

IMMUNOPRECIPITATION (IP) PROTOCOL

Immunoprecipitation is a method that enables the purification of a protein. An antibody for the protein of interest is incubated with a cell extract so that the antibody will bind 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. **Lysis buffers and other reagents**
2. **Preparation of lysates**
3. **Pre-clearing the lysates**
4. **Immunoprecipitation**
5. **Choosing the correct beads- summary table**

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. To optimize, these 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
10% glycerol
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 (RadiolImmunoPrecipitation 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.

50mM Tris HCl pH 8
150 mM NaCl
1% NP-40
0.5% sodium deoxycholate

0.1% SDS

The 10% sodium deoxycholate stock solution (5 g into 50 ml) 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:

10mM dithiothreitol or beta-mercaptoethanol

Protease inhibitors

15 U/ml DNase1

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 ug/ml) and aprotinin (1 ug/ml). For more details of protease and phosphatase inhibitors, please see our Western Blot Beginner's Guide.

Other reagents required:

Sterile PBS pH 7.4

Sterile PBS-BSA 1% (filtered)

TBST buffer

Loading/sample buffer for Western blotting

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

2. Preparation of lysates

Lysates from cell culture

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 (1ml per 10^7 cells/100mm dish/150cm² flask; 0.5ml per 5×10^6 cells/60mm dish/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 microfuge tube.
4. Maintain constant agitation for 30 minutes at 4°C.
5. Centrifuge in a microcentrifuge at 4°C.

You may have to vary the centrifugation force and time depending on the cell type. A guideline is 20 minutes 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.

Denaturing:

1. Add 100 ml denaturing lysis buffer per 0.5 to 2×10^7 cells.
2. Mix well by vortexing vigorously 2 to 3 seconds at maximum speed. Transfer the cell suspension to a microcentrifuge tube.

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

3. Heat samples to 95°C for 5 minutes to denature
4. Dilute the suspension with 0.9 ml non-denaturing lysis buffer. Mix gently.
(The excess 1% Triton X-100 in the nondenaturing 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, it can interfere with the separation of the pellet and supernatant after centrifugation.

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

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 microfuge 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, homogenize with an electric

homogenizer.

4. Rinse the blade twice with another 300 µl lysis buffer for each rinse, then maintain constant agitation for 2 hours 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 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.

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

3. Centrifuge for 20 min at 12,000 rpm at 4°C in a microcentrifuge. 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 be a lowering of background and an improved signal-to-noise ratio. However, if the final detection of the protein is by immunoblotting, pre-clearing may not be necessary, unless a contaminating protein is interfering with visualization of the protein of interest.

1. Add 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 1 hour on ice.

1. Add 100 µl of bead slurry to the lysate.

2. Incubate for 10 to 30 minutes at 4°C with gentle agitation.

3. Spin in centrifuge at 14,000 x g at 4°C for 10 minutes.

4. 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 for antigen. 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 it is not (bands seen at 50 and 25 kD 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

1. On ice, in a tube add 10-500 μg cell lysate plus the recommended amount of antibody. 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 where a fixed amount of protein is precipitated by increasing amounts of antibody.

You can 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 to 1 μl ascites fluid (monoclonal antibody)
 20 to 100 μl culture supernatant (monoclonal antibody)

2. Incubate the sample with the antibody between 1 hour to overnight (depending again on the amount of protein and affinity properties of the antibody), at 4 °C, preferably under agitation.

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. If the beads come as a powder incubate 100 mg of beads in 1 ml PBS 0.1M, wash for one hour so they swell up, then centrifuge, remove the supernatant and discard. Add 1ml PBS-BSA 1% w/v, mix for one hour and rinse in PBS twice. 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 extensively the beads on the day of use and make up in 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 Goat anti Mouse IgM (or polyvalent Ig, or anti-heavy chain) beads.

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 hours (the optimal incubation time can be determined in a preliminary experiment).

6. When the incubation time is over, centrifuge the tubes, remove the supernatant and wash the beads in lysis buffer three times (each time centrifuging at 4 °C and removing the supernatant).

7. Finally, remove the last supernatant and add 25-50 μl of 2x loading buffer. Boil at 95-100 °C for 5 minutes to denature the protein and separate it from the protein-A/G beads, then centrifuge and keep the supernatant where the protein is now. You can then freeze the samples or run them on a SDS-PAGE. See section I, WB protocol for further details.

Using loading buffer is the harshest elution, 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. Please also see separate procedure cross-linking antibody to Sepharose.



5. Choosing the correct beads- summary table

Species Immunoglobulin Isotype	Protein A	Protein G
Human IgG1	+++	+++
IgG2	+++	+++
IgG3	-	+++
IgG4	+++	+++
IgM	Use anti Human IgM	
IgE	-	+
IgA	-	+
Mouse IgG1	+	+++
IgG2a	+++	+++
IgG2b	++	++
IgG3	+	+
IgM	Use anti Mouse IgM	
Rat IgG1	-	+
IgG2a	-	+++
IgG2b	-	++
IgG2c	+	++
Chicken All isotypes	-	++
Cow All isotypes	++	+++
Goat All isotypes	-	++
Guinea Pig All isotypes	+++	++
Hamster All isotypes	+	++
Horse All isotypes	++	+++
Pig All isotypes	+	++
Rabbit All isotypes	+++	++
Sheep All isotypes	-	++

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.