ROCKLAND™
antibodies & assays

Scientific Excellence

Tips and tricks for improving a variety of research specialties

Table of Contents

Selection Tips

Bench Tips

Application Tips

For over fifty years now, Rockland has assembled an outstanding team of scientists and technicians with a singular dedication to making great antibodies fit for the exacting needs of scientific discovery.

We understand how important your research is. With our elite group of scientists, we are able to share our best tips and tricks with you – to help make your work more efficient. From performing a western blot, to diluting antibodies, or even choosing the right antibody – our scientists are here to help during each step of the way.

Use this book as a guide to your excellence. And know that Rockland's team is there with you as you tackle even the most aggressive tasks that a young scientist can face.

Meet Our Scientists

Dr. Carl A. Ascoli, Chief Science Officer.

Dr. Carl Ascoli is the Chief Science Officer at Rockland. Carl's responsibilities include all divisions of the laboratory including production, research and development, quality control, quality assurance and technical service.

Dr. Karin Abarca Heidemann, Director of Research and Development

Dr. Karin Abarca Heidemann's team at Rockland focuses on the development and production of new products and the expansion of new and existing product portfolios. Karin also manages critical relationships with collaborators, significant bio-pharmaceutical companies and academic research institutions.

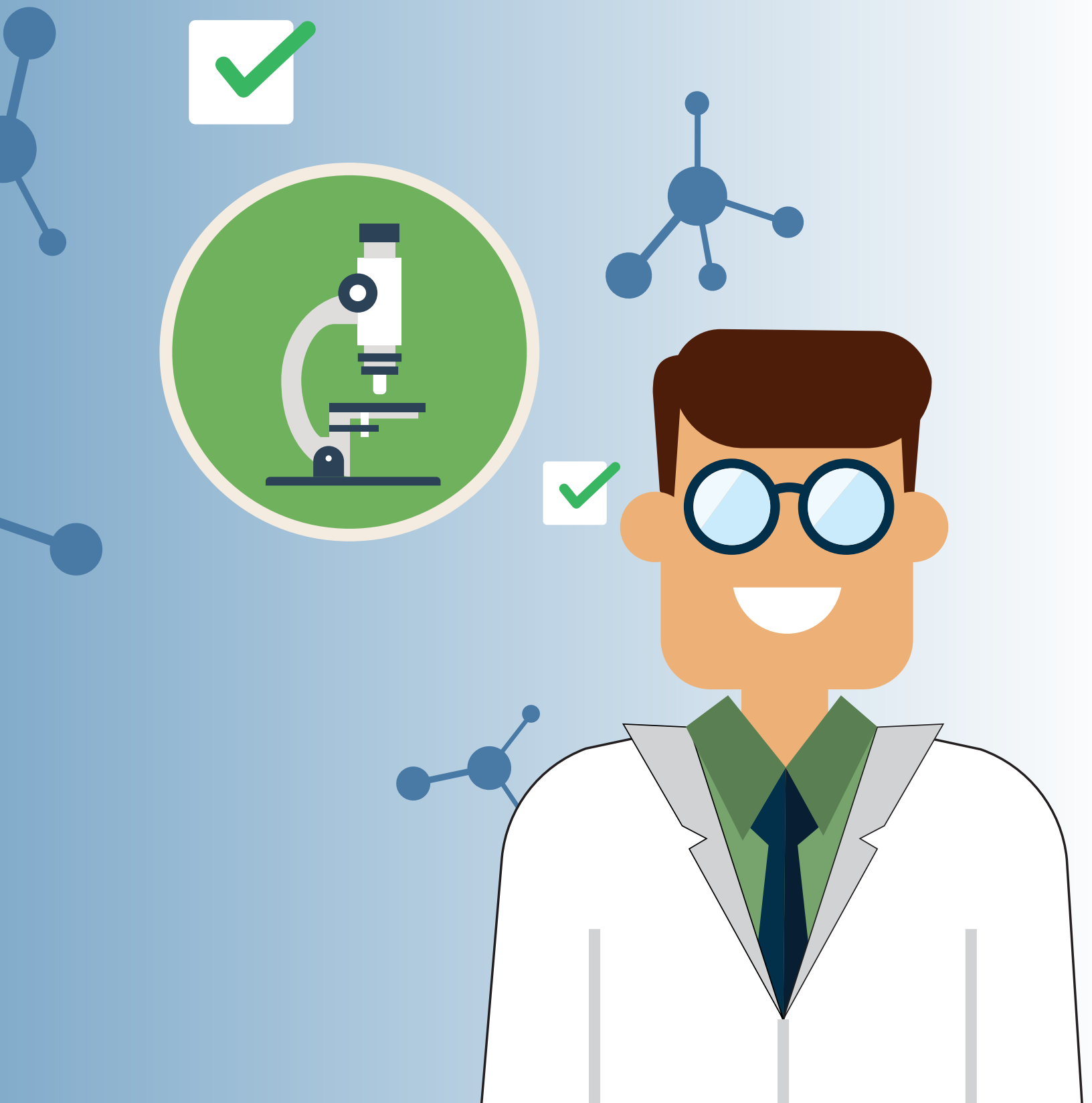
Dr. Camilo Moncada, Director of Quality Control

Dr. Camilo Moncade is the Director of Quality Control and the head of the custom polyclonal antibody development group. Camilo also performs as a senior scientist, providing technical guidance and support to Rockland's lab staff in different areas including molecular and cellular biology, protein science and immunoassays.

Dr. Jahan Ara, Senior Scientist in Research and Development

Dr. Jahan Ara is a Senior Scientist in Research and Development at Rockland Immunochemicals, Inc. Jahan leads and oversees the expansion of new and existing products and direct activities associated with identifying, designing, and promoting new products. She also executes pre-launch, and launch processes for product portfolios and antibody based tools into the developing marketplace.

Selection Tips



Antibody Basics

Antibodies have become essential tools for research, diagnostic and therapeutic purposes because of their high specificity, high binding affinity, long half-lives and low toxicity. Antibodies comprise those secreted by a single clone of B lymphocytes, termed monoclonal antibodies, and those produced by a mixture of various B lymphocyte clones, termed polyclonal antibodies. Antibodies are invaluable reagents for antigen detection and purification, e.g. immunoblotting, immunoprecipitation, immunohistochemistry, ELISA and immunoaffinity chromatography.

Antibodies are generated by immunizing host animals with an immunogenic material. Antibodies can be produced against a wide range of different immunogens. These immunogens can be full-length proteins, protein fragments, peptides, whole organisms (for example, bacteria), or cells. Thus, given enough time, any foreign substance will be recognized by the immune system and induce specific antibody production. However, this specific immune response is highly variable and depends much in part on the size, structure, and composition of antigens.

Proteins or glycoproteins are generally considered the most suitable antigens due to their structural complexity and size and are thus strongly immunogenic. Lipids usually are not immunogenic but can be made immunogenic by conjugation to a carrier protein. Similarly, nucleic acids are poor immunogens but can become immunogenic when coupled to a carrier protein. Immune responses against small substances and peptides (haptens), can be generated by chemically coupling them to a larger carrier protein, such as bovine serum albumin, keyhole limpet hemocyanin (KLH), or other synthetic matrices.

Successful production of antibodies depends upon careful planning and implementation of critical steps that may influence the outcome of the effective antibody responses. The several important steps and considerations involved in the production of antibodies include the following:

1. Selection of the Right Immunogen

A key action in developing an antibody that works in the intended assay is the selection of an appropriate immunogen. The three characteristics that a substance must have to be immunogenic are foreignness, high molecular weight, and chemical complexity. The most natural immunogens are macromolecules composed of protein, carbohydrate or a combination of the two. Peptides may have the complexity essential to be antigenic, but their small size typically renders them ineffective as immunogens on their own. Peptides are often covalently coupled to carrier proteins to insure that they induce an immune response.

If adequate supply of the full-length protein is available, immunization with the full length protein in the form of native, recombinant, fusion, gel band, etc. may be a convenient and cost effective option. One characteristic of large antigen molecules is that they induce the activation of numerous antibody-producing B cell clones. This polyclonal mixture of resulting antibodies may then recognize multiple epitopes on the antigen. As a result, there is a very high probability that antibodies against at least one of these epitopes will bind with the native protein in the target assay.

The disadvantage with this approach is that since antibodies are being generated against multiple epitopes, there is a higher chance that antibodies against some of these epitopes could recognize other proteins that contain homologous epitopes. As a result, non-specific cross reactivity against homologous epitopes might be problematic when assaying with antibodies developed against the full length protein. Additionally, recombinant expression of a protein is sometimes costly and the expressed protein may not completely resemble all the characteristics of a native protein.

The second strategy is to immunize with a peptide sequence corresponding to a specific region of the full length protein. With this approach, synthetic peptides are generated which mimic selected regions of the protein of known amino acid sequence. Peptides are frequently favored, since they are easy to synthesize in high purity and are easy to work with. Using this approach, antibodies can be raised against selected regions, such as highly conserved regions, active sites, extra- or intra-cellular domains or regions of post-translational or chemical modifications.

The disadvantage of using a peptide sequence is that it is identical to a very specific region of the native protein and this region may not be accessible in the protein's native conformation in a particular assay.

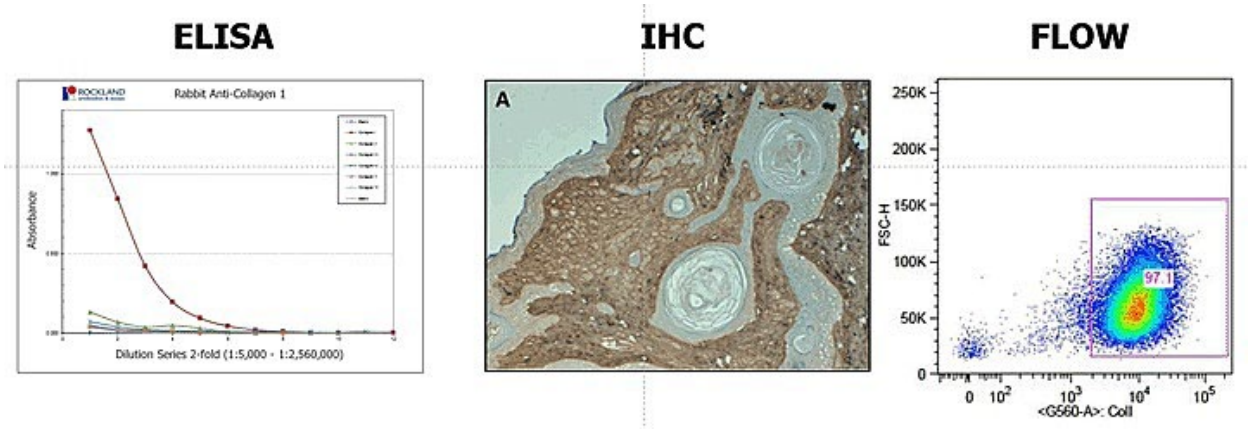
Some general key considerations in designing de novo or native-based peptides which will increase the probability of generating a successful working antibody are as follows:

- Choose areas of structural stability and chemical complexity within the molecule.
- Select sequences lacking extensive repeat units.
- Avoid complex and inaccessible regions such as alpha helices and beta sheets. Aim for accessible regions of native protein that are surface oriented, hydrophilic and flexible.
- N and C-terminus are often exposed parts of the protein with a high degree of flexibility and are a good choice for generating anti-peptide antibodies directed against the intact protein.
- Avoid domains that are present in other proteins as these may increase cross reactivity.
- Peptide length in general should be in the range of 10-20 amino acid residues. Peptide sequences of this length minimize synthesis problems, are reasonably soluble in aqueous solution and may have some degree of secondary structure.
- Determine the regions that should be avoided or targeted, for example, post-translational modification sites such as phosphorylation, glycosylation, ubiquitination, methylation, acetylation, proteolysis.
- Examine the protein sequences for which the resulting antibody should or should not cross react with.
- Avoid N-terminal glutamine or asparagine and C-terminal proline or glycine in a peptide.
- Avoid internal cysteine (which can be replaced with serine) as cysteine is susceptible to rapid oxidation which can negatively influence the cleavage of protecting groups during synthesis and the subsequent peptide purification.

2. Selection of the Right Detection Method

Antibodies for research applications are most commonly used to identify and localize intracellular and extracellular proteins. Antibodies are used in flow cytometry to differentiate cell types by the proteins they express. They are also used in Western blot analysis to detect proteins separated by electrophoresis, in immunoprecipitation to separate proteins and any bound molecules (co-immunoprecipitation) in a cell lysate, and in immunohistochemistry or immunofluorescence to study protein expression in tissue sections or to localize proteins within cells. Proteins can also be detected and quantified with antibodies using ELISA.

It is important to determine the best application for the research need as not all antibodies will work with every application. Qualitative and quantitative applications have vastly different antibody requirements and a selection of an appropriate antibody that works in the intended assay is a key requirement.



For efficient interaction between the target antigen and the antibody, the epitope must be readily accessible for binding. If the target molecule is denatured, e.g., through fixation, reduction, pH changes, or during preparation, the epitope may be changed and this may affect its capability to interact with an antibody. Some antibodies, for example, are ineffective in Western blotting but are appropriate for immunohistochemical applications, because, in immunohistochemistry, a complex antigenic site might be maintained in the tissue, whereas in the Western blotting, the proteins are subjected to denaturing conditions which changes the protein conformation sufficiently to destroy the antigenic site, and hence eliminates antibody binding. Thus the antibodies produced against native proteins are likely to react best with native proteins (e.g. immunoprecipitation or flow cytometry) and antibodies produced against denatured proteins react with proteins subjected to denaturing conditions (e.g. Western Blotting).

Optimally, an antibody that identifies a linear epitope on the surface of a normally folded protein will work well in both non-denaturing and denaturing procedures. Thus, the epitope may be present in the antigen's native, cellular environment, or it may be exposed only when denatured. In their normal form, antigens may be cytoplasmic, membrane-bound, or secreted. The number, location and size of the epitopes depend on how much of the antigen is presented during the antibody development process.

3. Selection of the Right Clonality

Antibodies are produced and purified in two basic forms for use as reagents in immunochemical techniques; polyclonal and monoclonal. Normally, the immunological response to an antigen is heterogeneous, resulting in many different cell lines of B lymphocytes producing antibodies to the same antigen. As a result of this heterogeneous response, several antigen specific antibody clones, potentially of several different immunoglobulin classes and subclasses are produced. An antibody purified from this heterogeneous collection of antigen-binding immunoglobulins, is called a polyclonal antibody.

An individual B lymphocyte produces and secretes a homogenous population of antibodies called monoclonal antibodies. All antibodies secreted by a B cell clone are identical, providing a source of homogenous antibody with a single defined specificity. Monoclonal antibodies can be raised by fusion of B lymphocytes with immortal cell cultures to produce hybridomas. Hybridomas produce many copies of the exact same antibody. This remarkable phenomenon has been instrumental in the development of antibodies for diagnostic applications since monoclonal antibodies react with one epitope on the antigen.

Additionally, synthetic antibodies called recombinant antibodies can be created using synthetic genes expressed in an in vitro mammalian cell line. Recombinant antibodies are monoclonal antibodies which do not need hybridomas and animals in the production process. The technology involves isolating antibody genes from source cells, amplifying and cloning the genes into an appropriate phage vector, introducing the resulting vector into expression host such as bacteria, yeast, or mammalian cell lines, and attaining expression of sufficient amounts of functional antibody. Recombinant antibodies can be used in all applications where classical monoclonal antibodies are used.

Properties of Polyclonal, Monoclonal, and Recombinant Antibodies

Polyclonal Antibody

- Have a broader specificity and often recognize multiple epitopes, making them less sensitive to minor antigen changes (for example, polymorphism, heterogeneity of glycosylation or slight denaturation). Hence, they can identify proteins of high homology or from different species.
- Frequently the preferred choice for detecting denatured proteins.
- Relatively easy to generate, less complex, and are more cost-effective.
- May be generated in a variety of species, including rabbit, goat, sheep, donkey, chicken, and others, giving the users many options in experimental design.
- Target multiple epitopes on the same protein and thus usually provide more robust detection.
- Sometimes used when the nature of the antigen in an untested species is not known.
- Can amplify signals from a target protein with low expression levels, as the target protein will bind more than one antibody molecule.
- Compatible with a wider range of applications.

Monclonal Antibody

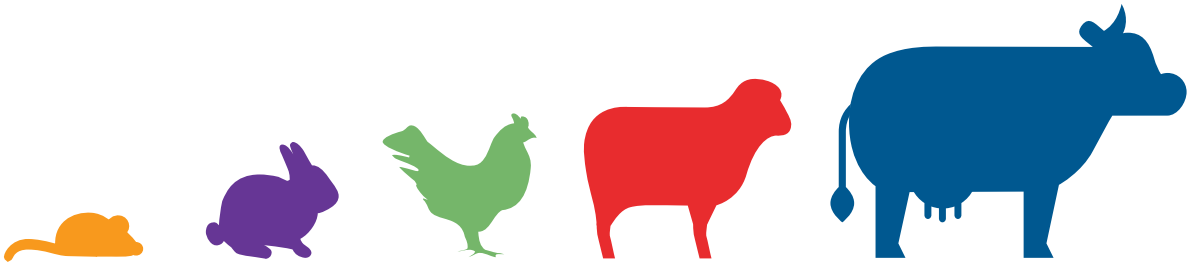
- Highly specific and detect only one epitope on the antigen.
- Due to their specificity, monoclonal antibodies are excellent as the primary antibody in an application, or for detection of antigens in tissue, and often minimize background signal and eliminate cross-reactivity.
- The highly specific nature of the monoclonal antibody permits the development of assays where two very closely related antigens can be distinguished from each other.
- Homogeneity of monoclonal antibodies is very high and they provide consistent, reproducible results if experimental conditions are kept constant.
- Hybridoma cells can serve as an infinite source of the monoclonal antibody.
- Essential for research, diagnostic and therapeutic applications.

Recombinant Antibodies

- For research purposes can be produced in significantly less time than hybridoma-based methods.
- Developed from a unique set of genes, making them more reliable, and providing controlled and reproducible results.
- Can be readily optimized, as their nucleic acid sequences are defined and easily available. A high degree of control is possible, making selection of recombinant antibodies that bind in a particular pH, salinity, or in other specific buffer conditions precise and practical.
- Mass production of recombinant antibodies can be achieved at a shorter timeframe and does not require the use of animals - thus overcomes ethical concerns over animal distress, discomfort, and pain.
- A required recombinant antibody fragment can readily be converted into a different species, isotype or subtype by adding the appropriate constant domain. This makes it easier to switch antibodies into a more desirable format.

4. Selection of the Right Host

A number of animals may be applied as hosts for production of antibodies, including rabbits, chickens, goats, sheep, cows, mice, guinea pigs, and rats. Selection of the most suitable animal depends on factors such as presence of a homologous protein in the immunized species, the amount of antibody required, the amount of protein available for immunization and the time period required to obtain an antibody response.



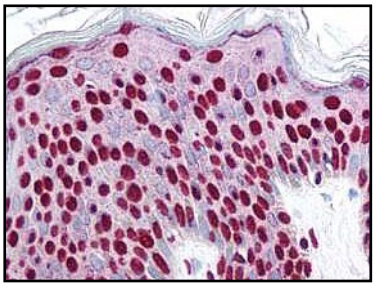
The most cost effective hosts are chickens and rabbits. While chickens are now the second most used host, rabbits are traditionally the most common host animals for polyclonal antibody production. Rabbits are used in 95% of the cases, since they have the capability to respond to broad classes of antigens and provide good yields in a short period of time.

Chickens are considered as the host if it is essential for the phylogenetic relationship between the antigen donor and the antibody producer to be distant. Chickens transfer high quantities of immunoglobulins, (IgY), into the egg yolk, which eliminates the need for invasive bleeding procedures.

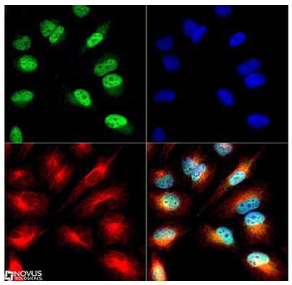
Goat/sheep is the host of choice for producing polyclonal antibodies in larger quantities. Goat/sheep give strong immune responses against most antigens. The goat produces about 7-8 times the amount of serum compared to the rabbit. Furthermore, the goat serum contains about 20 mg/ml of total IgG, which is about 2-3 times more than rabbit serum. This makes the goat the more cost effective choice of the two when greater amounts of antibody are required.

Mice/Rats are used as hosts for screening potential antigens prior to immunization into a larger host. They only provide small amounts of serum.

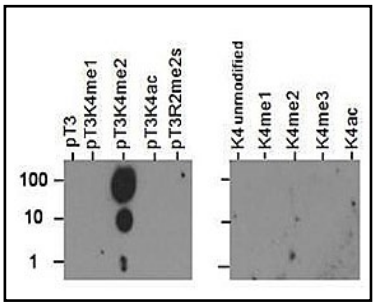
The most common host for monoclonal antibody production is the mouse. Balb-c mice are usually an obvious choice; they are an inbred strain preferably suited to monoclonal antibody work.



Anti-SMAD3 pS423/pS425



Anti-Histone H3 K9-Ac/pS10



Anti-Histone H3 K4me2/pT3

5. Selection of Post-Translational Modification Antibodies

Most proteins are frequently subjected to some form of modification following translation. These post-translational modifications (PTMs) result in mass changes that are detected during analysis. Post-translational modification of proteins increases their functional diversity by the covalent addition of functional groups or proteins, proteolytic cleavage of regulatory subunits or degradation of entire proteins. These modifications include phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation, proteolysis and influence almost all aspects of normal cell biology and pathogenesis. Therefore, the analysis of protein post-translational modifications is particularly important in the study of cell biology and disease treatment and prevention such as heart disease, cancer and diabetes.

The most widespread and useful tool for tracking post-translational changes is the modification-specific antibody. Such modified-protein-specific antibodies can be used in a wide range of biochemical assays, such as Western blotting, chromatin immunoprecipitation and flow cytometry.

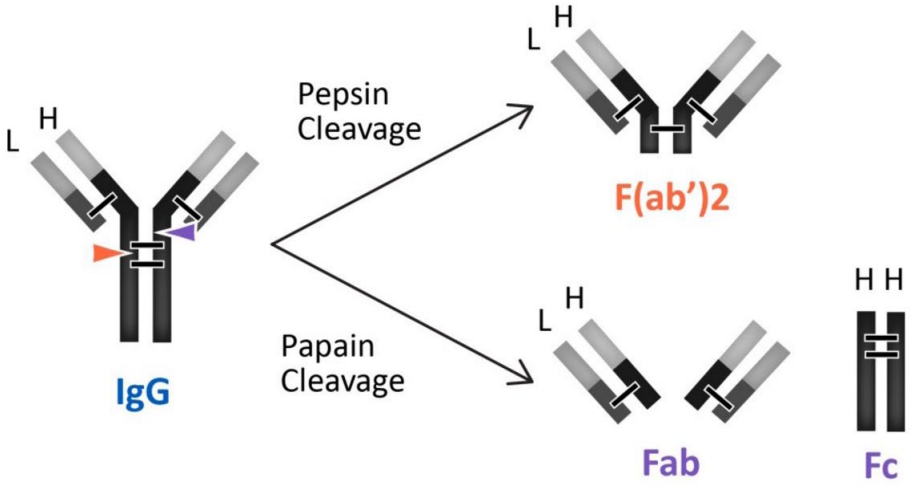
Antibodies against PTMs are generated using synthetic peptides designed against a short, specific region of the protein, largely eliminating the issue of specificity compared to antibodies generated using large constructs as immunogens. However, it is critical that the antibody is tested against established positive and negative controls to ensure specificity for the modification.

PTM specific antibodies may be either monoclonal or polyclonal. The latter are easier and faster to develop, but both require significant validation. PTM antibodies are purified to remove antibodies that react to the non-modified protein by subtraction against the unmodified peptide, followed by affinity enrichment for antibodies that react to the modified protein. The antibodies bound to the modified-peptide are then eluted and tested under various experimental conditions.

6. Antibody Fragments and Subclasses

A range of applications where Fc mediated effects are not required and are even undesirable, the antibody fragments that lack the FC domain are used. The antibody fragments of primary interest are antigen-binding fragments such as Fab' and F(ab')2. Antibody fragments are smaller than whole IgG molecules and are often used to block a signaling molecule or receptor. The smaller size of these antibody fragments offers better tissue delivery resulting in improved staining such as immunohistochemistry and immunocytochemistry. Fab and F(ab')2 fragment antibodies eliminate non-specific binding between Fc portions of antibodies and Fc receptors on cells such as macrophages, dendritic cells, neutrophils, NK cells, and B cells. Furthermore, antibody fragments are frequently used as the starting point for drug molecules because of their lower immunogenicity than intact antibodies.

The antibodies produced by plasma cells are classified by their isotypes that differ in function and antigen responses. Five major antibody isotypes have been identified; IgA, IgD, IgE, IgG, and IgM. The antibody isotypes IgG and IgA are further divided into subclasses (e.g. human IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) based on additional differences in their amino acid heavy chain sequences. Determining the class and subclass identity of an antibody is important for selecting the method to purify and use these antibodies in immunoassays. For example, if an antibody is determined to be IgM, it cannot be purified effectively with Protein A or G, and it will most likely need fragmentation for use in immunohistochemical procedures.

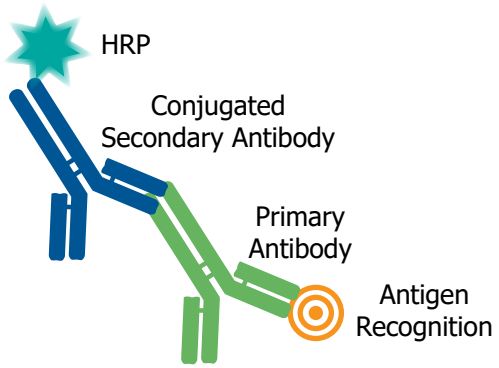


Antibody isotyping is a critical and valuable characteristic of hybridoma development. Screening is one of the most important stages during hybridoma development to ensure that the most productive and positive clones are selected for further evaluation. Antibody isotype screening at the various development stages of fusion, cloning and subcloning by ELISA, and flow cytometry assays, provides an accurate and specific identity of heavy and light chains produced by hybridomas. Several individual hybridoma samples can be tested by ELISA for anti- IgG1, IgG2a, IgG2b, IgG2c or IgG3, IgA, and IgM as well as kappa and lambda light chains. If a monoclonal antibody is determined to be IgG1 composed of kappa light chains, there is an option to use immobilized Protein L to purify it from culture supernatant without contamination of bovine immunoglobulins from the serum supplement.

7. Selection of Right Reporting System

All antibodies require systems to detect the binding of antigens. The method of detection can be 'direct" if the label is conjugated to the primary antibody or 'indirect' if the label is attached to another molecule, called a 'secondary reagent or antibody', and may generate a fluorescent or chromogenic signal. It is important to select a secondary antibody that has specificity for the antibody species and isotype of the primary antibody and is conjugated to a suitable detectable tag or label for detection. Detection tags or labels that may be conjugated to purified antibodies include enzymes, fluorophores, or haptens, such as horseradish peroxidase (HRP), alkaline phosphatase (AP), rhodamine, fluorescein isothiocyanate (FITC), DyLight™, AlexaFluor™, Atto dyes or biotin. Labeling strategies result in the covalent attachment of molecular labels to the target protein in order to facilitate the detection of a labeled protein and its binding partners. While multiple types of labels are available, their diverse uses are preferable for specific applications. Therefore, the type of label and the labeling strategy used must be considered carefully and tailored for each application.

The choice of whether to use direct or indirect detection is often dictated by the level of antigen expression. The detection of a highly expressed antigen might be possible using a primary antibody directly conjugated to a label. The direct labeling approach is simple and avoids the problems of non-specific binding with labeled secondary antibodies. Additional advantages of using conjugated primary antibody is the ability to multiplex with antibodies from same species. Indirect detection methods generally have a higher level of sensitivity and generate a more intense signal. The signal is amplified because several secondary antibodies carrying multiple labels bind to primary antibody, resulting in signal amplification. Furthermore, labeled secondary antibodies are readily available.



8. Setting Limits of Specificity and Sensitivity

There is a growing need for antibodies that are both highly specific and highly sensitive. Specificity refers to the properties of an antibody to bind to one or more antigens (a qualitative measurement) and sensitivity refers to how much antibody is needed to elicit a reaction (a quantitative measurement). However, when choosing antibodies, there is usually a trade-off between sensitivity and specificity. Polyclonal antibodies can offer great sensitivity by virtue of being able to recognize multiple epitopes on a specified antigen target, but this advantage also presents potential drawbacks such as increased nonspecific binding. Monoclonal antibodies, on the other hand, provide the maximum specificity because they only recognize a single epitope, but this restricted targeting results in fewer binding sites for the antibody on an antigen, which translates to reduced overall sensitivity. Validating each antibody using rigorous standards including multiple experimental controls, and, when available, multiple cell types in the recommended applications can verify the sensitivity, specificity, and reproducibility of antibodies.

9. Considering Antibody Lifespan

The amount of antibody needed and the characteristics of the antibodies being made depends on the intended use of the resulting antibody. If a large volume diagnostic product is the objective, or if the extensive research utilizing resulting anti-serum for a long term is required, immunizing large animals (sheep, goat, cow) is a viable approach since these can provide large volumes of antiserum from a single bleeding. On the other hand, if the antiserum is to be used for the analysis of a dozen Western blots, immunizing small animals (guinea pig, rabbit) is a feasible approach. For most routine work where small volumes of antiserum are required, e.g. <100 ml, the rabbit is the most common species for polyclonal production, while goats and sheep are the species of choice for large-scale production of antiserum. Rabbits typically yield 25 ml/bleed or 50-70 ml/month of antiserum and the expected yield of antiserum from goat is 800 ml/bleed or 2-2.5 liters/month. If the antigen of interest is a protein that is conserved in mammals, and a host with a larger phylogenetic distance is required, then chicken is the host of choice. The chicken is more cost effective and will yield in the order of 400 mg total IgY.

10. Benefits of Antibody Pools

Antibodies that are in high demand often need to be re-manufactured, beginning with the immunization of a host animal. Consequently, the specificity and affinity of these antibodies can vary from batch to batch. Since the specificity of the polyclonal population may drift over time during immunization, this drawback can be addressed by immunizing multiple animals at the same time followed by screening and pooling of antiserum prior to preparation or purification. To ensure consistency, the performance of the final antibody can be compared to previous batches.

In a multiple cohort study, the antiserum may be pooled based on different collection dates from the same host, or the same collection date from multiple hosts. To avoid increased background, antibodies from the same host should be pooled and tested for consistency.

Tips for Selecting the Best Secondary Antibody

Antibody-based assays or immunoassays represent a widely used, valuable tool in areas of basic research, bioprocessing, diagnostics and clinical applications. Although successful detection of the target protein relies on multiple parameters, it is well recognized that the use of high quality antibodies critically affects assay performance. Because very often immunoassays use secondary antibodies to bind the primary antibody to assist in detection, sorting and purification of target antigens, not only are primary antibodies exhibiting high specificity and sensitivity for the intended antigen essential, but also high quality secondary antibodies are paramount in achieving meaningful results.

The following roadmap will guide you step-by-step through the selection process of the best secondary antibody for your experiments:

1. Match the host species of the primary antibody

The first step is to determine the host species that was used to generate the primary antibody. Then, select a secondary antibody specific for detection of the primary antibody species. For example, when using a polyclonal antibody produced by rabbit you will select an anti-rabbit secondary antibody that was raised in an alternative host species such as mouse, goat or donkey. Most primary antibodies are produced in commonly used host species such as rabbit, mouse, goat or chicken. Therefore, anti-mouse, anti-rabbit, anti-goat or anti-chicken polyclonal secondary antibodies are often used for detection. Remember, the species used to generate the secondary antibody should be always different from the host species of the primary antibody.

2. Select the correct reporter based on intended use

Once the source host has been selected, identifying the optimal secondary antibody requires knowledge of the detection assay. For commonly used techniques such as Western blot and ELISA, an enzyme conjugated secondary is most likely the best choice. Good examples are Peroxidase or Alkaline phosphatase. In the case of immunoassays such as immunofluorescence microscopy or flow cytometry (also called FACS) it is more typical to use a secondary antibody conjugated to a fluorochrome (i.e. FITC, DyLight™ or Cy™ dye). For immunoprecipitation experiments a special reagent that does not detect the precipitating antibody is essential for publication quality images. Rockland's TrueBlot® products are useful for the accurate detection of secondary antibodies used for immunoprecipitation followed by western blot.

3. Consider using a pre-adsorbed secondary antibody

Pre-adsorption (also cross-adsorption) of the secondary antibody is used to eliminate reactivity from immunoglobulins of undesired species, antibody fragments and/or cell and tissue samples, improving the specificity of an antibody. The degree of cross reactivity is determined by ELISA or Western Blot detection and is typically less than 1% of the desired signal. The secondary antibody is cross adsorbed against serum antibody protein from another species or is adsorbed against a mixture of serum antibody protein from several species (i.e., Pre-adsorbed). These highly cross-adsorbed antibodies show low levels of cross reactivity particularly required in multiple labeling experiments.

4. Define the class/sub-class of the primary antibody

Primary polyclonal antibodies are generated in rabbit, goat, donkey, or chicken and are usually gamma chain immunoglobulins (IgG isotype). Therefore, the secondary antibody should be an anti-IgG antibody that recognizes both heavy and light chain of the primary antibody (anti-IgG H&L). Primary monoclonal antibodies are normally raised in mouse, rat and Armenian hamster but even rabbit and human derived are also used. Because monoclonal IgG antibodies are subclass specific, it is very important you use the secondary antibody directed against that specific subclass. Despite the notion that any anti-mouse IgG should recognize any of the IgG subclasses, recent studies have shown potential bias toward specific subclasses, making the use of anti-mouse IgG subclass-specific essential for "robust and reliable multiplex labeling of target proteins in a variety of applications". See Manning et al. (2012) for an in depth review of the subject. When the sub-class of your primary antibody is unknown, you can use anti-IgG F(ab) or consider performing an isotyping assay.

5. Sometimes smaller is better

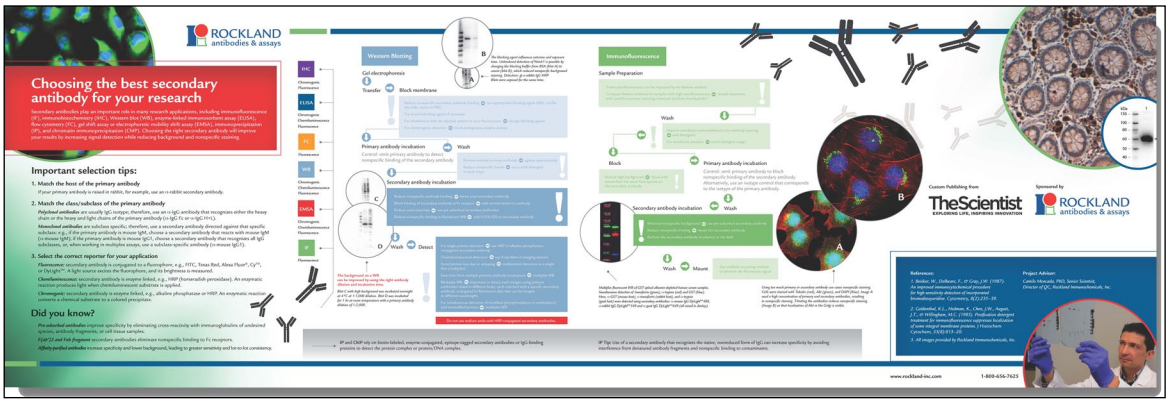
F(ab')2 fragment secondary antibodies are generated by pepsin digestion of whole IgG antibodies to remove most of the Fc region while leaving the hinge region intact. The resulting fragment is divalent with MW ~110 kDa. Fab fragment secondary antibodies are generated by papain digestion of whole IgG antibodies to remove the Fc region entirely generating a monovalent antibody of ~50 kDa. Both F(ab')2 and Fab fragment antibodies eliminate non-specific binding to the Fc receptors on cells and penetrate tissues more efficiently due to their smaller size. When working with tissues or cells that have Fc receptors (spleen, peripheral blood, hematopoietic cells, leukocytes, NK cells etc.), choose a F(ab')2 and Fab to eliminate non-specific binding to Fc receptors. Fragment conjugated secondary antibodies are ideal for Flow Cytometry, Immunohistochemistry and Immunofluorescence.

6. Choose the purity level of the secondary antibody

Affinity purified antibodies are isolated by separating monospecific antibodies from other antiserum proteins and non-specific immunoglobulins by solid phase affinity chromatography. Advantages of using an affinity purified antibody include increased specificity, low background, greater sensitivity and lot-to-lot consistency. Affinity purification reduces variation from one product to another, leading to more reproducible immunoassays. IgG fraction antibodies on the other hand are very robust and are prepared by a combination of salt fractionation and chromatographic methods with purity and specificity evaluated by different methods. The main benefit of using an IgG fraction is the presence of extremely high affinity antibodies that may result in a more potent secondary antibody reagent. This may or may not be the cause for affinity purified antibodies which usually exhibit improved specificity sometimes at the expense of affinity. Low abundance proteins or weakly detected primary antibodies detected using an affinity purified secondary antibody may be better recognized using an IgG fraction secondary antibody. Assays where high background or non-specific binding from the secondary antibody are apparent may be optimized using an affinity purified secondary.

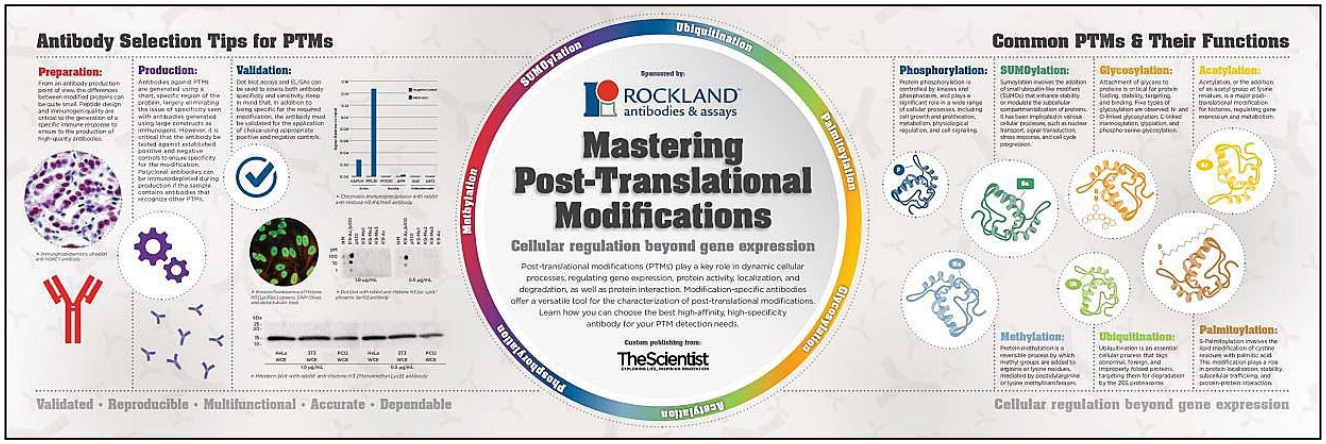
Featured Products for Secondary Antibodies

- Conjugated Secondary Antibodies
- Anti-Mouse Secondary Antibodies
- Anti-Rabbit Secondary Antibodies
- Anti-Goat Secondary Antibodies
- Peroxidase enzyme substrates for ELISA
- Peroxidase enzyme substrates for WB and IHC



Click to download
"Choosing a Secondary Poster"

Mastering Post-Translational Modifications



Click to download PDF

Post-translational modifications (PTMs) play a key role in dynamic cellular processes, regulating gene expression, protein activity, localization, and degradation, as well as protein interaction. At Rockland, scientists have developed proprietary methods for the development of highly specific PTM antibodies that can be used in a wide range of in vitro and in vivo studies of a modified protein, some of which are not easily performed by other approaches, such as mass spectometry (MS).

In this poster, we have teamed up with The Scientist to show you the most important tips for choosing the best high-affinity,

high-specificity antibody for your PTM detection needs.

Antibody Selection Tips for PTMs

1. Preparation

From an antibody production point of view, the differences between modified proteins can be quite small. Peptide design and immunogen quality are critical to the generation of a specific immune response to ensure to the production of high-quality antibodies.

2. Production

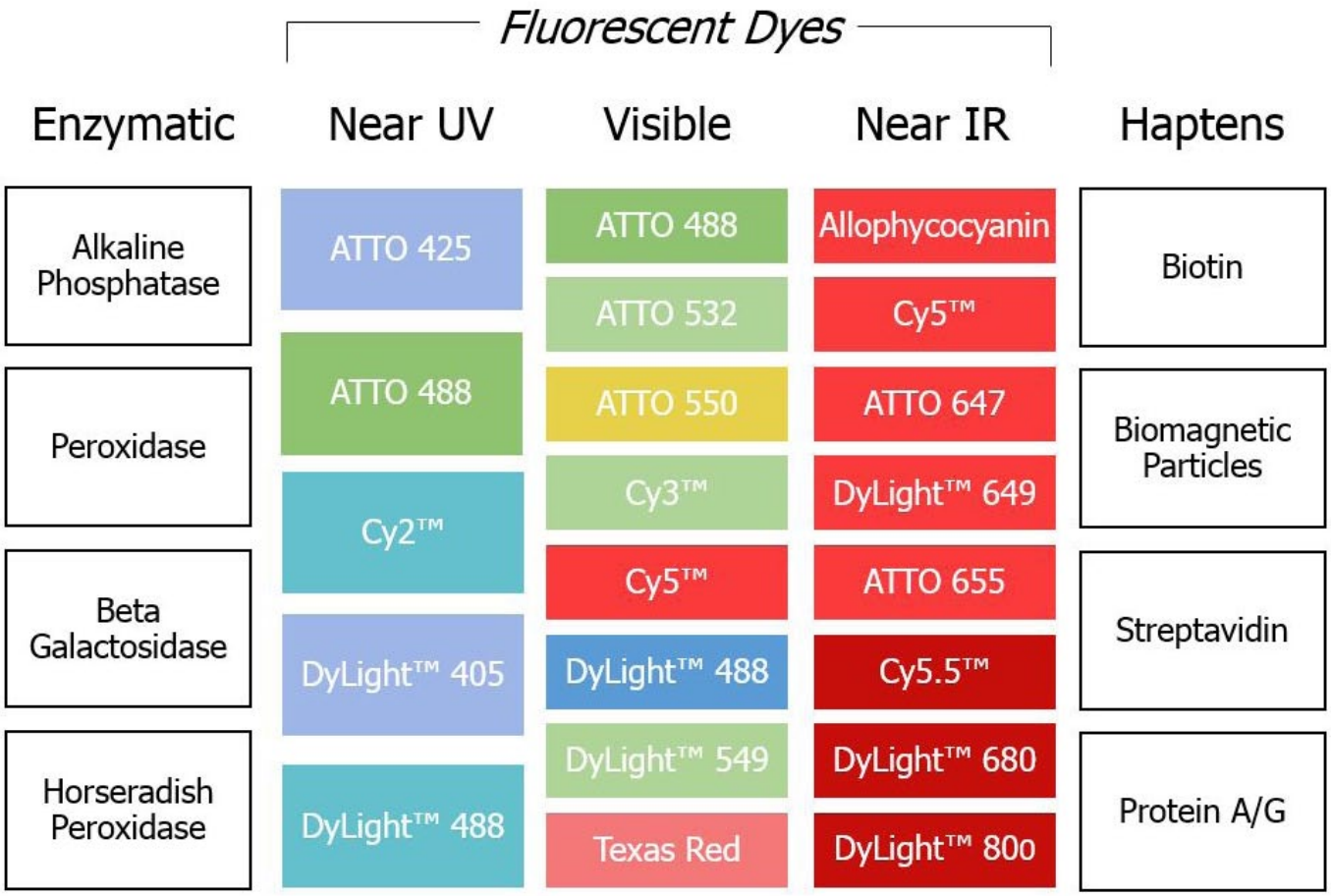
Antibodies against PTMs are generated using a short, specific region of the protein, largely eliminating the issue of specificity seen with antibodies generated using large constructs as immunogens. However, it is critical that the antibody be tested against established positive and negative controls to ensure specificity for the modification. Polyclonal antibodies can be immunodepleted during production if the sample contains antibodies that recognize other PTMs.

3. Validation

Dot blot assays and ELISAs can be used to assess both antibody specificity and sensitivity. Keep in mind that, in addition to being specific for the required modification, the antibody must be validated for the application of choice using appropriate positive and negative controls.

Tips for Selecting Conjugated Antibodies

Antibodies are used to detect and quantify antigens using an appropriate detection technique such as flow cytometry, ELISA, Western blotting, immunofluorescence and immunohistochemistry. Often for signal amplification and detection purposes, purified antibodies are conjugated to enzymes, fluorophores, or haptens, such as horseradish peroxidase (HRP), alkaline phosphatase (AP), rhodamine, fluorescein isothiocyanate (FITC), or biotin. Labeling strategies result in the covalent attachment of molecular labels to the target protein in order to facilitate the detection of a labeled protein and its binding partners. Labeling assays are classified as 'direct' if the label is conjugated to the primary antibody or 'indirect' if the label is attached to another molecule, called a 'secondary reagent'. While multiple types of labels are available, their diverse uses are preferable for specific applications. Therefore, the type of label and the labeling strategy used must be considered carefully and tailored for each application.



Direct or Indirect Detection

The choice of whether to use direct or indirect detection is often dictated by the level of antigen expression. The detection of a highly expressed antigen might be possible using a primary antibody directly conjugated to a label. The direct labeling approach is simple and avoids the problems of non-specific binding with labeled secondary antibodies. Additional advantages of using conjugated primary antibody is the ability to multiplex with antibodies from same species, reduction in the number of incubation and wash steps and production of better data quality. However, the direct detection lacks the signal amplification step which might result in weak or no signal if the target protein is present at low levels. Therefore, the use of directly conjugated antibodies is only recommended for the detection of very abundant target proteins.

Indirect detection methods generally have a higher level of sensitivity and generate a more intense signal. The signal is amplified because several secondary antibodies carrying multiple labels bind to primary antibody, resulting in signal amplification. Furthermore, labeled secondary antibodies are readily available. However, the use of labeled secondary antibody may compromise the required specificity and requires extra blocking and wash steps and additional controls.

Fluorescent Protein Conjugation

Fluorescence detection is based on the use of fluorophore that have a unique and characteristic spectra for absorption and emission -they emit a photon at one wavelength when excited by light of another shorter wavelength. The fluorochrome can be conjugated directly to the primary or secondary antibody. Fluorescent-dye conjugated antibodies provide a much needed tool for identifying proteins in many applications including fluorescent cell imaging, Western blotting, immunohistochemistry and more. The advantages of using a fluorescently labeled antibody include brighter signal, multiplexing capabilities, and ease of use (many are available pre-conjugated to many different color of dyes).

Fluorescein derivatives and their conjugates are the most common fluorescent reagents for biological research as they encompass several performance characteristics such as high absorptivity, excellent fluorescent quantum yield, and good water solubility.

When choosing fluorophores, the excitation and emission spectra of each fluorophore should be considered for each experiment. It is important to avoid overlapping emission spectrums if co-localization of two different proteins is desired.

It is recommended to select fluorophores with high extinction coefficient and with high quantum yields. One defining factor of a fluorophores brightness is its extinction coefficient; the higher the extinction coefficient, the brighter the fluorophore. The quantum yield is a read-out of the efficiency of the fluorescence process.

Photobleaching is a photochemical process that reduces the intensity of the fluorescence signal; for example FITC and R-Phycoerythrin are known to have a relatively high rate of photobleaching. Where possible we recommend to avoid fluorophores with a high susceptibility to photobleaching. Some of the photostable fluorophores include DyLight™, AlexaFluor™, or Atto dyes. Alternatively, antifade reagents that inhibit photobleaching can be used to preserve the signal of fluorescently labeled target molecule.

Many conventional fluorophores, such as FITC, are not suggested for staining protocols using acidic buffers as the fluorescence intensity signal is greatly sensitive to an acidic environment. Use new generation dyes that stay fluorescent over a broad pH range.

When performing multi-color immunofluorescence experiments, use fluorophores with narrow emission spectra in order to avoid spectral overlap or bleed-through (detection of one fluorophore in another fluorophore's filter set). Bleed-through makes it difficult to detect distinct fluorescence signals and complicates the assessment of co-localization experiments. Ideally, there should be no spectral overlap between the fluorophores.

In order to verify that the observed fluorescence is a result of staining rather than an unspecific artifact, we recommend to use appropriate controls with each experiment. As cells can display natural fluorescence, it is essential to check immunofluorescent samples microscopically before every staining experiment. Additionally, a label/fluorophore control should be included by performing the complete staining protocol without the addition of fluorophore conjugated antibodies. Use positive and negative controls (for example cell lines in which your protein of interest is either over-expressed or absent such as a knock-out cell line) with each experiment. If using secondary antibodies rather than directly fluorophore conjugated primary antibodies, a secondary antibody only control should be performed following the same protocol without the addition of a primary antibody. This will verify that the secondary antibody does not nonspecifically bind to certain cellular compartments. For multi-color immunofluorescence experiments we recommend the use of cross-adsorbed/pre-adsorbed secondary antibodies as these will minimize the risk of the secondary antibody reacting with endogenous immunoglobulins or an undesired primary antibody.

Enzyme Protein Conjugation

To facilitate chromogenic detection, the primary antibody or secondary antibody is conjugated to an enzyme. The enzyme reacts with a soluble organic substrate to generate an insoluble colored product that is localized to the sites of antigen expression. Chromogenic or precipitating substrates offer the simplest and most cost-effective method of detection. Various reporter enzymes, such as horseradish peroxidase (HRP), alkaline phosphatase (AP) and many others, can be attached to antibodies and proteins through the use of different coupling chemistries to ensure the maximum retention of activity of both enzyme and protein. HRP can be visualized by chromogenic reactions, for example diaminobenzidine (DAB), and TMB and AP signal is often measured through its colorimetric substrate PNPP. These enzyme-antibody conjugates can be used in applications such as ELISAs, blotting techniques, in situ hybridization, cytochemistry and histochemistry detection system.

Peroxidase is economical and a more stable enzyme than alkaline phosphatase. It has also become very popular for use in chemiluminescent detection systems. Alkaline phosphatase, on the other hand, is considered more sensitive than peroxidase particularly when colorimetric detection is used.

Chromogenic substrates exhibit low sensitivity and thus it is difficult to optimize them for detecting proteins of low abundance. Although the reaction can be allowed to develop for several hours or even overnight, this leads to increased background signal. It is recommended to use chromogenic substrates for applications where protein abundance is high.

Chromogenic detection is considered to be more sensitive than that of immunofluorescence, but is less convenient because it includes more incubation and blocking steps. Like immunofluorescence, chromogenic detection allows for the visualization of multiple antigens, but only if the antigens are localized at different sites in the cell or tissue because overlapping colors may obscure results. DAB chromogenic staining should be used if slides need to be stored for longer periods as the colored precipitate formed during the reaction between HRP and DAB is not sensitive to light.

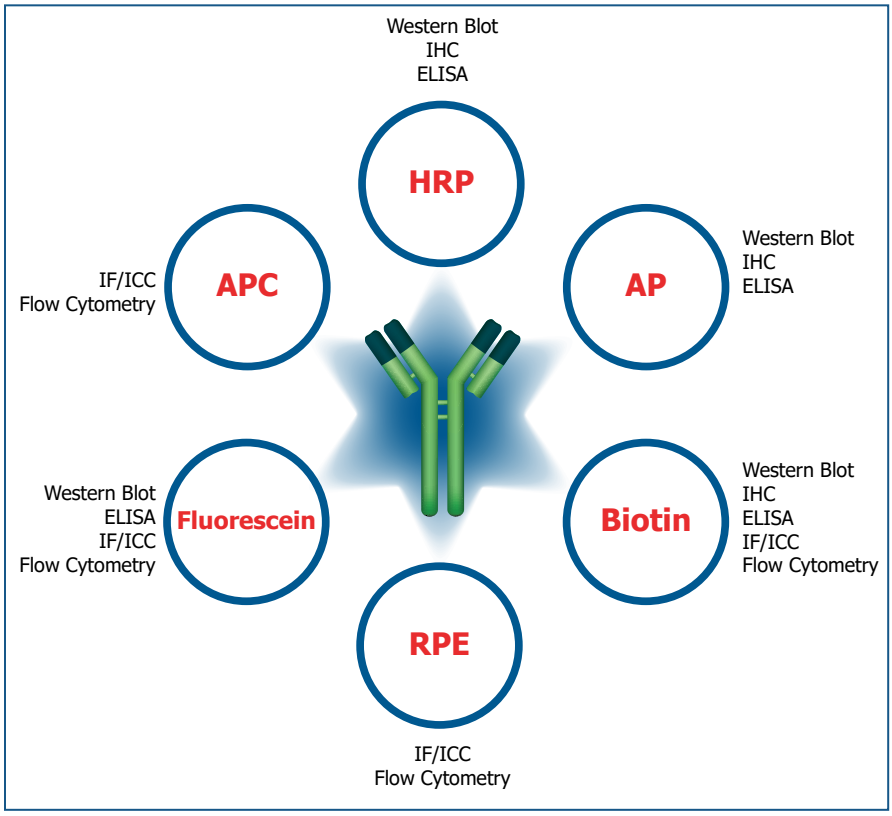
Biotin/Streptavidin Conjugation

Biotin/Streptavidin is commonly used when the target of interest is expressed at low levels and cannot be detected using labeled antibodies alone. Biotin is used in two-step detection systems in concert with conjugated streptavidin or avidin. Many biotin molecules can be conjugated to an antibody with the additional advantage of binding to streptavidin and avidin with extremely high affinity, fast-on-rate and high specificity.Through this amplification step and having the streptavidin bound to labels such as HRP or fluorescent probes, proteins which are expressed at low levels are more likely to be detected.

Streptavidin-based amplification techniques are commonly used in flow cytometry, western blotting, immunofluorescence, and microplate-based detection for increased signal and greater sensitivity. Fluorescent conjugates of streptavidin are used to detect biotinylated macromolecules such as primary and secondary antibodies, ligands and toxins, or bead-based detection. HRP and AP enzyme conjugates of streptavidin are commonly used in western blotting, ELISA, and in situ hybridization imaging techniques. Streptavidin-conjugated magnetic beads are used to isolate proteins, cells, and DNA.

It is recommended to use streptavidin over avidin as it is non-glycosylated and exhibits low levels of nonspecific binding. Avidin is a highly cationic glycoprotein and can cause nonspecific background signal in some applications due to its positively charged residues and oligosaccharide components.

For multicolor experiments, in some instances it may be necessary to simultaneously use primary antibodies from the same species. This could cause cross-reactivity between secondary antibodies. This cross-reactivity can be limited by using a biotinylated form of one of the primary antibodies. The biotinylated antibody is then incubated with streptavidin-conjugated fluorophore. This approach will ensure that the streptavidin-conjugate will only bind to biotin, thus limiting cross reactivity.

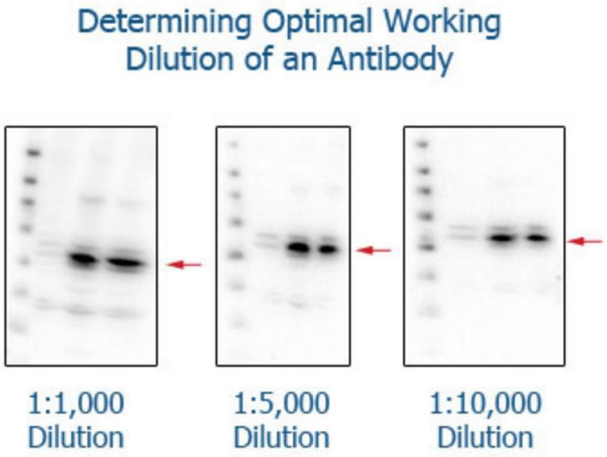


Bench Tips

Tips for Diluting Antibodies

1. Determining the optimal working concentration of each individual antibody

Correct dilutions of antibodies are best determined by first selecting a fixed incubation time and then making series of dilutions in a titration experiment. For example, if a product datasheet suggests using a 1:1000 dilution for Western blotting, it is recommended to make dilutions of 1:500, 1:1000, 1:2000, 1:4000, and 1:8000. This should determine the optimal dilution for your individual sample conditions.



Similarly for IHC, if the data sheet recommends using a 1:200 dilution, it is suggested to make dilutions of 1:50, 1:100, 1:200, 1:400, and 1:500.

Each dilution should be performed on the same type of sample in order to retain the same experimental conditions.

2. Effect of intrinsic affinity of an antibody on optimal antibody dilution

The rate of binding between antibody and antigen is also dependent on the intrinsic affinity of an antibody. When the titer is held constant, a high-affinity antibody will react faster with the antigen and will provide more intense signal or staining within the same incubation period than an antibody of low affinity. Thus the titers may vary between polyclonal antisera, monoclonal antibodies in culture supernatants, and monoclonal antibodies in ascites fluid.

In practical terms, for polyclonal antisera, the titers may vary from 1:100 to 1:2000; for chromatographically purified antibodies, the titers may vary from 1:500–1:10,000; for monoclonal antibodies in cell culture supernatants, the titers may vary from 1:10 to 1:1,000, and for monoclonal antibodies in ascites fluid, the titers may vary from 1:1000-1:100,000.

3. Determining the batch-to-batch consistency

Many antibodies will have comparable batch-to-batch consistency, therefore, in most cases only one titration experiment is required. However, for some antibodies, especially for polyclonal antibodies, when there is a change in the results of the staining between batches of the same antibody, another titration experiment should be performed.

4. Preparing antibody dilutions from concentrated stock solutions

Dilutions are typically expressed as the ratio of the more concentrated stock solution to the total volume of the desired dilution. For example, a 1:10 dilution is made by mixing one part of stock solution with nine parts of diluent. Two fold serial dilutions are prepared by consecutive 1:2 dilutions of the preceding dilution. In order to prepare a small volume of a highly diluted antibody solution, it may be necessary to make it in two steps. For example, to prepare 1.0 ml of a 1:1000 dilution, first prepare 1:10 dilution in100 µl volume (10 µl + 90 µl), and then prepare a 1:100 dilution in 1.0 ml volume by using 10 µl of an intermediate 1:10 dilution (10 µl + 990 µl).

5. Proper pipette usage

The use of adjustable pipettes for preparing dilutions allows for greater flexibility and more precise delivery. Tips supplied or approved by the manufacturer should always be used, since other tips may not fit on the pipette properly. An improper tip seal will cause inaccuracies in the amount of liquid transferred. When aspirating the reagent, the pipette must be held vertically, otherwise too much liquid will be drawn in. When dispensing the sample, the tip should be held at an angle against the container to draw out the liquid. To measure volumes in excess of 1.0 ml, serological or volumetric pipettes should be used.

Did You Know?

Western blotting and immunohistochemistry (IHC), antibody titer and dilutions are important for their effect on signal and staining quality. Correct dilutions of antibodies will contribute to the quality of signal/staining if prepared precisely and consistently. The optimum antibody titer is the highest dilution of antibody that results in maximum positive signal and specific staining without background or nonspecific reactions. Often the manufacturer recommends dilution ranges compatible with other variables such as method, incubation time, and temperature. However, occasionally these require some optimization. Furthermore, for custom antibodies or for those antibodies where this information is not provided, optimal working dilutions of antibodies must be determined.

Tips for Optimizing Protein Expression and Purification

Recombinant proteins are used throughout biological and biomedical science. The development of simple, commercially available systems has made the production of recombinant proteins more widespread. Most significantly, it has dramatically expanded the number of proteins that can be investigated both biochemically and structurally. Since every protein is different, the purification protocols and strategies must be worked out for each individual protein and with an eye to its intended use. We describe the various factors that have a large effect on soluble protein expression and describe how to change them in order to express folded, active proteins.

Influence of gene and/or protein sequence on expression and solubility

1. One of the most common reasons that heterologous proteins fail to express is the presence of "rare" codons in the target mRNA. This codon bias can be overcome by codon-optimized gene synthesis. One advantage of gene synthesis is the ability to change the codon bias of the gene to be more compatible with the recombinant host. For E. coli, expression strains supplemented with the rare tRNAs can overcome the codon bias of the recombinant gene.

2. The probability of successful soluble protein expression decreases with increasing molecular weight, especially for proteins that are > 60 kD. When using E.Coli as an expression host, it is advantageous to design constructs of individual protein domains, as opposed to full length protein and to use solubility-enhancing fusion tags as these tags will intensely aid in protein purification and seldom will adversely affect biological or biochemical activity.

3. The starting and ending residues of the target domain can also affect expression yield and solubility. The optimal boundaries for the protein domain construct should be determined using the available functional and structural data of the protein. For a protein of unknown domain structure, threading the target protein sequence onto a homologous protein structure can help in determining the optimal domain boundaries. When a homologous protein structure is not available, the prediction of secondary structural elements should be exploited.

Influence of vector on expression and solubility

DNA sequence elements that direct the transcription and translation of the target gene include promoters, regulatory sequences, the Shine-Dalgarno box, transcriptional terminators, and origins of replication etc. In addition, expression vectors contain a selection element to aid in plasmid selection within the host cell. Another critical feature of E. coli expression vector is the presence of fusion tag.

1. When selecting a promoter system, the nature of the protein target and its desired downstream use must be considered. If the protein target is a toxic protein, consider using promoter systems that have extremely low basal expression. Alternatively, for maximal protein yields, a strong promoter should be selected. For aggregation-prone proteins, a cold-shock promoter, in which expression is carried out at low temperatures, may be tested.

2. Larger bacterial and heterologous proteins fold more slowly and tend to aggregate. To prevent aggregation and facilitate folding in E. coli, protein chaperones and folding catalysts can be used. The target protein can be co-expressed with a second protein that is encoded on either the same plasmid or a separate plasmid.

3. Fusion tags are genetically fused to target proteins to increase protein solubility. It is often necessary to test multiple fusion tags to determine which tag results in the maximum yields of soluble proteins. The placement of the tag, either at N-terminus or C-terminus of target protein, is also important. N-terminal fusions are the most common and have the added benefit that they often enhance soluble protein expression more successfully than C-terminal fusions.

4. The presence of a fusion tag may interfere with the biological activity of the recombinantly expressed protein, and thus, it may be important to enzymatically remove the tag after the fusion protein has been purified. It is recommended to include a cleavage site for a sequence-specific protease to enable removal of the tag.

Influence of host strains on expression of heterologous proteins

Bacterial host strains have been developed to support the expression of heterologous proteins. Commercially available E. coli strains are specifically designed for the specific expression of proteins that are susceptible to proteolysis, contain rare codons, or require disulfide-bonds.

1. For proteins that are susceptible to proteolytic degradation, use of protease deficient strains such as E. coli BL21 or its derivatives are recommended.

2. Differences in codon frequency between the target gene and the expression host can lead to translational stalling, premature translation termination, and amino acid mis-incorporation. This difference may be overcome by supplying the rare tRNAs during expression. Bacterial strains that contain plasmids that encode rare tRNAs should be used to promote the efficient expression of genes that contain high frequencies of rare codons.

3. For proteins that contain disulfide bonds, expression in thioredoxin reductase (trxB) and/or glutathione reductase (gor) host strains will aid the formation of cytosolic disulfide bonds and will enhance the solubility of folded, disulfide-containing proteins. An alternative strategy to express disulfide-containing proteins would be to target the expressed protein to the E. coli periplasm which is highly oxidative and thus promotes the formation of disulfide bonds.

Improving solubility of proteins by changing expression conditions

The use of strong expression promoters and high inducer concentrations can result in high protein concentrations that would lead to protein aggregation before folding. Reducing the rates of transcription and/or translation will facilitate folding by allowing the newly synthesized protein to fold before it aggregates. Following are the common expression condition parameters that can be manipulated to enhance protein solubility.

1. **Temperature:** Lowering the expression temperature (15-25°C) will improve the solubility of recombinantly expressed proteins. At lower temperatures, cell processes slow down, and thus lead to reduced rates of transcription, translation, cell division, and reduced protein aggregation. Lowering the expression temperature also results in a reduction in the degradation of proteolytically sensitive proteins.

2. **Concentration of the inducer:** lowering the concentration ofthe induction agent, will reduce the transcription rate, thereby, improving the solubility and activity of recombinant proteins.

3. **Choice of media:** Batch culture is the most common method to cultivate cells for recombinant protein expression. All nutrients that are required for growth must be supplied from the beginning by inclusion in the growth medium.

Improving protein purification

1. Solubilize and purify the protein in a well-buffered solution containing an ionic strength equivalent to 300–500 mM of a monovalent salt, such as NaCl.

2. Use immobilized metal affinity chromatography (IMAC) as the initial purification step.

3. If additional purification is required, use size-exclusion chromatography (gel filtration). If necessary, use ion exchange chromatography as a final 'polishing' step.

4. The affinity tag may be removed to minimize non-native sequences in the recombinant protein and to achieve further purification.

Biotin, Avidin Streptavidin: Technical Tips for Success

The (strept)avidin-biotin system is a protein-ligand interaction present in nature that has been successfully used in a number of applications including detection of proteins, nucleic acids and lipids as well as protein purification. The avidin-biotin system is a simple yet elegant system to link proteins in immunoassays by exploiting the very high affinity of hen egg white avidin for biotin (vitamin B7). Streptavidin, isolated from bacteria, binds biotin equally well but lacks the glycoprotein portion found on avidin and therefore shows less non-specific binding. In immunoassays, antibodies and reporters like fluorochromes or enzymes that are used to localize or quantitate analytes are often coupled via an (strept)avidin-biotin bridge. Here, you will find some useful tips to keep in mind when working with the (strept)avidin-biotin affinity system.

1. Avidin or Streptavidin?

Although both avidin and streptavidin bind biotin with very high affinity, the major problem of using avidin in some applications is the high nonspecific binding, which is attributed to both the presence of the sugars and high pI. Thereby, significant nonspecific binding can be prevented with the use of streptavidin or alternatively, deglycosylated avidins that still preserve the same biotin-binding properties.

2. Biotin-streptavidin as a versatile detection system

The streptavidin-biotin system can be incorporated into virtually every immunoassay, whereby an antibody is conjugated to biotin and then detected with avidin or streptavidin conjugated to inexpensive, high quality variety of fluorochromes and enzymes, widely available from commercial sources. This makes biotinylated antibodies advantageous to signal amplification and increased sensitivity, but at the same time, demands optimization of antibody and conjugate dilutions.

3. Choose the right buffer system when working with biotinylated antibodies

In order to prevent high background and low signal to noise ratios, it is highly recommended to avoid fetal bovine serum (FBS) or biotin in blocking buffers and other solutions. A good substitute would be 0.1% – 2.0% BSA fraction V. In western blots, nonfat dry milk or casein should be limited to the initial blocking step because of residual biotin that will interfere with the assay. In this case, antibody solutions should then be prepared in just TBS-tween, which is compatible with both nitrocellulose and PVDF membranes. If background is still a problem, highly purified 0.2% – 6% casein may improve the results.

4. Reducing interference from residual biotin in samples

Sometimes non-specific bands might be observed by western blot when using the biotin streptavidin system. This is especially true for tissue preparations and cell lysates since they might contain enzymes with covalently bound biotin as a cofactor. In these cases it is useful to increase the ionic strength of buffers (~0.5 M NaCl) and/or perform a specific biotin blocking step before incubation with the primary antibody. In this instance, removal of free biotin and biotinylated enzymes from casein by using streptavidin agarose beads can significantly enhance sensitivity.

5. Biotinylation of cell surface for protein quantification

The biotin/streptavidin system can be successfully used in a noninvasive technique to chemically tag cell-surface proteins for direct, accurate quantitation of cell-surface expression, endocytosis, and recycling of a variety of plasma-membrane receptors and antigens. The amount of biotin bound to a specific receptor or antigen can be subsequently quantitated using ELISA or western blot. This technique constitutes a rapid and safe alternative to radioactive labeling.

6. Protein biotinylation

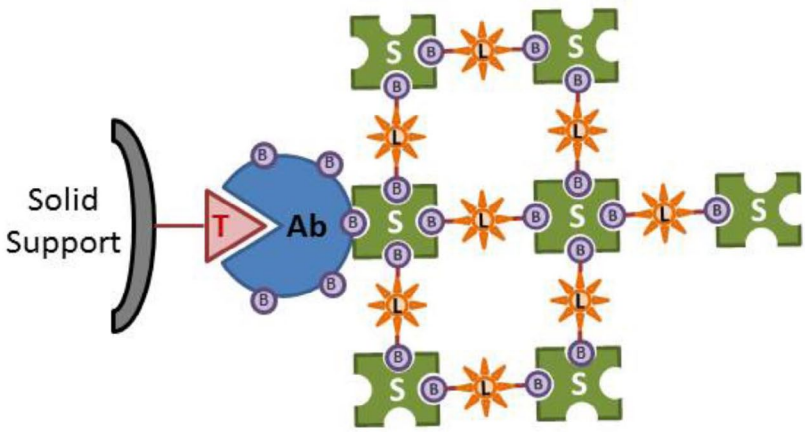
When preparing a reagent for biotin labeling of proteins it is advisable to conduct optimization assays under the system of study since the choice of an appropriate spacer and of the right chemistry to bind biotin to the target protein(s) may have a profound impact on the outcome of the experiment. The most widespread approach for protein biotinylation is based on modification of exposed primary amino groups in proteins with a succinimidyl (NHS) ester of biotin. When modification of free amino groups is not possible, proteins can still be biotinylated if they contain free thiol groups or if they are glycosylated. Moreover, it is also possible to biotinylate proteins using a commercially available photoreactive biotinylation reagent that activates following exposure to ultraviolet light.

7. Purification of biotinylated proteins

Since biotin-(strept)avidin complexes require strong denaturing buffers to break their interaction and release the biotin, purification of biotinylated proteins from immobilized (strept)avidin would also result in loss of native configuration and functional properties. For this purpose, consider using avidin analogs design to bind biotin with lower affinity and allowing elution to be carried out under milder conditions. Alternatively, 2-iminobiotin the cyclic guanidino analog of biotin, could be used since it exhibits a pH-dependent interaction with (strept)avidin that facilitates recovery under milder conditions as well. It retains high-affinity specific binding at high pH, whereas interacts poorly at acidic pH values. Alternatively, biotin with cleavable spacers can be also used (see tip 8).

8. Biotinylation including cleavable spacers

The use of cleavable spacers between the bicyclic ring of biotin and the chemical group responsible for carrying out the covalent binding to proteins have been introduced with the goal of facilitating the release of biotinylated proteins after capture on immobilized (strept)avidin and are readily available from different commercial sources. These include disulfide bridges that can be broken by reducing agents like 2-mercaptoethanol or dithiothreitol and photocleavable linkers. Each of these approaches, however, presents their own limitations and should be carefully optimized to particular experimental conditions.



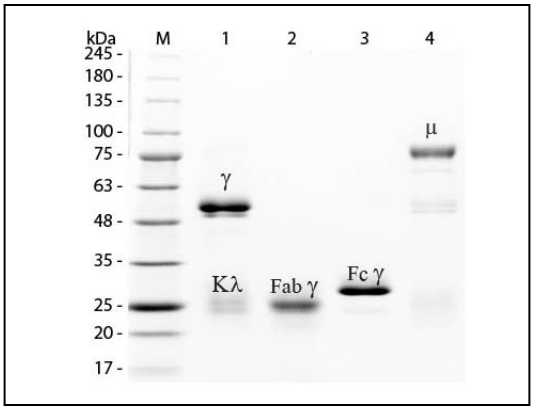
The target (T) is bound to a solid phase, i.e. ELISA plates, agarose beads, tissue sections. The binder (Ab) in this example is an antibody, but can be proteins, carbohydrates, nucleic acids or analytes. The binder is biotinylated (B) randomly at multiple sites. Unlabeled streptavidin (S) binds both to the biotinylated binder AND to the label (L) i.e. an enzyme, fluorochrome, nucleic acid, or analyte, which also contains multiple biotin moieties. This effect results in an extended polymeric structure that maximizes sensitivity in many assays.

Tips for Antibody Purification Using Immobilized Protein A and Protein G

The basis for purification of IgG, IgG fragments and IgG subclasses is the high affinity of protein A and protein G for the Fc region of polyclonal and monoclonal IgG-type antibodies. Protein A and protein G are bacterial proteins, which, when coupled to chromatography matrix such as Agarose or Sepharose, generate exceptionally useful, easy to use media (resin) for many routine applications. Protein A/G is a recombinant of Protein A and Protein G that has the additive binding properties of both proteins. Protein A, protein G and protein A/G can be used for purification of monoclonal IgG-type antibodies, purification of polyclonal IgG subclasses, and the adsorption and purification of immune complexes involving IgG. IgG subclasses can be isolated from cell culture supernatants and serum and from ascites fluid.

1. Binding Strength

- The binding strength of protein A and protein G for IgG depends on the source species and subclass of the immunoglobulin. The dynamic binding capacity depends on the binding strength and on factors such as flow rate during sample application. Binding strengths should be tested with free protein A or protein G and can be used as a guide to predict the binding behavior to a protein A or protein G purification resin.
- Protein G Sepharose is a better choice for overall capture of antibodies. Protein G binds more strongly to several polyclonal IgG's as well as to human IgG3. Under standard buffer conditions, protein G binds to all human and all mouse IgG subclasses. Protein G also binds to rat IgG2a and IgG2b.
- Binding of the target protein may be made more effective by adjusting the sample to the pH and composition of the binding buffer (dilute the sample in binding buffer or perform a buffer exchange using a desalting column).



SDS-PAGE of Affinity Purified Rabbit IgG Whole Molecule

2. Sample Presentation and Application

- Samples should be clear and free from particulate material. Simple steps to clear up a sample before purification will prevent clogging the column, decrease the need for stringent washing and will extend the life of the chromatography resin.
- The column should be pre-equilibrated in binding buffer before sample application.
- Test for a flow rate that provides the most efficient binding during sample application since this parameter can vary according to the specific interaction between the target protein and the ligand as well as their concentrations.
- For strong affinity interactions between the ligand and the target molecule, sample can be applied at a high flow rate. For interactions with weak affinity and/or slow equilibrium, a lower flow rate should be used.
- If working with weak affinity interactions between target molecule and ligand, it may be beneficial to stop the flow after applying sample in order to permit more time for the interaction to take place. In some cases, applying the sample in aliquots may be useful.
- Do not begin elution of target proteins until all unbound material has been washed by the binding buffer. This will increase the purity of the eluted target substance.

3. Medium/Resin and Buffer Preparation

- Storage solutions and preservatives should be washed away thoroughly before using any affinity medium. Re-swell affinity media in the correct buffer as recommended by the manufacturer.
- Use high quality water and reagents. Solutions should be filtered through 0.45 µm or 0.22 µm filters.
- Reuse of affinity media (protein A or protein G Sepharose) depends on the nature of the sample and should only be considered when dealing with identical samples to avoid cross-contamination.
- If an affinity medium is used routinely, care should be taken to confirm that any contaminants from the crude sample are removed by procedures that do not damage the ligand.
- Binding and elution buffers are specific for each affinity medium. Some affinity media may also require a specific buffer in order to make the medium ready for use again.
- Avoid using magnetic stirrers as they may damage the matrix. Use mild rotation or end-over-end stirring.

4. Elution Conditions

- To improve recovery of tightly bound substances to affinity medium, it may be useful to stop the flow for some time (10 min to 2 h after applying eluent) before continuing elution.
- The optimal flow rate to achieve efficient elution may vary according to the specific interaction between the target protein and the ligand and should be determined when necessary.
- Leakage of ligands from an affinity medium may occur, especially if harsh elution conditions are used. The multi-point attachment of ligand to affinity medium results in very low leakage levels over a varied range of elution conditions.
- IgG's from most species and subclasses bind protein A or protein G near the physiological pH and ionic strength. Avoid excessive washing if the interaction between the protein of interest and the ligand is weak, since this may decrease the yield.
- Most immunoglobulin species do not elute from Protein G Sepharose until pH 2.7 or less. If biological activity of the antibody or antibody fragment is lost due to the low pH, try protein A Sepharose.
- If low pH must be used, collect fractions into neutralization buffer such as 1 M Tris-HCl, pH 9.0 (60–200 µl per ml eluted fraction) to return the fraction to a neutral pH. The column should also be re-equilibrated to neutral pH immediately.
- Single step purification based on Fc region specificity will co-purify host IgG and may also bind trace amounts of serum proteins. To improve purity, perform multi-step purification such as affinity purification step followed by gel filtration step. Purity can also be improved by optimizing binding and elution conditions.

Application Tips

Western Blot: Technical Tips

Western blot or immunoblotting is a rapid and sensitive technique that uses antibodies for the specific detection of proteins separated by polyacrylamide gel electrophoresis (PAGE) and immobilized onto a nitrocellulose, nylon or PVDF membrane. Western blot requires successive steps including transfer of the PAGE-separated proteins onto the membrane using either a wet or semi-dry system, pre-incubation on a blocking buffer that will help to reduce non-specific background signal, and incubation with a primary antibody that specifically binds the antigen of interest. Positive reactivity can be evidenced by the signal generated from a reporter enzyme or fluorophore conjugated to a secondary antibody that recognizes the primary antibody. At Rockland, we routinely perform western blots for analysis of gene expression, antigens and antibodies. These are some useful tips to share for successful results:

1. Determine the best ratios of the target protein and primary antibody

Although general guidelines for protein loading and antibody dilution are recommended by the literature and antibody manufacturers, the relative abundance of the protein of interest as well as the titer of the antibody used sometimes require further optimization of those parameters. In general, 1 µg of purified protein or 10 µg of a mixture of proteins (i.e. lysate) containing the protein of interest should be enough to be detected by a solution containing 1 µg/mL of primary antibody. Nevertheless, visualization of low abundance proteins in a cell lysate might require as much as 50 µg of total protein and at least 2 µg/mL of antibody. Conversely, high background or undesired cross-reactivity can be modulated by adjusting these parameters in the other direction.

2. Keep up the protein transfer efficiency

In general, proteins can be successfully transferred by applying ~14V overnight in a wet transfer system or a maximum current of ≈0.8 mA/cm2 of gel area in a semi-dry system. Some proteins requiring improved transfer efficiency onto the membrane include large proteins exceeding 100 kDa or very hydrophobic proteins. They can be subjected to extended transfer times at high power using the semi-dry system but will require cooling to keep a constant transfer temperature of ~20°C. Also, the transfer buffer can be modified to increase transfer efficiency by adding SDS at a concentration of 0.1% (w/v). If using nylon membranes, SDS and methanol should not be used.

3. Anticipate the effect of gel thickness in western blot

Gel thickness has a double effect in immunoblotting, influencing both quantity and quality of antigen detection. In general, the thickness and acrylamide percentage of the gel inversely correlates with protein transfer efficiency and band diffusion, with gels 0.5 – 0.75 mm transferring more efficiently than thicker 1.0 – 2.0 mm gels. Also, the protein bands from thinner gels usually resolve better and provide crisper, well-defined detection.

4. Make sure to equilibrate membranes and gels on transfer solution

Always remember to equilibrate the membrane 10 to 15 min in transfer buffer before transfer and since PVDF membranes are hydrophobic and will not wet from just being placed into transfer buffer, first immerse 2s in 100% methanol, then equilibrate 10 to 15 min with transfer buffer. If the membrane dries out, wet once again with methanol and then transfer buffer. Following electrophoresis, it is recommended to equilibrate the gel 30 min at room temperature in transfer buffer to prevent a change in the size of the gel during transfer. Changes in gel dimension usually result in a blurred transfer pattern.

5. Cleaner blots with the right blocking solution

One of the most critical parameters to obtain clean western blots is the choice of an appropriate blocking agent. Blocking solutions work better when supplemented with a mild detergent like Tween-20, usually between 0.05% and 0.5% (v/v). A number of blocking agents can be used and these include immunoanalytical grade non-fat dry milk (blotto), BSA fraction V or normal serum at working concentrations ranging between 0.5% - 5%. Serum may be the best solution for very problematic backgrounds, apparently by reducing unspecific interactions between the primary antibody and the blocking agent. When using serum, it should be from the same species as the primary antibody or from the same species as the secondary when secondary antibody detection is used. Other applications including fluorescence detection should be performed using fluorescence dedicated reagents for optimal results.

6. Optimize your incubation time

The potency of a primary antibody might be leveraged by properly adjusting the incubation time with the antigen. In many cases, one hour incubation should be enough to visualize the protein of interest, however, overnight incubation at 4ºC will allow enough time for the antigen-antibody reaction to occur and result in detection of a positive signal. One hour incubation at room temperature is usually enough for the conjugated secondary antibody and certainly, it should not be extended more than three hours since it might generate high background during detection.

7. Sometimes antibodies won't recognize denatured proteins

Consider performing gel electrophoresis under non-denaturing conditions when you have your antibody working in other immunoassays but not in western blot. Since proteins are usually separated under denaturing conditions during gel electrophoresis, this restricts the detection of proteins by antibodies recognizing structural epitopes in non-denatured proteins.

8. Western blot of phosphorylated proteins

Some of the factors to consider when performing immunoblotting with phospho-specific antibodies are buffer compatibility, antibody specificity and protein abundance. The use of Blotto or other blocking mixtures containing dry milk is unsuitable because phospho antibodies could bind to a number of protein constituents in milk. Instead consider using BSA-based or alternative blocking buffers. A common problem related to phospho detection is that the phospho antibody is unable to detect the low abundant proteins of interest in a cell lysate. This problem can be overcome by means of immunoprecipitation as described in tip #9.

9. Detection of low abundance proteins

Detection of low abundant proteins by western blot can be achieved by immunoprecipitation of the target protein using a specific antibody, enabling more of the protein of interest to be loaded in the sample lane. Depending on the amount of lysate used in the immunoprecipitation, strong amplification of signal can be achieved by this method. Since the target protein can co-migrate with the heavy or light chain of the immunoprecipitating antibody that will react with the HRP-conjugated secondary antibody and obscure the signal from the protein of interest, it is necessary to use a qualifying reagent, i.e. TrueBlot, which avoids such interference and provides clear, unambiguous protein detection. Also, study of low abundant proteins by western blot can be aided by the use of enhanced chemiluminescence (ECL) that allows detection of pico and femto amounts of target protein.

10. Stripping and re-probing membranes

When the same membrane is required for testing of several proteins using different antibodies, stripping and re-probing is always possible although it might need to be empirically optimized for a particular assay since each antigen-antibody interaction is always distinct. In general, stripping buffers are reagents that combine low pH, detergents, reducing agents and/or heat in order to remove residual antibodies. Although repeated re-probing can lead to loss of signal, several re-probings are generally possible. Biotin-streptavidin interactions cannot be dissociated by this method but the whole complex can be removed away from the bound protein on the membrane.

Immunohistochemistry: Technical Tips

The acceptability of scientific results depends on the accuracy and sensitivity of the appropriate methods used, as well as controlled specificity of reagents and processes. This is particularly valid for immunohistochemistry, where sensitivity and specificity of the antibodies, as well as methodological procedures are critical to avoid false-positive and false-negative results.

In immunohistochemistry, several factors can cause false-positive or false-negative results and all should be verified as much as possible for each experimental set-up. The main steps that can lead to false-negative and false-positive results include:

- Detection of the antigen of interest by the primary antibody
- Detection of the primary antibody by secondary antibodies
- Tissue preparation

1. Highly Specific Primary Antibodies

Primary antibodies can fail to detect their target antigen for many reasons, such as conformation changes induced by fixation/embedding, steric hindrance by interacting proteins/post-translational modifications, low affinity of the antibody for the target, or failure of the antibody to penetrate into the tissue. Likewise, antibodies can bind non-specifically to other targets or tissue components.

- The most stringent specificity test can be performed by using tissue that is devoid of the antigen of interest (e.g. knockout mouse). When not possible, the best alternative is to use two antibodies raised against different epitopes of the antigen of interest yielding the same staining pattern.
- Another control includes inactivation of the antibody by incubation with its antigen prior to use for immunohistochemistry. This control, however, does not exclude that several targets sharing a common epitope are detected by this antibody.
- Manufacturer's datasheet should be used to confirm that the antibody has been tested in the specific immunohistochemical method intended to be used.
- When using an antibody for the first time, determine the optimal antibody dilution by performing staining with multiple antibody concentrations. This should be done for both the primary and secondary antibody.

2. Highly Cross-adsorbed Secondary Antibodies

Secondary antibodies are raised against immunoglobulins (typically IgGs) of the species in which primary antibodies were raised. These antibodies are used in fairly high concentrations, and as such they bind non-specifically to tissue components (extracellular matrix proteins, blood vessels, etc.) than to primary antibodies. Further, they might cross-react with IgGs from other species, which is particularly pertinent to multiple-labeling experiments. To minimize cross-reactivity, it is best to use highly cross-adsorbed secondary antibodies.

- To test for specificity, secondary antibodies should be applied in the absence of primary antibodies: all residual staining should be considered as non-specific.
- In immunofluorescence experiments, autofluorescent molecules may be contained in tissues, their presence can be detected best in the absence of secondary antibodies.
- Directly conjugated antibodies should be used only for the detection of very abundant target proteins (e.g. beta-actin and alpha-tubulin).
- For medium to low abundant proteins, use secondary antibodies for detection as binding of multiple secondary antibodies to a single primary antibody will amplify the signal.
- For very low abundant proteins, use biotinylated secondary antibodies in combination with conjugated avidin/streptavidin.
- When using biotinylated antibodies, ensure endogenous biotin is blocked prior to primary antibody incubation.
- When selecting a fluorophore conjugated secondary antibody, ensure that the microscope is able to excite and detect the fluorophore appropriately.
- Select photo-stable fluorophores such as DyLight Fluorophore dyes and Alexa Fluor® rather than FITC and PE, which are highly susceptible to fading/photo-bleaching.
- When conducting experiments with multiple fluorescent labels, ensure that each fluorophore can be spectrally separated and that one fluorophore does not get detected in another fluorophore's channel (a process known as bleed-through).

3. Proper Tissue Preparation

Relative to antibody specificity, influence of tissue preparation is more versatile and complex and it can result in both, false-positive and false-negative results, even when using highly specific and well-characterized antibodies. These effects arise mainly from epitope masking due to fixation-induced conformational changes and failure of the antibody to penetrate the tissue.

- Tissue samples can be frozen or fixed. Freezing the sections generally maintains the conformation of the target antigen allowing superior antibody binding, but small ice crystals that form in the tissue renders these sections unsuitable for long-term storage. Fixed and embedded tissue is a better alternative for long-term storage.
- Antigens can be masked as a result of the fixation process. The unmasking can be reversed with epitope retrieval/ antigen unmasking, which is either mediated by heat (heat-induced epitope retrieval) or proteases (proteolytic-induced epitope retrieval). The latter method acts by degrading the peptides masking the epitope, however, this might also result in alterations to the tissue morphology or the antigen itself. Heat-induced epitope retrieval acts by restoring the secondary and tertiary structure of an epitope and is more frequently used than proteolytic-induced antigen retrieval.
- Follow the antibody supplier recommended antigen retrieval protocol. If no specific protocol is available, use heat-induced antigen retrieval rather than a proteolytic-induced antigen retrieval protocol.
- For antigen retrieval protocol, use neutral staining solution; if this does not yield a good staining, alkaline or acidic antigen retrieval buffers should be tested.
- In addition to pH, other parameters to be optimized are temperature and duration.
- Blocking should be performed prior to incubation with the primary antibody to prevent non-specific antibody binding. Note that in perfusion-fixed tissue, good results can be obtained without any pre-blocking step.

Flow Cytometry: Technical Tips

1. Reducing noise from your fluorescent signal

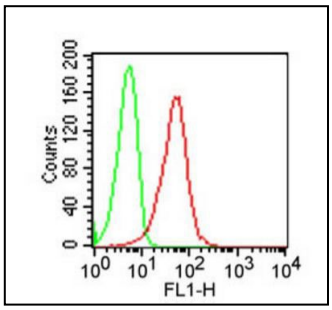
A critical step during optimization of flow cytometry experiments is reagent (antibody) titration. For optimal results you must determine the minimum amount of antibody required to achieve antigen binding saturation. This will help you improving the specificity and intensity of your fluorescent signal while minimizing background. If you are simultaneously staining with multiple colors, this titration step will also allow you to detect unexpected cross-reactivity between primary and labeled secondary antibodies.

2. Choosing the right controls for flow cytometry

Always remember to include negative controls of the same isotype as the labeled antibody so that you can determine the extent of background signal in your experiments. This is true when staining with a labeled primary antibody only or when using a combination of primary antibody and phycoeryhtrin labeled secondary antibody. For best results remember to always include a sample of unstained cells (incubated in parallel with your stained samples) so that you can control for background derived from auto-fluorescence. Whenever possible include cells known to express the antigen of interest as well as cells known to lack the same protein since they will help to determine the specificity of the antibodies used.

3. Optimize permeabilization and fixation to improve detection of intracellular proteins

Intracellular targets entail a more challenging detection since they have additional requirements beyond antibody specificity, including the ability to successfully cross the permeable cell membrane. Optimization of cell fixation and permeabilization is a critical parameter that needs to be empirically determined in order to balance the integrity of the intracellular structures with cell permeability. Always use freshly prepared solutions of high purity paraformaldehyde for fixation and initially try mild detergents (i.e., Tween 20) for permeabilization. If your fixation and permeabilization steps need further improvement, try increasing the formaldehyde concentration gradually up to 4% or try ethanol or methanol and using other detergents including Triton X-100, or saponin without fixation or alcohol fixation.



Mouse IgG2a Isotype Control
Fluorescein 010-0241

4. Stain dead cells to obtain meaningful data from viable cells

Since dead cells can bind non-specifically to any antibody, it is imperative to keep those out of the analysis. The best way to do this is by using a fluorescent dye that will pass through damaged plasma membranes and thus stain dead cells. In most cases and especially when performing staining of intracellular targets, it is advisable to use dyes that covalently bind and stain non-viable cells so that they won't leak out once the cell is permeabilized.

5. Keeping fluorescent signal at high intensity

When performing staining of cell surface markers always consider the possibility that extracellular antigens can be internalized upon antibody binding. This naturally occurring phenomenon can have a significant negative effect on the intensity of your fluorescent signal and can be prevented by:

- Working with aggregate-free antibody solutions
- Keeping your samples ice-cold or refrigerated
- Including sodium azide in the staining buffer so that cellular metabolic activity is down-regulated during staining. Also consider using monovalent antibodies in the form of F(ab) fragments.

Immunofluorescence Microscopy: Technical Tips

Immunofluorescence microscopy (also known as IF microscopy) is a very useful technique for detection and localization of cellular proteins and other antigens via fluorescent-labeled antibodies. By revealing the spatial distribution of antigens in the cells, immunofluorescent labels coupled to highly specific antibodies provides information on the dynamics of protein trafficking between cell compartments as wells as between the cell and the extracellular space. In most cases, cells are fixed and permeabilized, blocked and successively incubated with a primary and a fluorescently labeled secondary antibody. Despite being a straightforward procedure, several variables significantly contribute to successfully achieve remarkable staining patterns and thereby publication quality images.

1. Cell fixation and permeabilization

To guarantee optimal antibody detection, cells need to be properly fixed and permeabilized. These are critical, cell dependent parameters that need to be balanced for antibodies to reach their targets while maintaining cell and antigen integrity with as much fine structure preserved as possible. In general, you will find that at least one of the following should accomplish this goal: cell fixation with 2% - 4% paraformaldehyde followed by permeabilization with one of the following detergents 0.1% saponin or alternatively, 0.2% Triton X-100. Usually the former is a more mild treatment but might be ineffective with nuclear targets which are better reached when using Triton. When using saponin, be aware that contrary to Triton, it causes only reversible permeabilization of the cell membrane, meaning you'll have to include it not only during the initial permeabilization step but with each antibody incubation. Alternatively, cells can be simultaneously fixed and permeabilized with ice-cold methanol, circumventing the use of detergents at all.

2. Antibody specificity

Perform IF only with antibodies that show high specificity for the antigen of interest. This will reduce high background and unreliable patterns of protein localization. Affinity purified antibodies should work well in most cases but sometimes even affinity purified antibodies exhibit more than a single reactivity in cell lysates. Including the right controls will always help to determine the specificity of the antigen being recognized. Always use slides stained with secondary antibody only to determine the threshold of background signal and also include cells lacking the antigen of interest whenever possible. The use of an isotype of the primary antibody is also suitable as a control. If working with anti-serum, a useful control is including cells stained with pre-immune serum.

3. Use the appropriate antibody dilution

It is possible to improve staining by adjusting the antibody dilution. Usually, 1 µg/mL of purified antibody or 1:100 to 1:1000 of anti-serum should be enough to achieve specific staining. It is always possible to enhance the intensity of the signal as long as the background remains low. If it is the first time you are either using an antibody or characterizing an antigen, it is highly advisable to titer the reagents through a series of dilutions.

4. Optimizing buffers and blocking agents

Although most antigens will stain well in common buffers like PBS, a significant improvement in few targets might be achieved by switching to buffers with different compositions of ions like calcium, magnesium and potassium. A variety of blocking agents have also been successfully used in IF but 5 – 10 % fetal bovine serum is in general a good start. Other blocking agents like BSA fraction V, gelatin or serum from the same species as the secondary antibody can also be used. Rockland produces an optimized blocking buffer for IHC that also works very well for IF microscopy.

5. Use an appropriate cell density

Choose a number of cells that will result in about 50% cell confluence at the time of staining. When the cell number is excessive, the cell architecture might not be well appreciated and also may have the detrimental effect of higher background at low magnifications. Low cell numbers will reduce the probability of finding a field with the optimal pattern. Also, because non-adherent and weakly attached cells might be tricky to grow on glass surfaces, they can be attracted into adhering by coating the coverslips (12 mm, thickness #1) with poly-lysine or extracellular matrices like collagen or laminin.

6. Multiple staining

When the conditions for successful staining have been established independently, it is possible to study the expression and co-localization of two different antigens in the same sample by incubating them with their corresponding antibodies simultaneously. In this case, each primary antibody should be generated in different species so that it is possible to use secondary antibodies conjugated to fluorophores detected by separate channels. These fluorophores can range from small molecules like DyLights, ATTO, or Cy to bigger proteins like RPE. Alternatively, samples can be doubled stained sequentially, in which case all blocking, primary and secondary incubations are completed first for one antigen and then performed for the second one. In general, the sequential approach seems to generate better images and fewer artifacts.

7. Secondary Antibodies

It is highly advisable that you use pre-adsorbed secondary antibodies when performing IF. This is not only mandatory when performing multiple/double staining but highly recommended with single antigen staining. It is also preferable that you work with secondary antibodies from the same species.

8. Reducing Background

High background may be problematic during IF. Different ways to circumvent this issue include blocking with serum (from the same species of the secondary antibody) instead of BSA, reducing the amount of antibody (especially secondary) and/or reducing incubation times as well as having at least three 5-minutes washes (PBS + 0.05% Tween, recommended) between incubation steps.

9. Mounting

The final step of the IF method normally involves treating the sample with mounting solutions. This step is necessary since it improves the refractive index and helps to preserve the sample. Nevertheless, applying the right amount of mounting media can be tricky since applying too much can overflow and blemish the edge of the coverslip whereas too little can result in air bubbles trapped between the coverslip and the slide. An optimal amount of mounting media should take about 30s to spread out when the coverslip is placed on the slide.

10. Data Interpretation

Always include your secondary antibody only as well as isotype controls and other negative controls where the antigen is not present so that you can differentiate artifacts from auto-fluorescence and improper permeabilization that could lead to poor staining and even misleading patterns. More importantly, since IF results are usually a picture depicting few cells, make sure the picture truly represents the more common, representative pattern observed along the whole sample.

ChIP: Technical Tips

Chromatin immunoprecipitation **(ChIP)** is a powerful technique to determine protein interactions at particular regions of DNA in order to map the relative position of chromatin and DNA binding proteins such as histone modifications. Generally, the DNA-protein and protein-protein interactions neighboring chromatin (≈2 Å) in live cells are cross-linked upon treatment with formaldehyde (cross-link ChIP, **XChIP**) but it can be also performed without cross-linking (native ChIP, **NChIP**). Following cell lysis, chromatin is sheared by either sonication or enzymatic digestion with micrococcal nuclease (MNase) to generate ~500 bp DNA fragments. The clarified cell extract is then used for immunoprecipitation (IP) with an antibody that recognizes a target protein, modified peptide (e.g., acetylated, phosphorylated, methylated), or epitope tag. DNA sequences that directly or indirectly cross-link with a given target are selectively enriched in the immunoprecipitated sample. The obtained DNA is de-crosslinked, purified and analyzed by PCR, quantitative qPCR, labeling and hybridization to genome-wide or DNA microarrays (**ChIP-on-chip**), molecular cloning and sequencing or direct high-throughput sequencing (**ChIP-seq**). Recent advances on ChIP have simplified the technique in terms of sample requirements, handling and timelines while improving reproducibility and applicability.

1. Choosing the right antibody control for ChIP experiments

As with many other experiments involving antibodies, it is always advisable that you use an isotype control for your antibody to determine the specificity of the observed signal. It is advisable that you perform a mock IP in parallel with an isotype control or, alternatively, with an antibody that is non-related to your protein of interest or the same antibody used in ChIP that is blocked with specific peptide. If working with an epitope-tag protein, then you can also do the mock IP with a lysate that doesn't contain the tagged protein. If multiple antibodies are used with the same chromatin preparation, then a single isotype control is sufficient as long as all the antibodies are from the same species.

2. Always use the right antibody

When performing ChIP assays always make sure that your antibody has been tested for ChIP applications. If a ChIP validated antibody against your protein of interest is not available, you can also try an antibody that has been tested for IP. Remember that antigen binding can be significantly affected by loss of epitope accessibility and/or recognition resulting from the cross-linking step. If you suspect this could be a problem, consider using NChIP.

3. Make sure to have a chromatin preparation that suits your downstream applications

When study transcription factors and other chromatin associated proteins, including those with weak DNA binding, it is recommended that you use XChIP. Instead, mapping of post-translationally modified histones and histone variants in the genome is better achieved with nChIP.

4. Chromatin Fragmentation

Because the size of DNA fragments (~500 bp) generated for ChIP constitute a critical parameter in achieving good mapping resolution and efficient solubilization upon cell lysis, it is essential to estimate fragmentation by 1.2% agarose gel electrophoresis following DNA purification. This is a variable highly dependent on the extent of cross-linking that would otherwise generate larger, less soluble fragments when done excessively. Thereby, this confirmation step should be performed every time that fixation conditions are changed. Since it is not possible to check the efficiency of shearing in this way with less than ~100,000 cells, it is recommended to optimize the shearing on higher amount of cells before doing ChIP on actual samples of fewer cells. If using MNase, adjusting the concentration of the enzyme will result in different fragment sizes.

5. Optimize the ratio of antibody to chromatin used in your IP

Performing preliminary experiments to empirically determine the lowest antibody concentration that depletes >90% of the protein of interest from the extract will help you to improve the IP step. These experiments should be analyzed by western blot after trying different dilutions of antibody with chromatin devoid of cross-linking. If working with abundant proteins, 1 – 2 µg of antibody per ChIP should be enough whereas low abundant targets might require as much as 10 µg. This will help to optimize the efficiency and specificity of the enrichment in your actual ChIP experiment, especially if using cross-linked chromatin that is reported to exhibit ~50% reduction in IP efficacy as a result of epitope modification.

6. Preserving post-translational modifications

When mapping the location of PTM histones and other chromatin-associated proteins, you might see a several-fold enhancement in the enrichment efficiency by using specific inhibitors that prevent the degradation of the target modification. This is particularly relevant for some labile post-translational modifications including histone acetylations and phosphorylations that can be efficiently preserved by using histone deacetylase and phosphatase inhibitors in all the solutions used before fixation and thereafter.

7. Confirm protein enrichment of your target sequence

When working with your ChIP data it is always advisable to compare the obtained enrichment of an immunoprecipated genomic region to several other unrelated regions in the same experiment. Those irrelevant regions should generate a typical background of ~0.025 % to 0.05 % for IP efficiency and resulting in between >5-fold to 100-fold enrichment for regions bound by the protein of interest.

8. False negatives

Depending on how the protein of interest is bound to DNA and other associated proteins, it is possible that the target epitope is not always accessible to the antibody during IP, resulting in false negatives. This might be the case in those ChIP experiments where no enrichment is observed for any genomic region. The use of a polyclonal antibody or an epitope tag can help to diminish this problem by either increasing the possibility of recognize more epitopes or using an epitope which is expected to be rather inert.

9. Shorten the time for ChIP

One of the drawbacks of traditional ChIP is the length of the whole procedure. Several advances in this subject have led to time optimization of some stages, including combining distinct steps for cross-link reversal, proteinase K digestion and DNA elution into a single 2h step and shortening the antibody incubation time during IP to 15 min when performed in an ultrasonic bath.

10. Always verify the quality of your ChIP DNA

Verify that a minimum of 5-fold enrichment of target DNA is measured in your ChIP DNA by either PCR or qPCR when compared to the isotype control and remember that as with any other qPCR assay, the efficiency of the primers used is critical. For a problematic PCR, you can also include a PCR on genomic DNA as positive control. A frequently recommended starting dilution for the ChIP sample and controls is 1:100.

Immunoprecipitation: Technical Tips

Immunoprecipitation (IP) is a well-established technique used to isolate a specific protein or group of interacting proteins from a complex mixture of many different proteins using an antibody immobilized on a solid support. These solutions are often in the form of a crude lysate of cells, an animal tissue or a plant. IP is an important step in many proteomic studies designed to explore the presence, relative abundance, protein function, protein-protein interactions, post-translational modifications and expression profiling of proteins. Purified proteins obtained by immunoprecipitation can be analyzed by variety of techniques, such as ELISA and Western blotting.

Although IP technique is procedurally simple, the variables affecting the success of any specific experiment are many. IP conditions can be optimized to successfully isolate adequate amounts of specific protein. Consideration of main factors involved in IP can help to identify the components that are most likely to affect particular experiments.

Following we describe the various factors that have the large effect on desired yield and purity of target proteins.

1. Lysate Preparations

The quality of the sample used for immunoprecipitation critically depends on the right lysis buffer. The ideal lysis buffer will stabilize native protein conformation, inhibit enzymatic activity, prevent antibody binding site denaturation and ensure maximum release of proteins from the cells or tissue for capture and analysis. Nonionic detergents such as NP-40 and Triton X-100 are less harsh than ionic detergents such as SDS and sodium deoxycholate. Other variables that can affect the success of IP include salt concentration, divalent cation concentration, and pH. Non-denaturing buffers containing non-ionic detergents can be used if the IP antigen is detergent-soluble and the antibody can recognize the native form of the protein. Denaturing buffers, such as radio-immunoprecipitation assay (RIPA) buffer, are more stringent buffers because of the addition of SDS or sodium deoxycholate. While these buffers do not maintain native protein conformation, proteins that are difficult to release, such as nuclear proteins, can be released with denaturing buffers. Detergent-free buffers can also be used if the target protein can be released from cells by physical disruption, such as mechanical homogenization or heat.

Proteolysis, de-phosphorylation and denaturation can start as soon as cell lysis occurs, this can be slowed down by keeping the samples on ice or at 4°C at all times and by adding protease and phosphatase inhibitors to the lysis buffer.

2. Preclearing Lysates

Lysates are complex mixtures of proteins, lipids, carbohydrates and nucleic acids, and thus some amount of non-specific binding to the IP antibody, or the beaded support will occur and negatively affect the detection of the immunoprecipitated proteins. In most cases, preclearing the lysate by incubating the prepared lysate with the beaded support before commencing immunoprecipitation is a way to remove potentially reactive components that are binding non-specifically to beads components (i.e. coupled secondary antibodies) or the beads themselves. Another preclearing technique involves the addition of a non-specific antibody of the same species of origin and isotype as the capture antibody. This process will remove anything that might also bind non-specifically to the capture antibody during immunoprecipitation. The end result will be a lowering of background and an improved signal-to-noise ratio.

3. Choosing Antibodies

For immunoprecipitation, the antibody used for purification is an important factor that can affect the yield. Polyclonal antibodies, where possible, should be considered for the capture of a target protein. Polyclonal antibodies bind multiple epitopes on the target protein, and form tighter binding immune-complexes with higher retention rates.

The use of antibody pairs, such as a capture antibody from one species, and a detection antibody for Western blotting from another species, is an additional factor to consider for successful immunoprecipitation. In addition to the origination from different species, the antibody selection process should ensure that both antibodies recognize different epitopes of the target protein. A combination of a polyclonal capture antibody and a monoclonal antibody for detection will guarantee maximum capture efficacy with high detection specificity.

4. Binding and Wash Buffers

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). Although IgG's from most species and subclasses bind protein A or protein G near the physiological pH and ionic strength, specific protein A and protein G binding buffers can increase binding. However, such buffers may not always be appropriate 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.

When selecting a wash buffer for an IP application, it is important to create conditions in which the desired protein interactions are maintained but non-specific protein binding is prohibited. Ordinarily starting point for wash buffer optimization in protein purification methods is either PBS or TBS, which have physiological concentrations of salt and pH levels. Multiple washes (ideally at 4°C) with simple wash buffers such as PBS or TBS either alone or with low detergent concentrations (typically 0.5-1.0% of NP-40, Triton X-100, or CHAPS) or by moderate adjustments to salt concentrations, can be used to reduce background. If non-specific interactions persist and the desired interaction is still strong, the stringency may be further increased by increasing the sodium chloride concentration to 0.5 M or 1 M. Low levels of reducing agents (such as 1-2 mM DTT or β-mercaptoethanol) can help disrupt non-specific interactions mediated by disulfide bridges.

5. Elution Buffers

To ensure that the target protein is eluted from the beads, elution buffer at the correct strength and pH must be chosen for elution of proteins. For analysis of immunoprecipitated proteins by reducing SDS-PAGE and Western detection, elution of proteins directly in reducing SDS-PAGE sample buffer would be ideal. This buffer, designed to denature and reduce proteins for electrophoresis, is very effective in dissociating the affinity interactions. However, elution in SDS-PAGE sample buffer will cause multiple non-specific proteins to co-elute with the antigen. Fragments of the immobilized solid support (e.g., subunits of protein A/G) may be stripped from the beads with harsh elution buffers. In such cases, elution in a milder buffer (0.1 M glycine, pH 2.5) and neutralizing before loading to SDS-PAGE gel, will prevent this contamination. The low pH condition dissociates most antibody-antigen interactions as well as the antibody-Protein A/G interactions.

6. Choosing Secondary Antibodies

Secondary antibodies that recognize the heavy and light-chain of the primary antibody for Western blot detection of IP samples will always result in two bands (the heavy-chain at 50kDa and the light-chain at 25kDa). It is therefore difficult to detect the protein of interest if it migrates around either the 50kDa or 25kDa markers. To avoid interference by the antibody chains we recommend using Trueblot, which only recognize primary antibodies in their native (non-reduced) state, and thus eliminate the detection of the denatured primary heavy and/or light chains during Western blotting.

ELISA: Technical Tips

ELISA represents one of the most widely used antibody applications from basic research to diagnostics. This assay is the preferred method to determine the titer of an antibody but can also be successfully used to quantitative antigen or analytes in a sample. Moreover, ELISA provides an economical, rapid and highly sensitive method for screening a large number of samples often referred to as high throughput screening (HTS). The assay is based upon an antigen-antibody interaction and subsequent enzymatic action on a substrate yielding a soluble colored product. Variations of the basic method exist for specialized applications including detection and quantification of antibodies, proteins, peptides and even small molecules.

Here, you will find some important recommendations and useful tips to achieve outstanding ELISA results.

1. Determine the best ELISA format for your purposes

Indirect ELISA is the method of choice for determination of antibody titers and can be used with either anti-serum, hybridoma supernatants or purified antibody. In this assay, the observed changes in signal intensity are proportional to the amount (dilution) of the tested antibody.

Direct ELISA is a faster format in which the coated antigen is directly incubated with a conjugated antibody. It is commonly used for titering conjugated secondary antibodies and very useful to estimate antigen cross-reactivity.

Sandwich ELISA is an ideal format to quantify soluble antigen either pure or in a complex matrix. It is usually a very sensitive antigen assay that requires a pair of specific antibodies (capturing and detecting antibodies). In this assay, one antibody is used to coat the plate and capture the antigen present in the sample solution. Then, the second specific antibody (conjugated to a reporter system) is used to detect the captured antigen. Probably the most sensitive ELISA format.

Competitive ELISA is an excellent approach to assess antibody specificity of an antibody. This assay is a slight variation of the direct and indirect formats, where the antibody is pre-incubated with pure antigen and then added to the antigen pre-coated plate. It can be also used to quantify antigens or cross-reactivity between secondary conjugated antibodies and antigens.

2. Before you start: determine the optimal reagent concentrations

It is highly recommended to optimize the amount of a reagent (antigen and/or antibody) in the assay by performing a checkerboard titration. This is accomplished by serial dilution of one reagent across the plate and serial dilution of the other reagent down the plate. This design permits you to analyze different concentrations of the two reagents in each well and to obtain the optimal combination of both reagents.

3. Improve the performance of your assay by optimizing plate coating

It is important that the coating solution is absolutely free of detergents because competition for binding may cause low and/or uneven binding. Excessive concentrations of coating protein may actually lead to less coating. Although the coating solution doesn't need to be pure antigen, this shouldn't be less than three percent of the total protein in the coating solution. Also, sensitivity of some assays might benefit from including BSA in the coating solution.

4. The choice of blocking buffer is critical in obtaining genuine intensity signals and reduced backgrounds

For this purpose and to account for unspecific binding between conjugated secondary antibody and the antigen, it is also necessary to include controls of antigen coated wells incubated with the conjugate only. Efficient blocking buffers for ELISA include BSA and gelatin.

5. Optimize the amount of conjugated secondary antibody to use

IDifferent conjugates will provide different ranges of signal depending on how much antibody they will bind. Because the goal is to bind the entire antigen-antibody complex present in each well, you want to make sure you will use the right amount of conjugate in your assay by performing a serial dilution assay in advance (see tip #2). Always use high quality antibody conjugates. For alkaline phosphatase conjugates use TBS as a replacement for PBS.

6. Adjust Incubation times to improve the performance of your assa

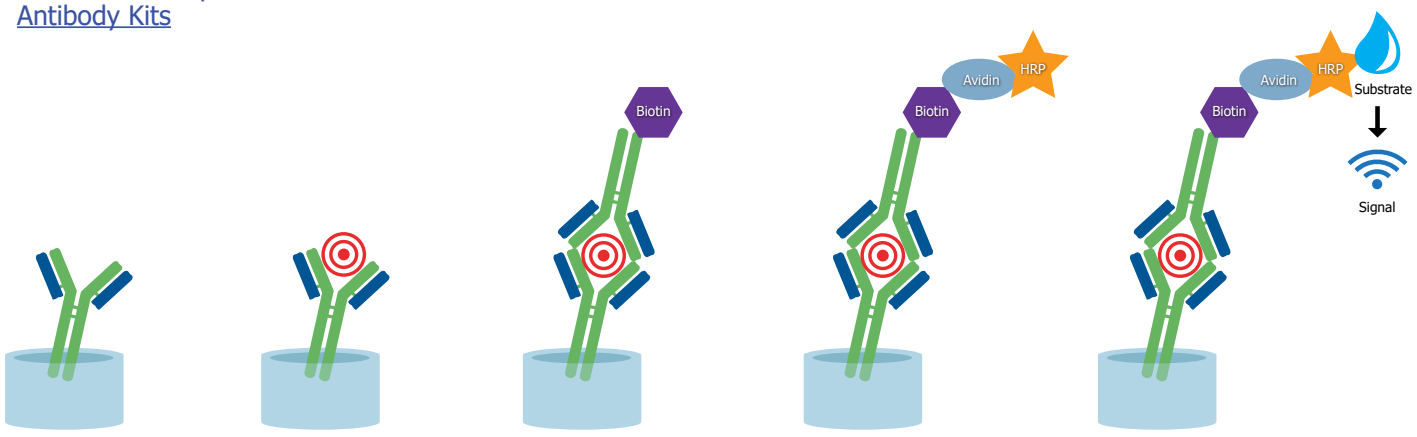
In general, antigen-antibody complexes will form within two hours incubation at room temperature but in some cases significant stronger specific signal might be obtained with longer incubation times. Following the addition of the substrate, it is important to read the plate within the recommended time for the specific reporter system being used. Chromogenic and chemiluminescent substrates, particularly those used by peroxidase-conjugated antibodies can saturate the signal intensity and reduce the dynamic range of the assay.

7. Once your assay is optimized, always keep the conditions unmodified throughout all steps

Because ELISA is a very sensitive assay, even minor changes in buffer composition, volumes, washing times, antigen/antibody concentrations, temperature or incubation times can have a significant impact in the performance of the assay. It is highly advisable to run all assays in replicates (duplicates or triplicates) and include controls that help to account for plate to plate variability.

Featured Isotype Controls for Flow Cytometry

- Blocking Buffer Formulations
- Secondary Antibodies
- Enzyme Substrates
- Primary Antibodies
- Proteins and Peptides
- Antibody Kits



Tips for TrueBlot® Immunoprecipitation and Western Blotting

Western blotting (WB) using immunoprecipitated samples can frequently result in detection of undesirable, high background signals. This problem arises because the same antibody (or an antibody from the same species) is often used for both immunoprecipitation (IP) and primary Western blot detection. The heavy chain and light chain of the immunoprecipitation capture antibody is also present in the eluted immunoprecipitated samples and appear at 50-55 kDa and 25-30 kDa on Western blot membranes following SDS-PAGE size-fractionation. The secondary antibody (horse radish peroxidase (HRP)-conjugated, species-specific antibody) used to detect the primary antibody on Western blots will then detect the heavy and light chain bands on WB membranes. If the antigen size is comparable to either the heavy or the light chain, then the antigen band may be masked, since heavy and light chain molecules are typically present in greater amounts than the immunoprecipitated antigen. This methodological artefact can be circumvented by using conjugated secondary antibodies which only recognize primary antibodies in their native (non-reduced) state, thereby, eliminating the detection of the denatured primary heavy and/or light chains during Western blotting. Rockland's TrueBlot® for IP secondary antibodies are immunoblotting reagents that enable the trouble-free detection of immunoblotted target protein bands, without interference from denatured IgG.

We provide guidelines for detection of immunoprecipitated samples by Western blotting utilizing TrueBlot® immunoblotting reagents. These readily available reagents provide flexibility to accommodate proteins with overlapping molecular weights to the heavy and light chain fragments in the IP experiments. These options offer cleaner WB membranes and therefore target signals that are simple to interpret.

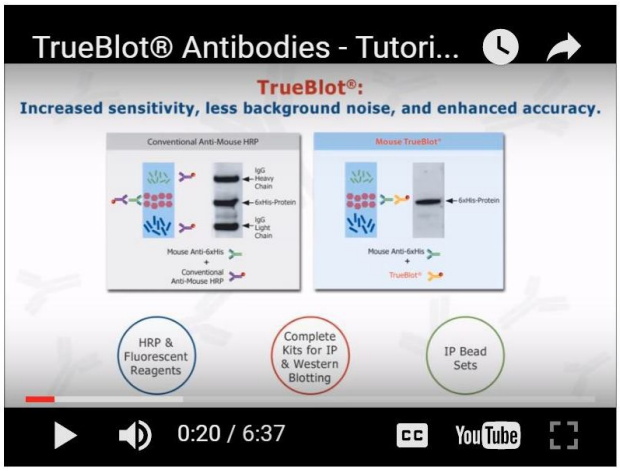
1. Sample Preparation

Cells or tissues may be lysed using any standard cell lysis protocol compatible with your starting material.

Proteolysis, de-phosphorylation and denaturation can start as soon as cell lysis occurs, this can be slowed down by keeping the samples as cool as possible by carrying out all the steps on ice or at 4°C and by adding protease and phosphatase inhibitors to the lysis buffer.

2. Preclearing Lysates

The pre-clearing step should be incorporated into the procedure to reduce the amount of non-specific contaminants in the cell lysate and to remove proteins with high affinity for beaded support prior to the specific immunoprecipitation.



Click Here to View

3. Choosing Immobolized Beaded Support

Choosing immobilized beaded support for the precipitation step is critical to the success of the procedure. We recommend to use Rockland's Trueblot IP beads (rabbit 00-8800-25 or mouse 00-8811-25) for best results.

If using Protein A or Protein G bead support, do not use excessive amounts of Protein A or Protein G bead slurry. Protein A or G binds non-specifically to IgG in Western blotting and can cause nonspecific bands. Furthermore, Protein A and Protein G are not interchangeable detection reagents; their use is determined by the isotype of the primary detection antibody as they exhibit differing isotype selectivity. Protein G binds well to mouse IgG1 and most subclasses of rat and human IgG's, whereas Protein A has a much higher affinity for mouse IgG2a, IgG2b, and IgG3.

We recommend that the IP beads vial be inverted several times to get the beads into suspension. Beads should be homogenous before use. It is advisable to cut the end of pipette tip at a 45° angle using a sharp blade to facilitate pipetting the bead slurry and to prevent damage to the beads. To maintain suction, only a very small section of pipette tip need to be removed.

When using IgM antibodies, do not use protein-A or protein-G conjugated beads. Use anti-IgM coupled protein A or Protein G beads. The IgM will then bind to the beads by binding to the anti-IgM antibody.

4. Choosing Primary Antibodies

The success of immunoprecipitation depends on the affinity of the antibody for its antigen. In general, while polyclonal antibodies are best, purified monoclonal antibodies, or hybridoma supernatant can also be used. As polyclonal antibodies bind multiple epitopes on the target protein, they retain a greater amount of the protein. Since IP procedure requires the washing of unwanted proteins and constituents, the retention rate of the protein need to be high so that washing does not remove the target protein away. Therefore, a good-quality polyclonal should be the first choice for an IP procedure.

5. Wash and Elution Buffers

When selecting a wash buffer for an IP experiment, it is essential to create conditions in which the desired specific protein interactions are retained but non-specific protein binding is prevented and washed away. If a particular IP experiment is afflicted with troublesome amounts of background, experimental testing is necessary to determine a wash condition that is effective.

The starting point for wash buffer optimization is either PBS or TBS, which have physiological levels of salt and pH. Multiple washes (ideally at 4°C) with simple wash buffers such as PBS or TBS either alone or with low detergent concentrations of 0.5-1.0% of NP-40, or Triton X-100, or CHAPS can be used to reduce background.

For analysis of immunoprecipitated proteins by reducing SDS-PAGE and Western detection, elution of proteins directly in reducing SDS-PAGE sample buffer is ideal. This buffer, designed to denature and reduce proteins for electrophoresis, is very effective in dissociating the affinity interactions. However, elution in SDS-PAGE sample buffer will cause multiple non-specific proteins to co-elute with the antigen. Fragments of the immobilized solid support (e.g., subunits of protein A/G) may be stripped from the beads with harsh elution buffers.
The most generally effective, nondenaturing elution buffer for immunoprecipitation 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).

6. Western Blot Analysis

Many blocking buffers can be successfully used for immunoblot detection. We recommend Rockland's MB-070. You can also use 0.5-5% casein, or up to 5% non-fat dry milk, or up to 3% BSA dissolved in TBS-Tween.

7. Blocking Buffer

Many blocking buffers can be successfully used for immunoblot detection. We recommend Rockland's MB-070. You can also use 0.5-5% casein, or up to 5% non-fat dry milk, or up to 3% BSA dissolved in TBS-Tween.