TECH TIP # 64

Thermo SCIENTIFIC

Immunoprecipitation (IP) technical guide and protocols

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Introduction

Immunoprecipitation (IP) and co-immunoprecipitation (Co-IP) are methods used to enrich or purify a specific protein or group of proteins from a complex mixture using an antibody immobilized on a solid support. IP is an important step in many proteomics workflows designed to investigate the presence, relative abundance, size, up-regulation or down-regulation, stability, post-translational modification and interactions of proteins. Purified antigen(s) obtained by IP can be analyzed by a variety of techniques, such as ELISA and Western blotting, and the isolated proteins can be quantified or identified by mass spectrometry using enzymatic digestion patterns based on the primary sequence.

The earliest methods of immunoprecipitation to be developed involved direct labeling of total protein using radioactive precursors such as amino acids added to the medium of cells growing in culture. Cells were lysed and the antigen purified from the mixture using a specific antibody immobilized on a beaded support. Purified antigens were then visualized by SDS-PAGE followed by exposure of the gel to autoradiography film.

This radio-immunoassay method is still performed, but safety, regulatory and cost concerns regarding the use of radioactive material have led to the development of non-isotopic methods. Instead of pre-labeling the antigen, it is now more common for the antigen to be purified from the lysate, resolved by SDS-PAGE and then transferred to a membrane, where it can be probed and detected by Western blotting. The advent of sensitive chemiluminescent substrates means this approach can easily match the sensitivity of radioactive techniques and provide extra specificity based on the affinity of the antibody.

General Description of IPs, Co-IPs and Pull-downs

A. Immunoprecipitation (IP)

The term "immunoprecipitation" generally refers to any assay in which proteins are affinity-purified on a small scale using a binding protein immobilized on a solid support. More precisely, IP is an assay designed to purify a *single antigen* from a complex mixture using a *specific antibody* attached to a *beaded support* (nearly always meaning agarose resin). Assembly of the immobilized protein complex can be accomplished sequentially or in one step (Figure 1). A common sequential method is to incubate antibody and sample (e.g., cell lysate), followed by addition of affinity beads to capture the antibody-antigen complex. Alternatively, the antibody may be incubated first with the beads (where it becomes bound either directly or indirectly through an IgG binding protein such as Protein A, G or A/G), followed by addition of the antigen-containing sample. After binding antigen, antibody and support, the beads are washed extensively and the antigen eluted from the support using an appropriate elution buffer. There are a number of factors that should be considered when designing an IP experiment (including order of addition) and these are discussed in subsequent sections of this guide.

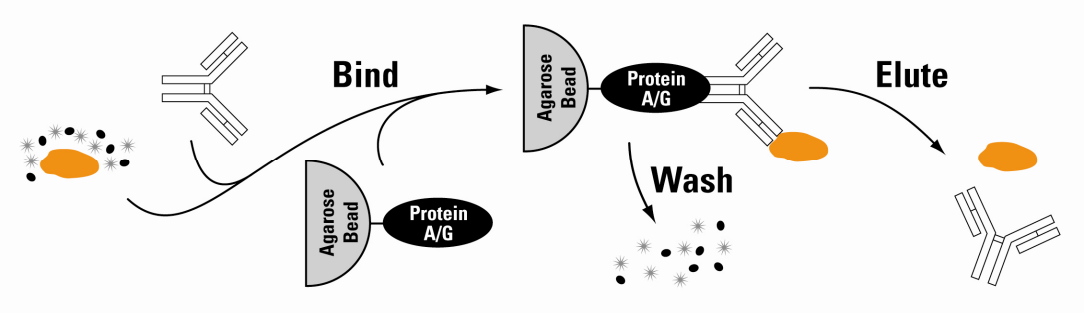

Figure 1. Diagram of a basic immunoprecipitation procedure. Antigen-containing sample (usually a cell lysate), antibody and beaded agarose affinity beads (usually Protein A or G) are allowed to bind. Non-bound sample components are washed away, and then antibody and antigen are eluted with a buffer that disrupts the binding interactions. If reducing SDS-PAGE sample buffer is used for elution, antibody and antigen products will be denatured and reduced to component polypeptide fragments.

B. Co-Immunoprecipitation (Co-IP)

Co-immunoprecipitation assays, or Co-IPs are very similar to IPs because the basic technique uses an immobilized antibody specific to an antigen of interest; however whilst the purpose of an IP is to purify a single antigen, a Co-IP is designed to isolate the antigen *along with any proteins or ligands that are bound to it*. In such instances the known antigen is termed the bait protein, and the protein(s) it interacts with are called the prey protein(s). These may be complex partners, signaling molecules, structural proteins, co-factors etc, and the strength of the interaction may range from highly transient to very stable. The basic Co-IP protocol is the same as that described for IP, and indeed any system designed for IP should also work for Co-IP. There are a number of additional factors to consider, however; for example, optimization of binding and wash conditions must include consideration of effects on bait-prey interactions as well as on that between antibody and bait.

C. Pull-down Assays

A pull-down assay is similar in concept to a Co-IP because it is performed in order to investigate proteins or ligands that bind to a known bait protein. The assay is performed either to prove a suspected interaction between two proteins or to investigate unknown proteins or molecules that may bind to a protein of interest. Pull-down differs from IP or Co-IP in that it is not based on an antibody-antigen interaction; it is not an immunoassay. The bait protein (or ligand) is captured to the solid support (beads) by a non-antibody affinity system, either by covalent attachment to an activated beaded support or through an affinity tag that binds to a receptor molecule on the support. For example, immobilized metal affinity chromatography (IMAC) chemistry can be used to perform pull-down assays with histidine-tagged bait proteins. Optimization of pull-down assays requires consideration of the peculiar characteristics of affinity system used.

Factors Affecting Immunoprecipitation

Although IP methods are logically and procedurally simple, the variables and factors affecting success of any specific experiment are as numerous and peculiar as the specific differences between different individual proteins and different primary antibodies. Immunoprecipitation involves purification of a protein or complex of proteins using specific binding conditions. Empirical testing is nearly always required to before IP conditions can be optimized to successfully isolate adequate amounts and purity of specific protein. Nevertheless, consideration of the main factors involved can help to identify the components that are most likely to affect particular experiments. A list of such factors and associated variables is described in Table 1, followed by a discussion of several pertinent issues. The protocol section at the end of this guide includes step-by-step instructions and suggestions for optimization.

Table 1. Factors that affect assembly of the purification complex

Factor	Variable Characteristics
Method Format	Column vs. batch method; spin vs. gravity columns
Type of Support	Physical characteristics, capacity, non-specific binding
Immobilization of Antibody	Amount, orientation, method of attachment
Immobilization of Bait	Tag, affinity ligand
Order of Addition	For beads, antibody/bait and antigen/prey
Lysate Pre-clearing	Non-specific binding
Binding Buffer	Components, stringency
Wash Buffer	Components, stringency
Elution Buffer	Components, elution strength

A. Method Format

Column method vs. batch method

Immunoprecipitation as performed by the batch method simply involves mixing the components of the reaction in a reaction vessel (usually a microcentrifuge tube) for a period of time to allow them to interact. At each step, the beads are separated from the solutions (nonbound sample, wash buffer and finally elution buffer) by centrifuging the tube to pellet the beads and carefully pipetting to remove the supernatant.

Column methods involve incubating IP components with beaded resin that is packed in a plastic or glass column. The sample is either allowed to pass the column by gravity or centrifugation (see next paragraph) or the column is capped and the sample incubated with the resin (with optional mixing) to allow the antibody and antigen more time to bind. In either case, the sample solutions are separated from the beads by gravity-flow or centrifugal collection from the column tip.

Gravity flow versus spin columns

Large scale IPs (>10 ml resin) are generally limited to gravity-flow because of the impracticality of centrifuging large columns, especially if they are not designed to fit in a collection tube. Conversely very small scale applications require centrifugation, as just a few microliters of solution will not flow through a filter by gravity alone. Most medium scale IPs can be performed by either gravity-flow or centrifugation so long as suitable columns and collection tubes are available, and the beaded support is compatible with the increased pressures associated with centrifugation (see below).

The use of spin columns has a distinct advantage over both gravity columns and batch methods because almost all of the residual solution can be spun through the filter allowing cleaner separation of the solid and aqueous phases. Gravity columns require constant monitoring to make sure the resin does not run dry and form air bubbles. In addition the antigen is eluted in multiple fractions, each of which must be monitored for the presence of antigen. Fractions containing antigen are normally pooled, therefore the volume will end up being much greater than the original sample and the antigen may require concentration. A disadvantage of the batch method is the formation of the resin pellet, which contains a significant volume of solution that cannot be removed by pipetting; additional wash and elution steps are necessary to obtain good purity and yield.

B. Type of Support

Agarose

The most prevalent type of beaded support used in research scale IP type applications is crosslinked beaded agarose (Figure 2). This is an easy-to-use, versatile support that can be modified for activation or coupling to an appropriate ligand. The resin is durable and robust, able to withstand centrifugation up to 5000 × *g*, pressures of 100 psi (depending on the

degree of crosslinking) and temperatures up to 120°C without significant loss of structure or flow rate. Agarose exhibits low non-specific binding in complex samples and is not harmed by moderate levels of most detergents, salts, most organic solvents or extremes of pH (will tolerate pH range 3-14 for extended periods without suffering measurable hydrolysis).

Other supports

Popular alternatives to agarose beads include crosslinked acrylamide/bis(acrylamide) resins such as Thermo Scientific UltraLink® Biosupport, which have distinct advantages such as resistance to microbial attack, good chemical and pH stability and low non-specific binding. UltraLink Biosupport can also withstand pressures up to 1000 psi. There are some disadvantages compared to agarose - chiefly poor flow rates, mechanical stability and a tendency to shrink or swell in various buffers and solvents.

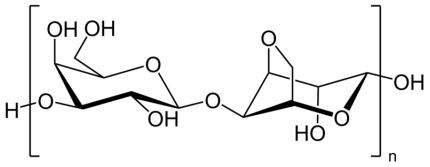

Figure 2. Chemical structure of agarose.

A variety of other solid supports have also been used for IP-scale affinity purification, including ferrous (iron oxide) magnetic beads, cellulose resin, polystyrene particles or microplate wells and controlled pore glass. Magnetic beads have become popular in recent years because they offer easy bench-top separation without a centrifuge and are amenable to high-throughput, automated solutions (e.g., Thermo Scientific KingFisher® Instrument).

C. Immobilization of Antibody (IP and Co-IP)

Protein A, Protein G or Protein A/G immobilization of antibody

Protein A, Protein G and the recombinant Protein A/G are immunoglobulin (Ig) binding proteins that, when attached to as affinity ligands to beaded support, comprise the most popular antibody-binding platforms for IP applications. Protein A was originally isolated from the cell wall of the bacterium *Staphylococcus aureus*, and Protein G from the surface of group C and G streptococcal bacteria. Both are now mass-produced as recombinant proteins expressed in *E. coli*. Protein A/G is an engineered recombinant protein combining four Protein A and two Protein G antibody binding sites.

Protein A binds exclusively to the Fc region of antibody heavy chains, effectively orientating the molecule with antigen-binding sites facing outwards. Protein G also preferentially binds to Fc region, but has also been reported to bind light chains in some instances. Protein A and G both have a high affinity for antibodies of multiple but not necessarily identical subclasses and species of Ig (Table 2). Protein A/G binds all of the subtypes to which Protein A and G bind individually.

Table 2. Binding characteristics of immunoglobulin binding proteins

Antibody	Prot. A	Prot.G	Prot. A/G	Antibody	Prot. A	Prot.G	Prot. A/G
Human IgG1	S	S	S	Rat IgG1	W	M	M
Human IgG2	S	S	S	Rat IgG2a	NB	S	S
Human IgG3	W	S	S	Rat IgG2b	NB	W	W
Human IgG4	S	S	S	Rat IgG2c	S	S	S
Human IgM	W	NB	W	Cow IgG1	W	S	S
Human IgE	M	NB	M	Cow IgG2	S	S	S
Human IgD	NB	NB	NB	Sheep IgG1	W	S	S
Human IgA	W	NB	W	Sheep IgG2	S	S	S
Human IgA1	W	NB	W	Goat IgG1	W	S	S
Human IgA2	W	NB	W	Goat IgG2	S	S	S
Human Fab	W	W	W	Chicken IgY	NB	NB	NB
Human ScFv	W	NB	W	Hamster IgG	M	M	M
Mouse IgG1	W	M	M	Pig IgG	S	W	S
Mouse IgG2a	S	S	S	Horse IgG	W	S	S
Mouse IgG2b	S	S	S	Rabbit IgG	S	S	S
Mouse IgG3	S	S	S	Cat IgG	S	W	S
Mouse IgM	NB	NB	NB	Monkey IgG*	S	S	S

Key: W = weak binding, M = medium binding, S = strong binding, NB = no binding, * = Rhesus monkey

Immobilized Protein A, G and A/G (hereafter collectively called "Protein A/G") are effective tools for attaching antibodies to a beaded support for IP applications (Figure 3). Innovations in manufacturing of prepared Protein A/G resins have yielded commercially available supports that have very high binding capacities, enabling excellent immunoprecipitation results to be obtained with very small volumes of beads. Thermo Scientific Protein A/G Agarose products are available in regular and high-capacity formats to suit nearly any scale of IP procedure. Binding capacities of 30-50 milligrams of Ig per milliliter of resin with very low non-specific binding are possible with these affinity resins.

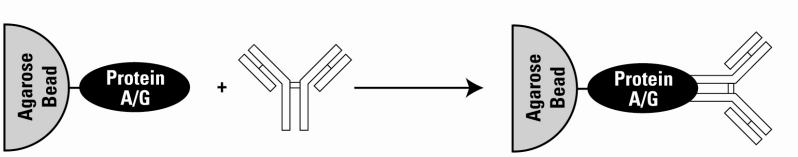

Figure 3. Diagram of antibody binding to Protein A/G agarose resin, the basis for traditional immunoprecipitation and co-immunoprecipitation methods. The Thermo Scientific Pierce® Classic IP Kit uses this method (see page 12).

Direct immobilization of antibody

Protein A/G supports are not compatible with certain IP experimental systems, such as when the species or subclass of IP antibody does not bind to these proteins (see Table 2 above) or when the antigen-containing sample is serum (which contains immunoglobulins that would compete with the IP antibody for binding). Fortunately, alternative immobilization strategies exist. Antibodies (regardless of species or subclass) can be chemically conjugated directly to an activated beaded support (Figure 4). Thermo Scientific AminoLink® Plus Resin is beaded agarose that has been activated with aldehyde groups. These aldehyde groups are extremely reactive towards primary amines (-NH_2), which are abundant on antibodies and other proteins (i.e., the side-chain of lysine residues). The initial reaction of amine and aldehyde groups forms an intermediate known as a Schiff base. Reduction of this intermediate with sodium cyanoborohydride yields an extremely stable secondary amine bond, resulting in permanent (effectively leak-proof) immobilization of the antibody. Coupling capacities of up to 15 mg protein per ml of support can be achieved.

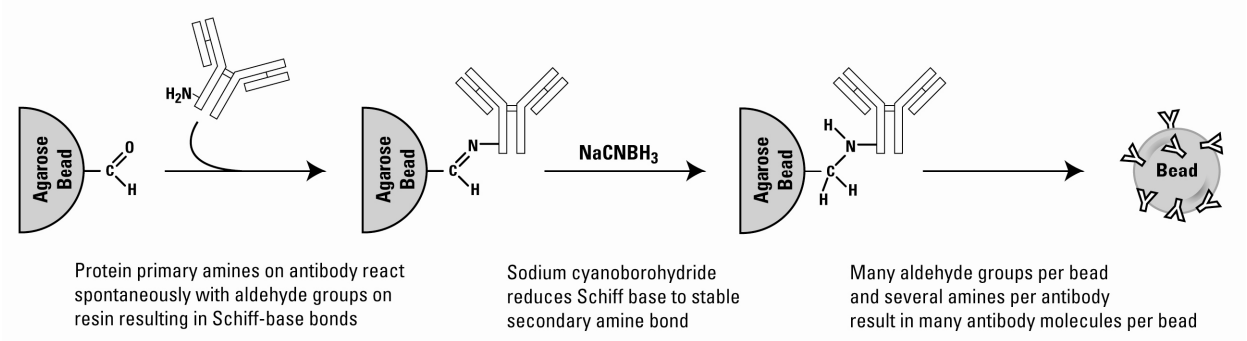

Figure 4. Diagram of AminoLink Reaction Chemistry (reductive amination), a method for covalent, direct immobilization of IP antibody. The Pierce Direct IP Kit uses this method (see page 12).

Although this method couples antibodies in random orientation (based on whichever surface-amines contact the reactive aldehyde groups), this usually has only slight effects on the antigen-binding function and capacity of the IP antibody. Besides eliminating dependence on Protein A/G, this direct immobilization method provides several advantages for immunoprecipitation. Most importantly, with the appropriate (non-reducing) elution buffer, the IP antibody will not co-elute with the antigen because the antibody is covalently attached to the beads. Co-elution of IP antibody fragments and their co-migration with antigen in SDS-PAGE is a significant obstacle in analysis of traditional IP results. In theory, this also means that the antibody support can be reused several times. Two noteworthy constraints of the direct immobilization method include the following: 1) With regard to order of addition, antibody must be immobilized to the beads first, then incubated with the sample; 2) Because immobilization is based on reaction to primary amines, the IP antibody must be supplied in pure form (i.e., in an amine-free buffer devoid of stabilizer proteins like BSA or gelatin). Desalting or clean-up steps may be necessary before certain antibodies can be used in this method.

Crosslinking immobilized antibody

It is also possible to covalently attach antibodies to Protein A/G supports using a crosslinker (Figure 5). Examples of such crosslinkers include DSS and BS3, which are short carbon chains with reactive *N*-Hydroxysuccinimidyl (NHS) ester groups at each end. NHS esters react with primary amines (side chain of lysine residues in proteins) to form covalent amide bonds. If an antibody is first bound to a Protein A/G support and then mixed with a crosslinker solution, the crosslinker molecules can react to covalently link adjacent amines of the antibody and Protein A/G.

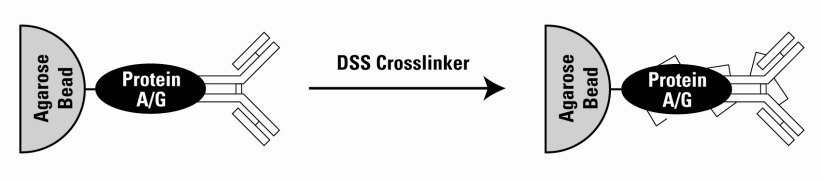

Figure 5. Diagram of the antibody immobilization to Protein G agarose by crosslinking with disuccinimidyl suberate (DSS). The Pierce Crosslink IP Kit used this method (see page 12).

As with the direct immobilization method, the crosslink method eliminates co-elution of antibody fragments and potentially enables the antibody support to be reused several times. For obvious reasons, this method can only be used for antibodies that successfully bind to Protein A or G. However, by contrast with the direct method, the crosslink method does not require pure antibody; stabilizer proteins and amine-containing buffer components of the original antibody solution can be washed away after binding and *before* adding the amine-reactive crosslinker. Because antibodies contain multiple amine groups that are not exclusively limited to the Fc region, and it is important to optimize the dosage of crosslinker. If too little or too much crosslinker is used, the antibody may not become successfully linked to the Protein A/G agarose or too many of the amine groups in the antibody binding site may become modified, rendering the antibody unable to bind antigen.

D. Immobilization of Bait (Pull-down)

IMAC/Histidine tag systems

In pull-down assays a protein or ligand of known identity (bait) is immobilized and used to isolate binding proteins (prey) from a complex mixture. One of the most common tags used for purification or binding of expressed proteins is a short sequence of six histidine residues known as a hexa-histidine tag, often abbreviated to "His-tag". Such a tag is usually co-expressed at the C-terminus or N-terminus of the protein, and has a unique ability to bind to divalent cations such as nickel, cobalt or copper. When Ni^{2+}, Co^{2+} or Cu^{2+} ions are immobilized via a chelating agent onto a solid support the chemistry provides a powerful system to bind and immobilize His-tagged proteins. Although the tag affinity system is more commonly used for bulk purification of recombinant proteins, it can be harnessed for immobilization and screening applications in pull-down assays. The Thermo Scientific PolyHis Protein Interaction Pull-Down Kit (Product No. 21277) uses this method.

Glutathione/GST systems

GST stands for Glutathione-S-Transferase, a 26 kDa enzyme that is commonly used as an expression tag in recombinant protein expression. GST binds tightly to its substrate glutathione, a three amino acid peptide which, when immobilized, represents an attractive strategy for anchoring a bait protein for use in pull-down assays. The system is generally not considered as robust as His-tag methodologies because GST is an enzyme and must remain functional in order to bind the glutathione substrate. However, GST's relatively large size sometimes has the advantage of conferring solubility to otherwise insoluble bait proteins making them easier to purify and manipulate in pull-down assays. The Thermo Scientific GST Protein Interaction Pull-Down Kit (Product No. 21516) uses this method.

Avidin/biotin systems

Biotin is a 244 Da vitamin comprising a double ring structure with a short carbon tail that ends in a carboxyl group (Figure 6). Although biotin cannot be co-expressed with a recombinant protein, it is easy to biotinylate proteins using activated derivatives of the molecule. The biotin carboxyl group can be chemically modified with reactive groups such as NHS esters, maleimides or hydrazides that target protein moieties such as amines ($-NH_2$), sulfhydryls (-SH) or aldehydes (>C=O), respectively. Thermo Scientific EZ-Link® Biotinylation Reagents and Kits (e.g., Product No. 21435) provide numerous options for labeling proteins with biotin.

Biotinylated proteins are especially easy to immobilize because of the high affinity of biotin for binding sites in the tetrameric glycoprotein avidin, and its low-sugar analogs streptavidin and Thermo Scientific NeutrAvidin™ Protein. The strength of the interaction is extremely high, so immobilized forms of avidin are especially useful for anchoring biotinylated bait proteins for use in pull-down assays. The Thermo Scientific Protein Interaction Pull-Down Kit (Product No. 21115) uses this method.

When the biotinylated bait protein is an antibody, the biotin-based pull-down is actually a form of immunoprecipitation. This is another method for performing IP without having to use Protein A/G (e.g., for species or subclasses of immunoglobulin that do not bind to Protein A or G).

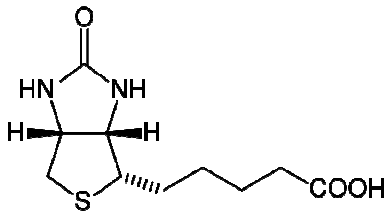

Figure 6. Chemical structure of biotin.

Direct immobilization

If a tag- or ligand-affinity system is not available for prospective bait proteins or the tag is needed for other functions in the experimental system, another method must be used to immobilize the bait protein for pull-down assays. Pure proteins can be covalently immobilized directly to an activated support in the same manner as described on page 5 for antibodies. The Pierce Direct IP Kit or one of the AminoLink Plus Resins can be used for this purpose.

E. Order Of Addition

Regarding the order of assembly of the immobilized antibody/antigen complex, there are three different approaches used in IP applications with conventional Protein A/G supports. The first approach is to add the antibody to the protein sample and allow it to interact with the antigen, followed by the addition of the support to bind the antibody and precipitate the protein complex. This option is generally considered to give the highest yield of antigen, but has the disadvantage that the antibody will be co-eluted with the antigen. A second approach is to bind the antibody to the support then mix this with the protein sample. This usually gives very slightly decreased antigen yield and purity compared to the first method; in other cases, it decreases nonspecific background. A third approach is to mix all three components together in a single reaction; although this may be a slightly faster method, it generally produces the lowest antigen purity and yield.

For obvious reasons, the second option (i.e., bind antibody to support first) must be used when performing IP by the either the direct or crosslink method. In general, the advantages offered by the direct and crosslink methods (e.g., antigen elution without antibody contamination) outweigh the any slight disadvantages of this constraint on the order of addition.

F. Sample Pre-Clearing (and Controls)

Sample pre-clearing is an optional step designed to remove proteins and ligands that bind non-specifically to the beaded support. It is performed on the sample before the binding complex is assembled. Pre-clearing generally involves the incubating the sample with plain beaded agarose (i.e, the base support to be used for the IP reaction) before performing the IP experiment with Protein A/G agarose and the IP antibody. If successful, off-target proteins or other components of the sample that bind non-specifically to the resin will be removed by this pre-clearing step so that they will not co-purify with the target antigen in the actual IP experiment.

Plain beads (or Protein A/G beads used without IP antibody) can also be used as a negative control for an IP or co-IP experiment. Any products obtained with these control conditions can be attributed to non-specific (off-target) interactions. One advantage of the direct immobilization method is its complete removal of Protein A/G as a component and potential source of non-specific binding interactions in the assay system.

G. Binding Buffer

Assembly of a protein binding complex is depends upon compatibility of the IP binding buffer with all of the component binding interactions. In traditional IP, this includes the affinity interaction between antibody and Protein A/G and the affinity interaction between antibody and antigen. In a co-IP, the affinity interaction(s) between bait antigen and prey protein(s) are also involved. In most cases, antibody-antigen interactions are fairly robust and will occur in any standard buffer of near-

neutral pH, such as phosphate-buffered saline (PBS) or Tris-buffered saline (TBS). By contrast, bait-prey interactions range in strength and time from irreversible and long-lived to labile and transient. If bait and prey proteins are known in advance to require some specific molecule or cofactor in order to associate *in vivo*, it may be necessary to add such components to the binding buffer also. Many researchers use identical solutions for binding buffer and wash buffer - in these instances the buffer can be optimized simultaneously (see Wash Buffer section).

When using Protein A, G and A/G, antibody binding can be enhanced using certain binding buffers. Although interaction will occur in most physiological buffers, specific Protein A and Protein G binding buffers can increase binding, in some cases more than doubling the binding capacity (see product list at the end of this article), although such buffers may not always be suitable for antigen or binding protein interaction. For example, Protein A binds IgG best at pH 8.2, while maximum IgG binding occurs with Protein G in buffers at pH 5.0.

In most cases, empirical testing is required to optimize binding conditions, especially for co-immunoprecipitation. One advantage of direct or crosslink immobilization methods is that these remove the antibody-Protein A/G interaction as a factor that must be accommodated in the final IP binding reaction.

H. Wash Buffer

When selecting a wash buffer for an IP application, it is essential to create conditions in which the desired specific protein interactions are maintained but non-specific protein binding is prevented and stripped away. Agarose is a carbohydrate and, even in its beaded form, can bind certain proteins and other cellular components. If a particular IP experiment is plagued with troublesome amounts of background, empirical testing is necessary to discover a wash condition that is effective.

Unless peculiarities of the particular interactions are known, the default starting point for wash buffer optimization in protein affinity methods is either PBS or TBS, which have physiological levels of salt and pH. If the desired interaction is observed, low levels (typically 0.5-1.0%) of NP®-40, Triton®-X-100, CHAPS or other mild detergent may be included to reduce background. If non-specific interactions persist yet the desired interaction is still strong, the stringency may be further increased by increasing the sodium chloride (NaCl) concentration to 0.5 M or even 1 M to reduce ionic and electrostatic attractions. Low levels of reducing agents (such as 1-2 mM DTT or BME) can help disrupt non-specific interactions mediated by disulfide bridges or nucleophilic attractions. Other system-dependent additives may be useful, as in the case of pull-down assays using His-tagged proteins, where low levels of imidazole or EDTA are commonly evaluated.

I. Elution Buffer

Traditional IP for downstream analysis by reducing SDS-PAGE (polyacrylamide gel electrophoresis) and Western blot detection typically involves elution directly in reducing SDS-PAGE sample buffer. This buffer – designed as it is to denatured and reduce proteins for electrophoresis – is very effective for dissociating the affinity interactions upon which IP is based. Other downstream applications for IP products are not compatible with this buffer system, nor is it possible to take advantage of certain IP methods (e.g., antigen elution without antibody fragment contamination in the direct or crosslink IP methods) when this elution buffer condition is used.

The most generally effective, nondenaturing elution buffer for protein affinity purification methods, including IP, is 0.1 M glycine at pH 2.5-3. The low pH condition dissociates most antibody-antigen interactions (as well as the antibody-Protein A/G interaction, assuming it has not been crosslinked). Low-pH glycine is not universally effective; some antibody-antigen interactions do not dissociate with this buffer, and conversely, some antibodies and target antigens denature or become inactive in this buffer. Several alternative types of elution buffers have been developed and used effectively for affinity purification, and these are described in greater detail in the related Tech Tip # 27 titled “Optimize elution conditions for immunoaffinity purification.”

Pull-down assays require elution with system-specific conditions. In many systems, elution is accomplished by competitive exclusion with the excess tag or ligand. For example, free glutathione is used to elute protein complexes immobilized through a GST tag.

Pull-down based on the biotin tag is exceptional among affinity systems because the avidin-biotin binding interaction is so strong. Only very harsh conditions, such as 8 M guanidine•HCl, pH 1.5, can effectively dissociate the biotin tag from avidin or streptavidin resin. While this puts certain practical constraints on the use of avidin-biotin systems, it also offers a distinct advantage for IP and pull-down: biotinylated antibodies (or bait proteins) can be retained on streptavidin agarose beads while effectively eluting antigens (or prey) with low-pH glycine or other such buffer.

Common Occurrences and Explanations

A. Low Amount of Antigen Purified

Low binding of antigen

There are numerous possible causes for low antigen binding in IP applications. A crucial factor is the solution in which the binding reaction is performed. It is important to use an appropriate buffer, pH and salt concentration and to include any co-factors required for the interactions involved. Most IP applications are performed simple buffers such as PBS or TBS. Detergents, salts and other additives may reduce non-specific binding but may also decreased yields may occur. Empirical testing is required to optimize conditions, sometimes as a trade-off between yield and purity.

In the case of IP or Co-IP, the antibody used for purification is an important factor that can affect the yield. If the antibody is easily inactivated or binds only weakly to its antigen, it may be very difficult find sufficiently mild conditions to establish and stabilize the essential binding interaction for the duration of incubation and wash steps required to perform an IP. Some antibodies simply do not work in IP (native antigen), although they may be effective for assays such as Western blotting (denatured antigen). Whenever possible, purchase commercial antibodies that have been validated for IP.

If a polyclonal antibody has not been affinity purified, the antigen-specific antibody may represent only 1-2% of the total IgG present. While this type of antibody sample may work fine for Western blotting, it is problematic for IP because all IgG molecules (irrespective of antigen-specificity) in the sample will compete for the same Protein A/G binding sites on the beads. Most of the antibody bound to the solid support will be non-specific, and antigen yields will be low.

Low elution of antigen

In some instances, the antigen may bind to the antibody or binding protein, but the interaction may be so strong that conventional elution buffers are not effective in breaking the interaction. This can be especially problematic if the antigen must be eluted in a functional form using a mild elution buffer. If low antigen yield is observed and there is a suspicion that the antigen is still bound on the beaded support, the best way to confirm this is by boiling a small aliquot of the beads in an extremely harsh buffer such as SDS-PAGE sample buffer. The beads can then be centrifuged and the supernatant analyzed by SDS-PAGE/Western blotting to confirm the presence or absence of the antigen. If the antigen is present, it may be worth investigating different elution buffers of increasing strength to determine conditions whereby the antigen can be eluted without being inactivated. (See Tech Tip # 27 titled “Optimize elution conditions for immunoaffinity purification.”)

B. Low Purity of Antigen

Contamination with non-specific proteins

If the antigen is eluted but has low purity there are several ways to improve the results. Adding detergents or other components to the binding or wash buffers can decrease general non-specific binding (see previous discussion of binding and wash buffers). Pre-clearing the sample with plain beads can also minimize co-purification of off-target molecules. Alternatively, the beads can be blocked by incubating them with an irrelevant protein such as BSA. (This is analogous to blocking in Western blotting or ELISA.) Although a small amount of the blocker protein may co-elute with the antigen, its identity and molecular weight are known, making it less troublesome in downstream analyses.

Unnecessarily harsh elution buffers such as SDS-PAGE sample buffer will cause multiple non-specific proteins to co-elute with the antigen. Fragments of the immobilized affinity ligand (e.g., subunits of Protein A/G or streptavidin) may be stripped from the beads with harsh elution buffers. In such cases, using a much gentler buffer (e.g., 0.1 M glycine, pH 2.5) will prevent this contamination.

Contamination with antibody

The classic immunoprecipitation format using Protein A, G or A/G will result in antibody being co-eluted with the antigen. If this poses a problem for downstream analysis, switch to a format in which the antibody is covalently immobilized onto the beads, such as the Pierce IP Direct or IP Crosslink formats.

Alternatively, when detection by Western blotting is the only concern, the IP procedure does not have to be modified if one uses a Western blot probing method that does not detect the IP antibody fragments. Several strategies are effective: 1) use a different species of primary antibody for the IP and Western blot; 2) perform the traditional IP with a non-biotinylated form of the antibody but perform the Western blot using streptavidin-HRP secondary detection of a biotinylated form of the antibody; 3) use a specialized secondary detection reagent that binds only to native primary antibody but not denatured IP antibody fragments. Thermo Scientific Clean Blot Reagents (Product No. 21230 and 21233) use this latter approach.

General Immunoprecipitation Protocol

This is an example of a basic or "traditional" IP procedure using Protein A/G agarose beads and 2-10 µg antibody. Typically, this protocol will yield enough antigen to load 3-5 lanes of a gel for electrophoresis and Western blotting detection.

A. Material Preparation

- IP Lysis/Wash Buffer: 0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol, pH 7.4
- Saline Solution: 0.15 M NaCl
- SDS PAGE Sample Buffer (2X): Lane Marker Reducing Sample Buffer (Product No. 39000) diluted to 2X with ultrapure water or use 100 mM Tris pH 6.8, 40 mM DTT, 2% SDS, 20% glycerol, 0.2% bromophenol blue
- Elution Buffer: IgG Elution Buffer (Product No. 21004) or 0.1-0.2 M glycine•HCl at pH 2.5-3.0

B. Preparation of Immune Complex

Note: Accounting for the intended sample volume and presumed concentration of target antigen, use sufficient antibody and affinity resin. The amount of sample needed and the incubation time are dependent upon each specific antibody-antigen system and may require optimization for maximum yield.

Table 3. Volume of Pierce Protein A/G Plus Agarose needed to ensure complete antibody capture in IP assays.

Antibody	Settled Resin
2 - 20 µg	10 µl
25 – 50 µg	20 µl
50 – 250 µg	50 µl

1. Prepare protein sample. To prepare lysate from typical cultured mammalian cells, use the IP Lysis/Wash Buffer. Use 500 µl of IP Lysis/Wash Buffer per 50 mg of wet cell pellet (i.e., 10:1 v/w).
2. Combine 2-10 µg of affinity purified antibody with the cell lysate in a microcentrifuge tube. The suggested amount of total protein per IP reaction is 500-1,000 µg.
3. Dilute the antibody/lysate solution to 300-600 µl with IP Lysis/Wash Buffer.
4. Incubate for 1 hour to overnight at 4ºC to form the immune complex.

C. Capture of the Immune Complex

1. Gently swirl the bottle of Protein A/G Agarose to obtain an even suspension. Using a wide-bore or cut pipette tip, add 10 µl of the resin (for example if resin is supplied as 50% slurry, pipette 20µl slurry) into a spin column. Place the column into a microcentrifuge tube and centrifuge at 1,000 × *g* for 1 minute. Discard the flow-through.
2. Wash the resin twice with 100 µl of cold IP Lysis/Wash Buffer. Discard the flow-through after each wash.
3. Gently tap the bottom of the spin column on a paper towel to remove excess liquid and insert the bottom plug into the spin column.
4. Add the antibody/lysate sample to Protein A/G Plus Agarose in the spin column. Attach the screw cap and incubate the column with gentle end-over-end mixing or shaking for 1 hour.
5. Remove the bottom plug, loosen the screw cap and place the column in a collection tube. Centrifuge column and save the flow-through. Do not discard flow-through until confirming that the IP was successful.
6. Remove the screw cap, place the column into a new collection tube, add 200 µl of IP Lysis/Wash Buffer and centrifuge.

   Note: An alternative wash buffer (20X TBS Buffer) is supplied if a detergent-free wash is required. Dilute buffer to 1X before use.
7. Wash the resin three times with 200 µl IP Lysis/Wash Buffer and centrifuge after each wash.
8. Wash the resin once with 100 µl of Saline Solution

D. Elution of the Immune Complex

Note: There are two options for recovering the immune complex. Sample-buffer elution is ideal for Western blot analysis. The low-pH elution is ideal for enzymatic or functional assays after the low pH is neutralized.

SDS sample buffer elution

1. Place the spin column containing the resin into a new collection tube and add 50µl 2X SDS PAGE Sample Buffer. Keep the column unplugged in the collection tube, and incubate at 100ºC for 5-10 minutes.
2. Centrifuge to collect eluate. Allow the sample to cool to room temperature before applying to an SDS-PAGE gel.

   Note: After heating the resin with SDS sample buffer, the resin cannot be reused and must be discarded.

Low pH elution:

1. Place the spin column into a new collection tube and add 50 µl of Elution Buffer. Incubate for 10 minutes at room temperature. The column does not need to be closed or mixed.
2. Centrifuge the tube and collect the flow-through.
3. Perform additional elutions as needed. Analyze each eluate separately to ensure that the antigen has completely eluted.

   Optional: To neutralize the low pH of the Elution Buffer (e.g., for downstream enzymatic or functional assays), add 5 µl of 1 M Tris, pH 9.5 to the collection tube, which will neutralize the pH upon centrifugation. Alternatively, use a neutral pH elution buffer (i.e., Gentle Elution Buffer, Product No. 21027).

   Note: For a more concentrated eluate, less Elution Buffer may be used; however overall yield may be reduced. It is not recommended to use a volume of Elution Buffer less than the volume of the settled resin.

Modifications of the IP Protocol

A. Co-IP assays

No major modifications are required to perform a Co-IP compared to a standard IP assay, except that the binding conditions may need to be modified to account for peculiarities of the antigen-prey interaction(s). If the IP Lysis/Wash buffer is too stringent for the desired proteins to interact it can be diluted or an alternative binding buffer used. Controls are also more critical in a Co-IP compared to a standard IP. See the previous discussion of binding and wash conditions (page 7-8).

B. Pull-down assays

Pull-down assays require binding, wash and elution buffers designed for the specific tag-ligand affinity system used, as well as accommodate the target protein (bait-prey) interactions. Depending on the system, this will require few or many changes to the default protocol. Most tag-ligand interactions are well characterized and descriptions of optimal conditions are readily available in literature for the associated affinity supports. By contrast, the specific bait-prey interactions being investigated are less well characterized, and this often requires that different binding and wash buffers be tested. For elution of protein complexes from the resin, the low pH elution buffer described in the protocol is usually sufficient to break bait-prey interactions and may also disrupt tag-ligand interactions (e.g., GST/glutathione, His-tag/IMAC, but not biotin/avidin).

C. Optimizing binding and wash buffers

See the discussion of binding and wash conditions in a previous section (page 7-8).

D. Scaling up

The terms “immunoprecipitation” and “pull-down” generally refer to small scale (microcentrifuge tube) purification, most commonly for direct comparative analysis by Western blotting. The same principles of affinity purification can be applied for antigen purification at a larger “preparative” scale using columns containing 1-10 ml of affinity resin. However, large scale purification of antigens using commercial primary antibodies is usually prohibitively expensive, especially in the traditional IP method where the antibody is used only once. In most situations, large scale antigen purification is only practical with the direct immobilization method using Thermo Scientific AminoLink Plus or SulfoLink® Coupling Resins, whereby antibodies can be immobilized to produce an affinity resin that can be used for multiple rounds of purification.

Thermo Scientific Pierce IP, Co-IP and Pull-Down Kits

26146	**Pierce Classic IP Kit** For traditional IP using Protein A/G Plus Agarose and convenient microcentrifuge spin columns.
26147	**Pierce Crosslink IP Kit** For IP using the crosslink method, whereby antibodies are bound and then covalently crosslinked to Protein A/G Agarose using the amine-reactive crosslinker DSS. Provides for antigen elution without antibody fragments.
26148	**Pierce Direct IP Kit** For IP using the direct method, whereby antibodies of any species or subclass are covalently immobilized directly to beaded agarose using AminoLink Plus Coupling Resin. Provides for antigen elution without without antibody fragments.
23600	**Pierce Co-Immunoprecipitation Kit** For Co-IP using the direct immobilization method (see Direct IP Kit above). Uses the same components as the Direct IP Kit but includes additional information about controls and other conditions peculiar to Co-IP.
45350	**Pierce Protein A/G Coated Plate IP Kit** For IP using a coated 96-well microplate format instead of beaded agarose.
45360	**Pierce Streptavidin Coated Plate Kit** For IP with biotinylated antibodies using a coated 96-well microplate format instead of streptavidin agarose resin.
23610	**Pierce HA-Tag IP/Co-IP Kit** For immunoprecipitation of protein interactions involving HA-tagged bait proteins. Includes pre-immobilized anti-HA antibody agarose resin.
23620	**Pierce c-Myc-Tag IP/Co-IP Kit** For immunoprecipitation of protein interactions involving c-Myc-tagged bait proteins. Includes pre-immobilized anti-cMyc antibody agarose resin.
21115	**Biotinylated Protein Interaction Pull-Down Kit** For pull-down or immunoprecipitation of protein interactions involving biotinylated bait proteins or biotinylated antibodies.
21516	**GST Protein Interaction Pull-Down Kit** For pull-down of protein interactions involving GST-tagged bait proteins using immobilized glutathione agarose resin.
21277	**PolyHis Protein Interaction Pull-Down Kit** For pull-down of protein interactions involving His-tagged bait proteins using immobilized cobalt chelate agarose resin.

Current versions of product instructions are available at *www.thermo.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.