SCIENTIFIC Excellence

Tips for Collecting High Quality Data from Immunoassays

ROCKLAND™
antibodies & assays

TABLE OF CONTENTS

SELECTION TIPS

BENCH TIPS

APPLICATION TIPS

For over fifty-five years now, Rockland has assembled an outstanding team of scientists and technicians dedicated to making great antibodies that fit the exact 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 with you to help make your work more efficient. From performing a Western blot, 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 for collecting high quality data from immunoassays and know that Rockland's team is here to assist you as you tackle the everyday challenges scientists 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, VP of Research & 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 Custom Control

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

Dr. Yong Zhang, Research & Development Manager

Dr. Yong Zhang is a Senior Scientist of Research and Development at Rockland Immunochemicals, Inc. Yong leads and oversees the expansion of new and existing products and direct activities associated with identifying, designing, and promoting new products. He 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 secreted by a single clone of B lymphocytes are termed monoclonal antibodies and those produced by a mixture of various B lymphocyte clones are 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 (bacteria), or cells. Consequently, with 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 vastly 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, making them strongly immunogenic. Typically, lipids 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 on 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 necessary 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 ensure that they induce an immune response.

If an 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 has the ability to recognize multiple epitopes on the antigen. As a result, there is a high probability that the antibodies will bind with the native protein against at least one of these epitopes in the target assay.

Unfortunately, the antibodies are being generated against multiple epitopes, resulting in 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 that mimic selected regions of the protein of known amino acid sequences. 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.

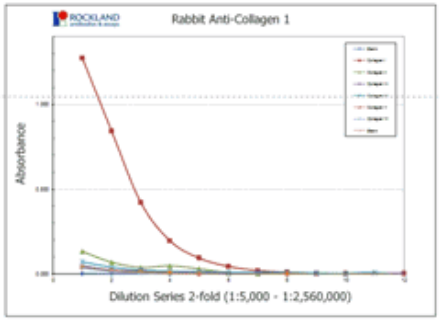
The disadvantage of using a peptide sequence is that it is identical to a very specific region of the native protein and this region may not be accessible in the protein's native conformation in a particular assay. Some general key considerations in designing *de novo* or native-based peptides that will 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 N-terminal glutamine or asparagine and C-terminal proline or glycine in a peptide.
- 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, and proteolysis.
- Examine the protein sequences for which the resulting antibody should or should not cross react with.
- Avoid internal cysteine (which can be replaced with serine) because cysteine is susceptible to rapid oxidation which could 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 and in Western blot analysis to detect proteins separated by electrophoresis. They are also used 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 and localize proteins within cells. Proteins can also be detected and quantified with antibodies using ELISA.

ELISA



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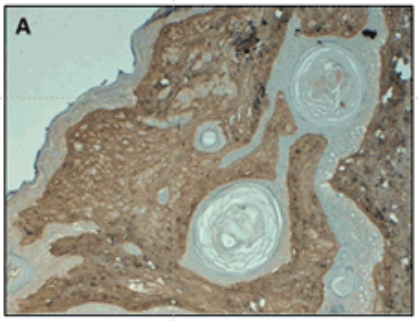
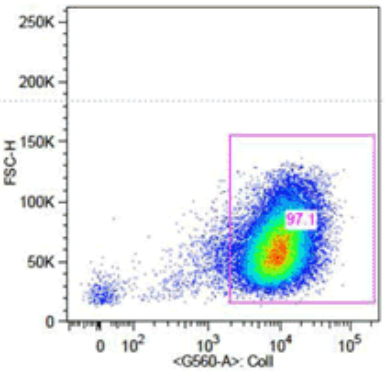


FLOW



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. This is 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 (immunoprecipitation or flow cytometry) and antibodies produced against denatured proteins react with proteins subjected to denaturing conditions (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, nuclear, 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 because 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 that 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, & RECOMBINANT ANTIBODIES

POLYCLONAL ANTIBODY

- Broader specificity and often recognizes multiple epitopes, making them less sensitive to minor antigen changes (e.g. polymorphism, heterogeneity of glycosylation, or slight denaturation). 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
- Target multiple epitopes on the same protein and thus usually provide more robust detection

- May be generated in a variety of species (See section 4 below) giving the users many options in experimental design.
- 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, which will bind more than one antibody molecule.
- Compatible with a wider range of applications.

MONOCLONAL 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 is high by providing 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 and 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.
- Readily optimized, as their nucleic acid sequences are defined and easily available. A high degree of control is possible with the selection of recombinant antibodies that bind in a particular pH, salinity, or in other specific buffer conditions.
- Mass production of recombinant antibodies can be achieved at a shorter time frame and does not require the use of animals—thus overcoming ethical concerns over animal distress, discomfort, and pain.
- A required recombinant antibody fragment can be converted into a different species, isotype, or subtype by adding the appropriate constant domain, making 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.

MOST COMMON POLYCLONAL

RABBITS

Rabbits are traditionally the most common host animals for polyclonal antibody production.

Rabbits are used in 95% of cases. They have the capability to respond to broad classes of antigens and provide good yields in a short period of time, making them one of the most cost effective options.

CHICKENS

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.

GOATS/SHEEP

Goats/sheep are the host of choice for producing polyclonal antibodies in larger quantities. They give strong immune responses against most antigens.

The goat produces about 7-8x the amount of serum compared to the rabbit and contains about 20 mg/mL of total IgG, which is about 2-3x more than rabbit serum. This makes the goat the most cost effective choice of the two when greater amounts of antibody are required.

MOST COMMON MONOCLONAL

MICE/RATS

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

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, and 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.

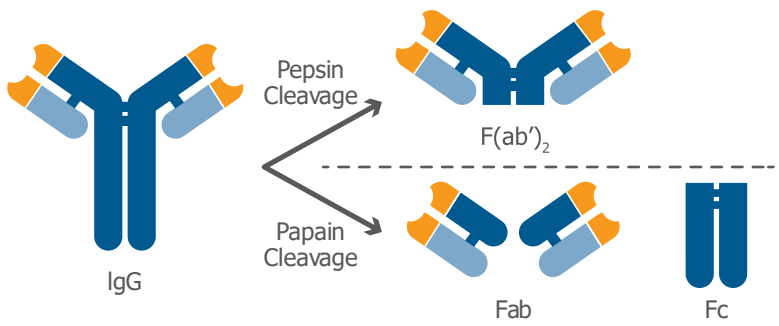
PTM specific antibodies may be either monoclonal or polyclonal. The latter are easier and faster to develop but both require significant validation. PTM antibodies are purified to remove antibodies that react to the non-modified protein by subtraction against the unmodified peptide, followed by affinity 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 & SUBCLASSES

When 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 IgG_1, IgG_2, IgG_3, IgG_4, IgA_1 and IgA_2) 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- IgG_1, IgG_2a, IgG_2b, IgG_2c or IgG_3, IgA, and IgM as well as kappa and lambda light chains. If a monoclonal antibody is determined to be IgG_1 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, 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 & 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 non-specific 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 bleed. 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 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.

|SELECTING THE BEST SECONDARY ANTIBODIES

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. Oftentimes, immunoassays use secondary antibodies to bind the primary antibody to assist in detection, sorting, and purification of target antigens. In these cases, not only are primary antibodies exhibiting high specificity and sensitivity for the intended antigen essential, but the high quality secondary antibodies are also paramount in achieving meaningful results. The following steps will guide you 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 chains 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". (Manning, Bundros, & Trimmer, 2012) for an in depth review of the subject. When the sub-class of your primary antibody is unknown, you can use anti-IgG Fab 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. This generates 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 that are 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

| MASTERING POST-TRANSLATIONAL MODIFICATIONS

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.

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).

ANTIBODY SELECTION TIPS FOR PTMS

PREPARATION	PRODUCTION	VALIDATION
1	2	3
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.	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.	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.

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 DYES

	Abs (nm)	Em (nm)		Abs (nm)	Em (nm)
DyLight™ 405	400	420	ATTO 550	554	576
Aminomethylcoumarin (AMCA)	353	442	Cy3.5™	581	596
ATTO 425	436	484	Texas Red®	596	620
Cy2™	489	505	ATTO 594	601	627
DyLight™ 488	493	518	Allophycocyanin	650	660
ATTO 488	501	523	Cy5™	650	667
Fluorescein	495	528	ATTO 647N	644	669
ATTO 532	532	553	DyLight™ 649	646	674
Cy3™	552	565	ATTO 655	663	684
DyLight™ 549	550	568	Cy5.5™	689	703
Rhodamine (TRITC)	550	570	DyLight™ 680	682	715
R-Phycoerythrin (RPE)	488	575	DyLight™ 800	770	794

FLUORESCENT PROTEIN CONJUGATION

Fluorescence detection is based on the use of fluorophore that has a unique and characteristic spectra for absorption and emission—emitting 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 fluorescent-labeled antibody include brighter signal, multiplexing capabilities, and ease of use (many are available pre-conjugated to many different colors of dye).

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

EXTRA TIP

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 using 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 (e.g. 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 non-specifically 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.

1. 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), tetramethylbenzidine (TMB) and AP signal is often measured through its colorimetric substrate p-Nitrophenyl Phosphate, Disodium Salt (PNPP). These enzyme-antibody conjugates can be used in applications such as ELISA, 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 may lead to an increased background signal. It is recommended to use chromogenic substrates for applications where protein abundance is high.

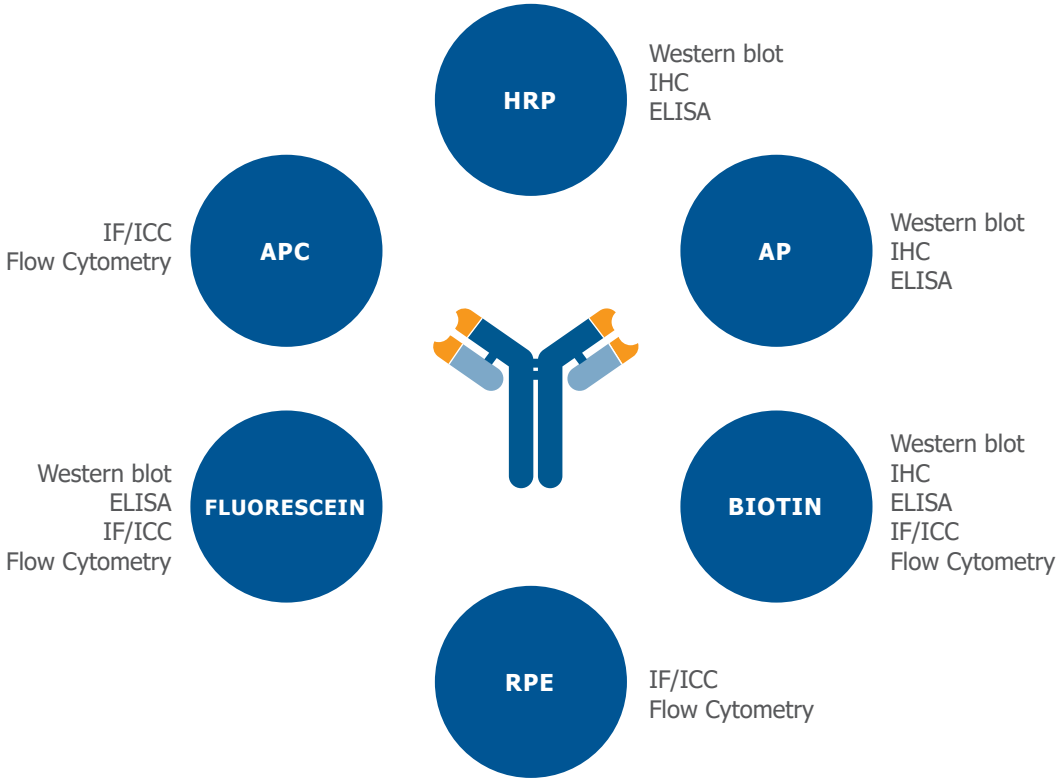
Like immunofluorescence, chromogenic detection allows for the visualization of multiple antigens, but only if the antigens are localized at different sites in the cell or tissue because overlapping colors may obscure results. DAB chromogenic staining should be used if slides need to be stored for longer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

2. 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 non-specific binding. Avidin is a highly cationic glycoprotein and can cause non-specific background signal in some applications due to its positively charged residues and oligosaccharide components.

For multi-color 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

SIGNAL & STAINING QUALITY

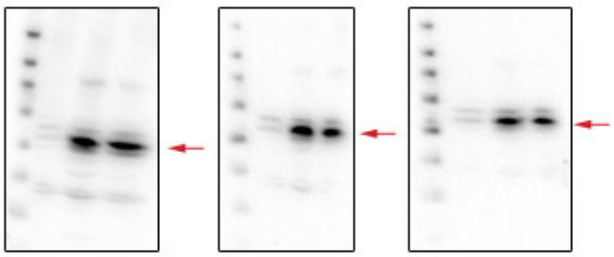
Western blotting and immunohistochemistry (IHC), antibody titer, and dilutions are important for their effect on signal and staining quality. Correct dilutions of antibodies will contribute to the quality of signal/staining if prepared precisely and consistently. The optimum antibody titer is the highest dilution of antibody that results in maximum positive signal and specific staining without background or non-specific reactions. Oftentimes, 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.

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.

Determining Optimal Working Dilution of an Antibody



1:1,000 Dilution | 1:5,000 Dilution | 1:10,000 Dilution

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 1:100–1:2000. 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 1:10–1:1,000, 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 in 100 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

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.

1. INFLUENCE OF GENE/PROTEIN SEQUENCE ON EXPRESSION & SOLUBILITY

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.

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.

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.

2. 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, 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.

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.

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.

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.

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.

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

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.

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.

4. 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.

- 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.
- Concentration of the inducer: Lowering the concentration of the induction agent will reduce the transcription rate; thereby, improving the solubility and activity of recombinant proteins.
- 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.

5. STEPS FOR IMPROVING PROTEIN PURIFICATION

STEP 1	STEP 2	STEP 3	STEP 4
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.	Use immobilized metal affinity chromatography (IMAC) as the initial purification step.	If additional purification is required, use size-exclusion chromatography (gel filtration). If necessary, use ion exchange chromatography as a final 'polishing' step.	The affinity tag may be removed to minimize non-native sequences in the recombinant protein and to achieve further purification.

| BIOTIN, AVIDIN, & STREPTAVIDIN

The streptavidin-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 to 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 streptavidin-biotin bridge. Here, you will find some useful tips to keep in mind when working with the streptavidin-biotin affinity system.

1. AVIDIN OR STREPTAVIDIN?

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

2. STREPTAVIDIN-BIOTIN 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. It is then conjugated to inexpensive, high quality variety of fluorochromes and enzymes that are 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 TBS-Tween, which is compatible with both nitrocellulose and PVDF membranes. If background is still a problem, highly purified 0.2%–6.0% 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 streptavidin-biotin 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

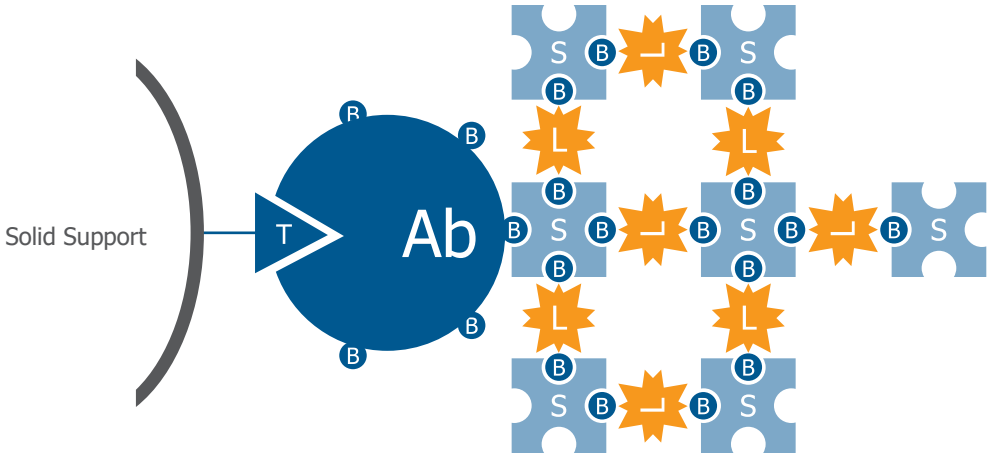
When preparing a reagent for biotin-labeling of proteins, it is advisable to conduct optimization assays under the system of study. This is because the choice of an appropriate spacer and the right chemistry to bind biotin to the target protein(s) may have a profound impact on the outcome of the experiment. 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 after exposure to ultraviolet light.

7. PURIFICATION OF BIOTINYLATED PROTEINS

Since streptavidin-biotin complexes require strong denaturing buffers to break their interaction and release the biotin, purification of biotinylated proteins from immobilized streptavidin would also result in loss of native configuration and functional properties. For this purpose, consider using avidin analogs designed 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 streptavidin that facilitates recovery under milder conditions as well. It retains high-affinity specific binding at high pH, but interacts poorly at acidic pH values. Alternatively, biotin with cleavable spacers can be also used.

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 has been introduced with the goal of facilitating the release of biotinylated proteins after capture on immobilized streptavidin. These include disulfide bridges that can be broken by reducing agents like 2-mercaptoethanol or dithiothreitol and photocleavable linkers, which are readily available from different commercial sources. Each of these approaches, however, presents its own limitations and should be carefully optimized to particular experimental conditions.



The target (T) is bound to a solid phase, e.g. 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) e.g. 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.

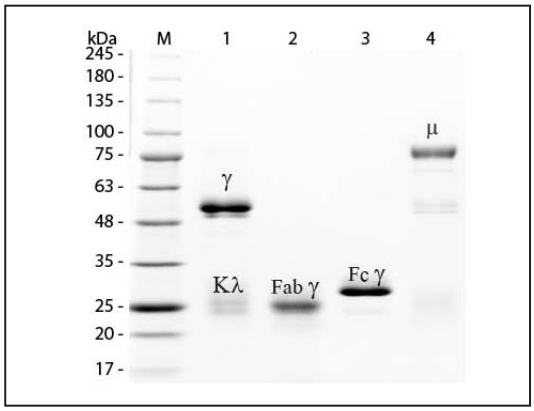
ANTIBODY PURIFICATION WITH IMMOBILIZED PROTEIN A & PROTEIN G

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, serum, and 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 antibodies as well as to human IgG_3. Under standard buffer conditions, Protein G binds to all human and all mouse IgG subclasses. Protein G also binds to rat IgG_2a and IgG_2b.
- 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 & 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 a binding buffer before sample application.
- Test for a flow rate that provides the most efficient binding during a 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, a 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 a target molecule and ligand, it may be beneficial to stop the flow after applying a 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 & 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; instead, 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 minutes to 2 hours 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 antibodies 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 an affinity purification step followed by a gel filtration step. Purity can also be improved by optimizing binding and elution conditions.

CULTURING HUMAN MELANOMA CELL LINES

Q: WHAT IS THE RECOMMENDED GROWTH TEMPERATURE FOR CELL SURVIVAL?

ANSWER:
The melanoma cells grow best at temperatures slightly less than 37°C—36.5°C. They'll grow at 37°C, but not at temperatures higher than 37°C. Lower is better.

Q: WILL THE CELLS DIE IF THEY ARE AT 37°C FOR A LONG PERIOD OF TIME?

ANSWER:
Depending on the melanoma cells, some will die off if the temperature is greater than 37°C and some will slow their growth. Usually they can be recovered, split, and transferred into a smaller dish, then increasing the amount of serum. It is possible to recover them.

Q: DO ALL OF THE CELL LINES REQUIRE THE SAME AMOUNT OF CO^2?

ANSWER:
Melanoma cells grow best at 5% CO^2, and that has to do with buffering the pH. The pH of the media should be slightly basic at 7.6, and if you keep the CO^2 at 5%, that will work best.

Q: SHOULD I BE WORRIED ABOUT MY MEDIA CHANGING TO BROWN?

ANSWER:
Melanoma cells are derived from melanocytes, which produce melanin. Some melanoma cells retain this capability and produce melanin that changes the pigment color or the color of the media to a brownish color. In some melanoma cells, the pigment remains in packages. If you look under the microscope, you'll see a lot of brown specs among your cells. That is perfectly normal.

Q: WHAT TYPE OF CELL CULTURE MEDIA DO THESE CELL LINES REQUIRE?

ANSWER:
Our melanoma cells grow best in tumor 2% media, that's what they were derived in. Also, calcium is very important.

Click here to watch "Tips for Culturing Human Melanoma Cell Lines"

Q: WHAT IS THE RECOMMENDED SEEDING DENSITY PER FLASK?

ANSWER:
Melanoma cell lines are very unique. Some grow fast and can be split by 1:16, giving you a confluent flask in a week. Other melanoma cells should be kept at a high density, otherwise they start to senesce. These would need to be split by 1:2 or 1:3 and would take 2–3 weeks before they're confluent again. Since they are all very unique, there is no general recommendations for all of the melanoma cells that would hold.

Q: DO ANY OF THE CELL LINES REQUIRE SPECIAL ATTENTION?

ANSWER:
There are some melanoma cell lines that require special care. A couple of cell lines will require insulin and there are some that are BRAF inhibitor resistant that need to be cultured with the BRAF inhibitor. If the BRAF inhibitor is removed they will revert to a sensitive phenotype.

Q: IS RECOVERY AN ISSUE WHEN TRANSFERRING THE CELLS FROM LIQUID NITROGEN STORAGE?

ANSWER:
When thawing the melanoma cells, each line has a different viability. The more pigmented a cell line is, the worse the viability and a longer recovery. Because most of the cells are dead, plate them in a small flask to start. Be sure to change the media the following day and watch them. You may need to cut back and split them into a smaller flask because of cell death. Those lines are sometimes more difficult to get started but once they're growing, they grow great—they just don't like thawing process.

Q: WHAT CAN BE DONE TO VERIFY THE INTEGRITY OF THE CELLS?

ANSWER:
If your cells are no longer responding in an experiment the way they have before, the first thing to check is mycoplasma. That will alter a cells response in an experiment. The second thing to check is to see if you are working with the same melanoma cell line as you thought you were, and that would be by performing DNA fingerprinting. The DNA fingerprint profiles are available online; you can compare the original results with the ones that you receive.

Q: ARE THERE PAIRS OF CELLS AVAILABLE FROM A SINGLE PATIENT?

ANSWER:
We have cell lines that match a patient's primary metastasis, while we also have ones that match several metastasis stages. We've been producing melanoma cell lines for 40 years, so we don't have extensive clinical data on some of these. In some cases, we don't know whether the metastasis is a secondary occurrence from the same site or if it's from another spot on the body, but either way you can still use those to look at heterogeneity.

|COLLAGEN

Collagen represents a large family of proteins that are the essential components of the extracellular matrix, the chief structural component of skin, and the most abundant protein in the human body, representing approximately 30% of the total dry weight. Collagen is an attractive substance for various medical applications, such as implants, transplants, organ replacement, tissue equivalents, vitreous replacement, plastic and cosmetic surgery, surgical suture, and dressings for wounds and burns among others.

Based on their supramolecular structures, the collagens are divided into two main classes, fibril-forming collagens (type I, II, III, and V) and non-fibril-forming collagens (type IV and VI). Different collagen types are necessary to confer distinct biological features to the various types of tissues in the body.

1. COLLAGEN STANDARDS

Collagen proteins exhibit an amino acid composition rich in glycine, proline, and hydroxyproline residues. Because of the relatively high sequence similarity between different types of collagen, it is necessary to choose collagen standards with native 3D structure instead of denatured structure. Under physiological conditions, only the native collagen networks can self-assemble in a highly type-specific, three-dimensional architecture to entrap other ingredients, and be expected to function as cellular scaffolds for tissue engineering. The use of properly folded type-specific collagen that exhibits native tertiary structure is recommended as a control for functional assays and testing specificity of anti-collagen antibodies.

2. COLLAGEN EXTRACTION

Collagen is insoluble in organic solvents. Water soluble collagen represents only a small fraction of total collagen and it depends on the age and type of tissue extracted. Limited digestion using proteolytic enzymes such as pepsin is a convenient method to extract collagen, although the resulting collagen could be partially digested. Continuous refrigeration throughout collagen extraction is paramount to avoid degradation and denaturation.

3. COLLAGEN-BASED ASSAYS

In general, collagen-based assays require special attention to buffer pH, temperature, and concentration. Acidic pH is critical to maintain collagens' stability and solubility. Since collagens are usually more unstable than other proteins, assays should be designed to perform at temperatures <10°C. Optimal concentrations of collagen are needed to obtain reliable measurements, but if it's too high it can result in spontaneous polymerization (gelatin formation) commonly occurring with collagen. An example of an ELISA assay for collagen can be found here.

4. TESTING COLLAGENS BY SDS-PAGE & WESTERN BLOT

The best separation of collagen by electrophoresis requires the addition of detergents as well as reducing chaotropic agents at optimal concentration. This ensures maximal resolution of the characteristic chains allowing for unambiguous identification of specific collagen types. Due to the variable size of different collagen antibodies, a 6% acrylamide gel can be used for collagens I, II, and III, while a 10% acrylamide gel is more suitable for collagens IV, V, and VI.

When performing Western blot for collagens it is highly recommended to use standards as positive and negative controls for the collagen type to be measured along with a validated antibody for this application. The quality of the antigen is important for good results and therefore attention to collagen extraction, sample preparation, and denaturing SDS-PAGE as described above is critical.

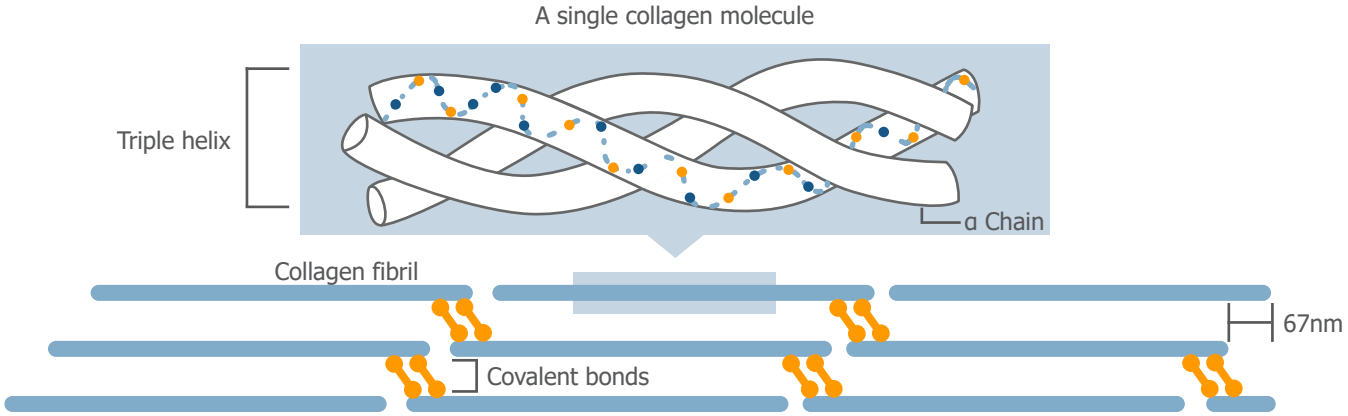
5. COLLAGEN SYNTHESIS & DYNAMICS

Collagen is first synthesized as a larger precursor molecule called procollagen which contains additional peptides at both ends. An important, early post-translational modification of collagens is the hydroxylation of selected proline and lysine amino acids in the procollagen protein. Other modifications include glycosylation by the addition of galactose and glucose. As the procollagen is translocated to the cell surface, specific enzymes called procollagen proteinases remove both of the extension peptides from each ends of the molecule.

6. COLLAGEN CROSSLINKING

In the extracellular spaces, crosslinking in collagen takes place as the triple helical collagen molecules line up and begin to form fibrils and then fibers. This is thought to affect the mechanical properties of collagen fibers-composed tissue such as muscle or bone. The degree of cross-linking can affect the yield of collagen during extraction and also affect the exposure of some epitopes, thus requiring further optimization of antigen retrieval methods when performing immunohistochemistry. A useful method to determine the extent of collagen cross-linking in a tissue is using specific dyes like picrosirius red.

THE STRUCTURE OF A TYPICAL COLLAGEN MOLECULE:



The top panel represents a single collagen molecule, which always consists of three protein chains. Each chain is an alpha helix, and in type I collagen molecules there are two alpha 1 chains and one alpha 2 chain, which is abbreviated as [α1(I)]2 [α2(I)]. The three protein chains (wound into a triple helix) make up one collagen molecule. The red line shown in one of the strands of the triple helix represents the alpha helical winding of the peptide backbone of that peptide chain. As shown in the bottom panel, each triple-helical collagen molecule is 300 nm long and forms a rod. Collagen molecules spontaneously assemble in a head-to-tail alignment with a small gap that separates the 'head' of one molecule with the 'tail' of the next molecule in a staggered side-by-side arrangement. Adjacent collagen molecules are displaced about 67 nm, or slightly less than one-fourth of the length of a single molecule. The side-by-side interactions are stabilized by covalent bonds (red bars) between the N-terminus of one molecule and the C-terminus of an adjacent molecule.
[Adapted from Mathews CK, Van Holde KE, Ahern KG. Biochemistry. San Francisco, CA: Benjamin Cummings, 1999.]

| ELISA KITS

Rockland offers a wide variety of ELISA kits in sandwich assay formats. This extensive portfolio includes over 800 complete, ready-to-use ELISA kits for detection of specific growth factors, cytokines, chemokines, hormones, glycoproteins, and neurotrophic factors that are common targets of research interest. Each enzyme linked immunosorbent assay is rigorously tested to ensure high precision, accuracy, sensitivity, and specificity. Each ELISA kit is optimized for accurate quantitation of targets in various sample types, including serum, plasma, saliva, cell lysates, and cell culture supernatants. In order to get the best performance from your ELISA kits, we suggest the following:

1. KIT COMPATIBILITY

When choosing an ELISA kit, it is important that you understand whether a given kit is known to be compatible (or incompatible) with the nature of your sample (matrix). As kits are developed, their performance is evaluated in different matrices and often reported in the instructions manual and other accompanying documentation. Make sure you understand how well the ELISA kit has been characterized in the matrix that is relevant to your samples (serum, plasma, tissue culture, urine, etc.). The fact that a kit has not been tested in your matrix of interest does not mean it will not work, but rather that additional validation assays would need to be run in order to determine its performance.

2. HAVE A THOROUGH UNDERSTANDING OF THE ASSAY BEFORE STARTING

Although most commercial ELISA kits follow the same sandwich ELISA format, differences in a range of parameters including the reporter system, type of conjugates (detection antibody), recommended incubation times, and temperatures, are not unusual among kits detecting different targets, even when they come from the same manufacturer. It should not be assumed that because a particular brand of ELISA kit against one target has been used before, the same conditions will apply to a different kit of the same brand against another target. Because the nature of each analyte and the reagents used is unique to each target, it is imperative that you review and understand in advance each particular protocol. Prepare accordingly and follow all directions carefully.

3. REPRODUCIBILITY

Once you fully understand the proper handling and the optimal conditions and parameters for your ELISA kit, follow these steps to avoid irreproducibility issues:

- Before running the assay, allow about 30 minutes for all the kit reagents to reach room temperature. Frozen samples should also be allowed to thaw completely before being used and repeated freeze-thaw cycles should be minimized, less than three times is preferably.
- Make sure environmental conditions including temperature and humidity are kept constant throughout the assay and between assays.
- Work only with properly calibrated equipment including pipettes, plate washers, and readers.
- Always confirm proper aspiration, withdrawal, and addition of reagents by direct visual inspection of tips and wells throughout the assay.
- Always use freshly prepared substrate solutions and do not hold them for prolonged periods of time.

Altogether, these recommendations should favor more consistent binding kinetics and color development between assays of the same type.

4. ACCURACY

In addition to having a properly generated standard curve with replicates, samples should also be tested in at least duplicate or triplicate, if possible. Whenever possible, prepare samples at several dilutions to ensure at least one of them will follow within the linear range of the assay. Samples with optical densities (OD) above or below the linear range of the standard curve will result in target concentrations to be underestimated or overestimated, respectively. Follow these guidelines every time you use your ELISA kit to result in improved accuracy of quantification for the collagen type that's measured along with an antibody validated for this application. The quality of the antigen is important for good results and therefore attention to collagen extraction, sample preparation, and denaturing SDS-PAGE, as described above, is critical.

5. STANDARD CURVES

ELISA kits require the generation of a standard curve for quantification of the target molecule. Because assays can never be identical but rather kept within acceptable range of variation, the accuracy of your data will result with a standard curve obtained under the same conditions your sample is measured. This means that a standard curve should be produced for each set of samples assayed. Avoid using the standard curves generated from other plates or other days. Make sure the standard curve is generated within the dilution range recommended by the manufacturer and that you have enough data points along such range. Run standards in duplicates or triplicates.

6. MAXIMIZE YOUR SAMPLES

Valuable samples are often available only in limited quantities and the ideal situation of running multiple dilutions in duplicates or triplicates is not feasible. In this regard, it is advisable not to use all your samples (especially the most valuable) until you have run a couple of preliminary assays—one including standard curves and one or two control samples at several dilutions that will give you a more reliable idea of the optimal dilutions to use when running your real samples. Always plan in advance the layout of your plate, keeping in mind the number of samples and dilutions to use and decide the most effective way to run your assay. This will maximize not only your samples but also the use of your kit.

7. CROSS CONTAMINATION

Avoid cross contamination of samples or reagents by changing tips between samples, standard and reagent additions, and using new disposable reagent reservoirs for each transfer. When planning your experiments as described before, determine the amount of reagents to be used in the planned assay to ensure you will only prepare enough reagent as required and avoid having excess. You will have to make sure is discarded appropriately and never returned back to stock containers. Similarly, avoid reusing any wash or reagent reservoirs or trays.

8. DATA ANALYSIS

Make sure you understand how data should be used for calculations. ELISA kits usually indicate how correction factors for background should be applied, how to plot the data, and the type of integration required to generate an appropriate standard curve. Once you decide the best way to run your assay and plate layout, prepare in advance a spreadsheet template that can generate your standard curve automatically and calculate sample protein concentrations.

Application 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. The following pages share useful tips for successful results.

1. DETERMINE THE BEST RATIO OF TARGET PROTEIN & 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 (e.g. 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/cm^2 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 & GELS ON TRANSFER SOLUTION

Always remember to equilibrate the membrane 10–15 minutes in transfer buffer before transfer since PVDF membranes are hydrophobic and will not wet from just being placed into transfer buffer. First immerse for two seconds in 100% methanol, then equilibrate 10–15 minutes 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 minutes 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%–5.0%. 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 4°C 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. It should not be extended more than three hours since it could 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.

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, (TrueBlot®), which avoids such interference and provides clear, unambiguous protein detection. Also, studying 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 & 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 may 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. streptavidin-biotin interactions cannot be dissociated by this method but the whole complex can be removed away from the bound protein on the membrane.

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 (IHC), 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 impossible, the best alternative is to use two antibodies raised against different epitopes of the antigen of interest, thus 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 IgG antibodies) 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 IgG antibodies 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 the 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 AlexaFluor™ 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

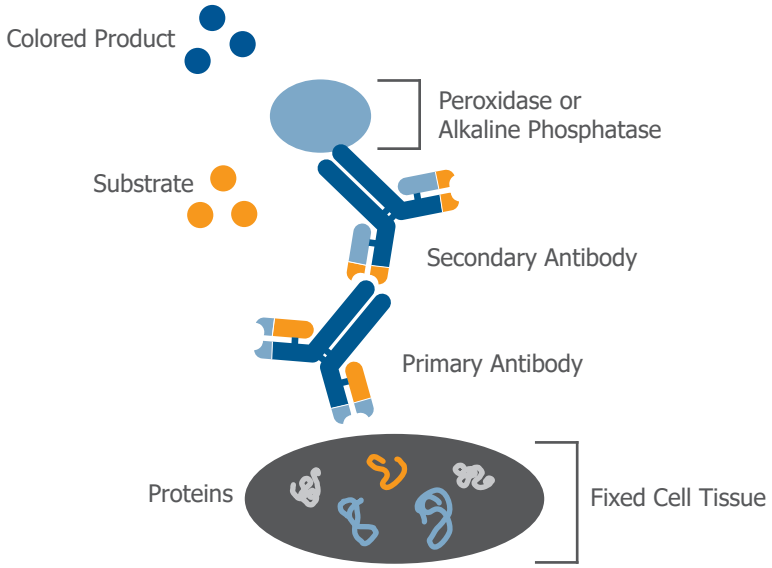
Relative to antibody specificity, influence of tissue preparation is more versatile and complex and it can result in both, false-positive and false-negative results, even when using highly specific and well-characterized antibodies. These effects arise mainly from epitope masking due to fixation-induced 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's 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 a pre-blocking step.

IMMUNOHISTOCHEMISTRY SCHEMATIC



IHC SAMPLE PREPARATION

The antigen is fixed and adhered to a glass microscope slide. Formaldehyde fixation is suggested as the routine initial method of choice for tissue and cell fixation, but other methods are also suitable. Background staining is reduced with a blocking buffer to block non-specific sites to which the antibodies may bind. Rockland offers common blocking buffers including BSA, normal serum, non-fat dry milk, and an optimized proprietary blocking buffer.

IHC SAMPLE LABELING

Labeling is achieved by the reaction of a primary antibody with the immobilized antigen to form an antigen-antibody complex. A second biotinylated antibody specific for the primary antibody host species reacts with the complex. Streptavidin conjugated to the enzyme peroxidase or alkaline phosphatase reacts with this complex. Finally, substrate (view substrates) is added, causing a reaction with the enzyme causing colored precipitate to form on the slide at the location of the antigen. The slide is viewed through a standard light microscope and photographed. A common manipulation in IHC fixing is use of the antigen retrieval technique, a simple method of heating or boiling paraffin-embedded tissue sections to enhance the observed signal.

1. REDUCING NOISE FROM YOUR FLUORESCENT SIGNAL

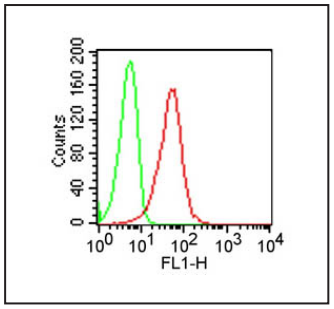
A critical step during optimization of flow cytometry experiments is reagent (antibody) titration. For optimal results, you must determine the minimum amount of antibody required to achieve antigen binding saturation. This will help you improve the specificity and intensity of your fluorescent signal 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 the 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 because they will help to determine the specificity of the antibodies used.

3. OPTIMIZE PERMEABILIZATION & 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 (e.g. 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 IgG_2a 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 effects on the intensity of your fluorescent signal and can be prevented by:

- Working with aggregate-free antibody solutions
- Keeping 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 Fab fragments.

FEATURED ISOTYPE CONTROLS FOR FLOW CYTOMETRY

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

| IMMUNOFLUORESCENCE MICROSCOPY

BASIC DEFINITION

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 well 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 fluorescent-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 & 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 that 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 a secondary antibody only and include cells lacking the antigen of interest whenever possible to determine the threshold of background signal. 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. USING THE APPROPRIATE ANTIBODY DILUTION

It is possible to improve staining by adjusting the antibody dilution. Usually, 1 µg/mL of purified antibody or 1:100–1:1000 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 & 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 generally 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. USING 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 a 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 DyLight™, 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 minute 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 30 seconds 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. This way, 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 most representative pattern observed along the whole sample.

| CHROMATIN IMMUNOPRECIPITATION

BASIC DEFINITION

Chromatin immunoprecipitation (ChIP) is a powerful technique that determines protein interactions at particular regions of DNA 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 also be 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 IP 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 a specific peptide. If working with an epitope-tag protein, you can also do a 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. USING 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. USING 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 (PTM) 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. OPTIMIZING 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. CONFIRMING PROTEIN ENRICHMENT OF YOUR TARGET SEQUENCE

When working with your ChIP data it is always advisable to compare the obtained enrichment of an immunoprecipitated 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 recognizing more epitopes or using an epitope which is expected to be rather inert.

9. SHORTENING 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 2 hour step, and shortening the antibody incubation time during IP to 15 minutes when performed in an ultrasonic bath.

10. VERIFYING 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

BASIC DEFINITION

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.

The following elements describe the factors that have a greatest 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. PRE-CLEARING LYSATES

Because lysates are complex mixtures of proteins, lipids, carbohydrates, and nucleic acids, non-specific binding to the IP antibody or beaded support will occur and negatively affect the detection of the immunoprecipitated proteins. In most cases, pre-clearing 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 (e.g. coupled secondary antibodies) or the beads themselves. Another pre-clearing 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. 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 a 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 & 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 antibodies 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, the 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 blot detection, elution of proteins directly in reducing SDS-PAGE sample buffers 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 buffers 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. 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 recognizes primary antibodies in their native (non-reduced) state, and thus eliminates the detection of the denatured primary heavy and/or light chains during Western blotting.

BASIC DEFINITION

Enzyme-Linked Immunosorbent Assay (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 and peptides, and even small molecules.

Below you will find some important recommendations and useful steps 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. This is 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 also be used to quantify antigens or cross-reactivity between secondary conjugated antibodies and antigens.

2. 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 ASSAY PERFORMANCE 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. CHOOSE A BLOCKING BUFFER

This is a critical step 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

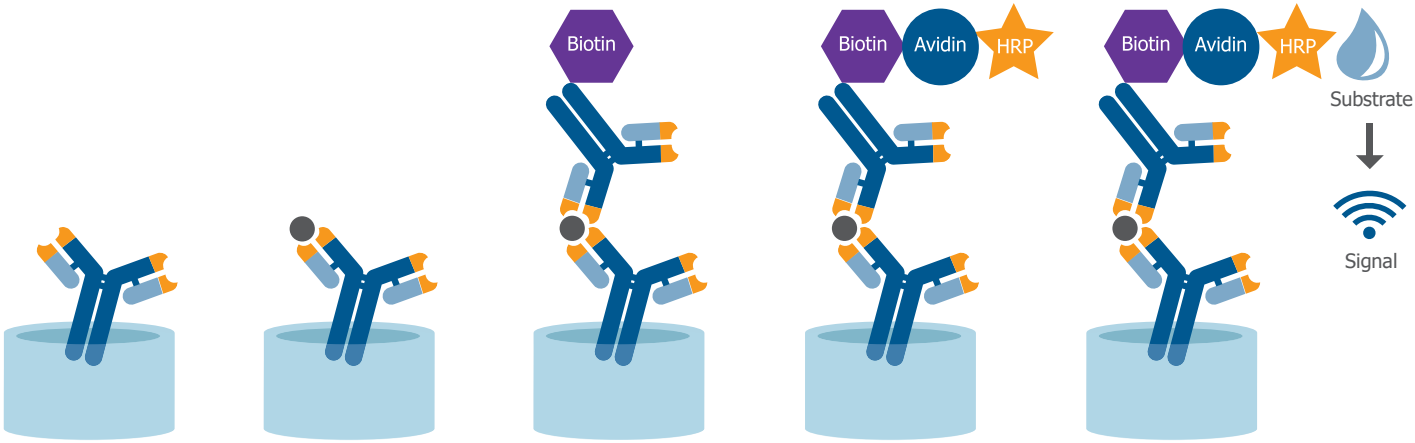
Different conjugates will provide different ranges of signal depending on how much antibody they will bind. Because the goal is to bind the entire antigen-antibody complex present in each well, you want to make sure you will use the right amount of conjugate in your assay by performing a serial dilution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

6. ADJUST INCUBATION TIMES TO IMPROVE ASSAY PERFORMANCE

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 OPTIMIZED, KEEP CONDITIONS UNMODIFIED

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.



| TRUEBLOT® IMMUNOPRECIPITATION & 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 (horseradish 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 artifact can be circumvented by using conjugated secondary antibodies that 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.

Click here to watch "TrueBlot® Antibodies - Tutorial for IP / Western blot"

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. PRE-CLEARING 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.

3. CHOOSING IMMOBILIZED BEADED SUPPORT

Choosing immobilized beaded support for the precipitation step is critical to the success of the procedure. We recommend using 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 non-specific 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 IgG_1 and most subclasses of rat and human IgG antibodies, whereas Protein A has a much higher affinity for mouse IgG_2a, IgG_2b, and IgG_3.

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, needs 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. While polyclonal antibodies are generally the 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 needs 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 & ELUTION BUFFERS

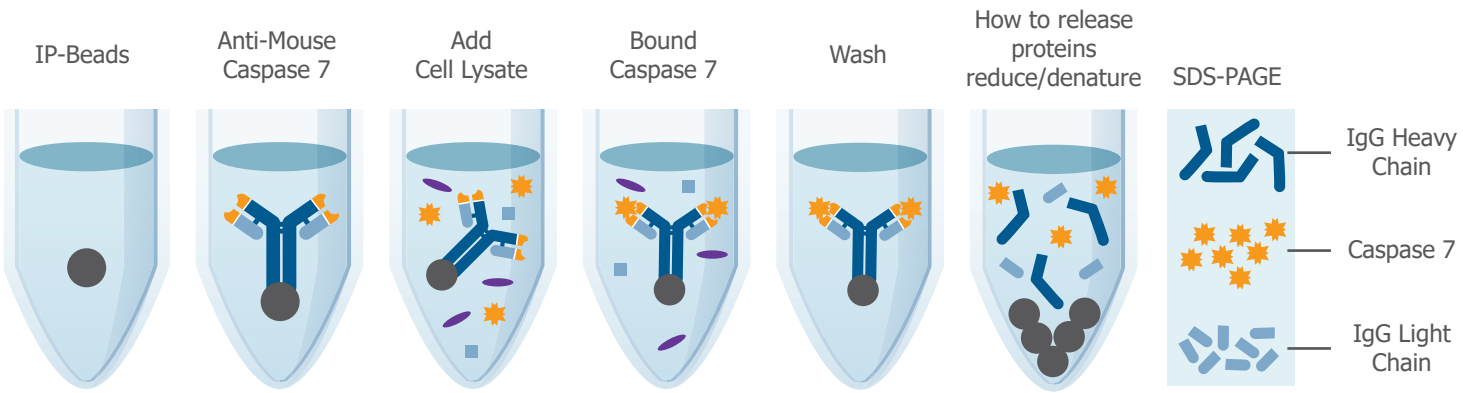
When selecting a wash buffer for an IP experiment, it is essential to create conditions in which the desired specific protein interactions are retained but non-specific protein binding is prevented and washed away. If a particular IP experiment is afflicted with troublesome amounts of background, experimental 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 blot 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, non-denaturing elution buffer for immunoprecipitation is 0.1 M glycine at pH 2.5–3.0. The low pH condition dissociates most antibody-antigen interactions (as well as the antibody-Protein A/G interaction).

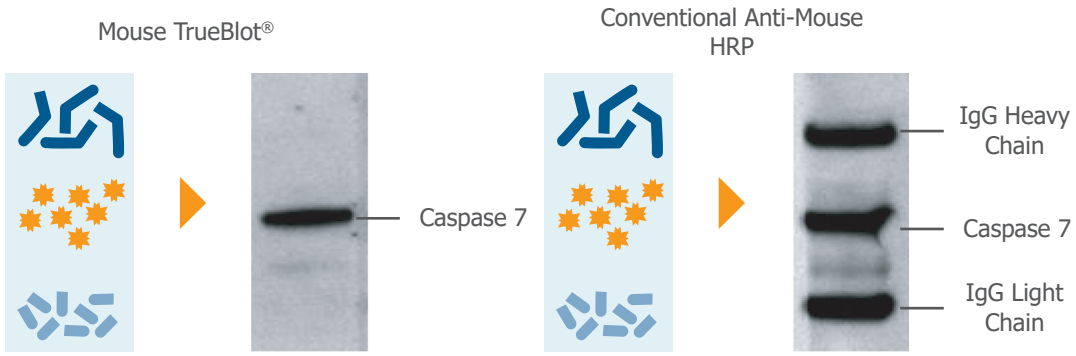
6. BLOCKING BUFFER

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.

USE IN CONVENTIONAL IP



USE IN WESTERN BLOT



| OPTIMAL SDS-PAGE SEPARATION

BASIC DEFINITION

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate mixtures of proteins of variable complexity and allows you to determine sample protein composition, purity, whether you have a target of interest in your sample, and even some of its structural characteristics. Gel electrophoresis is a basic tool in itself, but also is the stepping stone to other important techniques in the lab going hand in hand with Western blotting (WB). You can't have a good Western blot without first having a good SDS gel. In this particular tips segment, we are discussing protein gel electrophoresis using precast gels and the parameters involved. Below are some pointers for optimizing your SDS-PAGE results.

1. SAFETY FIRST

Please remember that typical voltages and currents used in electrophoresis are dangerous and possibly lethal. Make sure that all cables and connections are routinely inspected for cracks and other defects from normal wear and tear that result in exposed wires and replace immediately. Avoid inserting or removing high-voltage leads with both hands to prevent shunt electricity through the body and make sure to always handle those when the power supply is turned off. When disconnecting the system, first wait until the voltage and current meters reach zero, turn off the power supply and then disconnect the gel box.

2. CHOOSING THE RIGHT GEL

Choosing the correct percentage gel is key. Rule of thumb, the larger the protein the lower percentage gel and vice versa. With that in mind, what is the size of your target? If you know your target's molecular weight then you can get away with one percentage, but if you don't, gradient gels are better for this purpose. Commonly used are 4–20% gradient gels that can cover a vast range of molecular weight sizes. Proteins ≥ 200 kDa will resolve better in 4–8% gels.

3. HOW MUCH TO LOAD

Consider the concentration of your purified protein, lysate, or culture sample. Ideally, it is best to load ≤2 µg per well of a purified protein or ≤20 µg of a complex mixture like whole cell lysates if you are doing Coomassie stain only. Protein loading can be adjusted accordingly for more sensitive stains like silver and fluorescent staining or when doing WB where you can do lower amounts. Loading too little will result in an incomplete picture of your target such as any contaminants or accurate detection of your target protein. Overloading can result in smears or streaks due to protein precipitating or aggregating. In general, improper loading will lead to inconclusive results.

4. TIMING

After samples are loaded, you want to begin to run your gel immediately after. Not doing so will result in your samples diffusing out of the wells. If you need to leave for a short amount of time, you can run your gel at low voltage to keep your samples from diffusing until your return. Now as for actually running your gel, follow manufacturer's recommendations of gel apparatus and pre-cast gel instructions. Typical conditions include runs at 100–150 volts for 40–60 minutes or until the dye front has reached the bottom of the gel. Letting it run too long will result in losing your lower molecular weight bands. Running too short and you will have poor resolution, especially in the low molecular weight range.

5. HEATING SAMPLES DURING DENATURATION

Although not all samples need heating, it is critical for certain preparations like those containing membrane proteins. Thoroughly heating your sample not only completes the denaturing process but also ensures the dissociation of hydrophobic interactions, like those

involving lipids. Not heating long enough results in incomplete denaturing, heating too long can cause aggregation. In general, heating at 95°C for 5 minutes should suffice. Following the denaturation step, spin down of samples at max speed for 2–3 minutes is critical to separate any particulates or aggregates that would otherwise interfere with gel loading and electrophoretic separation.

6. TO REDUCE OR NON-REDUCE

When considering protein molecules, which are built up of complex tertiary, secondary, and primary structures, we want to denature our samples to its relative simplest structure for electrophoresis. While SDS helps to break these structures it also confers a negative charge to the amino acids giving a constant charge to mass ratio to all proteins and allowing separation to occur based on size. Nevertheless, SDS works well for secondary/tertiary structures but for more complex structures like quaternary, we need to apply DTT (dithiothreitol) or BME (2-mercaptoethanol). These reducing reagents break disulfide bonds which are covalent and do not normally break using SDS alone. Both reducing agents work well, advantage of DTT is it has less of an odor as BME, but breaks down faster. You can make your sample buffer with BME and have it freeze, thaw over and over due to its stable properties. If you are looking to analyze molecular weight complexes then you would be performing a non-reducing electrophoresis and would not use reducing agents.

7. PROTEIN PROPERTIES

One must consider the protein's properties when preparing the sample for SDS-PAGE. Typically, you add your sample of known concentration to some amount of SDS sample buffer which is typically made up of SDS, glycerol, Tris-HCI pH 6.8, EDTA with or without reducing agent. In general a 2X buffer stock can be used unless you have a much diluted sample, in this case would be beneficial to use a more concentrated stock (e.g 5X or 6X) that allows you to load more samples in the same final volume. Proteins can also be further concentrated using trichloroacetic acid (TCA), acetone, or methanol at the expense of some net loss. Depending on the gel's well size, you want to keep volume in mind, leading up to our next tip.

8. GEL LOADING

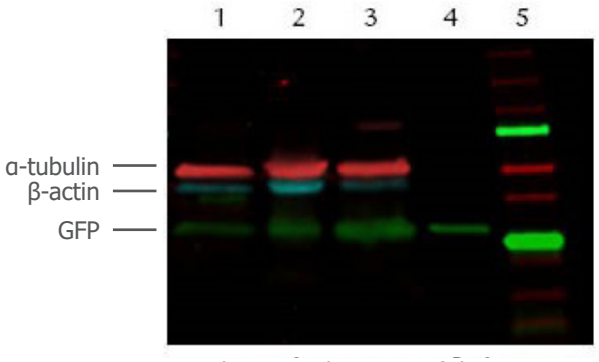
When loading your gel, it may be easier to use your standard tips that you use on a daily basis. However, by using gel loading tips you actually have more control of your sample. The tip is designed to go into the gels with narrow wells. Gel tips allow dispensing your sample accurately and precisely without the risk of overflowing (and cross-contaminating) into the next well. Always run gel electrophoresis markers along with your samples since these are not only necessary to determine accurately protein sizes but also serve as a control for the separation.

9. GEL TEMPERATURE

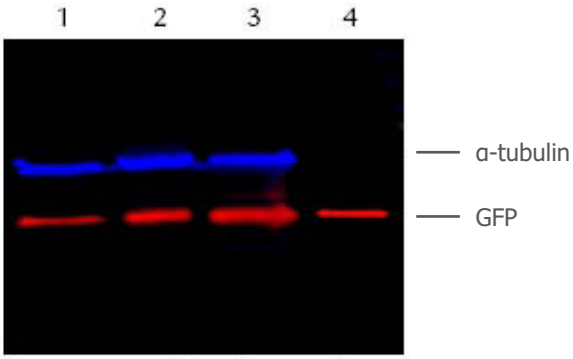
Maintaining a constant temperature between 10°C–20°C during separation is paramount in obtaining even migration of proteins. Otherwise, outer lanes will move slower than center lanes resulting in artifact known as "smiling". To prevent this negative effect, you can ensure efficient heat transfer by completely filling the outer chamber of the electrophoresis apparatus with SDS running buffer and constantly stirring the buffer with a magnetic stirrer. Alternatively, heat can be reduced by lowering the running current during separation.

| MULTIPLEX FLUORESCENT WESTERN BLOTTING

Fluorescence-based multiplex Western blot is suited for simultaneous detection and quantification of specific proteins in a biological sample. Using a combination of two or three antibodies, fluorescent detection enables simultaneous quantitative analysis of multiple proteins within the same sample on the same blot. The fluorescent dyes, when conjugated to secondary antibodies, offer a variety of benefits over traditional colorimetric and chemiluminescent detection methods. Fluorescent multiplexing reduces complete workflow by minimizing the requirement for multiple blots and allows for a more reliable quantitation of multiple proteins. Other benefits of fluorescent Western blotting include increased sensitivity, excellent signal stability over time as well as precise quantitative analysis. Multiplex fluorescent Western blotting also reduces or eliminates the need to strip and re-probe. In addition, due to their exceptional photostability, fluorescent dye conjugates can be archived and visualized several times without a decrease in signal. Two or three-color detection requires careful selection of primary and secondary antibodies.



Simultaneous detection of three proteins (α-tubulin, β-actin, and GFP on a single blot using Rockland DyLight™-labeled secondary antibody conjugates.



Simultaneous detection of α-tubulin and GFP on a single blot using Rockland DyLight™-labeled secondary antibody conjugates.

Guidelines

- Optimize primary and secondary antibody concentrations by incubating the membranes in several dilutions of each antibody. Select the dilution that yields the highest signal-to-background ratio.
- Use primary antibodies from distantly related host species such as mouse, rabbit, or chicken to minimize non-specific binding when using in direct detection. Avoid using primary antibodies derived from mouse and rat together. Cross-reactivity may occur because these species are so closely related.
- Before combining primary antibodies in a two or three-color experiment, always perform separate preliminary blots with each primary antibody to determine the expected banding pattern and possible background bands. The more proteins combined on a blot, the more complex optimization becomes. Single target detection helps determine the banding pattern of each antibody prior to multiplex analysis.
- For the primary antibodies that are derived from host species (for example, mouse and chicken), fluorescent dye-IgG secondary antibodies derived from the same host and labeled with different fluorophores must be used (e.g. DyLight™ 800 Anti-Mouse (GOAT), Dylight™ 488 or Dylight™ 649 Anti-Rabbit (GOAT)).
- Use highly cross-adsorbed secondary antibodies. Failure to use highly cross-adsorbed antibodies may result in cross-reactivity.
- Use fluorophore conjugates with non-overlapping emission spectra to avoid cross-channel fluorescence or bleed-through. Due to higher wavelength and lower auto-fluorescence, optimal detection of lower abundance targets may be achieved by detecting targets in the 800 channel, with normalization in the 649 channel.
- To minimize auto-fluorescence, substitute standard PVDF membranes for low fluorescence PVDF membranes. These membranes increase signal-to-background ratios per channel for transferred proteins, producing bright clean bands.
- Use high quality reagents and buffers. Filter sterilize all buffers as particulates in buffers can settle on membranes and create fluorescent artifacts.
- Run the dye front sufficiently far away from the expected protein size as the bromophenol blue in loading dye has the tendency to fluoresce.
- Use a clean, dust free, incubation box for each step of the fluorescent Western blotting procedure.
- To minimize fluorescent artifacts, avoid using inks to mark membranes, don't handle while wearing powdered nitrile gloves, and make sure to be gentle when handling membranes.
- While there's no risk to photo-bleaching fluorescent-labeled antibodies in working daylight conditions, all fluorescent-labeled antibody stocks should be stored in the dark.

GLOSSARY OF TERMS

Antibody
Avidin
Biotin
Blocking Buffer
ChIP
Collagen
Controls
DyLight™
ELISA
Enzymes
Epitope Tag
Flow Cytometry (FACS)
Fluorescent Dye
Fragmentation
Horseradish Peroxidase (HRP)
Immunoblotting
Immunocytochemistry
Immunofluorescence Microscopy (IF)
Immunohistochemistry (IHC)
Immunoprecipitation (IP)
In Vitro Diagnostics
Isotype
Monoclonal Antibody
Polyclonal Antibody
Primary Antibody
Proteins and Peptides
Reagent
Recombinant Antibody
SDS-PAGE
Secondary Antibody
Streptavidin
Substrates
TrueBlot®
Western blot (WB)

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