



# Using Antibodies

A LABORATORY MANUAL

**Ed Harlow**

MASSACHUSETTS GENERAL HOSPITAL CANCER CENTER  
HARVARD MEDICAL SCHOOL

**David Lane**

DUNDEE UNIVERSITY



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The underlying strategy behind the immunoprecipitation technique is to use the high affinity of antibodies for their antigens as a method to locate and bind target molecules in solution. Once the antibody-antigen complexes are formed in solution, they are collected and purified by using small agarose or polyacrylamide beads with covalently attached protein A or protein G. Both protein A and protein G specifically interact with conserved regions of the antibodies, thus forming an immobilized complex of antibody-antigen bound to beads. Irrelevant molecules in the starting solution are removed by washing the beads. The purified antigens can then be analyzed by any of a number of methods.

Immunoprecipitations are normally one of the first methods used to characterize new protein antigens. When immunoprecipitations are combined with SDS-PAGE, they can be used to determine the relative molecular weight of a protein, look for protein-protein interactions, detect the presence or regulation of posttranslation modifications, or determine the rate of protein degradation. Immunoprecipitated proteins can be used for many different tests and are commonly used to check for intrinsic or associated enzymatic activities or as preparative steps for other assays such as immunoblots or immunizations. This method is one of the most versatile for collecting diverse information about new antigens.

### Major constraints

The success of immunoprecipitations depends on the level of purification and the ease of preparing antigens. These are influenced by two major factors: (1) the abundance of the antigen in the original preparation and (2) the affinity of the antibody for the antigen.

The immunoprecipitation procedure is a very effective purification method used at an analytical scale. Using immunoprecipitations with the proper care and controls, all but the rarest soluble polypeptide can be purified to a sufficient degree that it can be seen as a specific band on an SDS-polyacrylamide gel. Insoluble or highly polymerized antigens may not be able to be studied using immunoprecipitations, because they pellet in the centrifugation steps regardless of whether or not specific antibodies are present. Obviously, abundant soluble proteins are easy to detect and analyze by immunoprecipitation. When rare cellular proteins are studied, the immunoprecipitation procedure must achieve purifica-

tions in the order of 100,000- to 1,000,000-fold. Although this level of purification would be difficult using other techniques, when immunoprecipitation is coupled to a technique such as SDS-PAGE such rare polypeptide chains can be identified. Immunoprecipitations themselves can achieve 10,000-fold purifications, and by resolving the resulting proteins on protein gels, a further 10- to 100-fold purification can be achieved, thus allowing the examination of even the rarest of cellular proteins.

The second factor that affects the ease of purification is the affinity of the antibody for the antigen. Comparing the affinities of different monoclonal antibodies provides an excellent example of this problem. Unlike other assays that present the antigen in a highly concentrated local environment, immunoprecipitations rely on the formation of the antibody-antigen complex in solution at relatively low concentrations of the antigen. In practice, this means that quantita-

tive immunoprecipitations normally require antibody affinities of  $10^8$  liter  $\text{mol}^{-1}$  or higher. Affinities of  $10^6$  to  $10^7$  liter  $\text{mol}^{-1}$  may allow the antigen to be detected, but it will not be possible to remove the antigen quantitatively from the solution. These observations mean that some monoclonal antibodies that are positive in other tests may not be usable in immunoprecipitations. The use of

polyclonal antibodies or pools of monoclonal antibodies may avoid this problem by interacting with multiple epitopes on the antigen. The effect of antibody affinity on immunoprecipitation is discussed in detail in Chapter 2, and the variables for monoclonal versus polyclonal antibodies are discussed in Chapter 3 and below.

## Choosing the correct antibody

Three types of antibody preparations can be used for immunoprecipitations. These are polyclonal antibodies, monoclonal antibodies, and pooled monoclonal antibodies. Their relative advantages and disadvantages are summarized in Table 7.1 and discussed in detail below. Chapter 3 contains a more thorough discussion of the problems of choosing the correct antibody along with a consideration of how to evaluate different antibody sources. The major complicating factors that should influence your choice between available antibodies are the extent of cross-reactions with unrelated antigens, a common problem found with about one-third of monoclonal antibodies, and bad backgrounds, a problem that is more common with polyclonal antibodies. Nonspecific backgrounds can normally be kept to a minimum by titrating the amount of antibody to the lowest amount that does not lower the strength of the antigen band (essentially still staying in antibody excess) and by carefully following the preclearing protocols described below.

Table 7.1 *Antibody choice*

	<b>Polyclonal antibodies</b>	<b>Monoclonal antibodies</b>	<b>Pooled monoclonal antibodies</b>
<b>Signal strength</b>	Excellent	Antibody dependent (poor to excellent)	Excellent
<b>Specificity</b>	Usually good, but some background	Excellent, but some cross-reactions	Excellent by avoiding antibodies with cross-reactions
<b>Good features</b>	Stable, multivalent interactions	Specificity Unlimited supply	Stable, multivalent interactions Specificity Unlimited supply
<b>Bad features</b>	Nonrenewable Background	Need high affinity for antigen	Not commonly available

### Immunoprecipitations using polyclonal antibodies

Polyclonal antibodies are the most commonly used reagents for immunoprecipitations. Normally they contain antibodies that bind to multiple sites on the antigen and therefore have a much higher avidity for the antigen (see Chapter 2). Having more than one antibody bound to an antigen also has other important advantages. When the immune complexes are collected on any of the solid-phase matrices, such as protein A beads, the availability of multiple binding sites for the protein A molecules provides a more stable antigen-antibody-protein A complex. Together, multiple antibody-antigen interactions and multiple antibody-protein A interactions provide a multivalent complex that is easy to prepare, stable, and can be treated relatively harshly during the washing procedure.

Although using polyclonal antibodies for immunoprecipitations often produces stable multivalent interactions, their use also yields higher nonspecific backgrounds than the use of other types of antibodies. Multiple interactions that lead to forming large complexes are more apt to trap or bind nonspecific proteins. Because polyclonal antibodies normally are used as whole sera, they contain the entire repertoire of circulating antibodies found in the immunized animal at the time the serum was collected. Therefore, serum may contain antibodies that specifically recognize spurious antigens. Because this type of contamination is specific, it cannot be removed by methods that are designed to lower nonspecific background (e.g., preclearing, adding BSA). In these cases, the easiest method to remove these activities is to switch antibody sources. Other antisera are unlikely to contain identical spurious reactions. In some cases, it may also be possible to block the specific antibodies by preincubating the serum with a solution that contains the contaminating proteins (e.g., an acetone powder from a source that does not express the antigen being studied, p. 437).

Because of contaminating activities and increased nonspecific interactions, immunoprecipitations using polyclonal antibodies normally have higher backgrounds than other antibody preparations. Many of these problems are inherent in this technique, but some of the background can be effectively removed by titrating the amount of antisera needed to immunoprecipitate the antigen. By providing the smallest amount of serum necessary for the quantitative recovery of the antigen, the background can be kept to a minimum. In addition, because of the stability of the complexes, nonspecific background problems may be lessened by more stringent washing.

### Immunoprecipitations using monoclonal antibodies

The biggest advantage of using monoclonal antibodies for immunoprecipitations is the specificity of their interactions. Because monoclonal antibodies bind to only one epitope, they provide an excellent tool to identify a particular structure on an antigen. Given the right antibody, they can be used not only to detect an antigen, but also to distinguish among different forms of the antigen, including conformational changes or posttranslational modifications. In addition, because the immune complexes formed using monoclonal antibodies are not usually multimeric and are smaller than

those formed when using polyclonal antibodies, there is less of a problem with non-specific binding. Therefore, the backgrounds are normally cleaner.

Although using monoclonal antibodies for immunoprecipitations may solve or lessen some of the problems found when using polyclonal antibodies, their use also creates another set of difficulties. The most worrisome problem is affinity. Because the antigen is held only by one antibody-antigen interaction (except when the antigen is multimeric), the affinity of the antibody for the antigen is critically important (see discussions of affinity on p. 28). Monoclonal antibodies with affinities lower than about  $10^8$  liter mol<sup>-1</sup> are difficult to use in immunoprecipitations. Because many screening techniques for hybridoma fusions detect antibodies with affinities as low as  $10^6$  liter mol<sup>-1</sup>, not all monoclonal antibodies work well in immunoprecipitations.

A second problem with using monoclonal antibodies is the possibility of detecting spurious cross-reactions with other polypeptides. Because an epitope can be a relatively small protein structure, often composed of only 4 or 5 amino acids, there is a reasonable chance that a similar epitope can be found on another polypeptide. In some cases, the common epitopes form part of an important structural similarity between antigens, and monoclonal antibodies can be used to detect related antigens. Alternatively, the antibodies may detect small structural similarities confined only to the antibody combining site. This is particularly true for antibodies that recognize denaturation-resistant epitopes. Presumably this occurs because these antibodies recognize features found in the primary structure of the polypeptides. Depending on the set of hybridomas, as many as one in three monoclonal antibodies have been shown to display these types of cross-reactions. Because of the frequency of these cross-reactions, the precipitation of an unexpected polypeptide should be treated as a contaminant until proven otherwise.

### Immunoprecipitations using pooled monoclonal antibodies

Using pools of monoclonal antibodies in immunoprecipitation takes advantage of the best properties of both polyclonal and individual monoclonal antibodies. The monoclonal antibodies provide specificity, and the use of multiple antibodies allows the formation of stable multivalent complexes. Consequently, pooled monoclonal antibodies are the best choice of reagents for most immunoprecipitations. Unfortunately, not all antigens have been studied in enough detail to have a set of monoclonal antibodies available for pooling. However, even the use of two antibodies specific for two separate epitopes will greatly increase the avidity for the antigen as well as for protein A or protein G. Therefore, whenever possible, pooled monoclonal antibodies should be used for immunoprecipitations.

## Immunoprecipitation protocols

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Each immunoprecipitation has a core set of steps that are discussed in detail below. However, immunoprecipitations are often combined with other methods to make the assays more versatile. For example, immunoprecipitations are often performed on lysates from radiolabeled cells. This allows the identification of proteins from uncharacterized sources and allows investigators to ask about such issues as relative molecular weight, associated molecules, protein modifications, or protein half-lives. When lysates are prepared from unlabeled sources, the immunoprecipitated proteins can easily be quantitated by immunoblotting or checked for enzymatic activities.

The various modifications of the immunoprecipitation method can be used to determine many important characteristics of antigens. Table 7.2 lists these variations and the strategy for determining these characteristics.

### An overview of the basic immunoprecipitation procedure

The actual procedures for immunoprecipitations are quite simple. The method can be divided into three stages: (1) preparation of the antigen solution, (2) preclearing the lysate of nonspecific background, and (3) forming and purifying the immune complexes. The different techniques for each of the three steps are described below beginning on p. 230.

The first step is to prepare the antigen solution. Any solution can be used as a source for performing an immunoprecipitation; however, immunoprecipitation is normally performed on a lysate prepared from a cell or tissue. Lysates can be prepared by any number of methods, but for most purposes some type of mild detergent lysis of cells, such as treating with a nonionic detergent, is the preferred method. This removes the membranes, breaks apart many weak interactions, and releases most antigens from the cell. Importantly, it is mild enough not to destroy the conformation or enzymatic activity of most antigens. If correct conformation or activity of the antigen is not required or if the antigen is more tightly bound, more extreme conditions can be used to prepare the lysate. This can be as violent as boiling in a strong denaturant, which is later diluted or removed prior to immunoprecipitation. Once the cell lysate is prepared, it is ready for the preclearing step.

Because immunoprecipitations are normally used to determine the detailed biochemical characteristics of an antigen, the degree of purification of the final antigen preparation normally needs to be as extensive as possible. Because the interaction between the antibody and antigen behaves essentially according to the inherent properties of the antibody and antigen solutions, the easiest way to improve the signal-to-noise ratio in this technique is to lower the background. This is done by removing any nonspecific binding proteins from the antigen solution by pretreating with a nonspecific antibody that does not bind to the chosen antigen. This method of doing the immunoprecipitation—essentially doing the immunoprecipitation twice, once to lower the background with a nonimmune antibody and once with the antibody under study—is the most effective method for achieving clean immunoprecipitations.

Table 7.2 Variations on immunoprecipitation procedure

Goal	Variations	Recommended approach
Relative molecular weight of antigen		<ol style="list-style-type: none"> <li>1. Radiolabel cells with [<sup>35</sup>S]methionine in methionine-free media</li> <li>2. Immunoprecipitate</li> <li>3. SDS-PAGE and locate by film or imager</li> <li>4. Plot migration versus standards</li> </ol>
Quantitation of antigen	Abundant antigen	<ol style="list-style-type: none"> <li>1. Immunoblot</li> </ol>
	Abundant antigen	<ol style="list-style-type: none"> <li>1. Immunoprecipitate</li> <li>2. SDS-PAGE</li> <li>3. Stain by Coomassie blue or silver stain</li> </ol>
	Rare antigen	<ol style="list-style-type: none"> <li>1. Immunoprecipitate unlabeled protein</li> <li>2. SDS-PAGE</li> <li>3. Immunoblot with specific antibody</li> </ol>
Rate of degradation of antigen		<ol style="list-style-type: none"> <li>1. Radiolabel cells with short pulse of [<sup>35</sup>S]methionine in methionine-free media</li> <li>2. Chase with cold methionine</li> <li>3. Immunoprecipitate</li> <li>4. SDS-PAGE and examine by film or imager</li> </ol>
Posttranslational modifications	Any phosphorylation	<ol style="list-style-type: none"> <li>1. Radiolabel cells with [<i>ortho</i>-<sup>32</sup>P]phosphate in phosphate-free media</li> <li>2. Immunoprecipitate</li> <li>3. SDS-PAGE and locate by film or imager</li> </ol>
	Tyr-phosphorylation	<ol style="list-style-type: none"> <li>1. Immunoprecipitate unlabeled protein</li> <li>2. SDS-PAGE</li> <li>3. Immunoblot with anti-phospho-tyr antibody</li> </ol>
	N-linked sugar	<ol style="list-style-type: none"> <li>1. Radiolabel cells with [<sup>3</sup>H]mannose or [<sup>3</sup>H]galactose in glucose-free media</li> <li>2. Immunoprecipitate</li> <li>3. SDS-PAGE and locate by film or imager</li> </ol>
	O-linked sugar	<ol style="list-style-type: none"> <li>1. Radiolabel cells with [<sup>3</sup>H]mannose or [<sup>3</sup>H]galactose in glucose-free media</li> <li>2. Immunoprecipitate</li> <li>3. SDS-PAGE and locate by film or imager</li> </ol>
Associated proteins	Looking for potential new or studying known associated proteins	<ol style="list-style-type: none"> <li>1. Radiolabel cells with [<sup>35</sup>S]methionine</li> <li>2. Immunoprecipitate</li> <li>3. SDS-PAGE and examine by film or imager</li> </ol>
	Checking on known association	<ol style="list-style-type: none"> <li>1. Immunoprecipitate unlabeled protein</li> <li>2. SDS-PAGE</li> <li>3. Immunoblot with antibody for protein partner</li> </ol>
Enzymatic activity	Looking for intrinsic or associated enzyme activity	<ol style="list-style-type: none"> <li>1. Immunoprecipitate unlabeled protein</li> <li>2. Wash with reaction buffer</li> <li>3. Add substrate and perform assay</li> </ol>
Clear an antigen from a lysate	Removing a known antigen from solution	<ol style="list-style-type: none"> <li>1. Immunoprecipitate unlabeled protein using excess antibody</li> <li>2. Use supernatant from step 1 and repeat immunoprecipitation twice more</li> <li>3. Final supernatant is source for study, but use immunoprecipitates from each step to verify removal (by any method)</li> </ol>

After preclearing, specific antibodies are added to the lysate. Because of the high affinity of antibodies for their respective antigens, the antibody-antigen complexes form rapidly and easily. Then the immune complexes are purified on a solid-phase matrix that has either protein A or protein G linked to an agarose or polyacrylamide bead. Both protein A and protein G have high affinity for the Fc portion of the antibody. After the protein A/G-antibody interaction occurs, the unbound proteins are removed by washing the beads, leaving the purified antibody-antigen complexes bound to the matrix.

After molecules that are not bound to the antigen-antibody-protein A/G beads are removed by washing, the resulting immunoprecipitated proteins are available for further assays. The most common next step is to separate these proteins by SDS-polyacrylamide electrophoresis. This greatly extends the purification by separating the antigen and any remaining associated or contaminating proteins by their relative mobilities in SDS-PAGE. Individual proteins can then be identified by their relative molecular weight and any other specialized property of the antigen. Methods for SDS-PAGE are described on p. 410.

### Lysing cells

Cells and tissues can be lysed by several different techniques. The correct choice of lysis method depends on the types of cells or tissues being studied and the final use of the antigen. Cells without a cell wall can be easily lysed by treating with mild detergents. Cells that have a cell wall, are most often lysed by removing the cell wall by some type of mechanical shearing or enzymatic treatment. If the final preparation of the antigen need not retain its normal three-dimensional shape or biochemical activity, the cells can be lysed by using harsh denaturing conditions and then handled by diluting the denaturants before adding the specific antibody.

Many extraction conditions release proteases in the lysis buffer. If protease digestion becomes a problem during immunoprecipitations, two approaches can be used to lessen its effects. First, care should be taken to keep the samples cold. Temperature has a profound effect on the rate of degradation by most proteases. Second, the lysis buffers can be supplemented with protease inhibitors. The sidebar lists some of the commonly used protease inhibitors. In immunoprecipitations, the two most commonly used inhibitors are aprotinin and phenylmethylsulfonyl fluoride (PMSF); however, a mixture of several of the different protease inhibitors is better, particularly as a starting point for further tests that would identify exactly the right inhibitors to use with your source of antigen.

Protease inhibitor	Working conc.
Aprotinin	1 $\mu\text{g/ml}$
Leupeptin	1 $\mu\text{g/ml}$
Pepstatin	1 $\mu\text{g/ml}$
PMSF	50 $\mu\text{g/ml}$

#### Caution

Aprotinin, leupeptin, pepstatin, PMSF, see Appendix IV.

### Lysis buffers

A number of different lysis buffers can be used to release protein antigens from cells, but no one buffer is sufficient for all purposes. In choosing a buffer, there are two important considerations. The antigen must be released efficiently, and it must still be recognizable by the antibody. When beginning the analysis of a new antigen, it is best to test a number of different extraction buffers to identify the most efficient conditions for the release of the antigen. A good strategy is to lyse cells with a relatively strong buffer. If the antigen is released, then begin to alter the composition of the lysis buffer until the mildest conditions to release the antigen are determined (see sidebar p. 231).

### Build your own lysis buffer

*Variables that can drastically affect the release of polypeptide antigens include salt concentration, type of detergent, presence of divalent cations, and pH. Salt concentrations between 0 and 1 M, nonionic detergent concentrations between 0.1 and 2%, ionic detergent concentrations between 0.01 and 0.5%, divalent cation concentrations between 0 and 10 mM, EDTA concentrations between 0 and 5 mM, and pH values between 6 and 9 should all be monitored to determine the optimal conditions for extraction. Other possible additions to lysis buffers that may affect some antigens include RNases or DNases. Appendix III contains a short discussion on various detergents that may be chosen.*

Quantitative release should be judged both by testing the amount of antigen in the lysate and by testing the amount of antigen remaining in the cell debris. This can be done most easily by analysis on immunoblots.

In general, the conditions used for lysis should be as gentle as possible to retain the antibody-binding sites and to avoid solubilizing background proteins, but harsh enough to ensure quantitative release of the antigen. For detergents this normally means choosing nonionic detergents over ionic, lower concentrations over higher, and single detergents over mixes.

Probably the two most common extraction buffers for immunoprecipitations are NP-40 and RIPA lysis buffers. They release most soluble cytoplasmic or nuclear proteins without releasing the chromosomal DNA. This strength of release is a good starting point for most studies. It is preferable not to release the DNA if possible, because of the problems that are caused by the viscosity due to the DNA in the resulting solution.

#### NP-40 lysis

150 mM Sodium chloride  
1.0% NP-40  
50 mM Tris, pH 8.0

This is probably the most commonly used lysis buffer. It relies on a nonionic detergent, NP-40, as the major solubility agent. In place of NP-40, Triton X-100 can be used with similar results. Useful variations include lowering the detergent concentration, raising the salt concentration, or switching to other detergents such as saponin, digitonin, or CHAPS.

#### RIPA lysis

150 mM Sodium chloride  
1.0% NP-40  
0.5% Sodium deoxycholate  
0.1% SDS  
50 mM Tris, pH 8.0

This is more denaturing than the NP-40 lysis buffer described above. In addition to the nonionic detergent NP-40, two ionic detergents, sodium deoxycholate and sodium dodecyl sulfate, have been included. This lysis buffer releases most proteins in the cells, but will also break apart many protein-protein interactions.

#### Caution

Sodium deoxycholate, SDS, see Appendix IV.

### Purifying the immune complexes

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Forming an antibody-antigen complex is the simplest step in an immunoprecipitation. Antibodies are added to the antigen solution, and the antibodies bind to their cognate antigen. Then protein A or protein G beads are added to the solution. This slurry is mixed and the proteins that do not bind to the beads are removed by washing. The variables to be considered are the amount of antibody to be added, the final volume of the immunoprecipitation, and whether to use protein A or protein G.

#### Amount of antibody

Although determining how much antigen can be bound by a given amount of antibody is always a useful parameter to learn for any antibody preparation, most investigators do not need to go to extensive lengths to find this out. If you are just trying to characterize your antigen, we recommend the use of 1  $\mu$ l of serum, 50  $\mu$ l of tissue-culture supernatant, or 0.5  $\mu$ l of ascites fluid. For most high-affinity antibodies this will be sufficient to immunoprecipitate a large portion of the available antigen, and the levels of antibody are low enough that you should not experience extensive problems with background or distortion of the gel near the position of the heavy chain.

In all cases where quantitative measurements are needed or when the backgrounds must be kept to a minimum, the amount of antibody to be added should be determined by titration. This amount depends on how much antigen is present, the affinity of the antibody for the antigen, and the volume in which the immunoprecipitation takes place. The correct amount of antibody to be used can be determined by titrating the volume of antibody versus a constant amount of antigen. A general starting titration range is between 0.05  $\mu$ l and 5  $\mu$ l of a polyclonal serum, 1  $\mu$ l and 100  $\mu$ l of a hybridoma tissue culture supernatant, or 0.01  $\mu$ l and 1  $\mu$ l of an ascites fluid. At the midpoint of these three suggested ranges, the amount of heavy chain will be approximately 1  $\mu$ g. This is sufficient that the heavy chain can be detected easily by Coomassie blue staining of an SDS-PAGE. The presence of a stained band is used for troubleshooting because it indicates that efficient binding of the antibody to the protein A or protein G beads has occurred.

Another advantage of titrating the amount of antibody is that this often helps to lower the nonspecific background.

#### Volume of the immunoprecipitation

Several competing factors need to be considered when choosing the final volume of the immunoprecipitation. The final volume determines the rate at which the binding equilibrium between the antibody and antigen is reached. Small volumes reach this equilibrium more quickly than larger volumes. Although one might assume that as small a volume as possible is the preferred approach, smaller volumes also promote the affinity of nonspecific interactions. So using larger volumes can be an effective method of keeping the backgrounds down. Also, it is important to get good mixing after the addition of the protein A or protein G beads. Larger volumes promote the ease of keeping the beads in a good mix with the antigen/antibody solution. To balance



these variables, we recommend using a final volume of approximately 0.5 to 0.75 ml in a 1.5-ml conical tube. In general, most of the antibodies that have been selected for use in immunoprecipitations have a relatively high affinity for the antigen, making the volume of the reaction less important. In addition, for most investigators, the background problems are more worrisome than making certain that 100% of the antigen is captured. Increases in the final volume can be made by the addition of lysis buffer. If desired, this diluent can be made with 1% bovine serum albumin to help reduce any nonspecific protein binding.

In settings where the background is not a problem or when trying to remove all of the antigen from a solution, keeping the volumes as small as possible will drive the antigen/antibody interaction toward completion.

Finally, there may be cases where the types of antibodies you are using should be considered. When using a polyclonal antibody, the volume of the reaction is seldom a concern. In fact, the avidity is normally high enough to allow efficient binding and, eventually, removal of the antigen from solution even with dilute samples. With monoclonal antibodies of high affinity, similar approaches are possible. However, with low-affinity monoclonal antibodies, using high concentrations of the antibody in low volumes will help drive the reaction toward completion.

## Protein A versus protein G

All of the methods for purifying immune complexes rely on secondary reagents that bind to the antibody. The antibody is removed from the solution by binding to the secondary reagent, and the antigen remains associated with the antibody throughout the purification. The earliest versions of this method used anti-immunoglobulin antibodies to form a large complex of antibody-anti-immunoglobulin antibodies. This multicomponent complex of molecules is known as a lattice. When a lattice is suitably large, it can be removed from solution by centrifugation. This procedure is still one of the best at yielding clean immunoprecipitations. However, the ability to form large enough complexes to be collected by centrifugation is critically dependent on the molar ratio of anti-immunoglobulin antibodies to primary antibodies. For quantitative removal of the immune complexes, the ratio must be determined empirically for each primary antibody that is used. To some degree, the size of the lattice also depends on the amount and type of antigen. A large quantity of a multimeric antigen changes the potential size of any lattice work compared with a rare, monomeric antigen. A monoclonal antibody gives a different set of problems to forming the lattice.

As a solution to these problems, Kessler (1975) suggested the use of protein A-bearing *S. aureus* Cowan 1 (SAC) as a solid phase to collect the antibody-antigen complexes. Protein A forms a portion of the cell wall of these bacteria. The cell-wall proteins are fixed by treating with formaldehyde, the bacteria are killed by heat treatment, and the resulting particles form an excellent solid-phase matrix to bind to the antibodies. The protein A binds to the Fc domain of the antibody, thus, its attachment to the antibody does not affect the interaction with the antigen.

In recent years, the use of SAC has been replaced by the widely available recombinant protein A and protein G. They are available covalently coupled to a number of different bead matrixes. Use of this solid phase also lowers some of the background problems encountered with SAC. The choice of either protein A or protein G beads is based on the affinity of the antibodies for protein A or protein G.

### *Technical considerations for drug block determination of half-lives*

Many labs have begun to report the determination of protein half-lives by using protein synthesis inhibitors to initiate the chase period. Commonly this is done using cycloheximide at concentrations of 1–10  $\mu\text{g/ml}$  added from concentrated stocks. Protein levels are then determined by simple immunoblots at different times post treatment with the protein synthesis inhibitor. This methodology is appropriate only when preliminary tests show that pulse-chase results are correlated with those from the drug blocks. There are many examples now reported in the literature in which protein turnover is dramatically different in the presence of protein synthesis inhibitors. Therefore, the preferred technique is the pulse-chase methodology described above. However, there may be cases when many different conditions need to be tested for altering protein half-life or when use of radioactive precursors is inappropriate.

### **Checking for posttranslational modifications**

A number of proteins are modified after their synthesis, commonly by the addition of sugars, phosphates, or sulfates. These additions are used for many purposes including to mark proteins for particular intracellular or extracellular transport systems, to help ensure a specific protein-folding option, or as regulatory events that change a key feature of the target substrate. By growing cells in the presence of a radioactive precursor specific for a modifying group, immunoprecipitations can be used to identify proteins that undergo posttranslational modifications.

There are several hundred different potential posttranslational modifications, but many modifications are not commonly studied using specific radioactive precursors. This is because the correct precursors are not commercially available, the precursors cannot be taken up by cells, or the intracellular pools are too large to achieve high enough specific activities to identify the group. However, a number of modifications can be studied in this manner. Several of these are summarized below.

#### *Phosphorylation by radiolabeling*

Phosphorylation of proteins is one of the most common methods of regulating protein function. Phosphorylation is used to turn enzymes off and on, to mark proteins for transport, to stimulate interaction with other molecules, and to designate protein scheduled for proteolytic degradation. Phosphorylation of proteins is catalyzed by the transfer of the gamma-phosphate of ATP to a target site by a class of enzymes known as protein kinases. Phosphorylated proteins can be detected by growing cells in the presence of radiolabeled orthophosphate. Commonly this will be  $^{32}\text{P}$ , a beta-particle emitter, but  $^{33}\text{P}$ , a low-energy beta-emitter, is becoming more frequently used. It is more expensive than  $^{32}\text{P}$ , but the half-life of  $^{33}\text{P}$  makes it more useful in some settings. The phosphate is readily taken up by the cells and is converted to labeled ATP primarily through standard nucleotide synthesis pathways. Labeling cells with radioactive ATP is not possible, because ATP is not transported across the cell membrane.

Protein phosphorylation is detected by lysing the labeled cell population and immunoprecipitating the antigens using standard techniques. The labeled proteins are located after SDS-PAGE either by using film autoradiography (intensifying screens at  $-70^\circ\text{C}$  give the most sensitive film detection) or by detection using a phosphorimager.

Phosphate labeling is performed in phosphate-free media, which can be purchased from several different commercial suppliers. The highest specific activity  $^{32}\text{P}$  that can be obtained is normally used. This is because the intracellular pools of phosphate are relatively large, and this decreases the percentage of proteins that contain the labeled phosphate moiety. Fortunately, the detection of  $^{32}\text{P}$  is efficient, so high specific activity of the final phosphorylated proteins is not required. Labeling for most proteins is linear in mammalian cells only for about 1–2 hours and levels of incorporated phosphate rise only until about 4 hours. Labeling times beyond 4 hours should be avoided unless the medium is supplemented with 10% complete medium with phosphate. Commonly, researchers label with 100  $\mu\text{Ci}/100\text{-mm}$  dish to 1  $\text{mCi}/100\text{-mm}$  dish. The choice of amount of label is based on the abundance of the protein, the number of phosphorylated residues, and the turnover rate of these sites. Since this is often not known prior to the first phosphate labeling, the levels are commonly determined empirically. For initial tests, we recommend using 500  $\mu\text{Ci}/100\text{-mm}$  dish.

*A good starting point to examine phosphorylations of proteins would be to transfer a 100-mm dish of cells into 2 ml of phosphate-free medium for 15 minutes at normal growth conditions, then add 500  $\text{mCi}$  of  $^{32}\text{P}$ -orthophosphate. Incubate for 1 hour. Then wash cells twice with ice-cold PBS and process for a normal immunoprecipitation.*

One disadvantage of using  $^{32}\text{P}$  as a radionuclide for labeling proteins is that it causes DNA damage. This occurs in two manners. First, as expected, the  $^{32}\text{P}$  is incorporated into the DNA backbone, and its decay breaks the resident chain and the local decay also likely breaks the opposite strand, yielding a double strand break. In addition and more frequently, as all of the  $^{32}\text{P}$  decays, it exposes the cells to a local and very intense treatment of ionizing radiation. Both of these treatments lead to the induction of the normal DNA damage response in the cell. If the levels of  $^{32}\text{P}$  are sufficiently high, cell cycle arrest will occur. Therefore, shorter labeling times and lower levels of radiolabel are preferable.

### ***Tyrosine phosphorylation by immunoprecipitation/immunoblot***

Phosphorylation of proteins on tyrosine residues can be tested by using antibodies raised against the phosphotyrosyl moiety. This can be most easily tested by immunoprecipitating the antigen under study directly from unlabeled cells. The immunoprecipitated proteins can be separated by SDS-PAGE and transferred to nitrocellulose using standard immunoblotting methods (see Chapter 8). Develop the blot with antibodies specific for phosphotyrosines.

### ***Glycosylation***

Having specific antibodies that will immunoprecipitate your antigen is a major advantage in studying protein glycosylation. Glycosylation is normally first noticed when proteins migrate unusually on SDS-polyacrylamide gels. Immunoprecipitated proteins that are glycosylated run considerably larger in molecular weight than expected, perhaps as much as 30–50% larger than their predicted mass, and they will generate quite broad bands. Glycosylation is used for many purposes in cells, but it is primarily employed to mark proteins for particular stages of protein trafficking, to alter the structure of a protein to change its activity, or to provide extracellular binding domains that function in intercellular signaling and tissue development.

Glycosylation occurs in two general forms based on the type of linkage that is made to the polypeptide chain. N-linked glycosylation links a sugar, almost exclusively N-acetylglucosamine, to asparagine through its amino group. O-linked glycosylation links a wide range of sugars to serine or threonine reacting with the hydroxyl. Proteins are seldom modified by single sugars. What makes glycosylation difficult to study is the wide range of different sugars that appear in the glycan, the different orders in

which sugar subunits are added to the growing sugar backbone, and the variations in the extent and complexity of the branching.

There are two general strategies to study protein glycosylation. These are (1) to label cells with radioactive monosaccharides and allow these sugars to be processed and added to the sugar backbone or (2) to treat purified polypeptides with specific chemical or enzymatic cleavage agents to remove all or portions of the sugars. Both approaches are helped by the availability of specific antibodies. Following growth in labeled sugars, proteins can be precipitated and examined for the presence of the radioactivity after separation on SDS-polyacrylamide gels. Proteins that are marked by labeling with [<sup>35</sup>S]methionine or another amino acid that gets incorporated into the polypeptide chain can be immunoprecipitated and then left untreated or treated with chemical or enzymatic cleavage agents. These proteins can then be resolved on SDS-polyacrylamide gels and checked for changes in mobility. Both approaches have some difficulties. Radiolabeling is done by growing cells in the presence of [<sup>3</sup>H]galactose or [<sup>3</sup>H]mannose. Labeling times should be kept to the absolute minimum incubation—seldom longer than 1 hour—because sugars are quickly shunted into many different biosynthetic pathways and their labels will quickly appear in other macromolecules in addition to just sugar-containing compounds. Cleavage is equally problematic because there are no single methods that will hit all glycosylations or all N-linkages or all O-linkages. Therefore, a large number of potential agents have been discovered and are used frequently. Enzymatic cleavage of N-linked sugars is now done with peptide-N-glycosidase F, which will cleave most of the commonly studied N-linked glycans. Other useful enzymes include endoglycosidase H and endoglycosidase F. Chemical cleavage is more complicated technically but is believed by many workers to be more reliable once the method is set up and working in the lab. The most useful reagent to use for chemical cleavage is anhydrous trifluoromethanesulfonic acid.

For more detailed discussions of these problems, the reader is referred to Varki (1994) for radiolabeling approaches and one of the recent manuals on cell biology (e.g., *Cells: A Laboratory Manual* by Spector, Goldman, and Leinwand is an excellent compendium of methods) for appropriate conditions for enzymatic or chemical cleavage.

### **Prenylation**

It has been suggested that between 0.1% and 0.5% of all cellular proteins are modified by the addition of an isoprenoid. The known isoprenoids used for protein modification are farnesyl and geranylgeranyl. These hydrophobic moieties are used for such processes as anchoring proteins in lipid membranes. The modifications are found exclusively at carboxy-terminal cysteine residues, linked through a thioester.

Detecting prenylation can be done by metabolic labeling with a precursor of the isoprenoids, [<sup>3</sup>H]mevalonate. Four variations on the labeling method can be considered. Early experiments relied on relatively low levels of incorporation and were performed by adding high levels of [<sup>3</sup>H]mevalonate to the media (up to 500  $\mu$ Ci/ml were used). A variation that helps boost incorporation is to block the endogenous synthesis of mevalonate. In cells, mevalonate is synthesized from conversion of HMG CoA by HMG CoA reductase. In metabolic labeling experiments, HMG CoA reductase can be inhibited to slow this synthesis reaction and increase the incorporation of [<sup>3</sup>H]mevalonate into the precursors of farnesyl and geranylgeranyl groups. This now can be done by using a drug specific for the HMG CoA reductase, mevinoлин (available from Merck, Rahway, New Jersey). A third variation is to increase the rate of transport for

mevalonate. A transporter for mevalonate has recently been cloned (Kim et al. 1992), and this can be transfected into cells you wish to test. Finally, there are a few cell lines that have been selected for high levels of transport. Your gene of interest can be transfected into these cells and studied for prenylation there.

In addition, there are chemical detection methods that allow the identification of farnesyl and geranylgeranyl groups. For more details on these modifications, readers are referred to Spector et al. (1998).

### **Sulfation**

Many extracellular proteins are modified by the addition of sulfate. Some of the best characterized are the glycosaminoglycans, such as the heparin sulfate proteoglycans, and the granins. Sulfation can be detected easily by growing cells in small volumes of sulfate-free medium ( $\text{MgCl}_2$  replacing  $\text{MgSO}_4$ ) in the presence of [ $^{35}\text{S}$ ]sulfate. If serum needs to be included, use dialyzed and refiltered sterilized samples to remove the sulfate in the medium. Final concentration of the radiolabeled sulfate should be approximately  $0.5 \mu\text{Ci/ml}$ . Labeling times should be kept relatively shorter than those used for peptide chain elongation. Times as short as 5 minutes are used to label proteins still in the secretory pathway itself, whereas labeling incubations of 30–60 minutes are commonly used for secreted proteins and extracellular matrix proteins.

Secreted proteins can be studied by immunoprecipitating directly from the media. Intracellular proteins are released from cells after washing the cells and treatment with lysis buffers. Immunoprecipitations are performed using standard methods and sulfate-labeled proteins are detected on SDS-polyacrylamide gels by film autoradiography or phosphorimaging.

### **Protein-protein interactions**

When lysates are prepared under gentle conditions, specific antibodies immunoprecipitate not only the antigen under study, but also any other macromolecules that are bound to it. Immunoprecipitations are often the most useful method for studying these types of interactions, and thereby allow workers the possibility of studying heteropolymeric complexes.

Immunoprecipitations are still considered the gold standard for arguments of protein-protein interactions (Fig. 7.9). Because immunoprecipitations have many points at which specific and nonspecific contaminants can be detected, extreme care must be used before any band is classified as an associated protein. Good minimal evidence for association should meet several criteria. First, all antibodies that are used must be shown to be specific, that is, to recognize only one of the proteins in question. Second, the association needs to be demonstrated by more than one method. Useful combinations include:

1. Perform a second immunoprecipitation with an antibody specific for the potential binding partner. If the association can be demonstrated from both partners, this forms a strong argument for *in vivo* binding.
2. Use more than one independent antibody against the protein antigen. If two or more antibodies that can be shown to recognize independent epitopes can precipitate the potential interacting partner, this is reasonably good evidence for binding. Problems could be caused by unexpected cross-reactions between the antibodies,

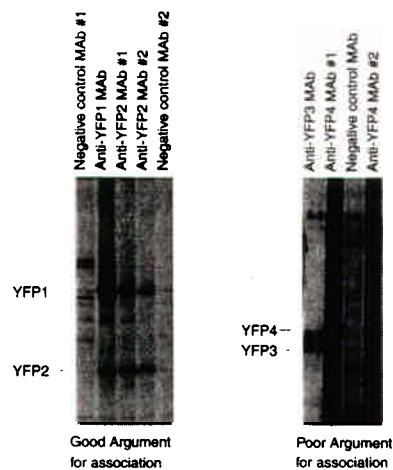


Figure 7.9  
Protein–protein interactions.

or by the two antigens having multiple epitopes in common, as seen with protein families or with spliced variants of the same gene.

3. Less convincing but still helpful are coprecipitation and copurification of the two proteins following one or more standard purification steps, such as gel filtration or velocity gradient sedimentation. This method is most easily interpreted when the proteins are mostly pure. Copurification from crude samples should only be used to support association claims.

Excellent evidence for association would be to include multiple examples from the above list. All claims should contain clear evidence of antibody specificity for only one member of the protein complex.

Other methods also provide helpful approaches to study protein–protein interactions. *In vitro* binding, binding following transfection of two expression constructs, or two hybrid interactions are useful first steps to look for interactions or good methods to map interaction domains, but they are prone to overinterpretation and should not be considered sufficient evidence to claim an *in vivo* interaction. Even clear evidence generated by immunoprecipitation from lysates needs a careful interpretation because proteins may bind after lysis of the cells and never touch each other *in vivo*.

In many cases, the choice of buffers or detergents used in the lysis step will determine whether a multimeric complex remains intact. The properties of various detergents are discussed in the Appendix (pp. 469–471). Try various combinations to test for weaker interactions.

### ***Technical approaches for identifying or studying protein–protein interactions***

There are two useful methods using immunoprecipitations to identify and study protein–protein interactions. Immunoprecipitation of radiolabeled proteins is an effective method to look for new interacting proteins. Because most proteins are labeled with an amino acid such as [<sup>35</sup>S]methionine, an immunoprecipitation with a specific antibody may detect the specific antigen and any other interacting protein. In this method, cells are grown in a radioactive amino acid precursor, and immunoprecipitation is per-



formed as usual. The immunoprecipitated proteins are resolved by SDS-PAGE. By examining the gels for bands (in addition to the antigen) that are specific for immune lanes but not present in the control lanes, one can look for potential associated proteins.

The second approach uses immunoblotting to study known proteins. Immunoprecipitations are performed from unlabeled sources. The immunoprecipitated proteins are resolved on SDS-PAGE and transferred to membranes, as described in Chapter 8. The blot is then developed with an antibody against a known protein. This method is very sensitive, because the immunoprecipitation step represents a powerful concentration step. Consequently, investigators must be careful that the experiments include controls for the total amount of each protein. Comparing this to the amount in the complex gives a good indication of the percentage of the protein that is associated. This can be done by running samples of lysates that have not been precipitated or by immunoprecipitating and immunoblotting with the same antibodies. Researchers should show that associations detected in this manner represent a reasonable proportion of the total protein present in the solution. It is a good practice to examine closely and be skeptical of complexes that represent less than about 5% of the total protein.

Several controls should be included when using either the radiolabeled or immunoblotting approaches. As mentioned above, the antibodies for these experiments must be well characterized to show that they are specific only for their cognate antigens. Appropriate controls should always include a negative antibody that does not recognize any of the proteins in the potential interactions.

### **Checking for intrinsic or associated enzymatic activities**

Because immunoprecipitated proteins can be prepared in relatively mild conditions, the final protein complexes often retain many of their biochemical properties. For example, immunoprecipitated kinases often retain their ability to recognize and phosphorylate their natural substrates. This allows the use of a variation of the immunoprecipitation method to examine not only the intrinsic enzymatic activities of any antigen, but also the activities of any proteins that are associated with the antigen under study.

#### ***Technical approaches to studying enzymatic activities***

Immunoprecipitated proteins or protein complexes often retain their enzymatic activities. Following the third wash, the immune complexes bound to the beads are washed one additional time in the reaction buffer. This last reaction buffer wash normally does not contain the intended substrate and should also omit any ATP or other energy source. This will remove most contaminating detergents of other agents, such as EDTA, that might block or slow enzymatic activities. The immune complexes and beads can then be resuspended in reaction buffer and aliquoted for the desired number of reactions.

Several points should be kept in mind while designing these experiments. First, remember that the enzyme kinetics will be altered dramatically in this setup. The enzymes are still bound to the beads, so they will not be able to diffuse through the solution and hence the reaction will follow essentially second-order kinetics. Second, the beads are dense and will drop to the bottom of your tube quite quickly. This may mean you will need to rock closed tubes during the reaction period. Alternatively and more easily, you can occasionally resuspend the beads by flipping the bottom of all the tubes during the reaction period.

To stop the reaction, several approaches can be used. The correct choice depends on the next step. If you will be detecting the transfer of a radioactive moiety to a protein substrate, such as the transfer of the [<sup>32</sup>P]gamma phosphate of ATP to a protein in a protein kinase reaction, you can stop the reaction by the addition of Laemmli sample buffer. In other cases, heating the reactions will kill enzymatic activity. Other choices might include adding EDTA to chelate divalent cations (EGTA for Ca<sup>++</sup>) or freezing the samples.

One other advantage of this approach that is not often used, but investigators should keep in mind, is that the enzymes in this setting are most often still bound to the beads. This is equivalent to a small-scale immunoaffinity purification. This will allow the simple separation of the enzyme from the substrates by centrifugation and removal of the supernatant.

### Clearing a lysate of an antigen

When immunoprecipitations are performed in conditions that lead to complete or near-complete removal of the antigen, they allow a rapid and effective method to clear an antigen and all molecules bound to it from a lysate. With the availability of the correct antibody, this allows an investigator to learn the effects of losing an antigen from a solution. Uses for this approach include removing an antigen and determining what proportion of an associated protein is bound to antigen versus what remains, and learning how the activities of a lysate, when depleted of an antigen, are changed.

This is a useful approach for analytical-scale projects. When large solutions are being studied, clearing is more easily done by using the methods described in Chapter 9.

#### Technical approaches to clearing a lysate of an antigen

Preclearing a lysate of an antigen is normally done by performing the immunoprecipitation in antibody excess and often is accomplished by performing the immunoprecipitation several times in succession with the same lysate (Fig. 7.10).

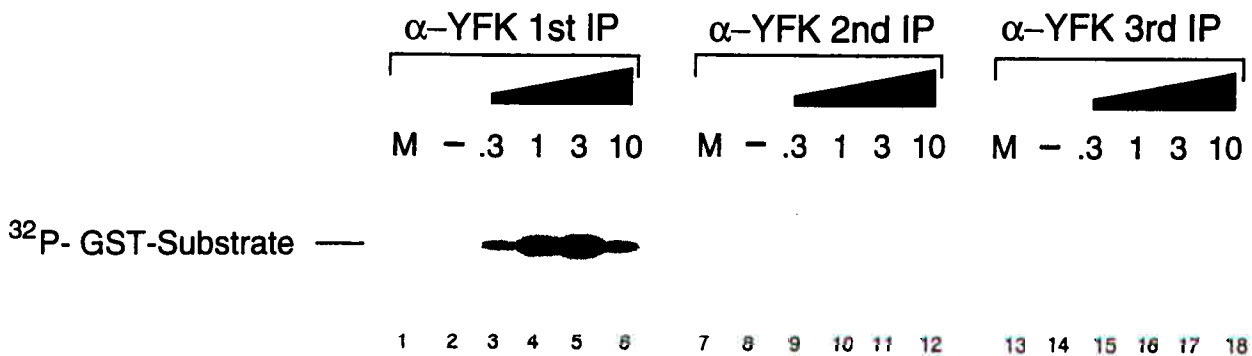


Figure 7.10  
Preclearing lysate of antigen.

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