1.b. Denaturing:

1. Add 100 µl denaturing lysis buffer to 0.5 to 2 x 107 cells.
2. Mix well by vortexing vigorously for 2 to 3 sec at maximum speed. Transfer the cell suspension to a micro centrifuge tube.

The solution can be viscous at this stage due to release of DNA.

3. Heat samples to 95°C for 5 min to denature.
4. Dilute the suspension with 0.9 ml non denaturing lysis buffer. Mix gently. (The excess 1% Triton X-100 in the non denaturing lysis buffer quenches the SDS in the original denaturing buffer).
5. Fragment the DNA by passing the lysed suspension 5 to 10 times through a needle attached to a 1 ml syringe.

Repeat mechanical disruption until the viscosity is reduced to manageable levels. If the DNA is not fully digested and fragmented, it can interfere with the separation of the pellet and supernatant following centrifugation.

6. Incubate on ice for 5 min.
7. Proceed with the immunoprecipitation

2. 2 Lysates from tissue

1. Dissect the tissue of interest with clean tools, on ice preferably, and as quickly as possible to prevent degradation by proteases.
2. Place the tissue in round bottom micro centrifuge tubes and immerse in liquid nitrogen to “snap freeze”. Store samples at -80°C for later use or keep on ice for immediate homogenization.
3. For a ~5 mg piece of tissue, add ~300 µl lysis buffer rapidly to the tube and homogenize with an electric homogenizer.
4. Rinse the blade twice with another 300 µl lysis buffer per rinse and then maintain constant agitation for 2 hr at 4°C (e.g. place on an orbital shaker in the refrigerator).

Volumes of lysis buffer must be determined in relation to the amount of tissue present. Protein extract should not be too dilute in order to avoid loss of protein and to minimize the volume of samples to be loaded onto gels. The minimum concentration is 0.1 mg/ml; optimal concentration is 1-5 mg/ml.

Note: If denatured samples are required, use denaturing lysis buffer and perform steps 2 to 5 from the denaturing protocol above.

5. Centrifuge for 20 min at 12,000 rpm at 4°C in a micro centrifuge. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice. Discard the pellet.

3. Pre-clearing the lysates

Pre-clearing the lysate can help reduce non specific binding of proteins to agarose or Sepharose beads. Pre-clearing with an irrelevant antibody or serum will remove proteins that bind immunoglobulins non-specifically. The end result will have a lower level of background and an improved signal to noise ratio. However, if the final detection of the protein is by western

blotting, pre-clearing may not be necessary, unless a contaminating protein is interfering with visualization of the protein of interest.

1. Add either 50 µl of irrelevant antibody of the same species and isotype as the IP antibody or normal serum (rabbit is preferred by some researchers, see Harlow and Lane, page 243) to 1 ml of lysate. Incubate for 1 hr on ice.
2. Add 100 µl of bead slurry to the lysate.
3. Incubate for 10 to 30 min at 4°C with gentle agitation.
4. Spin in micro centrifuge at 14,000 x g at 4°C for 10 min.
5. Discard bead pellet and keep supernatant for immunoprecipitation.

To increase the yield, the beads can be washed 1 or 2 more times in lysis buffer, and the supernatants collected together.
It is important to make sure that as much of the normal serum is removed as possible as this will compete with the specific antibody against the antigen of interest. To check for this, a test can be done with lysis buffer instead of sample, performing all pre-clearing steps as above. A coomassie stain of a gel in which the resulting supernatant is run will reveal if the serum Ig is being removed effectively. If serum has not been sufficiently removed, bands will be present at 50 and 25 kDa for heavy and light chains; its presence may contribute to a weak IP. Consider either decreasing the amount of serum or increasing the amount of beads incubated with your samples in the pre-clearing
step.

4. Immunoprecipitation

Immunoprecipitation can be performed using antibodies by different methods. The use of these methods is based on the requirements of end users. The first approach (4.2 Method A) is to mix antibody with protein sample and allowing it to interact with protein followed by addition of Protein A/G support. This method yields high purity of protein however the antibodies are also co-eluted with protein of interest which sometime creates difficulties in western blot detection. The second approach (4.2 Method B) is to bind antibody to the Protein A/G beads and then mix with the antigen. This method gives lesser yield than first one and avoids the problem of co-elution of antibodies. The third approach is to mix antibody, antigen and beads together; this method gives lowest yield and purity.

4.1 Method A

Immunoprecipitation with antibodies in solution

1. On ice, in a micro centrifuge tube add 10-50 µg cell lysate plus the recommended amount of antibody (see below). These amounts will be chosen depending on the abundance of the protein and the affinity of the antibody for the protein. Typically in a pilot experiment a fixed amount of protein is precipitated by increasing amounts of antibody.

Check the antibody datasheet for recommended antibody concentration. As a guideline use:

1-5 µl polyclonal antiserum
1 µg affinity purified polyclonal antibody
0.2-1 µl ascites fluid (monoclonal antibody)
20-100 µl culture supernatant (monoclonal antibody)

2. Incubate the sample with the antibody between 1-12 hr (overnight) at 4°C, preferably under gentle agitation or rotation. The length of the incubation period depends on the amount of protein and affinity properties of the antibody.
3. Meanwhile prepare the Sepharose beads. If using a monoclonal antibody choose protein G-coupled Sepharose beads. If using a polyclonal antibody, protein A-coupled Sepharose beads are usually suitable (please refer to ‘Choosing the protein beads’ table below).

If the beads come as a powder, incubate 100 mg of beads in 1 ml 0.1 M PBS, wash for 1 hr so they swell up, then centrifuge, remove the supernatant and discard. Add 1 ml PBS 0.1% BSA, mix for 1 hr using eppendorf rotator and rinse in PBS 2 X. Remove the supernatant and add 400 µl of buffer made with protease inhibitors (can be the same as the lysis buffer). The slurry is now ready for use. It can be stored at 4°C for a few days; for longer periods keep the beads in PBS with 0.02% azide (rinse the beads extensively on the day of use and make up in fresh lysis buffer). You can also buy pre-swollen beads as slurry ready for use.

It is advisable to use pipette tips with the end cut off to prevent damage to the beads.
IgM antibody: Do not use protein-A or protein-G conjugated beads. Use anti IgM coupled protein A or Protein G beads. The IgM will then bind to the beads by binding to the anti-IgM antibody.

Click here to view the procedure using IgM antibodies:

4. Mix the slurry well and add 70-100 µl of the beads to each sample. Always keep samples on ice. Beads will tend to stick to the sides of the tip so try to minimize the movement in the pipette and use a tip cut 5 mm from the top.
5. Incubate the lysate beads mixture at 4°C under rotary agitation for 4 hr (the optimal incubation time can be determined in a preliminary experiment).
6. Follow the steps in section 5 wash.

4.2. Method B

Immunoprecipitation with Antibody-Agarose conjugate

1. To prepare Protein A or G agarose/Sepharose beads, follow the step 3 in method A.
2. To the microcentrifuge tubes add approximately 70-100 µl of slurry of Protein A-, or G-, or L-agarose conjugate.
3. Add 10 µl of primary antibody. Use the dilution recommended on the antibody datasheet for IP as a guideline.
4. Incubate the antibody-beads mixture for 1-4 hours at 4°C by gently mixing the mixture on a suitable shaker.
5. Centrifuge at 1,000-3,000 g for 2 minutes at 4°C and discard the supernatant.
6. Add 1 ml lysis buffer to the mixture by keeping gentle agitation and then centrifuge at 3,000 g for 2 minutes at 4°C. Repeat this washing step twice.
7. After washing the beads and antibody mixture, add 10-50 µg of cell lysates.
8. Incubate the lysate-beads/antibody conjugate mixture at 4°C under rotary agitation between 4 hours to overnight as required (The optimal incubation time can be determined in a preliminary experiment).
9. At the end of the incubation, continue with wash steps given in section 5.

5. Wash

1. When the lysate-beads/antibody incubation time is over centrifuge the tubes, remove the supernatant from the beads and discard. The complex of interest should now be specifically bound to the antibody coating the beads.
2. Wash the beads with washing buffer or lysis buffer 3 times, to remove non-specific binding. For each wash, mix the beads gently with wash buffer, centrifuge at 4°C and remove the supernatant which can be discarded.
3. Finally, ensure to carefully remove as much wash buffer as possible from the beads. The complex is now ready for elution from beads.

Using loading buffer is the harshest elution method, and will also elute any non-covalently bound antibodies and antibody fragments, which will appear on western blot gels. Antigens can be gently eluted with a glycine gradient (up to 1 M) to reduce the amount of eluted antibody.

Click here to view separate procedure for cross-linking antibody to Sepharose.

6. Elution

One of the three common methods can be used to elute the complex from the beads. The SDS buffer is the harshest buffer which will also elute non-covalently bound antibodies and antibody fragments along with the protein of interest on the other hand Glycine buffer gently elutes the protein with reduced amount of eluted antibody.

6.1. Glycine Buffer Elution:

In this procedure the complex can be eluted from the beads by acidification. A glycine buffer containing 0.1-0.2 M Glycine pH 2.0-3.0 can be used. The low pH of glycine buffer helps to weaken the interaction between the antibody and the beads. This method is advantageous as beads can be reused after removal of the glycine buffer. However the eluted sample should be immediately neutralized with Tris, pH 8.0-8.5. After standard IP, follow these steps for glycine elution:

1. Elute the beads (50 µl) 3 X with 150 µl 0.2 M glycine pH 2.6 (1:1) by incubating the sample for 10 minutes with frequent agitation before gentle centrifugation.
2. Pool the eluate and neutralize by adding equal volume of Tris pH 8.0.

3. Repeat these steps 1-2 times and collect all the eluate.
4. Neutralize the beads by washing 2 X with 150 µl lysis buffer (without detergent) and pool with eluate.
5. Run the samples on a western blot to check the precipitation of proteins.

6.2. SDS Buffer Elution

In SDS elution the Ag-Ab complex is eluted from the beads by heating or boiling samples in loading buffer with denaturant SDS. This method is advantageous because the extraction method is highly efficient and the resulting sample is more concentrated.

1. Elute 50 µl of beads by heating in 50 µl of 2 X SDS without DTT for 10 min at 50°C.
2. Pellet beads, transfer supplement to a new tube and add DTT at 100 mM (Elution 1).
3. Add 50 µl 2 X SDS Buffer with DTT to pelleted beads (Elution 2).
4. Boil the elution samples for 5 min and analyze content of the sample by western blot.

Generally, there should be target protein in both elutions 1 and 2 collected above although amounts in each will be variable; Elution 2 will have more IgG contamination than Elution 1.

6.3. Urea Buffer Elution:

In this method the complex is eluted from the beads by the chaotrope urea. Beads should be resuspended in 2-5 volumes of urea elution buffer (6 – 8 M Urea, 20 mM Tris pH 7.5, and 100 mM NaCl). The purified complexes have now been released into the supernatant which should be collected from above the beads. This method is advantageous for mass spectrometry because the sample can be digested by proteolytic enzymes.

1. Wash beads with pre-urea wash buffer (50 mM Tris pH 8.5, 1 mM EGTA, 75 mM KCl). Remove all residual supernatant.
2. Add 100 µl urea elution buffer and rotate for 30 min at room temperature with frequent agitation before gentle centrifugation.
3. Repeat this process at least twice more to ensure that the entire captured complex has been released from the beads. Pellet beads and remove urea to a new tube.
4. Add 3 X sample buffer to run on a gel in western blot.

7. Choosing the correct beads- summary table

Species Immunoglobulin Isotype	Protein A	Protein G
Human IgG1	+++	+++
Human IgG2	+++	+++
Human IgG3	-	+++
Human IgG4	+++	+++
Human IgM	Use anti human IgM	
Human IgE	-	+
Human IgA	-	+
Mouse IgG1	+	+++
Mouse IgG2a	+++	+++
Mouse IgG2b	++	++
Mouse IgG3	+	+
Mouse IgM	Use anti Mouse IgM	
Rat IgG	-	+
Rat IgG2a	-	+++
Rat IgG2b	-	++

Rat IgG2c	+	++
Chicken all isotypes	-	-
Cow all isoptypes	++	+++
Goat all isotypes	-	++
Guinae Pig all isotypes	+++	++
Hamster all isotypes	+	++
Horse all isotypes	+	+++
Pig all isotypes	+	++
Rabbit all isotypes	+++	++
Sheep all isotypes	-	+++

Key: +++ = Strong binding, ++ = Medium binding, + = Weak binding, - = No binding

8. References

Harlow, Ed, and David Lane. Using Antibodies. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1999.

Bonifacino, Juan S. et al. Current Protocols in Immunology 8.3.1-8.3.28, New York: John Wiley, 2001.