

Analysis of Proteins by Immunoprecipitation

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Introduction

Immunoprecipitation is a procedure by which peptides or proteins that react specifically with an antibody are removed from solution and examined for quantity or physical characteristics (molecular weight, isoelectric point, etc.). As usually practiced, the name of the procedure is a misnomer since removal of the antigen from solution does not depend upon the formation of an insoluble antibody-antigen complex. Rather, antibody-antigen complexes are removed from solution by addition of an insoluble form of an antibody binding protein such as Protein A, Protein G or second antibody (Figure 1). Thus, unlike other techniques based on immunoprecipitation, it is not necessary to determine the optimal antibody dilution that favors spontaneously-occurring immunoprecipitates.

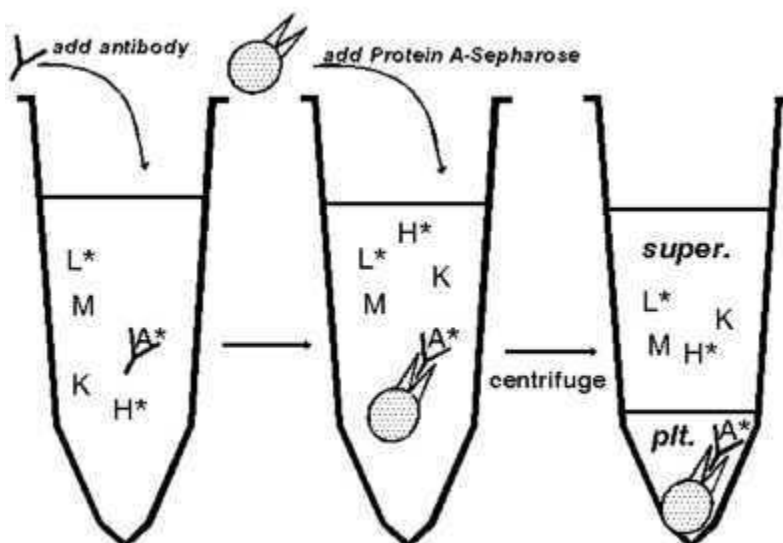


Figure 1. Schematic representation of the principle of immunoprecipitation. An antibody added to a mixture of radiolabeled (*) and unlabeled proteins binds specifically to its antigen (A) (left tube). Antibody-antigen complex is absorbed from solution through the addition of an immobilized antibody binding protein such as Protein A-Sepharose beads (middle panel). Upon centrifugation, the antibody-antigen complex is brought down in the pellet (right panel). Subsequent liberation of the antigen can be achieved by boiling the sample in the presence of SDS.

Typically, the antigen is made radioactive before the immunoprecipitation procedure, either by culturing cells with radioactive precursor or by labeling the molecule after synthesis has been completed (e.g., by radioiodination to iodinate tyrosine residues or by sodium [³H]borohydride reduction to label carbohydrate). Having a radioactive antigen is not required but interpretation of data are simplified since the antigen, and not the antibody, is radiolabeled. Analysis of the immunoprecipitate is usually by electrophoresis although other techniques can be used.

The choice of immobilized antibody binding protein depends upon the species that the antibody was raised in. Protein A binds well to rabbit, cat, human, pig and guinea pig IgG as well as mouse IgG_{2a} and

IgG_{2b}. Protein G binds strongly to IgG from cow, goat, sheep, cow, horse, rabbit and guinea pig and to mouse IgG₁ and IgG₃. Protein G can also bind bovine serum albumin (BSA). Thus, BSA should be added to buffers used with Protein G. Alternatively, recombinant Protein G without BSA binding sites can be used (Protein G Plus from Oncogene Science).

Applications

Immunoprecipitation can be used for many purposes. Among these are:

1. Determination of the molecular weight and isoelectric point of immunoprecipitated proteins by one-dimensional or two-dimensional SDS-PAGE.
2. Verification that an antigen of interest is synthesized by a specific tissue (i.e., that radiolabeled protein can be identified in tissues or cells cultured with radiolabeled precursors).
3. Determination of whether a protein contains carbohydrate residues by evaluating whether immunoprecipitated antigen from cells cultured with radioactive monosaccharides is radiolabeled.
4. Characterization of the type of carbohydrate present on glycoproteins - evaluate incorporation of different radiolabeled monosaccharides into immunoprecipitated protein during cell culture and test whether inhibitors of glycosylation alter the molecular weight of immunoprecipitated protein.
5. Determination of precursor-product relationships by performing pulse-chase labeling followed by immunoprecipitation.
6. Quantification of synthesis rates of proteins in culture by determining the quantity of immunoprecipitated, radiolabeled protein.

Procedures

The following are two methods that have been used in our laboratory. Both are similar in many respects. Other protocols will also give good results.

METHOD I

A. Reagents

IMP Buffer¹

50 mM Tris-acetate, pH 7.5²

1 mM phenylmethylsulfonyl fluoride (PMSF)³

1 mM EDTA

0.3 M NaCl

1 mg/ml BSA

2% IGEPAL CA-630 (v/v)⁴

0.02% NaN₃

Store at 4 C indefinitely

Washing Buffer

50 mM Tris-acetate, pH 7.5

0.3 M NaCl

0.5% IGEPAL CA-630

0.1% sodium dodecyl sulfate (SDS)⁵

0.02% NaN₃

Store at 25 C indefinitely

Protein A-Sepharose CL-4-B Suspension⁶

Protein A-Sepharose beads (Pharmacia or Sigma) are swelled in PBS or IMP buffer on a tube turner at room temperature. Beads are then washed twice in IMP buffer and diluted to 10% (v/v) in IMP buffer. Dilution can be performed by estimating the approximate volume of swollen beads in a graduated conical centrifuge tube and adding 9 volumes of buffer. Beads can be stored at 4 C indefinitely.

Notes:

- 1) Stocks can be made of Tris-acetate (1 M), EDTA (0.2 M), PMSF (0.1 M in ethanol) and NaN_3 (20%, v/v) to simplify making up buffers.
- 2) Note that Tris buffers are very temperature-sensitive and should be prepared using water at the temperature they will be used at. Other buffers such as phosphate can be used instead of Tris.
- 3) PMSF is very labile in water. A more expensive but more stable alternative is 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; Pefabloc®) from Boehringer-Mannheim (0.1-0.5 mg/ml). In addition to PMSF and EDTA, other proteinase inhibitors can also be included (for example, 10 $\mu\text{g/ml}$ leupeptin and 0.7 $\mu\text{g/ml}$ pepstatin). The Boehringer Mannheim catalog contains a very detailed summary of proteinase inhibitors available for use.
- 4) The protocol was originally used with Nonidet P-40, which is no longer available. IGEPAL CA-630 from Sigma is chemically indistinguishable from Nonidet P-40.
- 5) SDS tends to precipitate at 4 C although low concentrations used here may not be a problem; can replace with an equal amount of sarkosyl if it is desired to store in the refrigerator.
- 6) Second antibody coupled to Sepharose or Protein G-Sepharose can be used instead. It is also not crucial that Sepharose be used as a matrix. Other polymerized agaroses or even fixed strains of *Staphylococcus* cells expressing high amounts of surface Protein A can be used.

B. Procedure

Note: The procedure here assumes that a concentration step is required to obtain enough radioactivity for the immunoprecipitation analysis. Other procedures for concentration other are available besides the dialysis/lyophilization procedure described here (for example, use of Centricon devices from Amicon). For some applications, samples can be analyzed without concentration and after dilution with immunoprecipitation buffer.

- 1) Prepare radiolabeled protein mixture by culturing cells with radiolabeled precursor, radioiodination or other method.
- 2) For soluble antigens (i.e., conditioned medium), dialyze sample against water. For intracellular antigen, harvest cells, and lyse cells with appropriate detergent. Some that have been used include 1% (v/v) Triton X-100, IGEPAL CA-630, Renex 30, or CHAPS. If dialysis is to be used to concentrate protein, use a dialyzable detergent (example, deoxycholate or CHAPS). The buffer used for lysis should contain proteinase inhibitors (for example, PMSF, EDTA, aprotinin, and leupeptin).

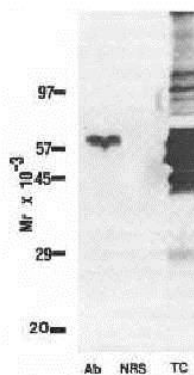


Figure 2. Representative result from immunoprecipitation. Conditioned cultured medium from endometrial explants cultured in the presence of [^{35}S]methionine was incubated with an antibody to an endometrial-specific protein called uterine milk protein (UTMP). Antibody-antigen complexes were absorbed using Protein A-Sepharose and analyzed by SDS-PAGE and fluorography. Lanes represent proteins

immunoprecipitated with rabbit antiserum to UTMP (Ab), normal rabbit serum in place of antiserum (NRS) and the total array of radiolabeled proteins present in the unabsorbed sample (TC). From Leslie and Hansen (1991).

- 3) For each sample tested, lyophilize 2 aliquants of 200,000 dpm of radiolabeled protein and 1 aliquant of 100,000 dpm in 1.5 ml microcentrifuge tubes. The samples of 200,000 dpm will be used for immunoprecipitation with antibody and control immunoglobulin and the 100,000 dpm tube (optional) will be used to determine the entire array of radiolabeled proteins present in the sample.

Note: To determine radiolabeled protein, one must remove unincorporated radiolabel first. This can be done by dialysis or gel filtration. Alternatively, incorporation of radiolabel can be determined by trichloroacetic acid precipitation. Lower dpm than stated here can be used if needed but sensitivity will decrease. We have used as many as 6 million dpm to maximize sensitivity.

- 4) Put the 100,000 dpm samples aside until step #12.
- 5) After lyophilization, solubilize 200,000 dpm samples in 350 μ l IMP buffer.
- 6) Add 50 μ l of antibody or control (normal rabbit serum, mouse isotype control,, etc.) to each tube and incubate overnight for 6 h at room temperature.
- 7) Add 100 μ l of Protein-A-Sepharose solution (shake to suspend slurry before pipetting) and incubate on a tube turner for 6 h at room temperature.
- 8) Centrifuge of 1 min on microcentrifuge and save pellet.
- 9) Add 500 μ l washing buffer, vortex, spin 1 min on microcentrifuge and discard supernatant.
- 10) Add 1 ml washing buffer, vortex, spin 1 min, discard supernatant; repeat 3x.
- 11) Add 1 ml 10 mM Tris-acetate, pH 7.5, vortex, spin 1 min, discard supernatant.
- 12) Solubilize all samples (including 100,000 dpm samples) in 30-50 μ l of the gel solubilization buffer used for SDS-PAGE, boil for 3 min and centrifuge.
- 13) Save supernatant and analyze by SDS-PAGE and fluorography or autoradiography (125 I) or fluorography (3 H, 14 C, and 35 S).

Method II

A. Reagents

IMP Buffer

50 mM Tris-acetate, pH 7.5
150 mM NaCl
1% (v/v) Triton X-100
1% (w/v) deoxycholate
0.1% (w/v) SDS
4 mM AEBSF
1 μ g/ml aprotinin¹
10 μ g/ml leupeptin¹
0.02% (w/v) NaN₃

Notes:

1. Prepare from a 1 mg/ml stock in water. Store frozen in aliquots.

Protein G Plus-Agarose

From Oncogene Science (Cat # IP04). This product, which is ready to use, is 30% (v/v) agarose by volume and contains 20 mg IgG binding capacity/ml packed beads.

Procedure

- 1) Prepare samples as for Procedure I. For each sample tested, prepare 2 aliquants of 200,000 dpm each and 1 aliquant of 100,000 dpm in 1.5 ml microcentrifuge tubes.
- 2) Put the 100,000 dpm samples aside until step #11.
- 3) After lyophilization, solubilize 200,000 dpm samples in 300 μ l IMP buffer.
- 4) To preclear samples, add 50 μ l of control (normal rabbit serum, mouse isotype control, etc.) to each tube and 25 μ l of Protein G slurry (shake to suspend slurry before pipetting) and incubate for 1 h at 4 C.
- 5) Centrifuge of 1 min on microcentrifuge and save supernatant.
- 6) Add antibody or control (50 μ l maximum volume, diluted in IMP buffer) and incubate 1 h at room temperature.
- 7) Add Protein G agarose (25 μ l) overnight at 4C on a tube rotator.
- 8) Centrifuge 1 min on microcentrifuge, discard supernatant.
- 9) Add 1 ml IMP buffer, vortex, spin 1 min, discard supernatant; repeat 3x.
- 10) Add 1 ml 10 mM Tris-acetate, pH 7.5, vortex, spin 1 min, discard supernatant.
- 11) Solubilize all samples (including 100,000 dpm samples) in 30-50 μ l of the gel solubilization buffer used for SDS-PAGE, boil for 3 min and centrifuge.
- 12) Save supernatant and analyze by SDS-PAGE and fluorography or autoradiography (125 I) or fluorography (3 H, 14 C, and 35 S).

Some Problems

- 1) Like all immunochemical procedures, attention must be given to antibody crossreactivity with other antigens.
- 2) Nonspecific binding can be a problem especially if proteins that are immunologically distinct from the antigen are trapped in the pellets formed during immunoprecipitation. To reduce nonspecific binding, immuno-precipitation buffers usually have some detergent to reduce hydrophobic interactions, a protein to block nonspecific binding sites, and high salt to reduce ionic interactions. In many protocols, a preclearing step is performed to remove molecules that nonspecifically bind to the insoluble Protein A or Protein G. Despite these precautions, nonspecific binding can occur. It is crucial, therefore, to always perform a control reaction where antibody is replaced by a non-relevant immunoglobulin (i.e., normal serum for polyclonal antibodies, control mouse ascites fluid for ascites, and isotype controls for purified mouse monoclonal antibodies).
- 3) Proteolytic digestion can occur when cells are lysed and contents of lysosomes are mixed with other compartments of the cell. Accordingly, most immunoprecipitation buffers contain one or more proteinase inhibitors.
- 4) Care should be taken in using immunoprecipitation as a quantitative tool to determine rates of synthesis of proteins because the rate of incorporation of radiolabel into protein will depend upon rate of synthesis of a protein as well as the rate of dilution of radiolabeled precursor by the intercellular pool of precursor.
- 5) It has been the author's experience that sometimes scintillation spectrometry reveals little difference in the quantitative yield of radioactivity between an immunoprecipitation reaction and a control reaction (i.e., where antibody has been substituted with normal rabbit serum). Nonetheless, subsequent analysis by SDS-PAGE reveals precipitation of radiolabeled protein in the antibody reaction only. It is likely, therefore, that molecules too small or too large to be resolved by SDS-PAGE are sometimes trapped in the pellet formed by immobilized Protein A or Protein G. These molecules, while not interfering with analysis by SDS-PAGE, can make direct quantification of radiolabeled antigen by scintillation spectrometry problematic. Thus, immunoprecipitated protein should be quantified by densitometric analysis of autoradiographs or fluorographs.
- 6) Sensitivity can be a problem, especially when the antigen is a minor component of the protein pool. New advances in enhancing screen technology for low energy radioisotopes (Transcreen

LE enhancing screens by Kodak) should increase sensitivity greatly. In the author's lab, efforts are made to use as much protein in the immunoprecipitation reaction as possible.

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Further Reading

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Examples of Applications from the Author's Laboratory

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