Protocol

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Immunoprecipitation Protocol

Utilizing Agarose Resins

Introduction

Immunoprecipitation is a technique often used in protein characterization studies that gives insight into size, abundance and protein-protein interactions. In immunoprecipitation, a protein of interest is isolated from other proteins in a biological sample by promoting binding to an antibody specific for that protein. Immunoprecipitation consists of multiple stages, beginning with lysing of cells to release the antigen, followed by attaching an antibody to a matrix to use in the isolation and incubating the released antigen with the antibody/matrix, and lastly, washing of unbound proteins. The process detailed here utilizes Protein A/G resin bound antibodies to perform this separation from unwanted proteins and allows for elution of the purified proteins. To ensure proper binding of your antibody to protein of interest, use this Relative Affinity Comparison Chart.

Materials

Cells and solutions

- Protein A Agarose Resin (GoldBio Catalog # P-400) or
  Protein G Agarose Resin (GoldBio Catalog # P-430)
- Labeled or unlabeled cells in suspension
- PBS (GoldBio Catalog # P-271), ice-cold
- Nondenaturing lysis buffer, ice-cold (see below)
- Specific polyclonal antibody (antiserum or affinity-purified immunoglobulin) or monoclonal antibody (ascites, culture supernatant, or purified immunoglobulin)
- Control antibody of same type as specific antibody (e.g. preimmune serum or purified irrelevant immunoglobulin for specific polyclonal antibody; irrelevant ascites, hybridoma culture supernatant, or purified immunoglobulin for specific monoclonal antibody)
- 10% (w/v) BSA (GoldBio Catalog # A-420)
- Wash buffer, ice-cold
- Triton X-100
- Tris HCl (GoldBio Catalog # T-095)
- EDTA (GoldBio Catalog # E-210)
- PMSF (GoldBio Catalog # P-470)
- Leupeptin (GoldBio Catalog # L-010)

Non-denaturing lysis buffer

- 1% (w/v) Triton X-100 (store at room temperature in the dark)

- 50mM Tris HCl (GoldBio Catalog # T-095) at a pH of 7.4, dilute from 1M stock solution.
- 300mM NaCl
- 5mM EDTA (GoldBio Catalog # E-210), dilute from 0.5M stock solution.
- 0.02% (w/v) sodium azide

This solution can be stored for up to 6 months at 4°C. Immediately before use add:

- 10mM iodoacetamide
- 1mM PMSF (GoldBio Catalog # P-470), dilute from 1M stock solution.
- 2 µg/ml Leupeptin (GoldBio Catalog # L-010)

Elution Buffer

- 1% (w/v) SDS
- 100mM Tris HCl at a pH of 7.4
- This solution can be stored for up to one week at room temperature, add 10mM of DTT powder immediately prior to use.

Equipment

- Microcentrifuge with fixed-angle rotor
- Tube rotator (capable of end-over-end inversion)
- Pasteur pipette attached to a vacuum trap
- Ice or refrigeration to keep sample cold
- Heating block set at 95°C

Caution: When working with radioactivity, take appropriate precautions to avoid contamination of the experimenter and the surroundings. Carry out the experiment and dispose of wastes in an appropriately designated area while following guidelines provided by the local radiation safety officer.

Note: All solutions should be ice-cold and procedures should be carried out at 4°C or on ice.

Method

Preparation of Protein slurry

1. Combine 50% (v/v) Protein A or Protein G resin slurry in PBS containing 0.1% (w/v) BSA and 0.01% (w/v) sodium azide (NaN_3).

Preparation of cell lysate

1. Collect cells in suspension by centrifuging for 5 minutes, 400 x g at 4°C in a 15 or 50 ml capped conical tube. Place the tube on ice.

Note: Approximately 0.5-2 x 10^7 cells are required to yield 1 ml lysate, which is the amount generally used for each immunoprecipitation.

Note: Labeled cells are likely to have been pelleted earlier as part of the labeling procedure. If the cells are frozen, they should be thawed on ice before solubilization.

2. Aspirate supernatant with a Pasteur pipette attached to a vacuum trap.

   Caution: Dispose of radioactive materials following applicable safety regulations.

3. Resuspend cells by gently tapping the bottom of the tube. Rinse suspended cells twice with ice-cold PBS, using a volume of PBS equal to the initial lysate volume.

4. Add 1 ml ice-cold nondenaturing lysis buffer per sample tube (~0.5-2 x 10^7) and resuspend pellet by gentle agitation for 3 seconds in a vortex mixer set at medium speed.

   Note: Do not shake vigorously, as this could result in loss of material or protein denaturing due to foaming.

5. Keep the suspension on ice for 15 to 30 minutes and transfer to a 1.5 ml conical microcentrifuge tube.

   Note: Screw-capped tubes are preferred over flip-top tubes because they are less likely to accidentally open during subsequent procedures. They are also recommended for work dealing with radioactivity.

6. Clear the lysate by centrifuging for 15 minutes, maximum speed (16,000 x g) at 4°C.

   Note: Centrifugation can be carried out in a microcentrifuge placed in a cold room or in a refrigerated microcentrifuge. Take precautions to ensure that the 4°C temperature is maintained during the spin (e.g. use a fixed-angle rotor with a lid, as the aerodynamics of this type of rotor reduces generation of heat by friction). If it is necessary to reduce background, the lysate can be spun for 1 hour at 100,000 x g in an ultracentrifuge.

7. Transfer the supernatant to a fresh microcentrifuge tube using an adjustable pipette fitted with a disposable tip. Do not disturb the pellet, and leave the last 20 to 40 µl of the supernatant in the centrifuge tube. Keep the cleared lysate on ice until preclearing or addition of antibody beads.

Note: Resuspension of even a small amount of sedimented material will result in high nonspecific background due to carry-over into the immunoprecepitation steps. A cloudy layer of lipids floating on top of the supernatant will not adversely affect the results of the immunoprecipitation.

Note: When the lysate is highly radioactive (as is the case for metabolically labeled cells) the use of tips with aerosol barriers is recommended to reduce the risk of contaminating internal components of the pipette.

Note: Cell extracts can be frozen at -70°C until used for immunoprecepitation. However, it is preferable to lyse the cells immediately before immunoprecipitation in order to avoid protein degradation or dissociation of protein complexes. If possible, freeze the cell pellet from step 3 rather than the supernatant from step 7.

Preparation of antibody-conjugated beads

1. In a 1.5-ml conical microcentrifuge tube, combine 30 µl of 50% protein agarose bead slurry, 0.5 ml ice-cold PBS, and one of the following specific antibodies:
    a. 1-5 µl polyclonal antiserum
    b. 1 µg affinity-purified polyclonal antibody
    c. 0.2-1 µl ascetic fluid containing monoclonal antibody
    d. 1 µg purified monoclonal antibody
    e. 20-100 µl culture supernatant containing monoclonal antibody

    Note: The quantities of antibody suggested are rough estimates based on the expected amount of specific antibodies in each preparation. Quantities can be increased or decreased depending on the quality of the antibody preparation.

    Note: If the same antibody will be used to immunoprecipitate multiple samples (e.g. samples from a pulse-chase experiment), the quantities indicated above can be increased proportionally to the number of samples and incubated in a 15 ml capped conical tube. In this case, the beads should be divided into aliquots just prior to the addition of the cleared cell lysate.

    Note: Antibody-conjugated beads can be prepared prior to preparation of the cell lysate in order to minimize the time that the cell extract is kept on ice.

2. Set up a nonspecific immunoprecipitation control in a 1.5 ml conical microcentrifuge tube by incubating 30 µl of 50% Protein A or Protein G bead slurry, 0.5 ml ice-cold PBS and the appropriate control antibody:
    a. 1-5 µl preimmune serum as a control for a polyclonal antiserum.

b. 1 µg purified irrelevant polyclonal antibody (an antibody to an epitope that is not present in the cell lysate) as a control for a purified polyclonal antibody.
c. 0.2-1 µl ascetic fluid containing irrelevant monoclonal antibody (an antibody to an epitope that is not present in the cell lysate and of the same species and immunoglobulin subclass as the specific antibody) as a control for an ascetic fluid containing specific monoclonal antibody.
d. 1 µg purified irrelevant monoclonal antibody as a control for a purified monoclonal antibody.
e. 20-100 µl hybridoma culture supernatant containing irrelevant monoclonal antibody as a control for a hybridoma culture supernatant containing specific monoclonal antibody.

Note: The amount of irrelevant antibody should match that of the specific antibody and the antibody should be from the same species as the specific antibody.

3. Mix the suspension thoroughly. Tumble incubation mixtures end over end for an hour or longer at 4°C in a tube rotator.

Note: Addition of a 0.01% (w/v) Triton X-100 may facilitate mixing of the suspension during tumbling. The incubations can be carried out for as long as 24 hours. This allows preparation of the antibody-conjugated beads prior to immunoprecipitation.

4. Microcentrifuge for 2 seconds at maximum speed (16,000 x g) at 4°C.

5. Aspirate the supernatant (containing unbound antibodies) using a fine-tipped Pasteur pipette connected to a vacuum aspirator.

6. Add 1 ml nondenaturing lysis buffer and resuspend the beads by inverting the tube three or four times.

Note: Use of a repeat pipettor is recommended when processing multiple samples.

7. Wash by repeating steps 4-6 and then steps 5-6 once more.

Note: At this point the beads have been washed twice with non-denaturing lysis buffer and are ready to be used for immunoprecipitation. The antibody-bound beads can be stored up to 6 hours at 4°C until used.

Preclear lysate (Optional)

1. In a microcentrifuge tube, combine 1 ml of cell lysate from step 7 in the Preparation of cell lysate section and add 30 µl of 50% protein agarose slurry.

   Note: The purpose of this step is to remove from the lysate proteins that bind to protein A/G resins, as well as pieces of insoluble material that may have been carried over from previous steps. If the lysate was prepared from cells expressing immunoglobulins (such as spleen cells or cultured B cells), the preclearing step should be repeated at least three times to ensure complete removal of endogenous immunoglobulins.

   Note: If cell lysates were frozen and thawed, they should be microcentrifuged 15 minutes at 16,000 x g (maximum speed), 4°C, before the preclearing step.

2. Tumble end over end for 30 minutes at 4°C in a tube rotator.

3. Centrifuge 5 minutes at maximum speed (16,000 x g), 4°C.

Immunoprecipitate

1. Add 10 µl of 10% BSA to the tube containing specific antibody bound to protein beads and transfer this tube the entire volume of cleared lysate (from the final step of first or second section of procedure). If a nonspecific immunoprecipitation control is needed, divide the lysate in two ~0.4 ml aliquots, one for the specific antibody and the other for the nonspecific control.

   Note: In order to avoid carryover of beads with precleared material, leave 20 to 40 µl of supernatant on top of the pellets in the preclearing tubes. Discard beads and remaining supernatant. The BSA quenches nonspecific binding to the antibody-conjugated beads during incubation with the cell lysate.

2. Incubate for 1-2 hours at 4°C while mixing end over end in a tube rotor.

   Note: Samples can be incubated overnight, although there is an increased risk of protein degradation, dissociation of multiprotein complexes or formation of protein aggregates.

3. Centrifuge for 5 seconds at (16,000 x g), 4°C.

4. Aspirate the supernatant (containing unbound proteins) using a fine-tipped Pasteur pipette connected to a vacuum aspirator.

   Note: The supernatant can be kept up to 8 hours at 4°C or up to 1 month at -70°C for sequential immunoprecipitation of other antigens or for analysis of total proteins. To reutilize lysate,

remove the supernatant carefully with an adjustable pipette fitted with a disposable tip. Before reprecipitation, preabsorb the lysate with protein beads (via the preclear lysate section) to remove antibodies that may have dissociated during the first immunoprecipitation.

Caution: Dispose of radioactive materials following applicable safety regulations.

5. Add 1 ml of ice-cold wash buffer, cap the tubes, and resuspend the beads by inverting the tube 3 or 4 times.

   Note: Use of a repeat pipettor is recommended when processing multiple samples.

6. Centrifuge 2 seconds, maximum speed (16,000 x g) at 4°C.

7. Aspirate the supernatant, leaving ~20 µl supernatant on top of the beads.

8. Wash the beads, repeating the previous 3 steps (steps 5-7), 3 times.

   Note: Total wash time should be ~30 minutes, ensuring to keep the samples on ice for 3 to 5 minutes between washes if necessary.

9. Wash beads once more using 1 ml ice-cold PBS and aspirate supernatant completely with a drawn-out Pasteur pipette or an adjustable pipette fitted with a disposable tip.

   Note: The final product should be 15 µl of settled beads containing bound antigen.

   Note: Immunoprecipitates can either be processed immediately or frozen at -20° for later analysis. For subsequent analysis of the isolated proteins prior to electrophoresis, samples can be divided into two or more aliquots after addition of PBS. Transfer aliquots of the bead suspension to fresh tubes, centrifuge and aspirate as in the previous steps.

10. Analyze immunoprecipitates by one-dimensional or two-dimensional electrophoresis or immunoblotting.

Immunoprecipitation-Recapture (Optional)

In immunoprecipitation-recapture, an antigen is disassociated from the beads and reimmunoprecipitated with the same or a different antibody used in the first immunoprecipitation. If the same antibody is used, then immunoprecipitation-recapture allows for more accurate identification of the isolated protein. If a different antibody is used, then this procedure allows for analysis of subunit composition of multi-protein complexes.

1. Add 50 µl elution buffer to 15 µl beads containing bound antigen. Mix by vortexing.

Note: The DTT in the elution buffer reduces disulfide bonds in the antigen and the antibody while the SDS contributes to the unfolding of polypeptide chains.

2. Incubate for 5 minutes at room temperature then 5 minutes at 95°C in a heating block. Cool tubes to room temperature.

3. Add 10 µl of 10% BSA. Mix by gentle vortexing.

   Note: BSA is added to prevent adsorption of antigens to the tube, and to quench nonspecific binding to antibody-conjugated beads.

4. Add 1 ml nondenaturing lysis buffer.

   Note: The iodoacetamide in the nondenaturing lysis buffer reacts with the DTT and prevents it from reducing the antibody used in the recapture steps. The presence of PMSF and leupeptin in the buffer is not necessary at this step.

5. Incubate for 10 minutes at room temperature.

6. Clear the lysate, and perform a second immunoprecipitation procedure as described earlier in this protocol.

Associated Products

- Protein A Agarose Resin (GoldBio Catalog # P-400)
- Protein G Agarose Resin (GoldBio Catalog # P-430)
- PBS (GoldBio Catalog # P-271)
- Tris HCl (GoldBio Catalog # T-095)
- EDTA (GoldBio Catalog # E-210)
- PMSF (GoldBio Catalog # P-470)
- Leupeptin (GoldBio Catalog # L-010)
- BSA (GoldBio Catalog # A-420)

Troubleshooting

Problem	Cause	Solution
Gel is completely blank after prolonged autoradiographic exposure.	Poorly labeled cells: too little radiolabeled precursor, too few cells labeled, lysis/loss of cells during labeling, too much cold amino acid in labeling mix, wrong labeling temperature	Check incorporation of label by TCA precipitation; troubleshoot the labeling procedure
Only nonspecific bands present	Antigen does not contain the amino acid used for labeling	Label cells with another radiolabeled amino acid or, for glycoproteins, with titrated sugar
	Antigen expressed at very low levels	Substitute cells known to express higher levels of antigens as detected by other methods; transfect cells for higher expression
	Protein has high turnover rate and is not well labeled by long-term labeling	Use pulse labeling
	Protein has a low turnover rate and is not well labeled by short-term labeling	Use long-term labeling
	Protein is not extracted by lysis buffer used to solubilize cells	Solubilize with a different nondenaturing detergent or under denaturing conditions
	Antibody is nonprecipitating	Identify and use antibody that precipitates antigen
	Epitope is not exposed in native antigen	Extract cells under denaturing conditions
	Antibody does not recognize denatured antigen	Extract cells under nondenaturing conditions
	Antibody does not bind to immunoadsorbent	Use a different immunoadsorbent, use intermediate antibody
	Antigen is degraded during immunoprecipitation	Ensure that fresh protease inhibitors are present
Isolated lanes on gel with high background	Random carryover of detergent-insoluble proteins	Remove supernatant immediately after centrifugation, leaving a small amount with pellet; if resuspension occurs, recentrifuge

High background in all lanes	Incomplete washing	Cap tubes and invert several times during washes
	Poorly radiolabeled protein	Optimize duration of labeling to maximize signal-to-noise ratio
	Incomplete removal of detergent-insoluble proteins	Centrifuge lysate 1 hour at 100,000 x g
	Insufficient unlabeled protein to quench nonspecific binding	Increase concentration of BSA
	Antibody contains aggregates	Microcentrifuge antibody 15 minutes at maximum speed before binding to beads
	Antibody solution contains nonspecific antibodies	Use affinity-purified antibodies; absorb antibody with acetone extract of cultured cells that do not express antigen; for yeast cells, absorb antibody with null mutant cells.
	Too much antibody	Use less antibody
	Incomplete preclearing	Preclear with irrelevant antibody of same species of origin and immunoglobulin subclass bound to immunoadsorbent
	Nonspecifically immunoprecipitated proteins	Fractionate cell lysate (e.g. ammonium sulfate precipitation, lectin absorption, or gel filtration) prior to immunoprecipitation; after washes in wash buffer, wash beads once with 0.1% SDS in wash buffer or 0.1% SDS/0.1% sodium deoxycholate
Complete immunoglobulin or heavy and/or light chains visible in immunoblot	Protein conjugate or secondary antibody recognizes immunoprecipitating antibody	Use antibody coupled covalently to solid-phase matrix for immunoprecipitation; probe blots with primary antibody from a different species and the appropriate secondary antibody specific for immunoblotting primary antibody

References

Bonifacino, J. S., Dell'Angelica, E. C., & Springer, T. A. (2001). Immunoprecipitation. Current *Protocols in Immunology*, 8-3.