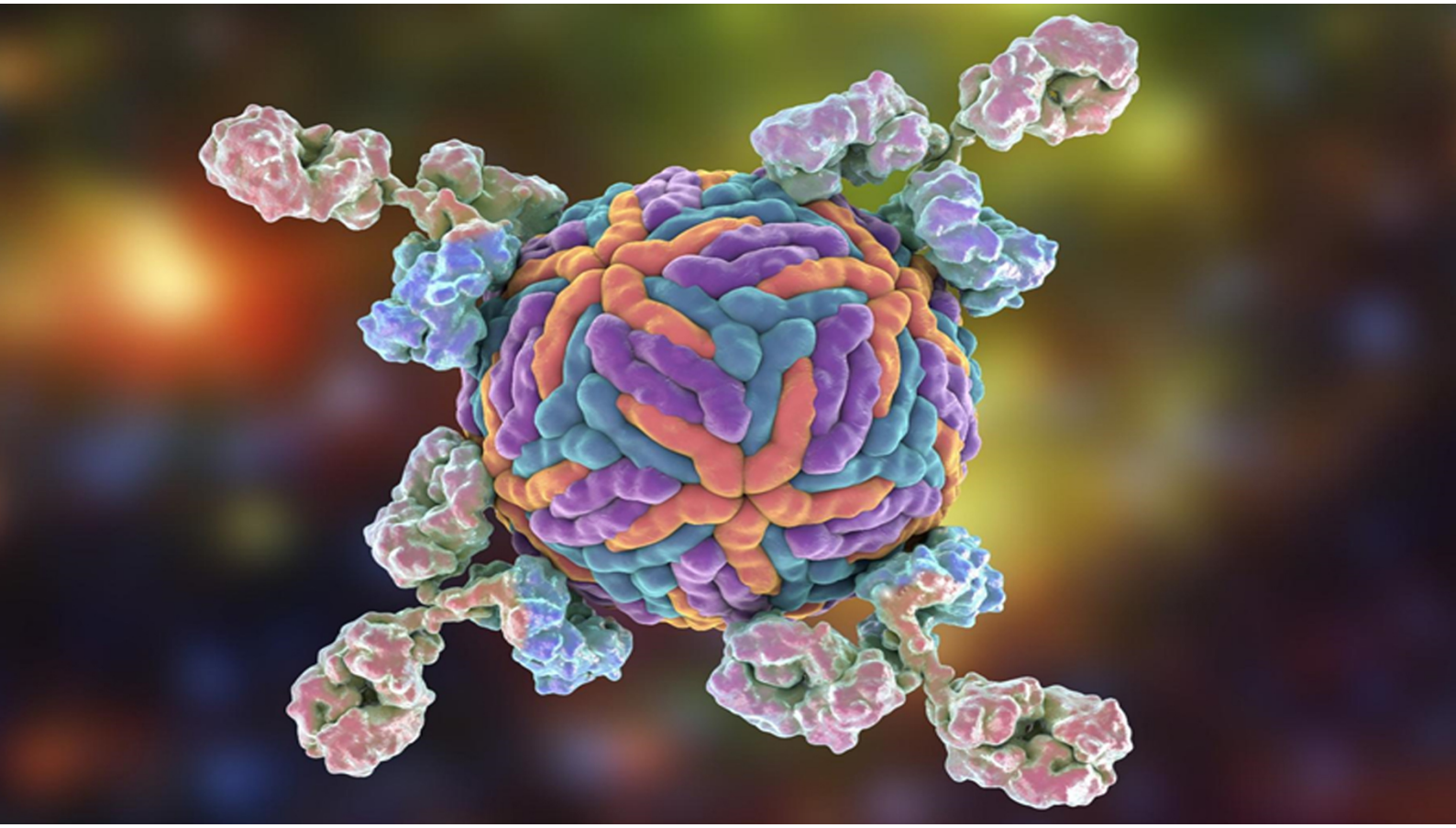
IMMUNOPRECIPITATION (IP) PROTOCOL



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IP Protocol

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Introduction to the Immunoprecipitation (IP) Protocol

Immune sedimentation is a method for purifying proteins. Antibodies to the target proteins are incubated with the cell extracts so that the antibodies bind to the proteins in solution. Protein A/G coupling agarose beads are then used to extract the antibody/antigen complex from the sample. This physically separates the protein of interest from the rest of the sample. Finally, the separated samples can then be analyzed by SDS-PAGE.

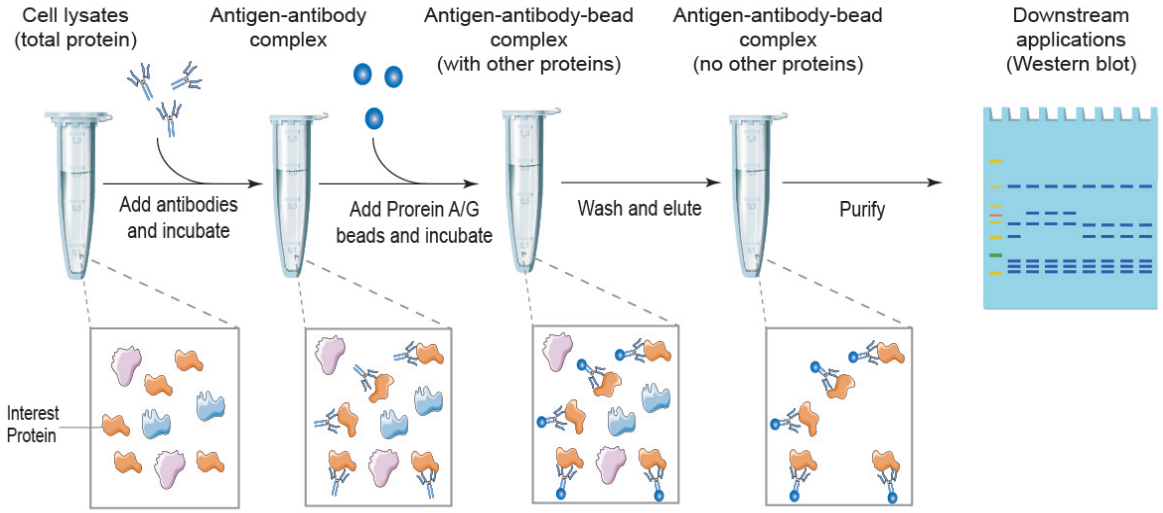

Figure 1. The process of Immunoprecipitation (IP) protocol.

1. Lysis buffers and other reagents
2. Preparation of lysates
3. Pre-clearing of lysates
4. Immunoprecipitation
5. Selection of the correct beadsSectioning and mounting

01 Lysis Buffers and Other Reagents

A. Lysis Buffers

An ideal lysis buffer would allow the protein to retain its natural conformation, minimize denaturation of the antibody-binding site, while releasing sufficient amounts of protein from the sample for subsequent analysis. Non-ionic detergents (such as NP-40 and Triton X-100) are less irritating than ionic detergents (SDS and sodium deoxycolate). Other variables that may affect the success of immune sedimentation include salt concentration, binary ion concentration, and pH. For optimization, tests should be performed within the following ranges:

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

Used for antigens that are soluble in detergents and can be identified by antibodies in natural form. Among them, the Triton X-100 can replace the NP-40.

20 mM Tris HCl pH 8

137 mM NaCl

10% glycerol

1% Nonidet P-40 (NP-40)

2 mM EDTA

Store at 4°C for up to 6 months

Add protein inhibitors before use.

2) RIPA (RadioImmunoPrecipitation Assay) Buffer

RIPA buffers have a stronger degeneration capacity than NP-40 or Triton X-100 lysis buffers, containing SDS and sodium deoxycolate as active ingredients, and are particularly suitable for disrupting nucleic membranes in nuclear extracts. The background of RIPA buffer is lower, but may cause enzyme degeneration.

50 mM Tris HCl pH 8

150 mM NaCl

1% NP-40

0.5% Sodium Deoxycholate

0.1% SDS

10% Sodium Deoxycholate Reserve Liquid (5 g/50 ml) must be protected from light.

3) Detergent-free Soluble Protein Lysis Buffer

Soluble proteins may not require the use of detergents. The buffer is used for mechanical disruption of cells, e.g., by repeated syringe passages or homogenization with a Dounce homogenizer.

PBS containing:

5 mM EDTA

0.02 % Sodium Azide

Store up at 4oC for to 6 months

Add protein inhibitors prior to use.

4) Denaturing Lysis Buffer/Buffer for Non-detergent Soluble Antigens

This buffer is used for antibodies that recognize denatured proteins. When cells are harvested and lysed, cells can be heated in this buffer. In addition, the buffer can also be used to extract antigens that cannot

be extracted from cells with non-ionic detergents. The use of DNase1 assists in the extraction of protein chromosomes.

1% SDS

5 mM EDTA

Store at room temperature for up to 1 week

Add immediately before use:

10 mM dithiothreitol or beta-mercaptoethanol

Protease inhibitors

15 U/ml DNase1

B. Other Reagents

Protease Inhibitors

Once cells are disrupted, protein hydrolysis, dephosphorylation and intracellular degeneration begin. By keeping the sample on ice or at 4°C and adding appropriate fresh enzyme inhibitors to the lysis buffer, these events can be greatly slowed. A mixture of proteinase and phosphatease inhibitors ("mixture") is commercially available. If the mixture is not used, the two most commonly used protease inhibitors in IP are PMSF (50 ug/mL) and inhibitor (1 ug/mL).

Other Reagents Required:

Sterile PBS pH 7.4

Sterile PBS-BSA 1% (filtered)

TBST buffer

Loading/sample buffer for Western blotting

Add 1.86 g EDTA to 40 ml H_2O to make a 100 mM EDTA reserve solution. Add the NaOH solution and adjust the pH to 7.4. Finally, adjust the total volume to 50 ml.

02 Preparation of Lysates

Lysates from Cell Culture

Non-denaturing:

1. Place the cell culture dish on ice and rinse the cells with cold PBS.
2. Drain the PBS and then add cold lysis buffer (1ml per 10^7 cells/100mm dish/150cm^2 flask; 0.5ml per 5x10^6 cells/60mm dish/75cm^2 flask).
3. Using a cold plastic cell scraper, scrape the adherent cells from the culture dish and then gently transfer the cell suspension fluid to the pre-cooled microfuge tube.
4. Maintain agitation at 4°C for 30 minutes.
5. Centrifuge at 4 ° C in the micro centrifuge. You may need to change the centrifugal force and time

depending on the cell type. The guiding principle is 12,000 rpm for 20 minutes.

6. Carefully remove the test tube from the centrifuge, place it on the ice, aspirate the supernatant, place in a new tube on ice and discard off the sediment.

Denaturing:

1. Add 100 ml Denaturing Lysis Buffer per 0.5 to 2 x 10^7 cells.
2. Vortex at maximum speed for 2 to 3 seconds and mix well. Transfer the cell suspension into the microcentrifuge tube.

   Tips: Due to the release of DNA, the solution may become sticky at this stage.
3. Heat the sample to 95°C for 5 minutes to degenerate.
4. Dilute the suspension with 0.9 ml non-denaturing lysis buffer. Mix gently.
5. Inject the lysed suspension 5 to 10 times by connecting the needle to the 1 ml injector.

   Tips: This procedure may fragment the DNA. Repeat the mechanical damage until the viscosity decreases to a manageable level. If the DNA is not completely digested, it may interfere with the separation of the sediment and supernatant after centrifugation.
6. Incubate for 5 minutes on ice.
7. Continue immunoprecipitation.

Lysates from Tissue

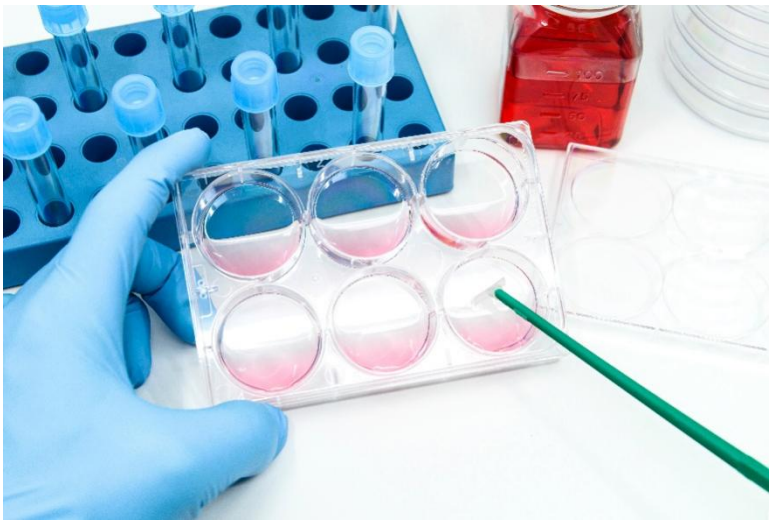

Figure 2. Cell collection was performed from the Petri dish.

1. Use clean tools to dissect tissues of interest on ice, and perform as soon as possible to prevent proteinase degradation.
2. The tissue is placed in the round bottom microfuge tubes, immersed in liquid nitrogen "fast freeze". Store at -80°C for later use or on ice for immediate homogenization.
3. For tissues of approximately 5 mg, rapidly add approximately 300 µL of lysis buffer to the tube and homogenize using an electric homogenizer.
4. Wash the blade twice with 300 µL of lysis buffer for each wash, then mix continuously at 4 °C for 2

hours.

5. The volume of lysis buffer must be determined according to the amount of tissue present. Protein extracts should not be too rare to avoid protein loss and to minimize the volume of samples on the gel. The minimum concentration is 0.1 mg/mL; the optimal concentration is 1-5 mg/mL.

6. In micro centrifuges, centrifuge at 4 °C, 12,000 rpm for 20 minutes. Carefully remove the tube from the centrifuge and place it on the ice, aspirate the liquid and place it in the new pipe on ice, discarding the sediment.

03 Pre-clearing the Lysates

Pre-cleaned lysates help reduce the non-specific binding of proteins to agarose or sepharose beads. Pre-cleaning with unrelated antibodies or serums will remove non-specific proteins that bind to immunoglobulin. The end result will be a reduction in background and an increase in signal-to-noise ratio. However, if the final detection of the protein is performed by immunoblotting, pre-clearance may not be necessary unless the contaminating protein interferes with the visualization of the target protein.

1. 50 µl of non-related antibodies or normal serums of the same species and isotype as the IP antibody are added to 1 ml of the lysate. Incubate for 1 hour on ice .

2. Add 100 µL of bead suspension to the lysate.

3. Incubate at 4°C for 10 to 30 minutes with gentle mixing.

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

5. Discard the bead and retain the upper liquid for immunoprecipitation.

To increase the yield, you can wash the bead 1 or 2 times or more in the lysis buffer and collect the upper liquid together.

It is important remove as much normal serum as possible as it will compete with the specific antibodies. To check this, the sample can be tested with a lysis buffer instead of a sample and all of the above pre-cleaning steps can be performed. Coomassie staining of the gel will show if serum Ig has been effectively removed. If not (heavy and light chains are seen at 50 and 25 kD), its presence may lead to weaker IPs. Consider reducing the serum volume or increasing the number of beads incubated with the sample during the pre-clarification steps.

04 Immunoprecipitation

1. 10-500 µg of cell lysate and the recommended amount of antibodies are added to the test tube on ice. These amounts are chosen based on the abundance of the protein and the strength of the antibody's affinity to the protein, usually in pre-experiments, where fixed amounts of protein are sedimented by increasing amounts of antibodies.

   You can refer to the antibody data table for recommended antibody concentrations as a guide:

   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 antibodies at 4°C for 1 hour to overnight (again, depending on the amount of the protein and the affinity of the antibody), preferably with agitation.

3. At the same time, prepare the Sepharose beads. If monoclonal antibodies are used, protein G-coupled Sepharose beads should be selected; and protein A-coupled Sepharose beads are usually suitable if polyclonal antibodies are used. If the beads are powdered, incubate 100 mg of the beads in 1 ml of 0.1M PBS, wash for 1 hour to expand, then decant, remove the liquid. Add 1ml of PBS-BSA 1% w/v, mix for one hour, then rinse twice with PBS. Add a buffer of 400 µL proteinase inhibitors (can be the same as a crack buffer). The slurry can be used. It can be stored at 4°C for several days; if it takes longer, store the bead in PBS with 0.02% azide. You can also purchase pre-dissolved beads for use as pulp at any time.

   Tips: It is recommended to use pipette tips with the end cut off to prevent damage to the beads.

   Tips: IgM antibodies: Do not use protein A or protein G binding beads. Use goat anti-mouse IgM (or polyvalent Ig, or anti-heavy chain) beads.

4. Mix the slurry thoroughly and add 70-100 µL of beads to each sample. Always keep the samples on the ice. The beads tend to stick to both sides of the suction head, so try to minimize the movement of the transmitter and use a 5 mm cut from the top.

5. Incubate the lysate bead mixture at 4 °C rotary agitation for 4h (the optimal incubation time can be determined in preliminary experiments).

6. At the end of the incubation time, centrifuge and remove the supernatant, and wash the beads 3 times in the lysis buffer (each time centrifuge at 4 ° C and remove 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 bead, then centrifuge and save the supernatant. You can then freeze the samples or run them on SDS-PAGE.

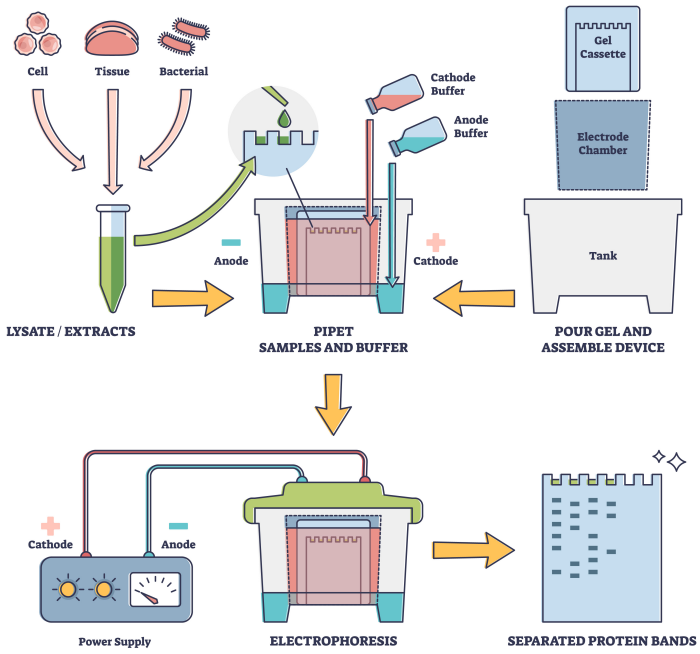

Figure 3. The process of Western blotting.

Tips: Use of the buffer above is the harshest elution and will also wash off any non-covalently bound

antibodies and antibody fragments present on the gel. The antigen can be gently washed with a glycine gradient (up to 1 M) to reduce the amount of antibody washed away.

References

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

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

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