3rd Edition

An Introduction to Antibodies and Their Applications

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An Introduction to Antibodies and Their Applications

Merck Millipore – your partner in Life Science research.

At Merck Millipore, our commitment to advancing scientific research defines us.
We understand the challenges faced by today's researcher and know the importance of research tools (**antibodies, proteins, enzymes, inhibitors, and other reagents**) for research, drug discovery, and publications. This technical guide on the theory and practical use of antibodies in biological research is a part of our continuing commitment to provide useful information and exceptional service to researchers.

The 3rd edition of **An Introduction to Antibodies and Their Applications** provides a concise overview of some of the key features for the use of antibodies and immunochemical techniques in biological research. This handy reference guide supplements the techniques described in literature, recorded in general laboratory procedures, and described on individual product data sheets. Antibody design, development, and production are our expertise. Stringent validation of our antibodies is only one component of a comprehensive process we undertake to provide the antibodies most cited by the research community (see section Antibody Quality on page 2 for an in-depth look at our expertise).

As every antibody and experimental design is unique, these general principles and suggestions should not be interpreted as applicable to all situations, but rather as an additional source of information. As always, individual assays must be optimized empirically and antibody titers must be established for every unique batch of antibody.

Whether you are a veteran researcher or just beginning your research career, we hope that you will find this guide to be useful in your research. Your suggestions and comments for further improvements are always welcome.

Merck Millipore products are among the best in the industry, and include the expertise of Chemicon®, Upstate®, Calbiochem®, and Novagen®. For more information about using any of our research products, including more than 10,000 antibodies and kits, or simply to get scientific advice for a variety of immunological applications, please contact us. In addition, you can find extensive general information and technical specifications on our website **www.merckmillipore.com**. Rest assured that our highly trained, exceptional customer and technical service support specialists are always available to support you in your research.

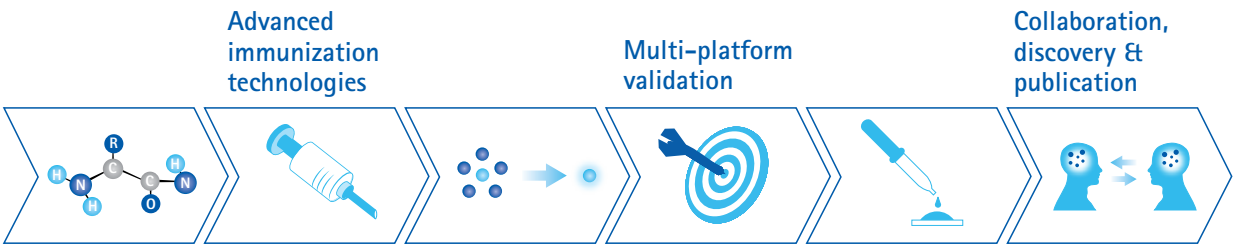
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A Note on Antibody Quality

It is often assumed that because specificity defines their function, antibodies must have some intrinsic high quality, which implies reliability and ease of use. Surprisingly, this is most often not the case. Although it is true that antibody binding to a specific amino acid sequence or conformational form can be theoretically modeled and is predictable in theory, decades of user experience has demonstrated that factors such as antigenicity and uniqueness of the immunogen sequence, antibody concentration, buffers, immunization and sample preparation techniques, contribute significantly to issues of cross-reactivity and nonspecific binding.

Since the early 1970s, when researchers began to use antibodies as protein probes, the problem of inconsistent antibody performance has plagued researchers. The pioneer developers and users of antibodies had fairly extensive knowledge of immunology to rely on. In today's research world, antibodies are used for an amazing variety of applications, from purification to blocking of cellular function. Today, antibody users range from classic medical anatomists to novice biochemists and biomedical engineers. Many users are unaware that immunogen design is fairly complex and that only certain, uncommon immunization protocols are robust enough to use the less immunogenic, but more specific peptides critical for generating a quality antibody.

Based on the combined strengths of Chemicon®, Upstate®, Millipore®, Calbiochem® and Novagen®, at Merck Millipore, we have decades of experience in innovative immunogen design, immunization, selection, screening, and validation to create many of our highly cited antibodies. Our commitment to produce high quality antibodies is based not only on innovation, but is also tempered with customer beta testing and feedback to the design/engineering team, prior to release. These efforts and collaborations have lead to the development of new validation techniques and novel antibody-based technologies, such as improved multiplexing and high resolution imaging flow cytometry. This cycle, from thoughtful design to field usage and report, is the basis of quality. Our technical and scientific background is what makes Merck Millipore a major designer and producer of quality antibodies, and not merely a distributor. Indeed, there is a whole lot of science in every vial of our antibodies.





The science inside your tube of Merck Millipore antibodies.

Antigen development and immunization

Our team of antibody research scientists continually monitors and reviews the latest publications and collaborates with leading research institutions to identify the most useful targets and antibodies. We design multiple immunogens, taking into consideration post-translational modifications, structure, cross-reactivity, and homology. We discuss target selection with leaders in key research areas, including neuroscience, cancer, epigenetics, and signaling research using their expertise to guide development and validation.

Monoclonal antibody development

Following immunization at our USDA-approved facility, we develop monoclonal and polyclonal antibodies using state-of-the-art processes. For monoclonal antibodies, we use a robotic cloning and screening system, which handles all the fusions and feeding steps for hybridomas. These highly automated processes use cutting-edge technology to ensure optimum throughput and maximum consistency. Using an array-jet automated microarray system, we test the fusion and identify positive clones. To ensure that the positive signal is from the antibody, we screen again using ELISA. Positives are screened using automated Western blotting of cells with endogenously expressed antigens. The pool of positives is repeatedly diluted to isolate the highest performer until our samples reach 100% clonality. This additional process is one way that Merck Millipore sets itself apart from the competition. With additional Western blotting, we select the best possible clone for further validation.

Polyclonal antibody development

Polyclonal antibodies are purified using fast protein liquid chromatography (FPLC) systems, using no column more than 10 times. Automated Western blotting is used to determine further purification steps. These new polyclonal antibodies are then validated in a number of applications and are also incorporated into kits and assays for Merck Millipore's protein and cellular analysis platforms.

Special validation

To support our multi-step, multi-application validation process, we have a tissue and blot library with over 1300 lysates, allowing us to precisely determine each antibody's specificity. At Merck Millipore, we have the advantage of having an entire cell analysis technology development team in-house. We validate antibodies for flow cytometry using our own guava easyCyte™ dual-laser microcapillary instruments. Similarly, our in-house bead-based immunodetection team helps us validate antibodies using the trusted Luminex xMAP platform. Our microarray system handles development and validation mainly for antibodies recognizing modified histones. Using confocal microscopes and high-throughput IHC instruments, we can obtain accurate data faster than manual imaging. Further scientific review determines whether staining patterns conform to published subcellular expression. For immunohistochemistry, we include negative controls, to confirm the signal.

Technical review

The final step in our process is a quality review carried out by an independent team of scientists. Before releasing the antibody to manufacturing, the team considers all antibody validation data, as well as data from scientists who participated in the beta testing. If our antibodies fail to meet our strictest specifications, they are discarded without question, even if they have hit initial targets. As an integral component of our quality process, only antibodies that pass this stringent review are made available to customers.

Customer experimentation and collaboration

Once produced and released for sale, we support customers' research efforts with a highly specialized team of technical support scientists and field engineers. We work closely with researchers to improve immunogen design and antibody performance. We have a growing network of beta testers to continue validation. And each and every one of our antibodies is backed by our 100% Antibody Performance Guarantee.

Merck Millipore antibodies are among the most cited, trusted, and highly validated on the market today. Their quality starts at inception and carries through manufacturing, production and distribution - into the lab and onto the bench. From start to finish, it's the science in every tube of Merck Millipore antibodies that assures confidence in the world's most reliable, defensible, and publishable antibody performance.

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Antibody Theory

1

1.1 An Introduction to Antibodies
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1.1 An Introduction to Antibodies

During the first half of the 20th century, a series of scientific discoveries resolved that antibody-mediated immunity is the cornerstone of the specific immune response. Since their first use as immunolabeling research tools in the early 1970s, antibody technologies have vastly improved, and antibodies have become critical tools for most areas of life science research. The basic principle of any immunochemical technique is that a specific antibody will combine with its specific antigen to generate an exclusive antibody-antigen complex. In the following pages we will discuss the nature of this bond, and the use of this robust and specific binding as a molecular tag for research.

1.2 Antigens

The term antigen is derived from antibody generation, referring to any substance that is capable of eliciting an immune response (e.g., the production of specific antibody molecules). By definition, an antigen (Ag) is capable of combining with the specific antibodies formed by its presence.

Generally, antigens are foreign proteins or their fragments that enter host body via an infection. However, in some cases, the body's own proteins may act as antigens and induce an autoimmune response. Bacteria and viruses contain antigens, either on their surface, or inside. These antigens can be isolated and used to develop vaccines.

Antigens are generally of high molecular weight, and commonly are proteins or polysaccharides. Polypeptides, lipids, nucleic acids, and many other materials can also function as antigens. Immune responses may also be generated against smaller substances, called haptens, if these are chemically coupled to a larger carrier protein, such as bovine serum albumin, keyhole limpet hemocyanin (KLH), or other synthetic matrices.

A variety of molecules such as drugs, simple sugars, amino acids, small peptides, phospholipids, or triglycerides may function as haptens. Thus, given enough time, just about any foreign substance will be identified by the immune system and evoke specific antibody production. However, this specific immune response is highly variable and depends much in part on the size, structure, and composition of antigens. Proteins or glycoproteins are considered as the most suitable antigens due to their ability to generate a strong immune response; in other words, they are strongly immunogenic.

Antigens are recognized by the host body by two distinct processes (1) by B cells and their surface antibodies (sIgM) and (2) by the T cell receptor on T cells. Although both B and T cells respond to the same antigen, they respond to different parts of the same molecule. Antibodies on the surface of B cells can recognize the tertiary structure of proteins. On the other hand, T cells require antigens that have been ingested and degraded into recognizable fragments by the antigen-presenting cells. Commonly employed antigen-presenting cells are macrophages and dendritic cells. The immune response is illustrated in Figure 1. For greater detail on the natural process of antibody production, a suitable immunology textbook should be consulted.

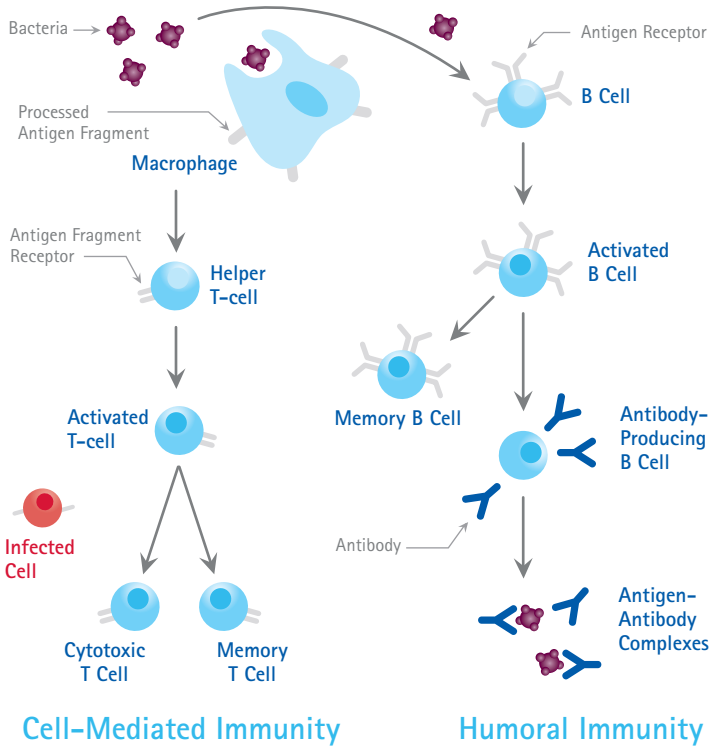


Figure 1.
The Immune Response.

Characteristics of a Good Antigen

- Areas of structural stability and chemical complexity within the molecule
- Significant stretches lacking extensive repeating units
- A minimal molecular weight of 8,000 to 10,000 Daltons, although haptens with molecular weights as low as 200 Da have been used in the presence of a carrier protein
- The ability to be processed by the immune system
- Immunogenic regions that are accessible to the antibody-forming mechanism
- Structural elements that are sufficiently different from those present in the host
- For peptide antigens, regions containing at least 30% of immunogenic amino acids: K, R, E, D, Q, N
- For peptide antigens, significant hydrophilic or charged residues

1.3 Epitopes

The small site on an antigen to which a complementary antibody may specifically bind is called an epitope or antigenic determinant. This is usually one to six monosaccharides or five to eight amino acid residues on the surface of the antigen. Because antigen molecules exist in space, the epitope recognized by an antibody may be dependent upon the presence of a specific three-dimensional antigenic conformation (e.g., a unique site formed by the interaction of two native protein loops or subunits). This is known as a conformational epitope. The epitope may also correspond to a simple linear sequence of amino acids and such epitopes are known as linear epitopes.

The range of possible binding sites on a target molecule (antigen) is enormous, with each potential binding site having its own structural properties derived from covalent bonds, ionic bonds, hydrophilic, and hydrophobic interactions. Indeed, this has important ramifications for antibody choice and performance. For efficient interaction to occur between the target antigen and the antibody, the epitope must be readily available for binding.

If the target molecule is denatured, e.g., through fixation, reduction, pH changes, or during preparation for gel electrophoresis, the epitope may be altered and this may affect its ability to interact with an antibody. For example, some antibodies are ineffective in Western blotting (WB) but are suitable for immunohistochemistry (IHC) applications, because, in the IHC procedure, a complex antigenic site might be maintained in the tissue, whereas in the WB procedure, the process of sample preparation alters the protein conformation sufficiently to destroy the antigenic site, and hence eliminates antibody binding.

Watch Out

A very important target, αVβ3 integrin will not work in Western blotting experiments because the epitope is formed by the proximal association of the αV and β3 subunits to each other—a conformation destroyed in the electrophoresis protocol.

In a denatured protein, only the linear epitope may be recognized. Hence, in protocols where a denatured protein is used, such as in Western blotting, an antibody that recognizes a linear epitope is preferred. Sometimes an epitope is on the interior of a folded protein. The epitope is then inaccessible to the antibody in a non-denaturing protocol, such as immunoprecipitation. A conformational epitope, by definition, is on the outside of the folded protein. An antibody that recognizes the conformational epitope is suitable for mild, non-denaturing procedures, such as immunoprecipitation or flow cytometry.

Optimally, an antibody that recognizes a linear epitope on the surface of a normally folded protein will work well in both nondenaturing and denaturing protocols.

Thus, the epitope may be present in the antigen's native, cellular environment, or it may be exposed only when denatured. In their natural form, antigens may be cytoplasmic (soluble), membrane-associated, or secreted. The number, location and size of the epitopes depend on how much of the antigen is presented during the antibody-making process.

Knowledge about the target protein, the epitope recognized by the antibody, sequence conservation, and the technique principles are valuable in making good antibody and protocol choices. Actual epitope mapping or sequence data, though useful, are not needed, however, to be confident in antibody specificity (see Publishing with Antibodies, section 2.7).

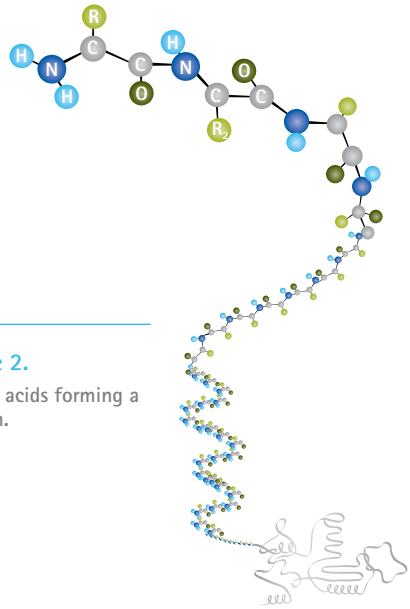


Figure 2.
Amino acids forming a protein.

1.4 Antibodies

An antibody is defined as "an immunoglobulin capable of specific combination with the antigen that caused its production in a susceptible animal." Antibodies are produced in response to the invasion of foreign molecules in the body. An antibody, abbreviated as Ab, is commonly referred to as an immunoglobulin or Ig. Human immunoglobulins are a group of structurally and functionally similar glycoproteins (82-96% protein and 4-18% carbohydrate) that confer humoral immunity.

Structure

Antibodies exist as one or more copies of a Y-shaped unit, composed of four polypeptide chains. Each Y contains two identical copies of a heavy chain and two identical copies of a light chain, named as such by their relative molecular weights. This Y-shaped unit is composed of the two variable, antigen-specific F(ab) arms, which are critical for actual antigen binding, and the constant Fc "tail" that binds immune cell Fc receptors and also serves as a useful "handle" for manipulating the antibody during most immunochemical procedures. The number of F(ab) regions on the antibody corresponds with its subclass (see below), and determines the valency of the antibody (loosely stated, the number of "arms" with which the antibody may bind its antigen).

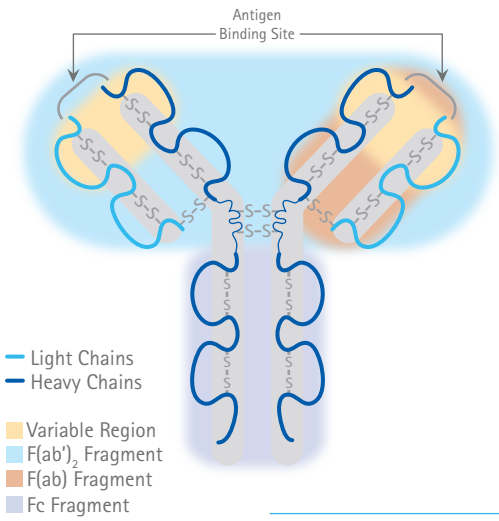


Figure 3.
Antibody Structure.

These three regions can be cleaved into two F(ab) and one Fc fragments by the proteolytic enzyme, papain, or into just two parts: one F(ab')2 and one Fc at the hinge region, by pepsin. Fragmenting IgG antibodies is sometimes useful because F(ab) fragments will not precipitate the antigen, and will not be bound by immune cells in live studies because of the lack of an Fc region.

Nice to know

Direct-conjugated antibodies are labeled with an enzyme or fluorophore in the Fc region. The Fc region also anchors the antibody to the plate in ELISA procedures and is also recognized by secondary antibodies in immunoprecipitation, immunoblots, and immunohistochemistry.

Often, because of their smaller size and lack of crosslinking (due to loss of the Fc region), F(ab) fragments are labeled for use in functional studies. Interestingly, the Fc fragments are often used as blocking agents in histochemical staining.

Subclasses

Antibodies can be divided into five classes: IgG, IgM, IgA, IgD, and IgE, based on the number of Y units and the type of heavy chain. Heavy chains of IgG, IgM, IgA, IgD, and IgE, are known as γ, μ, α, δ, and ε, respectively. The light chains of any antibody can be classified as either a kappa (κ) or lambda (λ) type (based on small polypeptide structural differences); however, the heavy chain determines the subclass of each antibody.

The subclasses of antibodies differ in the number of disulfide bonds and the length of the hinge region. The most commonly used antibody in immunochemical procedures is of the IgG class because this is the major immunoglobulin class released in serum.

IgA: In the blood IgA are present in low levels in monomeric form. They are most active at mucosal surfaces where they are present in dimeric form and provide the primary defense at mucosal surfaces. More IgA is produced in mucosal linings than all other types of antibody combined. Its major function is to act as a neutralizing antibody. High levels of IgA are present in saliva, tears, and breast milk. In humans two IgA subtypes are known to exist whereas in mice only one form is reported. IgA1 may account up to 85% of the total IgA in serum. Selective IgA deficiency is one of the most common immunodeficiency diseases that increases susceptibility to infections. IgA deficiencies are commonly seen in patients with autoimmune diseases and allergic disorders. IgA has a half-life of about 5 days.

IgD: It is a monomeric antibody with two epitope binding sites and is found on the surface of most B lymphocytes. Its precise function is still disputed, but is suggested to acts as an antigen receptor required for B cell activation. IgD is also reported to bind to basophils and mast cells and activate them to produce antimicrobial factors. It s also believed to play a role in eliminating B-lymphocytes that produce self-reactive autoantibodies. IgD is also produced in a secreted form that is found in serum in small quantities and contains two heavy chains of the δ class and two light chains. IgD has a half life of about 3 days.

Table 1: Immunoglobulin Subclasses

Class/Subclass	Heavy Chain	Light Chain	MW (kDa)	Structure	Function
IgA1 IgA2	α1 α2	λ or κ	150 to 600	Monomer to tetramer	Most produced Ig; protects mucosal surfaces; resistant to digestion; secreted in milk
IgD	δ	λ or κ	150	Monomer	Function unclear; works with IgM in B-cell development; mostly B cell bound
IgE	ε	λ or κ	190	Monomer	Defends against parasites; causes allergic reactions
IgG_1 IgG_{2a} IgG_{2b} IgG_3 IgG_4	γ1 γ2 γ2 γ3 γ4	λ or κ	150	Monomer	Major Ig in serum; good opsonizer; moderate complement fixer (IgG_3); can cross placenta
IgM	μ	λ or κ	900	Pentamer	First response antibody; strong complement fixer; good opsonizer

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IgE: This group of antibodies is effective at mucosal surfaces, blood, and tissues. It is present as monomer consisting of two heavy chains (ε chain) and two light chains. The ε chain contains 4 Ig-like constant domains. In serum, it is present in low concentrations contributing to only about 0.002% of total serum antibodies. Most IgE is tightly bound to its receptors on mast cells and basophils via the Fc region. It plays a crucial role in hypersensitivity reactions and its production is strictly controlled by cytokines. IgE has a half-life of about 2 days.

IgG: This is the most abundant class of antibodies in the blood, comprising up to 80% of the total serum antibodies. It is present in monomeric form. Four subclasses of IgG have been described depending on their abundance (IgG_1>IgG_2>IgG_3>IgG_4) and the subclass produced is dependent on the type of cytokine present. IgG_1 and IgG_3 exhibit high affinity for Fc receptors on phagocytes, while IgG_2 exhibits very low affinity and IgG_4 has moderate affinity for Fc receptors IgGs are capable of exiting the circulatory system and enter tissues. IgG_1, IgG_3, and IgG_4 can cross placental barrier to provide protection for newborns. IgGs are efficient at activating the complement system, and are very effective for opsonization using Fc receptors on phagocytes. Through its Fc region IgG can also bind to natural killer cells and participate in antibody-dependent cytotoxicity. IgG has a half-life ranging from 7 to 23 days, depending on its subclass.

IgM: This class of immunoglobulin is first to be produced in response to infection and is found either on membranes of B cells or as a 5-subunit macromolecule secreted by plasma cells. It is also the first immunoglobulin class to be synthesized by the neonates. The surface IgM differs from the secreted form in its Fc region. Surface IgM binds directly as an integral membrane protein and not to the IgM Fc receptor. Secreted IgM is a pentameric molecule where multiple immunoglobulins are covalently linked with disulfide bonds. This structure provides multiple binding sites. Each monomer consists of two light chains (either κ or λ) and two heavy chains. Because of its pentameric nature IgM is particularly suited for activating complement and causing agglutination. IgM has a half-life of about 5 days.

Nice to know

In a beautiful example of convergent evolution, cartilaginous fishes and camelid mammals, in addition to light and heavy chain antibodies, also have heavy chain-only versions, a smaller size that could be exploited as a research tool.

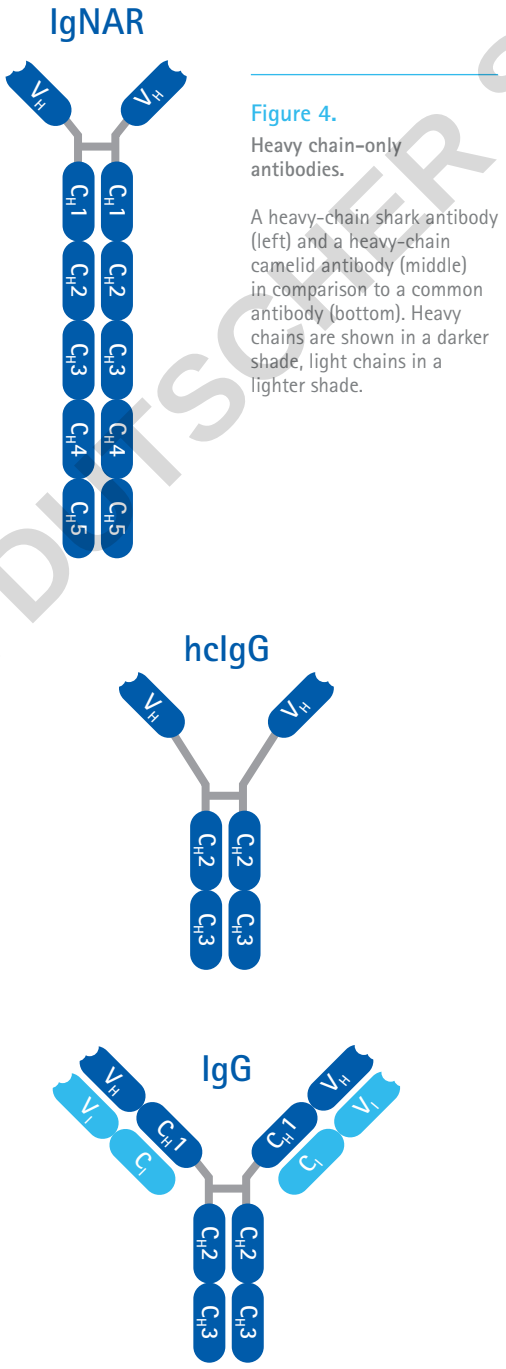
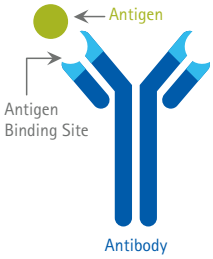


Figure 4.
Heavy chain-only antibodies.

A heavy-chain shark antibody (left) and a heavy-chain camelid antibody (middle) in comparison to a common antibody (bottom). Heavy chains are shown in a darker shade, light chains in a lighter shade.

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1.5 Antibody–Antigen Interaction

Now that you know what an antigen and antibody are, let us consider the interaction between them. The strength of interaction between antibody and antigen at single antigenic sites can be described by the affinity of the antibody for the antigen. Within each antigenic site, the variable region of the antibody "arm" interacts through weak noncovalent forces with antigen at numerous sites. The greater the interaction, the stronger the affinity. Avidity is perhaps a more informative measure of the overall stability or strength of the antibody-antigen complex. It is controlled by three major factors: antibody epitope affinity, the valence of both the antigen and antibody, and the structural arrangement of the interacting parts. Ultimately these factors define the specificity of the antibody, that is, the likelihood that the particular antibody is binding to a precise antigen epitope.

Cross-reactivity refers to an antibody or population of antibodies binding to epitopes on other antigens. This can be caused either by low avidity or specificity of the antibody or by multiple distinct antigens having identical or very similar epitopes. Cross-reactivity is sometimes desirable when one wants general binding to a related group of antigens or when attempting cross-species labeling when the antigen epitope sequence is not highly conserved during evolution. Cross-reactivity can result in over- or under-estimation of the antigen concentration and is problematic in immunoassays.

Nice to know

Researchers working with *Drosophila*, *Xenopus*, zebrafish, and other non-mammalian model organisms are often faced with antibodies validated only in mammals. Choosing polyclonal antibodies made against whole fusion proteins, or larger and conserved immunogen sequences, provides the best chances for interspecies cross-reactivity.

Immunochemical techniques capitalize upon the extreme specificity, at the molecular level, of each immunoglobulin for its antigen, even in the presence of high levels of contaminating molecules. The multivalency of most antigens and antibodies enables them to interact to form a precipitate. Examples of experimental applications that use antibodies are Western blot, immunohistochemistry and immunocytochemistry, enzyme-linked immunosorbent assay (ELISA), immunoprecipitation, and flow cytometry. Each is discussed in more detail in later sections of this reference guide.

Antibody–Antigen Interaction Kinetics

The specific association of antigens and antibodies is dependent on hydrogen bonds, hydrophobic interactions, electrostatic forces, and Van der Waals forces. These are of a weak, noncovalent nature, yet some of the associations between antigen and antibody can be quite strong. Like antibodies, antigens can be multivalent, either through multiple copies of the same epitope, or through the presence of multiple epitopes that are recognized by multiple antibodies. Interactions involving multivalency can produce more stabilized complexes; however, multivalency can also result in steric difficulties, thus reducing the possibility for binding. All antigen-antibody binding is reversible and follows the basic thermodynamic principles of any reversible bimolecular interaction:

$$K_A = \frac{[Ab\text{–}Ag]}{[Ab][Ag]}$$

where K_A is the affinity constant, [Ab-Ag] is the molar concentration of the antibody-antigen complex, and [Ab] and [Ag] are the molar concentrations of unoccupied binding sites on the antibody (Ab) or antigen (Ag), respectively.

The time taken to reach equilibrium is dependent on the rate of diffusion and the affinity of the antibody for the antigen and can vary widely. The affinity constant for antibody-antigen binding can span a wide range, extending from below 10^5/mol to above 10^{12}/mol. Affinity constants can be affected by temperature, pH, and solvent. Affinity constants can be determined for monoclonal antibodies, but not for polyclonal antibodies, as multiple bond formations take place between polyclonal antibodies and their antigens. Quantitative measurements of antibody affinity for antigen can be made by equilibrium dialysis. Repeated equilibrium dialyses with a constant antibody concentration, but varying ligand concentration are used to generate Scatchard plots, which give information about affinity valence and possible cross-reactivity.

When designing experimental procedures, it is important to differentiate between monoclonal and polyclonal antibodies, as these differences are the foundation of both advantages and limitations of their use.

1.6 Nature of Antigen-Antibody Bonds

The combining site of an antibody is located in the F(ab) portion of the antibody molecule and is assembled from the hypervariable regions of the heavy and light chains. The binding between this site and the antigen takes place with the following characteristics and processes:

- The bonds that hold the antigen to the combining site of any antibody are noncovalent, and, hence, they are reversible in nature.
- These bonds may be hydrogen bonds, electrostatic bonds, or Van der Waals forces.
- Usually there are multiple bond formations observed, ensuring relatively tight binding between antibody and antigen.
- The specific binding between the antigenic determinant on the cell (known as epitope) and the antigen-combining site (paratope) on the antibody involves very small portions of the molecules, usually comprising only a few amino acids.
- These sites are critical in antigen-antibody reactions as specific binding has to overcome repulsion between the two molecules.
- When the epitope comes in contact with paratope they are first attracted to each other by ionic and hydrophobic forces.
- These forces help them overcome their hydration energies and allow for the expulsion of water molecules as epitope and paratope approach each other.
- This attraction becomes even stronger when Van der Waals forces are employed later on to bring epitope and paratope even closer.

1.7 Factors Affecting Antigen-Antibody Reactions

The antigen-antibody reaction can be influenced by several factors. Some of the more common factors are:

Temperature

The optimum temperature for antigen-antibody reaction will depend on the chemical nature of the epitope, paratope, and the type of bonds involved in their interaction. For example, hydrogen bond formation tends to be exothermic. These bonds are more stable at lower temperature and may be more important when dealing with carbohydrate antigens.

pH

The effect of pH on the equilibrium constant of the antigen-antibody complex lies in the pH range of 6.5 and 8.4. Below pH 6.5 and above pH 8.4, the antigen-antibody reaction is strongly inhibited. At pH 5.0 or 9.5, the equilibrium constant is 100-fold lower than at pH 6.5 - 7.0. Under extreme pH conditions, antibodies may undergo conformational changes that can destroy the complementarity with the antigen.

Ionic strength

Effect of ionic strength on antigen-antibody reaction is particularly important in blood group serology. Here the reaction is significantly influenced by sodium and chloride ions. For example, in normal saline solution, Na^+ and Cl^- cluster around the complex and partially neutralize charges, potentially interfering with antibody binding to antigen. This could be problematic when low-affinity antibodies are used. It is well known that, when exposed to very low ionic strengths, γ-globulins aggregate and form reversible complexes with lipoproteins of red blood cells, leading to their sedimentation.

Nice to know

Prozone effect is a phenomenon when in an antibody-antigen laboratory testing false negative or false low results occur from the excess of antigen or antibody in a sample due to the inability of the analyte to bind to receptor sites.

In a typical immunoassay, antigens and antibodies bind to create a conjugate that can be detected and measured. However, when prozone effect occurs, excess antigens or antibodies can bind all of the receptor sites, leaving no molecules available to form conjugates. Hence, antibody-antigen conjugates cannot be detected and a false negative result is produced, which can go undetected. In a clinical setting, this could lead to misdiagnosis. If an error in results is suspected, one should always dilute the sample and retest.

1.8 Generation of Antibodies

Polyclonal and Monoclonal Antibodies

Antibodies are normally produced by B cells, which are part of the immune system, in response to the introduction of foreign substances, such as infectious agents, into the animal's body. The antibodies bind to the antigens that cause their generation and flag them for destruction, thus helping to fight infection. This inherent ability of the animal's body can be leveraged to generate antibodies that bind to specific molecules. Target-specific antibodies can be used to isolate and identify molecules of interest. Antibodies have become one of the most important tools in life science research, allowing the detection, quantitation, and determination of changes in proteins and other molecules with respect to time and other perturbations.

Many of the antibodies used in immunochemical techniques are raised by repeated immunization of a suitable animal, e.g., rabbit, goat, donkey, or sheep, with an appropriate antigen. Serum is harvested at the peak of antibody production. Specific IgG concentrations of approximately 1 to 10 mg/mL serum can be obtained by this method. Weakly antigenic molecules may require the addition of an adjuvant, which allows for the slow release of the antigen, making it more readily trapped by macrophages. Smaller molecules, such as drugs, must be coupled to more antigenic structures (i.e. carrier proteins) to stimulate an immune response.

One characteristic of large antigen molecules is that they induce the activation of many antibody-producing B cell clones in the immunized animal. This polyclonal mixture of resulting antibodies may then recognize a variety of epitopes on the antigen, which can be a useful feature in some experimental procedures. Because these polyclonal mixtures of antibodies react with multiple epitopes on the surface of the antigen, they will be more tolerant of minor changes in the antigen, e.g., polymorphism, heterogeneity of glycosylation, or slight denaturation, than will monoclonal (homogenous) antibodies.

Depending upon the antigen that is used to create the antibody, one may use polyclonal antibodies to identify proteins of high homology to the immunogen protein or to screen for the target protein in tissue samples from species other than that of the immunogen. Along the same lines, it is especially important when working with polyclonal antibodies to learn as much as possible about the immunogen that has been used for production of the polyclonal antibody and the potential for undesired cross-reactivity within the sample being analyzed. Peptide immunogens are often used to generate polyclonal antibodies that target unique epitopes, especially for protein families of high homology.

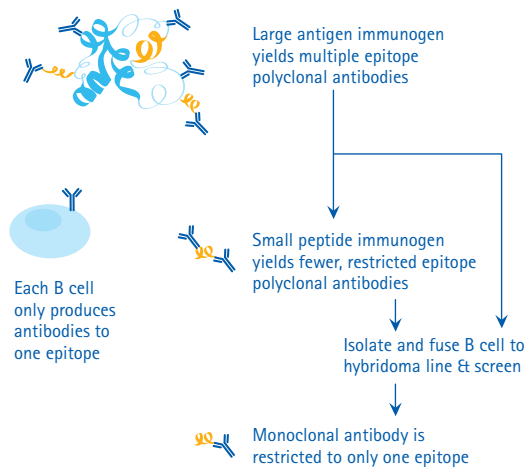


Figure 5.
Production of monoclonal vs. polyclonal antibodies.

A homogeneous population of antibodies (i.e. monoclonal antibodies) can be raised by fusion of B lymphocytes with immortal cell cultures to produce hybridomas. Hybridomas will produce many copies of the exact same antibody. This impressive phenomenon has been instrumental in the development of antibodies for diagnostic applications because monoclonal antibodies react with one epitope on the antigen. However, they are more vulnerable to the loss of epitope through chemical treatment of the antigen than are polyclonal antibodies. This can be offset by pooling two or more monoclonal antibodies to the same antigen.

Some Useful Properties of Polyclonal Antibodies

- Polyclonal antibodies often recognize multiple epitopes, making them more tolerant of small changes in the nature of the antigen. Polyclonal antibodies are often the preferred choice for detection of denatured proteins.
- Polyclonal antibodies may be generated in a variety of species, including rabbit, goat, sheep, donkey, chicken, and others, giving the users many options in experimental design.
- Polyclonal antibodies are sometimes used when the nature of the antigen in an untested species is not known.
- Polyclonal antibodies target multiple epitopes and so they generally provide more robust detection.

Some Useful Properties of Monoclonal Antibodies

- Because of their specificity, monoclonal antibodies are excellent as the primary antibody in an assay, or for detecting antigens in tissue, and will often result in significantly less background signal than polyclonal antibodies.
- When compared to that of polyclonal antibodies, homogeneity of monoclonal antibodies is very high.
- If experimental conditions are kept constant, results from monoclonal antibodies will be highly reproducible between experiments.
- Specificity of monoclonal antibodies makes them extremely efficient for binding of antigen within a mixture of related molecules, such as in the case of affinity purification.

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Table 2: Advantages and Disadvantages of Polyclonal and Monoclonal Antibodies

	Advantages	Disadvantages
Polyclonal Antibodies	Relatively easy to generate and more cost-effective.	Animal death can terminate the source of antibody.
	Multiple epitopes on the same protein can generate many antibodies. Hence, they provide more robust signals.	Different bleeds may give different results.
	Polyclonal antibodies can generate better signals with proteins expressed in low levels.	Immunization of a new animal with the same antigen may lead to different epitopes and different clones may be generated.
	They are compatible with a broader range of applications.	Shared epitopes on different proteins can lead to labeling of proteins other than the antigen protein.
	Polyclonal antibodies provide more flexibility in antigen recognition. For example, they may bind the antigen in spite of polymorphism, heterogeneity of glycosylation etc. Hence, they can identify proteins of high homology or from different species.	Greater batch-to-batch variability is possible.
	Better suited for the detection of denatured proteins.	May produce nonspecific antibodies that can add to background signal.
Monoclonal Antibodies	Different clones of antibodies can be generated to different epitopes on a single antigen.	Production of monoclonal antibodies is more labor-intensive. More work is required, especially in the cloning and selection process.
	Hybridoma cells can serve as an infinite source of the same antibody.	They may be limited in their applications.
	The high specificity of monoclonal antibodies minimizes background and eliminates cross-reactivity.	A vast majority of monoclonal antibodies are produced in mice because of a robust myeloma cell line.
	Their homogeneity is very high and they provide consistent, reproducible results.	High specificity of monoclonal antibodies limits their use in multiple species.
	They bind only to one antigen in a mixture of related proteins.	Monoclonal antibodies are more susceptible to the loss of epitope through chemical treatment of the antigen.
	Batch-to-batch variability is very minimal.	

Clone Numbers

Each clone number represents a specific cell line that was used to produce the antibody. Since antibodies are produced by more than one host, each cloned cell line receives a unique clone number. Each hybridoma cell clone produces only one single pure antibody type.

- An animal injected with an antigen will generate multiple antibodies to many epitopes. Since antibodies are produced by B cells, a single clone of B cells can produce antibodies to only a single epitope.
- Monoclonal antibodies are derived from a single clone of cells and can be generated in larger quantities.
- Polyclonal antibodies contain multiple clones of antibodies produced to different epitopes on the antigen. For example, if there are four epitopes on the antigen then four different clones of antibodies will be produced.
- Different antibody clones may have different properties and may even be of different isotypes. They may also work in different applications. Hence, it is best to select an antibody clone that will work optimally in your choice of application.
- It is important to recognize that a clone number is not synonymous with the lot number, which often indicates the date of manufacture.

1.9 Antibody Formats

As the name implies, the antibody format refers to the presentation or purification state of the antibody. Various formats are described below:

Polyclonal antibodies are often available in relatively unpurified formats, and are referred to as "antiserum" or simply as "serum". **Antiserum** refers to the blood from an immunized host from which the clotting proteins and RBCs have been removed. The antiserum, as its name suggests, still possesses antibodies/immunoglobulins of all classes as well as other serum proteins. In addition to antibodies that recognize the target antigen, the antiserum also contains antibodies to various other antigens that can sometimes react nonspecifically in immunological assays. For this reason, raw antiserum is often subjected to purification steps, to eliminate serum proteins and to enrich the fraction of immunoglobulin that specifically reacts with the target antigen.

Antiserum is commonly purified by one of two methods: Protein A/G purification or antigen affinity chromatography.

Protein A/G purification takes advantage of the high affinity of *Staphylococcus aureus* protein A or *Streptococcus* protein G for the immunoglobulin Fc domain. While protein A/G purification eliminates the bulk of the serum proteins from the raw antiserum, it does not eliminate the nonspecific immunoglobulin fraction. As a result, the protein A/G purified antiserum may still possess undesirable cross reactivity. See Protein A/G Binding Affinities in Appendix.

Antigen affinity purification takes advantage of the affinity of the specific immunoglobulin fraction for the immunizing antigen against which it was generated. This method may be used to remove unwanted antibodies from a preparation. The preparation of antibodies is passed through a column matrix containing antigens against which the unwanted antibodies are directed. The unwanted antibodies remain bound to the column, and the effluent contains the desired, affinity-purified antibodies. Alternatively, a column matrix coupled to the desired antigen can be used. In this case, antibody directed against the coupled antigen remains bound to the column and may be then eluted using a solution that disrupts antigen-antibody binding. Unlike protein A/G purification, antigen affinity purification results in the elimination of the bulk of the nonspecific immunoglobulin fraction, while enriching the fraction of immunoglobulin that specifically reacts with the target antigen. The resulting affinity purified immunoglobulin will contain primarily the immunoglobulin of desired specificity.

Typically, affinity purified antibodies exhibit lower backgrounds than unabsorbed antibodies and this purification process is particularly important for difficult, or state-dependent epitopes. When developing polyclonal antibodies that recognize targets with post-translational modifications, the use of modification specific antigen affinity columns during the purification process can significantly improve the specificity of the antibody for state-dependent target. Depleting unmodified target protein from the serum before affinity purification (using immobilized, modified target protein) increases the specificity for the modified target. Specificity testing can then be performed to confirm that the antibody only recognizes the post-translationally modified form of the protein.

Monoclonal antibodies may be grown in cell cultures and collected as hybridoma supernatants, or grown in mice or rats and collected as relatively unpurified ascites fluid. These can be purified through the use of protein A/G or specific antigen affinity chromatography as with polyclonal antibodies.

Unpurified antibody preparations vary significantly in specific antibody concentration. If the specific antibody concentration of a given unpurified antibody preparation is unknown, one may refer to the following "typical ranges" as a guideline for estimation:

Polyclonal Antiserum: Specific antibody concentrations will typically range from 1–3 mg/mL.
Hybridoma Supernatant: Specific antibody concentrations will typically range from 0.1–10.0 mg/mL.
Ascites Fluid (unpurified): Specific antibody concentrations will typically range from 2–10 mg/mL.

Tech Tip

Antibody concentrations of purified preparations should be determined prior to the addition of stabilizing protein such as BSA.

1.10 Biological Effects of Antibodies

Antibodies are widely used for protection from infectious agents. Most vaccines (microbial antigens) induce the production of antibodies that block infection or interfere with microbial invasion of the bloodstream. To achieve this, antibodies must be functional in the sense that they are capable of neutralization or opsonophagocytosis.

The membrane attack complex (MAC) cytolysis

MAC is formed on the surface of pathogenic bacterial cell as a result of the activation of the complement system (both alternative and the classical pathways). The MAC forms transmembrane channels in bacterial walls, disrupting their phospholipid bilayer and leading to cell lysis and death.

Neutralization of viruses

Antibodies can interfere with virion binding to receptors and block their uptake into cells. Many enveloped viruses are lysed when antiviral antibodies and the complement system disrupt membranes. Certain antibodies can also aggregate virus particles. Non-neutralizing antibodies are also produced following any viral infection. Although these antibodies bind specifically to virus particles, they do not neutralize them. On the contrary, they may enhance infectivity because the virus-antibody complex enters the cell by endocytosis. This can lead to viral replication.

The type of antibody produced can influence the outcome of viral infection. For example, poliovirus can elicit IgM and IgG responses in the blood, but mucosal IgA is vital for blocking infection. The IgA neutralizes poliovirus in the intestine, the site of primary infection. Hence, the live attenuated Sabin poliovirus vaccine is more effective because it elicits a strong mucosal IgA response.

Immobilization

An antibody can be directed against cilia or flagella of motile bacteria or protozoa that results in cessation of their motility and blocks their ability to move around and spread infection.

Cytolysis

Certain antibodies can cause disruption of the microbial membrane that result in death of bacterial cells. This requires the participation of the complement system.

Opsonization

In this process, the pathogenic organism is targeted for digestion by phagocytes. The antibody binds to a receptor on the cell membrane of the bacterium, attracting phagocytes to the site. The F(ab) portion of the antibody binds to the antigen, while the Fc portion of the antibody binds to an Fc receptor on the phagocyte, facilitating phagocytosis. This process is further enhanced by the complement system.

Neutralization of exotoxins

Antitoxin antibodies can be generated against microbial toxins. The F(ab) region of the antibody made against epitope of the binding site of an exotoxin can block the exotoxin from binding to the exotoxin receptor on the host cell membrane. This blocks the entry of the toxin into the cell.

Preventing bacterial adhesion to host cells

The body's innate defenses can physically remove bacteria by constant shedding of surface epithelial cells from the skin and mucous membranes. However, bacteria may resist this by producing pili, cell wall adhesin proteins, and biofilm-producing capsules. The F(ab) region of the antibody can bind to the adhesive tip of the pili, the cell wall adhesins, or the capsular molecules, and blocks bacterial adhesion to host cells.

Agglutination of microorganisms

The F(ab) sites of IgM and IgA antibodies can link microorganisms together and cause them to agglutinate. The agglutinated microorganisms can be phagocytosed more effectively.

Technology Highlight

Dot blot arrays for testing antibody specificity

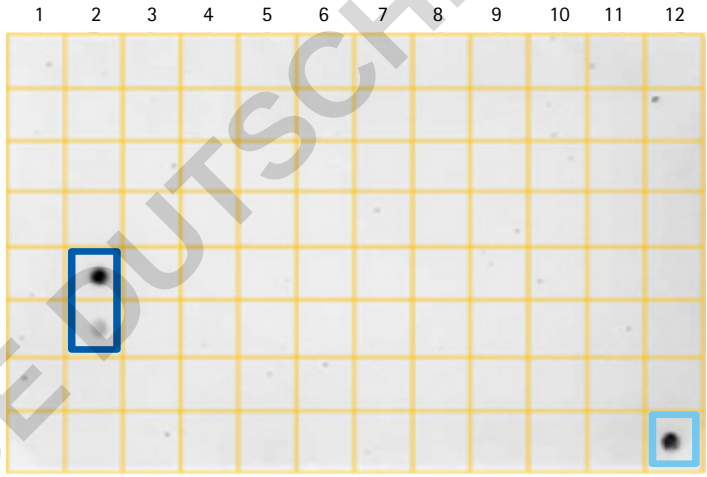
Antibody specificity is critical when performing chromatin immunoprecipitation or other sensitive antibody-dependent analyses. The diversity of post-translational modifications to histone protein targets makes unreliable antibody specificity and precision major sources of variation and error in data interpretation. Most antibodies are not tested to determine cross-reactivity among various modifications; however, even small changes in protein state, like dimethyl to trimethyl labeling, have important biological implications. Developing dot blot arrays is an effective way to screen antibodies for specificity.

AbSurance™ Histone Antibody Specificity Arrays employ the same technology used to screen Merck Millipore's highly characterized and extensively published histone antibodies. Built on easy-to-use Immobilon®-FL PVDF membranes, these arrays permit a detailed characterization of antibodies against key histone modification sites. The AbSurance™ screening process is performed using a simple, Western-blot–like procedure, followed by detection using either X-ray film or a CCD imager.

AbSurance™ Benefits

- High quality purified peptides (>95% purity)
- 89 peptides representing all key histone modification sites (acetyl, phospho, and mono-, di-, and trimethyl PTMs)
- Consistent and uniform spotting of peptides using a proprietary process
- Sensitive chemiluminescent detection using either film or CCD imagers
- Easy data analysis—no additional software required
- Built-in positive control primary antibodies from rat, mouse, sheep, and rabbit

A. Acetyl Histone H4 (Lys12)



B. Location of reactivity with the H4 antibody and Control IgG Tested

	1	2	3	4	5	6	7	8	9	10	11	12	
A	H2A 1-19 unmod	H2A 1-19 SIP	H2A 1-19 K5ac	H2A 1-19 K9ac	H2A 1-19 K13ac	H2A 110-129 unmod	H2A 110-129 T120P	H2A.X 124-142 unmod	H2A.X 124-142 S139P	H2A.X 124-142 Y142P	H2B 1-19 unmod	H2B 1-19 K5ac	100 ng
B	H2A 1-19 unmod	H2A 1-19 SIP	H2A 1-19 K5ac	H2A 1-19 K9ac	H2A 1-19 K13ac	H2A 110-129 unmod	H2A 110-129 T120P	H2A.X 124-142 unmod	H2A.X 124-142 S139P	H2A.X 124-142 Y142P	H2B 1-19 unmod	H2B 1-19 K5ac	10 ng
C	H2B 1-19 K5me1	H2B 1-19 K12ac	H2B 1-19 S14P	H2B 1-19 K15ac	H2B 107-125 unmod	H2B 107-125 K120ac	H4 7-26 unmod	H4 7-26 SIP	H4 7-26 R3me1	H4 7-26 R3me2a	H4 7-26 R3me2s	H4 7-26 K5ac	100 ng
D	H2B 1-19 K5me1	H2B 1-19 K12ac	H2B 1-19 S14P	H2B 1-19 K15ac	H2B 107-125 unmod	H2B 107-125 K120ac	H4 7-26 unmod	H4 7-26 SIP	H4 7-26 R3me1	H4 7-26 R3me2a	H4 7-26 R3me2s	H4 7-26 K5ac	10 ng
E	H4 1-19 K8ac	**H4 1-19 K12ac**	H4 11-30 unmod	H4 11-30 K16ac	H4 11-30 R17me1	H4 11-30 R17me2a	H4 11-30 R17me2s	H4 11-30 R19me1	H4 11-30 R19me2a	H4 11-30 R19me2s	H4 11-30 K20ac	H4 11-30 K20me1	100 ng
F	H4 1-19 K8ac	**H4 1-19 K12ac**	H4 11-30 unmod	H4 11-30 K16ac	H4 11-30 R17me1	H4 11-30 R17me2a	H4 11-30 R17me2s	H4 11-30 R19me1	H4 11-30 R19me2a	H4 11-30 R19me2s	H4 11-30 K20ac	H4 11-30 K20me1	10 ng
G	H4 11-30 K20me2	H4 11-30 K20me3	H4 11-30 R23me1	H4 11-30 R23me2a	H4 11-30 R23me2s	H4 82-100 unmod	H4 82-100 K19ac	100 ng			Rat IgG	Sheep IgG	100 ng
H	H4 11-30 K20me2	H4 11-30 K20me3	H4 11-30 R23me1	H4 11-30 R23me2a	H4 11-30 R23me2s	H4 82-100 unmod	H4 82-100 K19ac	10 ng			Mouse IgG	**Rabbit IgG**	10 ng

Specificity screening of histone H4 antibody.
A. The Histone H2A, H2B, H4 Array was probed with anti-acetyl histone H4 (Lys12) antibody (1:2000 dilution, Cat. #04-119). Peptides were visualized using a donkey anti-rabbit IgG, peroxidase conjugated, H+L (Cat. #AP182P) secondary antibody and a chemiluminescence detection system. B. Peptide map showing location of reactive peptide spots (Dark Blue). Control rabbit IgG is shown in lighter shade of blue.

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Notes

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Antibody Practice

2

2.1 Selection and Use
2.2 Antibody Titer and Concentration
2.3 Storage and Handling of Antibodies
2.4 Conjugated Antibodies
2.5 Use of Secondary Antibodies
2.6 Proper Controls
2.7 Publishing with Antibodies
2.8 Validating Antibodies

2.1 Selection and Use

Considerations when selecting an antibody for use in an experiment

Once you have identified your target antigen and have chosen your detection method, you must then choose one or more primary antibodies to detect your target. If more than one potential antibody is available for your target, it may be recommended to carry out key experiments using multiple antibodies (see section 2.7 on Publishing with Antibodies). Choose your antibodies based on the following considerations:

Determine the best application for your research need:

- Not all antibodies will work with every application.
- Determine if you are performing a qualitative or quantitative assay
- Check vendor's data sheet or website to see if the antibody is suitable for the specific application, such as immunoblotting, ELISA etc.

Type of sample being tested:

- Does your tissue or cell express the particular protein?
- Are you trying to detect a latent or activated protein? For example, phospho-specific antibodies may react only with activated phosphorylated proteins.
- If your protein has an intracellular location it will be necessary to perform a cell lysis.
- In flow cytometric analysis it may be necessary to use an antibody that recognizes cell surface molecules.
- If your protein has a tertiary structure and the epitope is obscured then sample has to be denatured because antibody will not recognize the native state.
- Some antibodies will work best only in frozen or on unfixed tissue and others will work in paraffin sections only after an antigen retrieval process.

Species from which the protein is to be detected:

- Select an antibody that is raised against the immunogen sequence derived from species of your interest.
- If the sequence is not derived from your species of interest, check to see if it will react with your sample. You may quickly check the sequence for specific proteins in the protein data bank: http://www.ncbi.nlm.nih.gov/protein.

Species in which the antibody is raised:

- This information will be of great advantage when selecting a secondary antibody. The secondary antibody should be phylogenetically as far apart as possible from a species from which your sample is derived.

Check for validation data available on data sheet or vendor website:

- Look at the validation data on data sheet or on vendor's website and examine the quality of data.
- Check to see if only a verification of the presence of antigen is provided (ELISA, Western blotting) or whether there are other in-depth data.
- Check to see what type of sample was tested (cell lysate, tissue homogenate etc). Just using purified recombinant protein may not give best results with real cell or tissue samples.

Guarantee and support:

- Is there an offer of guarantee from the vendor? It may be money-back or credit.
- What type of technical support is available? It is best to have access to live technical support as opposed to frequently asked questions on the website.

2.2 Antibody Titer and Concentration

The binding of antibody and antigen is dependent on the affinity constant, which, in turn, can be affected by temperature, pH, solvent composition, etc. Varying the relative concentrations of antibody and antigen in solution can also control the extent of antibody-antigen complex formation.

Concentration and titer are not equivalent. Concentration is the total amount of antibody contained in the solution. Usually, only a percentage of it represents the intact, active, and functional antibody with regard to its ability to bind the antigen, and determines its effectiveness. The titer is the highest dilution of the antibody that yields a response in the immunoassay. It is the degree to which the antibody-serum solution can be diluted and still contain detectable amounts of antibody.

In most cases, the concentration of antigen in a sample cannot be adjusted. Hence, the optimal working concentration (dilution) of the antibody must be determined empirically for a given set of experimental conditions.

For any assay, the optimum titer is that concentration (dilution) which gives the strongest reaction for positive tests with minimum background reaction (e.g., for negative controls). The optimal antibody concentration must be determined experimentally for each assay, and is typically determined by using a dilution series.

The optimal antibody concentration is best determined by first selecting a fixed incubation time and preparing a series of dilutions to test. Dilutions are usually expressed as the ratio of the more concentrated stock solution to the total volume of the desired solution. For example, a 1:10 dilution of antibody is created by mixing one part of antibody stock solution with nine parts of diluent, giving a total of ten parts.

Datasheets and protocols may suggest approximate dilutions for antibody use. When using an antibody for the first time, or when working with a new batch of antibody, it is advisable to try a dilution series to determine the optimal antibody dilution to use. For example, if a product data sheet suggests using a 1:500 dilution, making dilutions of 1:50, 1:100, 1:500, 1:1,000 and 1:10,000 can help determine the optimal dilution for a set of unique assay conditions. Especially in the case of polyclonal antisera, antibody concentrations may be significantly different from animal to animal or from one serum bleed to the next, and this kind of initial titration is essential in reducing inter-assay variations.

2.3 Storage and Handling of Antibodies

The proper storage and handling of antibodies is critical to their function and longevity. Properly stored antibodies show little degradation over long periods of time, extending their usefulness to several months or even years. Improperly stored antibodies, on the other hand, can denature in a matter of hours. Consider the following points when storing and handling antibodies and other biological reagents:

- In order to preserve maximum reactivity, reagents should be stored according to the manufacturer's instructions (e.g., avoid holding antibodies at room temperature when storage at 2–8°C is indicated).
- It is a good rule of thumb to store antibodies in tightly sealed containers in a non-frost-free refrigerator/freezer, away from tissue fixatives and crosslinking reagents.
- Antibodies are relatively stable proteins and are resistant to a broad range of mild denaturing conditions. Most antibodies are stable for years when stored properly as per manufacturer's recommendations.
- In most cases antibodies can be stored at -20°C without any loss in their binding capacity.
- It is best to avoid storing antibodies in a frost-free freezer. This is to avoid or minimize freeze-thaw cycles. Antibody solutions should not be frozen and thawed repeatedly, as this can lead to aggregation, causing a loss of activity. Hence, stock solutions should be aliquoted prior to storage.
- Undiluted antibodies should always be aliquoted prior to storage at -20°C to minimize repeated freeze/thaw cycles that can denature antibody. Storing antibody in concentrated form will either prevent or minimize degradation. A cryoprotectant, such as glycerol, to a final concentration of 50%, can be added to the antibody solution to prevent freeze/thaw damage. Do not store glycerol-containing antibodies at -80°C.

- Unless a stabilizing protein, such as BSA (1% w/v), has been added, antibodies should not be stored for extended periods at their working dilutions. Avoid storing diluted antibodies for extended periods.
- The major problem encountered during storage is contamination with bacteria or fungi. If antibodies are stored at 2-8°C for more than two to three days, it is advisable to filter-sterilization and/or add a bacteriostat/preservative, such as 0.05% sodium azide or 0.1% thimerosal.
- Sodium azide can interfere with various biological assays and with some coupling methods. Hence, in these applications it is best to either remove sodium azide by centrifugal diafiltration, dialysis, or gel filtration, or use azide-free antibodies. **NOTE**: Sodium azide is toxic. As with all laboratory reagents, consult a Material Safety Data Sheet (MSDS) for handling precautions.
- Generally, enzyme-conjugated antibodies are not frozen to prevent loss of enzyme activity and their binding capacity. It is best to store them at +4°C.
- Fluorescent conjugates are susceptible to photobleaching. Hence, fluorochrome-conjugated antibodies should be stored protected from light in a darker colored vial.
- When stored for a long period of time, some antibody solutions may produce an insoluble lipid component. The precipitate can be removed by a quick centrifugation at 10,000 g.

2.4 Conjugated Antibodies

A note on concentrations, storage buffers, and storage temperatures

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. The various antibody conjugates have differential stabilities and require different buffers and storage conditions to retain their maximal activity over time. The following table lists the standard antibody buffers and storage conditions for purified Merck Millipore antibodies and antibody conjugates. Note that these are general guidelines and that one should always consult the datasheet accompanying the antibody for specific storage conditions for that antibody.

Antibody Buffers

1. **Affinity-purified Monoclonal and Polyclonal Antibodies**
   0.02 M phosphate buffer, 0.25 M NaCl, 0.1% NaN_3, pH 7.6
   Same buffer without NaN_3 may be used as required.
2. **FITC Conjugates**
   0.02 M phosphate buffer, 0.25M NaCl, 15 mg/mL BSA, 0.1% NaN_3, pH 7.6
3. **HRP Conjugates**
   0.01 M PBS, 15 mg/mL BSA, 0.01% Thimerosal, pH 7.1
4. **Alkaline Phosphatase Conjugates**
   0.05 M Tris, 0.1 M NaCl, 0.001 M $MgCl_2$, 15 mg/mL BSA, 0.1% NaN_3, pH 8.0
5. **Biotinylated Conjugates**
   0.01 M PBS, 15 mg/mL BSA, 0.1% NaN_3, pH 7.1

Standard Antibody Concentrations

Purified and Monoclonal Conjugates	1 mg/mL
Polyclonal Affinity-purified Antibodies	2 mg/mL
Polyclonal FITC Conjugates	2 mg/mL
Polyclonal HRP/Alk Phos Conjugates	1 mg/mL

Antibody Storage Conditions

Polyclonal Affinity-purified Antibodies	4°C to 8°C
Fluorescent Conjugates (Store in dark)	4°C to 8°C
Enzyme Conjugates (Do not freeze)	4°C to 8°C
Hapten Conjugates (Do not freeze)	4°C to 8°C

2.5 Use of Secondary Antibodies

Secondary antibodies are often used to indirectly detect an antigen to which a primary antibody is first bound. Hence, it is important to select a secondary antibody that has specificity for the antibody species and isotype of the primary antibody and is conjugated to a detectable tag or label for detection. Consider the following points:

- The detectable tag could be an enzyme or a fluorochrome. Most commonly used tags are horseradish peroxidase, alkaline phosphatase, fluorescein isothiocyanate (FITC), rhodamine, Texas Red, phycoerythrin, and biotin.
- A proper selection of secondary antibody can improve staining and minimize false positive or negatives.
- Secondary antibodies are used when there are no conjugated primary antibodies available or the primary antibody is not conjugated to a desired enzyme or fluorochrome.
- Secondary antibodies are also used to increase the sensitivity of detection. Even though use of a secondary antibody involves extra steps, it does have the advantage of increased sensitivity due to the signal amplification from multiple secondary antibodies binding to a single primary antibody.
- Secondary antibodies are generated by immunizing a host animal with the antibody from a different species. For example, anti-goat antibodies are raised by injecting goat antibodies into an animal other than a goat. Accordingly, if the primary antibody is raised in mouse, then secondary antibody should be an anti-mouse antibody raised in another species (goat, donkey etc.)
- For ELISA detection, enzyme-conjugated antibodies are the better choice. For flow cytometry, it is best to use a fluorochrome-conjugated secondary antibody.
- A vast majority of primary antibodies belong to the IgG class. They can be detected with the relevant anti-species IgG secondary antibody. If the primary antibody is an IgM, then the secondary antibody specific for IgM should be selected.

Selecting an appropriate secondary antibody:

- Secondary antibody should be against the same species in which the primary antibody is raised. For example, if the primary antibody is raised in goat then secondary antibody should be anti-goat.
- Select an antibody labeled with a fluorochome or enzyme of your choice, and your expertise and the instruments available in your laboratory. More commonly used fluorochrome labels are fluorescein, rhodamine, Texas Red, phycoerythrin, etc, and enzyme conjugates could be horseradish peroxidase, alkaline phosphatase, etc.
- Biotin-conjugated antibodies provide greater sensitivity and more amplified signal when compared to fluorochrome- or enzyme-conjugated secondary antibodies.
- For best results, use secondary antibody that has been preadsorbed with serum from the same species as the sample. This will reduce the background. However, these preadsorbed antibodies may have reduced epitope recognition and may fail to recognize some IgG subclasses.
- Affinity-purified secondary antibodies will provide the least amount of nonspecific binding. However, sometimes IgG fractions are preferred when it contains high affinity antibodies. This is of great advantage when the antigen is present in very low levels.
- Select a secondary antibody that matches the class or subclass of the primary antibody used. For example, if the primary antibody is mouse IgM then it is best to use an anti-mouse IgM secondary.
- When the class or subclass of the primary mouse monoclonal antibody is unknown, then anti-mouse IgG may be used, because it will recognize most of mouse IgG subtypes.

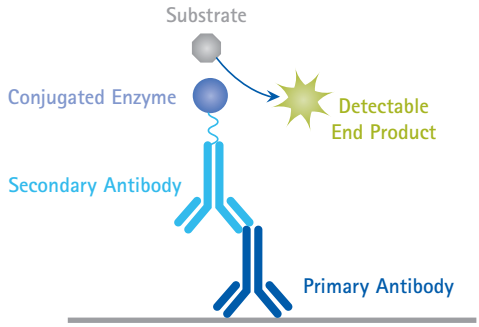


Figure 6.

Principle of antigen detection using primary and secondary antibodies

2.6 Proper Controls

The use of proper controls will help eliminate any false positive and false negative results and will enable better interpretation of the experimental data. The key to proving antibody specificity is often the correct use of controls. They will also be invaluable in troubleshooting throughout the experimental design process. Here are some considerations:

- Whenever possible, both negative and positive controls should be included in an assay.
- A positive control sample may be any tissue, cell line, or purified protein that is known to contain the antigen of interest, and has been previously demonstrated to be positive by a reliable method.
- A negative control sample is one that is known to be devoid of the antigen of interest. A cell line or tissue that is known not to express the protein of interest is a better negative control.
- In addition to sample controls, one should also use reagent controls.
- Remember to change only one experimental variable at a time.
- One should run separate controls for primary and secondary antibodies.
- Because antibodies from different animal bleeds or purification batches may have significantly different titer values, each new batch of antibody must be validated, and conditions optimized before use in an existing assay.

It should also be noted that an integral part of good laboratory practice is to keep complete documentation of all dilutions, diluents, incubation times, lot numbers, preparation dates of all reagents, and procedural steps. This information is highly valuable in efficient assay development.

Isotype controls are used to validate that the primary antibody binding is specific and does not result from background signal due to immunoglobulins binding nonspecifically. Typically, an isotype control is matched to the host species and isotype of the specific primary antibody. For example, IgG_{2a} type antibodies raised in mice can bind strongly to some human leukocytes. Hence, a mouse IgG_{2a} isotype control should be used when analyzing human cells and tissues. Isotype controls are most commonly used in immunoprecipitation, flow cytometry, and immunohistochemistry. In many flow cytometry applications, directly labeled primary antibodies are used. Here, it is important to use the isotype control that is conjugated to the same fluorochrome or label as the primary antibody.

Nice to know

Affinity maturation is an adaptive response to antigen exposure. It is a process of affinity-selected differentiation of activated B cells. When the host animal is repeatedly exposed to the same antigen it provokes greater antibody ligating affinity. With the passage of time, the antibodies produced are able to bind antigen more tightly and to deal more efficiently with the antigen.

2.7 Publishing with Antibodies

Let's return to the basic immunolabeling assumption that regardless of technique used, a positive signal infers that the specific antibody has bound to the specific antigen. As with any technique, it is good science to verify that your signal is indeed specific and reproducible. Reviewers of publications and grants are becoming increasingly critical of data analysis, and researchers are being challenged to think about the fundamental principles by which laboratory techniques work, and to be more careful about over-interpretation. The chart (on the next page) should help bolster confidence in immunodata.

Actual reviewer comments	Good practice
I am not convinced your antibody is specific	Use two or more different techniques to verify specificity; for example, WB can corroborate IHC data
	Use two or more antibodies made against different immunogens or regions of the protein and measure co-localization
	Test against relevant knockout samples
I am not convinced your antibody is not cross-reacting with related proteins	Use two or more antibodies made against unconserved epitopes of the same antigen to confirm results
Your Western blot shows more than one band or at the wrong size. How can you show specificity?	Cite published literature on cleavage products or glycosylation patterns
	Consider running a denatured vs. native gel
	Reprobe with a different antibody to same protein
Error bars are disturbingly large on your antibody-based data	Lock down your antibody protocol and then ensure you have enough antibody from the same lot number, so you don't have to re-optimize each experiment because of lot-to-lot variability
How much of the signal is actually background?	Optimize protocol and reduce variability (see above).
	Perform a peptide inhibition assay
	Perform experiment without the primary antibody to establish background
	Co-localize with direct fluorescent labeled primary
	Use species preabsorbed secondary antibodies
Repeat your experiment with monoclonal antibodies for better data interpretation	Many monoclonals are available for targets recognized by polyclonals
	Choose a polyclonal made from a short peptide thus minimizing clonality and epitope
	Choose a polyclonal antibody validated in multiple applications to demonstrate specificity across sample matrices, epitope treatments and detection environment
Redo experiment using antibody with known epitope.	Many antibody sequences are published by researchers or commercial suppliers and can be requested
	Sequenced epitopes are not necessary for verifying antibody specificity or experiment reproducibility
	Publish antibody catalog number and company to aid in peer validation of your data

2.8 Validating Antibodies

Validation is a process whereby, through the use of specific laboratory procedures, the performance and characteristics of an analytical technique are deemed suitable for the intended use.

Usually, the first test for antibody specificity is Western blotting of a variety of cell line lysates with known levels of target expression. Here, both positive and negative control cells are used. The next step in the validation process is often immunohistochemistry (IHC) or immunofluorescence (IF) to titer the antibody on tissue samples.

During validation, it must be shown that not only are the antibodies specific and selective, but they can also provide reproducible results. Hence, reproducibility is the final step in the validation process.

Antibody validation is particularly important in immunohistochemistry applications. Analysis of IHC data can be challenging due to pre-analytical, analytical, and post-analytical factors that affect staining, particularly with free-floating or paraffin-embedded sections. These factors are discussed in detail in section 3.7.

Notes

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Antibody Applications

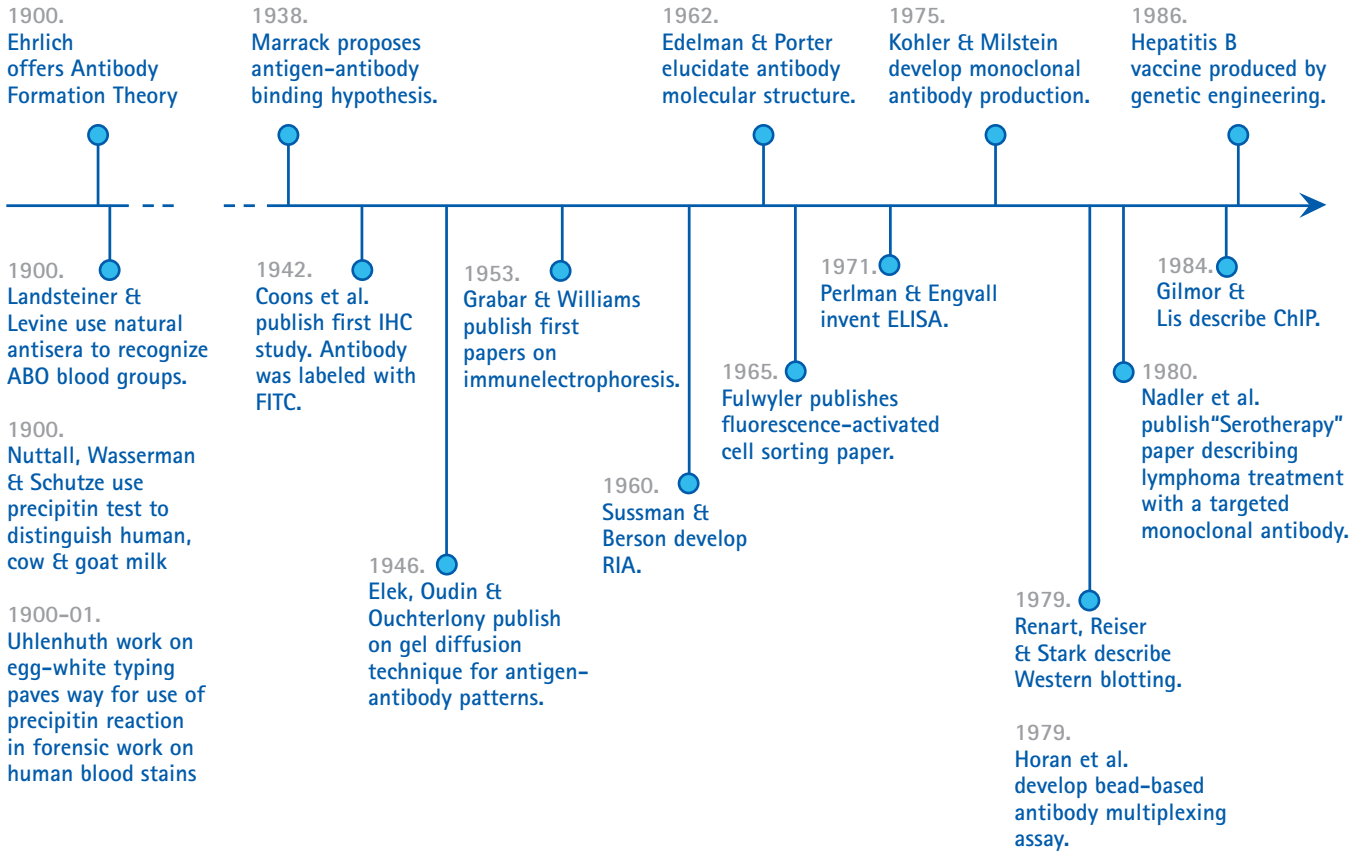
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3.1 Introduction to Antibody Applications
3.2 Immunoprecipitation
3.3 Chromatin Immunoprecipitation (ChIP)
3.4 Western Blotting
3.5 Enzyme-linked Immunosorbent Assays (ELISA)
3.6 Multiplexed Bead-based Detection
3.7 Immunohistochemistry/ Immunocytochemistry
3.8 Flow Cytometry
3.9 Functional Blocking and Stimulation Assays

3.1 Introduction to Antibody Applications

The recognition of the value of the specific antibody-antigen interaction at the end of the 19th century has led to the emergence of a variety of immunotechnologies, most of which are still in use today. From the precipitin test for analyzing blood components to highly specialized chromatin immunoprecipitation techniques to immunostaining in automated imaging flow cytometry, antibody-based tools continue to play an important role in biological and biomedical research. In this section, the theory and practice of the major modern immunotechniques used today will be discussed.

Timeline of Immunotools



3.2 Immunoprecipitation

Key Steps

3.2.1 Introduction
3.2.2 Antigen Labeling
3.2.3 Sample Preparation
3.2.4 Formation of the Antibody-Antigen Complexes
3.2.5 Precipitation of Immune Complexes
3.2.6 Analysis
3.2.7 Troubleshooting

3.2.1 Introduction

It is possible to use antibody-antigen precipitation, or immunoprecipitation (IP), to isolate a specific antigen from complex protein mixtures in cell or tissue lysates. Immunoprecipitation has proven to be an invaluable investigational tool that is routinely employed by many laboratories to ascertain critical information regarding a given antigen. These include small-scale antigen purification for functional studies, N-terminal sequence analysis, investigation of protein-protein interactions, and the determination of the relative abundance and stoichiometric distribution of the antigen within a cell or tissue. Success in an immunoprecipitation assay is dependent on two main factors: the abundance of antigen in the original sample and the affinity of the antibody for the antigen (normally requiring affinities of 10^8 M^{-1} or higher).

Before beginning the immunoprecipitation procedure, ensure that proper experimental controls are in place. Control antibodies should be as similar in nature to the specific antibody as possible. For a polyclonal antiserum, the ideal antibody negative control would be the pre-immune serum from the same animal. However, an equal concentration of non-immune (normal) serum from a different animal of the same species should suffice in its absence.

Immunoprecipitation can be divided into the following key steps:

- Antigen labeling (optional)
- Sample preparation (lysis of cells to release the antigen)
- Formation of the antibody-antigen (immune) complex
- Precipitation of the immune complexes
- Analysis

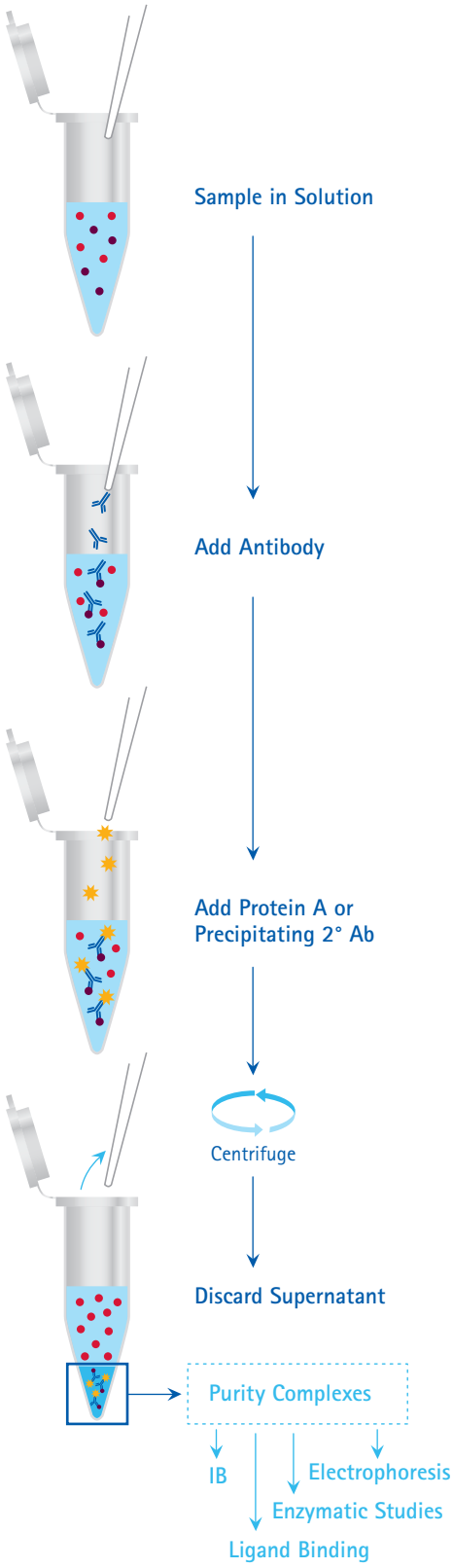


Figure 7.
These steps and troubleshooting will be discussed in greater detail below.

3.2.2 Antigen Labeling (optional)

Antigen may be labeled by incubating in a medium containing a radioactive precursor (such as ^{3}H-thymidine), by iodination or biotinylation of surface proteins, by treatment with radioactive sodium borohydride, or by other published techniques. Antigen labeling is optional, and is not required for most IP assays.

3.2.3 Sample Preparation

Prior to formation of antigen-antibody complexes, the antigen must first be efficiently extracted from the cell or tissue sample in a form that is still recognizable by the antibody. This requires taking into account the location of the antigen within the cell (nuclear, cytosolic, membrane-bound, etc.) and determining how best to extract the antigen with minimal effect on its structural integrity. Before initiating an extraction/cell lysis protocol, consider what information you hope to obtain from the immunoprecipitation. For example, is it necessary to extract functional protein for subsequent functional studies? Additionally, you may want to consider whether to disrupt protein-protein interactions or to co-immunoprecipitate proteins that may interact with the target antigen within the cell. Based on these and other considerations, decide upon an appropriate lysis strategy. Often, an effective lysis strategy is determined empirically.

Perhaps the most important aspect of the lysis procedure is the composition of the lysis buffer. The ionic strength (salt concentration), choice of detergent, and pH of the lysis buffer may significantly affect the efficiency of extraction and integrity of the antigen. Slightly alkaline pH and low ionic strength buffers typically favor protein solubilization, while high salt concentration and low pH may cause the antigen to denature and precipitate from solution. The choice of detergents is crucial and may be influenced by many factors, including (among others) the subcellular location of the antigen, and whether the goal is to preserve subunit associations and other protein-protein interactions.

In general, non-ionic (e.g., Triton® X-100, NP40) or zwitterionic (e.g., CHAPS) detergents tend to preserve noncovalent protein-protein interactions, while ionic detergents (e.g., SDS, sodium deoxycholate) tend to be more denaturing of protein-protein interactions, and may adversely affect the ability of the antibody to recognize the target antigen. For a thorough review of the characteristics of some commercially available detergents, please see Merck Millipore's Essential Biochemicals handbook (Available at: **www.merckmillipore.com/biochemicals**).

Regardless of the lysis strategy employed, the lysis buffer should always contain a relevant cocktail of protease inhibitors (see Appendix for recipe). to protect against proteolysis of the target antigen by proteases liberated during the cell lysis procedure. Additionally, all solutions should be pre-chilled, and all steps in the lysis procedure should be performed on ice.

The number of cells required for lysis will vary with cell line and the anticipated abundance of the target antigen in the sample. In general, lysates should be prepared from no less than 10^{7} cells at 10^{7} cells per mL of lysis buffer. Following preparation of the lysate, determine the protein concentration and adjust concentration of the lysate to between 2 to 5 mg/mL with lysis buffer or PBS. Extracts that will not be used immediately should be aliquoted, snap-frozen, and stored at -70°C for future use.



Watch Out

If Triton® X-100 was used in the lysis buffer, the protein concentration cannot be determined by absorbance of the solution at 280 nm as Triton® X-100 absorbs strongly at 280 nm.

In general, direct protein quantitation via an FTIR spectrometer, such as the Direct Detect® Quantitation System, enables accurate measurements of total protein in many lysates, even in the presence of detergents, reducing agents and lipids.

Tip



If the protein concentration of the extract is below 0.1 mg/mL, high quality BSA should be added to 1% (w/v) prior to freezing.

3.2.4 Formation of the Antibody–Antigen Complex

Once the antigen has been extracted, antibodies are added to the lysate to allow formation of the immune complex.

3.2.5 Precipitation of the Immune Complexes

To precipitate immune complexes, you may use:

- Protein A or Protein G Agarose,
- Precipitating secondary antibodies, or
- Protein A-bearing *S. aureus* cells.

The affinity of an antibody for Protein A or G is dependent on the subclass of the immunoglobulin and the species from which it came. For example, Protein A is exceptionally well-suited for immunoprecipitation of all rabbit primary antibodies, but not for chicken antibodies. (See Protein A/G Binding Affinities in Appendix).

To use protein A for immunoprecipitation of mouse primary antibodies, it is advisable to add 5 µg of rabbit anti-mouse IgG (secondary precipitating antibody) prior to the addition of Protein A/G (mix gently, and incubate for an additional 30 minutes at 4°C prior to adding Protein A/G). When Protein A or G Agarose is used for precipitation, 10–20 µL of a 50% Protein A/G agarose slurry should be sufficient to precipitate the quantity of antibody/antigen complex prepared according to the procedure above. Following addition of Protein A/G Agarose, incubate with gentle agitation for 30 minutes at 4°C, then wash three times (or more, if the antigen has been radiolabeled) by centrifugation and resuspension in immunoprecipitation buffer, and collect antibody-antigen-Protein A/G complex by centrifugation.

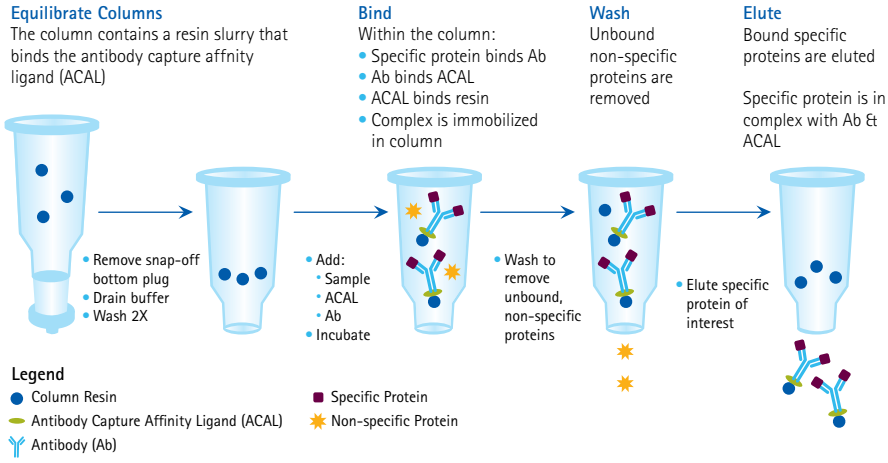
3.2.6 Analysis

Immunoprecipitated pellets to be used for electrophoresis may be resuspended with 2x SDS sample buffer and resolved by SDS-PAGE (for subsequent detection by Western blot; please see section 3.4). The purified immune complex may also be used for enzymatic studies, ligand binding, further immunizations, or for use in other immunochemical techniques. These methods, when used in conjunction with immunoprecipitation, can greatly increase the amount of information about an antigen.

Technology Highlight

Faster, Simpler, More Reproducible IP

Merck Millipore's Catch and Release® kit was designed to meet your needs by making IP faster, simpler, and more reproducible. With Catch and Release® kits, you can purify both native and denatured proteins using a variety of antibodies and sample types. The kits are even validated for Co-IP and IP-kinase assays.



Description	Qty	Cat. No.
Catch and Release® v2.0 Reversible Immunoprecipitation System	50 assays	17-500
Catch and Release® v2.0 Reversible Immunoprecipitation System	5 assays	17-500A
Catch and Release® v2.0 High Throughput (HT) Immunoprecipitation Assay Kit- 96 well	96 assays	17-501
Catch and Release® Phosphotyrosine, clone 4G10®	50 assays	17-502
Catch and Release® Phosphotyrosine, clone 4G10®	5 assays	17-502A

3.2.7 Troubleshooting

The most common challenge with immunoprecipitation is trying to lower the number and type of background proteins that contaminate the washed immune complexes. Background problems can arise from many different sources that may be either specific or nonspecific. Here are a few suggestions to deal with nonspecific background problems:

Problem	Possible Cause	Solution
High Background	Contaminating proteins in the lysate	Preclear the lysates with Protein A/G agarose beads prior to adding the primary antibody.
		Add saturating amounts of competitor proteins, such as BSA, gelatin, acetone powders, or nonfat dry milk.
		Spin the lysate at 100,000 x g for 30 min (discard pellet) prior to addition of primary antibody.
	Contaminants in antibody solution	Centrifuge the antibody at 100,000 x g for 30 min (discard pellet) and titrate.
	Precipitating antibody is nonspecific	Try a different antibody.
	Not enough wash time	Increase the number of washes. "Soak" solid phase in the wash buffers for 10 min per wash.
	Too much antibody	Decrease primary antibody concentration.
	Incubations too long	Decrease primary antibody incubation time.
	Secondary antibody is binding non-specifically	If using rabbit anti-mouse immunoglobulin (precipitating secondary antibody) in conjunction with a monoclonal antibody, check the background due to precipitating secondary antibody alone. Titrate if necessary.
	Protein A/G beads causing background	Run a control with only Protein A/G and without the primary antibody during Western blot analysis to determine if background bands are from the Protein A/G beads or extract alone.
	Need to optimize counting parameters (for radiometric assays)	Lower the number of counts per minute (cpm) of the radiolabel used to the minimum needed for antigen detection.
Multiple bands detected in Western blot analysis	Antigen may consist of more than one polypeptide chain or may have additional associated polypeptides.	Additional co-immunoprecipitated proteins are often useful in understanding antigen interactions, and must not be considered as a problem.
	Coeluted capture antibody's heavy or light chain interferes with target protein detection.	Use crosslinking IP (covalently linking capture antibody to Protein A/G) to avoid antibody coelution.
Multiple bands detected below the anticipated molecular weight	May indicate proteolytic degradation of sample, possibly due to protease inhibitor cocktail being old	Prepare new lysates with fresh protease inhibitors added. Protease inhibitor cocktails may be used for this step.

3.3 Chromatin Immunoprecipitation (ChIP)

3.3.1 Introduction

Key Steps
- Sample Preparation*
- Crosslinking Proteins to DNA*
- Cell Lysis and Chromatin Fragmentation*

3.3.2 Chromatin Immunoprecipitation

Key Steps
- Washing, Elution, and Crosslink reversal*
- DNA Purification and Cleanup*
- PCR Analysis*

3.3.3 Advances in ChIP Technology

3.3.4 Troubleshooting

3.3.5 FAQs

3.3.1 Introduction

Chromatin immunoprecipitation (ChIP) is a powerful technique classically used for mapping the *in vivo* distribution of proteins associated with chromosomal DNA. These proteins can be histone subunits, transcription factors, or other regulatory or structural proteins bound either directly or indirectly to DNA.

Successful ChIP requires high quality ChIP-validated antibodies that can specifically detect proteins associated with target regions of chromosomal DNA. Traditionally, endpoint and/or quantitative PCR (qPCR) are performed after ChIP to verify whether a particular DNA sequence is associated with the protein of interest. Using this classical approach, researchers can evaluate the interactions of the proteins of interest with a limited number of known target genes.

The ChIP experiment can be divided into the following main steps:

- Sample preparation*
- Crosslinking proteins to DNA*
- Cell lysis and chromatin fragmentation*
- Chromatin Immunoprecipitation
- Washing, elution, and crosslink reversal*
- DNA purification or cleanup*
- PCR analysis*

Epigenetics Research

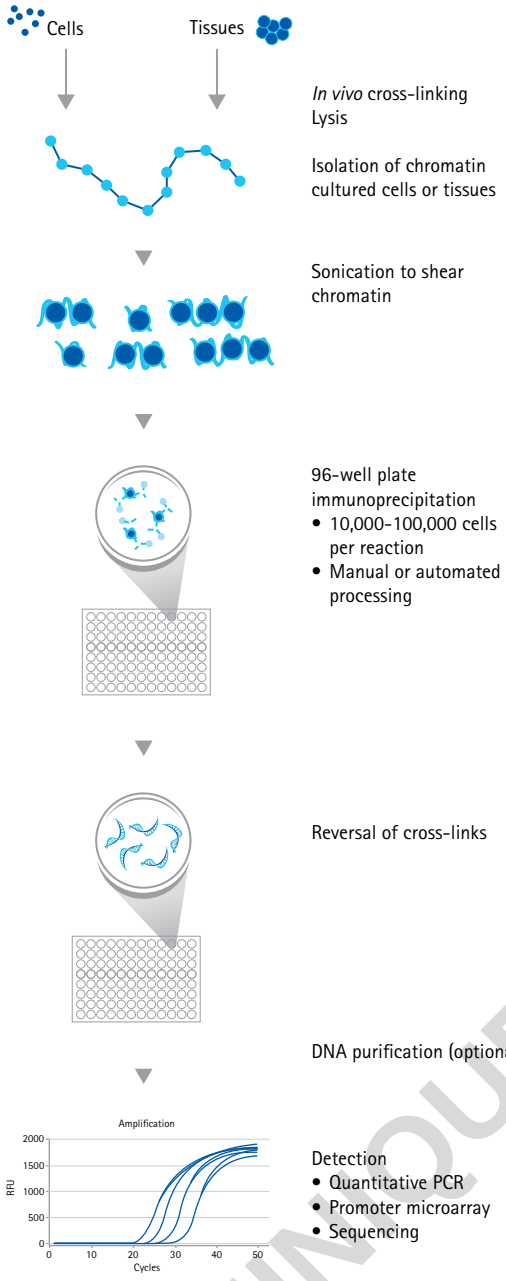
Epigenetics describes heritable changes in gene expression caused by non-genetic mechanisms instead of by alterations in DNA sequence. These changes can be cell- or tissue-specific, and can be passed on to multiple generations. Epigenetic regulation enriches DNA based information, allowing a cell to vary its response across diverse biological and environmental contexts. Epigenetic changes can effect transcriptional and post-transcriptional regulation via the following mechanisms:

- Histone modifications
- Positioning of histone variants
- Chromatin and nucleosome remodeling
- DNA methylation
- Small and non-coding RNA-mediated epigenetic regulation

These mechanisms, in cooperation with transcription factors and other nucleic acid-binding proteins, regulate gene expression, resulting in cellular diversity although DNA sequences are virtually identical from cell to cell. Epigenetic mechanisms of gene regulation impacts diverse areas of research—from agriculture to human health.

The use of antibodies in chromatin immunoprecipitation is discussed in greater detail in the following pages.

*** Resource:**
For a thorough discussion of key steps in ChIP, refer to **Merck Millipore's Guide to Chromatin Immunoprecipitation: Critical Factors for Success** (Literature Number TP5994EN00).



ChIP kits make chromatin immunoprecipitation a snap!

Chromatin Immunoprecipitation (ChIP) assays have become very easy with the availability of assay kits that contain reagents optimized for immunoprecipitation of transcriptionally active chromatin from mammalian cells. The detection of the gene or promoter of interest in immunoprecipitated chromatin must still be empirically determined by the researcher. Various detection techniques can be used: either quantitative PCR, sequencing, or Southern slot-blot analysis is recommended.

A Chromatin Immunoprecipitation (ChIP) Kit may include the following reagents:

- Control DNA
- Buffers for dilution, wash, incubation etc.

Including: Low salt, High salt, LiCl, Tris-EDTA, EDTA, NaCl, Tris-HCl, SDS Lysis Buffers

In addition, an antibody specific to the target of interest is required, such as an Anti-acetyl Histone H3 or H4.

Figure 8.
Chromatin Immunoprecipitation Workflow.

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3.3.2 Chromatin Immunoprecipitation

The goal of this step is to use appropriate antibodies to isolate target protein/DNA complexes from chromatin extracts prepared in previous steps.

Selecting an appropriate ChIP antibody is the one of the most critical steps toward a successful ChIP experiment. Even the highest quality antibodies, which may perform very well in typical Western blot validations, may not be suitable for ChIP. It is best to consider only antibodies that have been validated specifically in ChIP. If your antibodies are not specifically quality controlled and proven to perform in ChIP (e.g. ChIPAb+™ validated antibody primer sets) we suggest you evaluate several potential antibodies before selecting one for your actual ChIP experiments. Below are a few parameters to consider before selecting your antibody.

Monoclonal vs. Polyclonal Antibodies

Either monoclonal or polyclonal antibodies will work for ChIP. A monoclonal antibody recognizes a specific epitope on the target protein. Monoclonal antibodies provide the advantage of being highly specific with less of a propensity for nonspecific binding. In addition, they perform more consistently from batch to batch due low variability in their clonal nature. However, if the epitope recognized by the monoclonal is masked or altered by previous steps in the protocol, such as crosslinking, then monoclonal antibodies will not be effective in isolating your target protein and its associated DNA sequences. Fortunately, this masking rarely affects monoclonal antibodies.

In contrast, polyclonal antibodies recognize multiple epitopes of a target protein. A polyclonal antibody may therefore be more effective even if a few epitopes are masked by crosslinking. However, because polyclonals recognize multiple epitopes, this can increase the probability that nonspecific binding will occur. In addition, it is important to also consider that the specificity of the polyclonal population may drift over time during immunization, unless the serum from which the antibody is purified is pooled prior to preparation or purification. A related point is that most commercial polyclonal antibodies may differ from batch to batch. The degree of variation will depend upon the manufacturing and quality control practices of the vendor. For example, polyclonal antibodies to modifications have finite amounts of serum available.

Regardless of your choice of either monoclonal or polyclonal, when selecting a commercially prepared antibody for ChIP, the ideal antibody will have data demonstrating specificity as well data showing reliable performance in ChIP and other key applications.

Antibodies that are in high demand from commercial suppliers often need to be remanufactured, starting with the immunization of a host animal. Consequently, the specificity and affinity of these antibodies can vary from batch to batch. Larger manufacturers of antibodies such as Merck Millipore are able to address this by immunizing multiple animals followed by screening and pooling of materials demonstrating appropriate affinity and specificity. To ensure consistency, the performance of the final antibody can be compared to previous batches.

Tech Tips

- Whether you select a monoclonal or polyclonal antibody for your ChIP experiment, you must optimize the dilution of your antibody for your specific analysis. If you use excess antibody, you may succeed in immunoprecipitating your target protein, but you may also observe higher nonspecific binding or reduced specific signal. In contrast, if use you use too little antibody you will typically observe low recovery of your target.
- For the best results, ChIP antibodies should be well characterized, proven to bind to its target protein, rigorously tested for specificity, and ideally validated in ChIP.
- Just because an antibody works well in a Western blot does not always indicate it will perform well in chromatin immunoprecipitation. Unlike a Western blot that detects proteins that have been denatured, a ChIP antibody must recognize the target protein in its native state.

Control Antibodies for ChIP

Chromatin is a complex mixture of proteins bound to nucleic acid. To ensure valid results, ChIP experiments should include controls for nonspecific binding of chromatin to your antibody, immunoprecipitation beads, and the resultant ChIP'd DNAs to be analyzed. To control for specificity of your ChIP antibody, you should include the following controls in your ChIP experiment:

- Negative control antibody: Use a negative control IgG from the same species and format (e.g. purified, naive serum, or ascites) as your ChIP antibody. For example, if you use a normal, purified mouse monoclonal ChIP antibody, use a normal purified mouse IgG as your negative control. If you are using a purified rabbit polyclonal ChIP antibody, then use a normal, purified rabbit IgG as a negative control antibody. Alternatively, if an appropriate negative control IgG is not available, you may opt to use the "no antibody" or bead-only approach. Although the matched IgG is typically a better negative control, either the negative control IgG or a bead only control can be used for fold enrichment calculations.
- Positive control antibody: Use an antibody of the same species that recognizes an abundant protein target, such as RNA polymerase II or histone subunits (typically H3 or H4 N-term). The positive control antibody helps to validate aspects of the experiment not affected by selection of your ChIP antibody and can be helpful for troubleshooting experiments.

Specificity Testing

Before you select an antibody, it is important to understand the specificity of your antibody. This is particularly true when working with antibodies directed against post-translationally modified proteins, such as modified histones. It is important to confirm that the antibodies used detect only epitopes containing your specific modification site. There are multiple approaches to evaluate the specificity of an antibody, including: dot blots, peptide microarrays; peptide microarrays or peptide inhibition assays. Some labs will perform a number of validation methods, especially for modified histone peptides, to properly evaluate cross-reactivity to similar epitopes, for example between dimethyl and trimethyl adducts on the same histone subunit. For a reliable method of testing specificity for histone antibodies, we recommend AbSurance™ Histone Antibody Specificity Arrays (Cat. Nos. 16-665, 16-667, and16-668). These arrays provide a total of 89 high quality peptides of histones H2, H3, and H4 on PVDF membranes. The assay is a simple Western blot-like procedure that works with standard chemiluminescent detection systems and in contrast to microarray-based approaches, requires no additional software or expensive equipment for detection.

Validation Testing

Ideally an antibody will be validated in ChIP. However, the lack of ChIP data is not always an indication that an antibody will not work for ChIP. When selecting an antibody for ChIP studies, consider all application data available for that antibody. Typically the greater the variety of applications for which there is data, the higher the likelihood of an antibody performing well in ChIP. For example, an antibody validated in immunoprecipitation (IP), immunofluorescence (IF), or immunohistochemistry (IHC) is more likely to produce positive ChIP results than an antibody validated for only Western blot. However, validation in these applications does not guarantee the success of the antibody in ChIP, as successful ChIP antibodies must recognize accessible epitopes that are not affected by the crosslinking methods often used in ChIP.

Tech Tip

Select candidate ChIP antibodies that perform in immunocytochemistry, Western blot, and/or immunoprecipitation. Satisfactory performance in these other immunoassays increases the chances of performance in ChIP. Although this does not guarantee performance in ChIP, it can help prioritize candidate antibodies and screen out less desirable ones.

Application of ChIP Antibodies

The ChIP antibody may be conjugated directly to agarose or magnetic beads or it may be immobilized on beads conjugated to protein A, protein G or a protein A/G bead blend. Original ChIP protocols used agarose beads, but many long-time ChIP users have moved to magnetic beads. Magnetic beads enable rapid isolation of protein/DNA complexes from the crude chromatin mixture using a magnetic separation device.

Agarose beads perform well in the hands of many researchers and offer a less expensive, but more time-consuming option. These beads require centrifugation for separation and may exhibit nonspecific binding, which may require blocking and lysate preclearing.

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The choice of bead type is often influenced by the type of antibody one plans to use for the experiment. Protein A beads exhibit the highest affinity for rabbit polyclonal antibodies, whereas the protein G beads bind a wider range of antibodies including most, but not all, classes of mouse monoclonal IgGs. The protein A/G blend provides the most flexibility in terms of the type of antibody that you can use, because it combines the binding characteristics of both protein A and protein G. Our research and development team has found that the protein A/G blend typically produces lower backgrounds than protein A or G alone, for a wide range of antibodies without compromising the efficiency of immunoprecipitation.

Magnetic Beads

Advantages
Low nonspecific binding
Blocking and preclearing not required
Easy to handle during washes
Beads are visible in tube
Reproducible results
Disadvantages
Slightly higher cost
Magnetic rack required
Non-porous (binding capacity dependent on bead surface area)

Agarose Beads

Advantages
High capacity binding (porous), which may also increase nonspecific binding
Simple equipment required (centrifugation or filtration)
Slightly lower cost
Disadvantages
Blocking required
Preclearing required
Not visible in tubes
High probability of bead loss during handling

Regardless of your choice of beads, the order in which you apply magnetic or agarose beads to your ChIP reaction may affect your ChIP signal. One method is to incubate the beads with the capture antibody (few hours at room temperature, or overnight at 4°C) followed by addition of chromatin and further incubation (1 hour to overnight with rotation, at 4°C). Increasing the time of incubation may increase both the background and the ChIP signal; however, antibodies with low affinities for their targets generally do not produce significant ChIP signals without longer (overnight) incubations. Alternatively, some protocols involve either incubating the antibody with chromatin and then adding beads or adding all three components at the same time. Adding all three components often works, and reduces the time required to perform the overall reaction.

3.3.3 Advances in ChIP Technology

Epigenetic marks are as important as DNA sequences in determining phenotypes and their study has heightened focus on protein-DNA interactions versus traditional gene-based heredity studies. Characterization of these epigenetic patterns and regulatory networks has progressed beyond standard chromatin immunoprecipitation (ChIP) based techniques to include ChIP-chip, genome-wide microarray analysis of ChIP-isolated DNA, and ChIP-seq (ChIP followed by sequencing of the immunoprecipitated DNA).

These high content approaches provide insights into how regulatory and structural proteins, such as transcription factors and histone subunits, bind and interact with the genome. Immunoprecipitation of these protein-DNA complexes through high quality antibodies selective against post-translational modifications, combined with sequencing or microarrays, identify sequence-specific DNA binding sites with precise resolution.

3.3.4 Troubleshooting:

Problem	Possible Cause	Suggested Steps
High background in negative control (IgG or mock IP) samples	Excessive antibody resulting in binding to non-targets:	Optimize the concentration of the antibody.
	Nonspecific binding to beads:	Include a pre-clearing step to exclude these non-targets or add a blocking agent to the beads.
	Incomplete fragmentation of chromatin:	Optimize the fragmentation process to acquire chromatin lengths between 200-1000 bp. Separately optimize fragmentation for each cell or tissue type. Use siliconized or low retention tubes.
	Contaminated reagents:	Ensure that all reagents are freshly prepared and free of contaminants. Increase the number of washes.
		Run a "no DNA" PCR reaction to determine if your sample is contaminated with nucleic acids.
"No DNA" PCR reaction is showing signal		Use pipettes dedicated to PCR, and UV-irradiate pipettes prior to setting up PCR.
		Perform ChIP, DNA purification, and PCR reaction setup in three separate rooms/areas using dedicated pipettes, or set up reactions in a hood.
		Avoid using bottled or otherwise prepackaged water. Use freshly delivered Milli-QR water from a system containing a UV light source.
		Use aerosol-resistant pipette tips.
		Do not open tubes containing amplified PCR products anywhere near the location of future ChIP or qPCR experiments.
Low recovery of DNA	Ineffective or low affinity ChIP antibody:	Ensure that you are using an antibody that has been validated in ChIP For a complete selection of ChIP antibodies, see www.merckmillipore.com/epigenetics. If you are using a ChIP antibody, increase the incubation time of the antibody.
	Insufficient ChIP antibody:	Generally 1-10 µg of ChIP antibody is sufficient. However, the amount of antibody required may depend on the relative abundance of your target protein and the affinity of the antibody for the target.
	Insufficient starting sample:	Before crosslinking, prepare a separate plate to determine cell number. Re-evaluate your cell number per reaction. Increase your cell number especially if you are attempting to detect a low abundance target.
	Incomplete cell lysis and ineffective fragmentation:	Optimize these steps by varying parameters (see section 5.3) and assessing their effects on chromatin recovery. Use mechanical force such as a dounce homogenizer or glass beads to improve cell lysis. Optimize the fragmentation steps and avoid foaming.
	Over-crosslinking:	Long incubation in formaldehyde may mask epitopes required for recognition by ChIP antibody. This can be especially problematic if you are using a monoclonal antibody. Over crosslinking may also result in the formation of complexes that are resistant to sonication. Optimize crosslinking steps: the final concentration of formaldehyde should be 1%, and you should determine the most effective crosslinking time before proceeding with the experiment.
	Under-crosslinking:	Insufficient crosslinking may result in dissociation of target proteins from DNA during subsequent steps of the protocol. Unless you are studying histones and histone modifications, you should use an X-ChIP protocol to stabilize the associations of your target protein with DNA. Increase crosslinking time.
	Low affinity or low quality beads:	Protein G magnetic beads bind a wider range of antibodies, including mouse monoclonals. For the most flexibility with antibody choice, we recommend the protein A/G blend (Cat. No. 16-663).
	PCR primers:	Test the efficiency of the primers. Include appropriate controls and redesign primers if necessary.

For a more thorough Troubleshooting guide, refer to **Merck Millipore's Guide to Chromatin Immunoprecipitation: Critical Factors for Success** (Literature Number TP5994EN00).

3.3.5 Frequently asked questions:

Question	Answer
Should I use a monoclonal or polyclonal antibody?	Either monoclonal or polyclonal antibodies can work for ChIP. Monoclonals are frequently highly specific, but monoclonals can be sensitive to crosslinking conditions. Over crosslinking may mask the target epitope and careful optimization of crosslinking might be required. Polyclonals are less sensitive to over crosslinking conditions, and may produce better enrichment than comparable monoclonals, but polyclonals are more likely to bind to nonspecific targets.
How can I increase the chances of the selected antibody working in ChIP?	Test specificity/crossreactivity to identify the epitopes recognized by your antibody. You will also need to test the antibody in multiple immunoassays such as Western blot, immunocytochemistry, and immunoprecipitation, then make sure the antibody produces good fold enrichment of your target DNA in ChIP.
How much antibody should I use?	We recommend using 2-10 µg of your ChIP antibody depending on the abundance of your protein target and the affinity of your antibody for the target. More antibody does not always equal stronger signal. It is suggested that to get the best ChIP signal the amount of antibody be titrated.
How should I choose a commercial ChIP antibody?	Choose an antibody that has passed multiple specificity/crossreactivity tests, and validated in ChIP and multiple immunoassays. For screening of your histone antibodies, we recommend the AbSurance™ histone antibody specificity Arrays (Cat. Nos. 16-665, 16-667, and 16-668).
Do you recommend using tags if I cannot find a suitable ChIP antibody for my study?	Using a tagged antibody in ChIP is a way to get around antibody unavailability, variability and epitope masking in crosslinked chromatin. It is possible that a tag will interfere with transcription factor function. Tags should be evaluated on a case-by-case basis. Switching tags between N and C termini may be good controls.
What is a good control antibody?	We recommend using normal IgG from the same species as your ChIP antibody, so if you are using a mouse monoclonal, we recommend normal mouse IgG.
What is the advantage of using protein A/G bead blend?	Many antibodies bind to both protein A and G with varying affinity and specificity. Blending protein A and G beads eliminates the need to choose one over the other and to evaluate binding to both types for optimization. In most cases we have seen better fold enrichment and reduced background activity using a protein A/G bead blend compared to using similar quantities of pure protein A or protein G beads.
What is an acceptable % input range for the normal IgG control antibody?	The IgG pulldown can be quite variable and qPCR-assay dependent. The same mock IgG sample can have different percent input results in one location of the genome vs. another region based on sequence composition of the assay design. The signal may be the result of nonspecific binding of nucleic acid to tube, to beads, to antibodies. ChIP is relative so it is best not to attempt to conform to a specific percent of input value, but ideally, the IgG value should have Ct values that are nearest to the most dilute sample in your standard curve. Again, ChIP is relative so if your ChIP signal is higher than your IgG signal (within limits of variation in the assay), you have a positive ChIP result.

Technology Highlight

Reliable, Hassle-Free Results

Chromatin Immunoprecipitation Kits:

ChIP kits offer a ready-to-use and reliable approach to ChIP. At Merck Millipore we offer a variety of ChIP kits based on both magnetic beads as well as agarose beads. Magnetic beads utilize a magnetic separation device for processing, and are generally preferred by laboratories performing ChIP due to their ease of use along with better and more reliable recovery of input beads.

Magna ChIP® Kits Offer

- Full set of optimized and quality controlled reagents proven to work in ChIP
- Detailed protocols for cells and tissues
- Time savings: avoid making reagents and conducting multiple validation and troubleshooting experiments
- Faster protocols that enable ChIP in one day using magnetic beads
- Advanced protocols that enable up to 96 ChIP reactions at once in a single plate
- Positive and negative controls; EZ-Magna ChIP™ and EZ-ChIP™ kits come with IgG controls and well-designed qPCR primers
- Genome-wide kits for microarray and ChIP-seq analyses
- Expert technical support and troubleshooting

Description	Cat. No.
Magna ChIP® A/G Kit	17-10085
EZ-Magna ChIP™ A/G Kit	17-10086
Magna ChIP® HT96 Kit	17-10077
EZ-Magna ChIP™ HT96 Kit	17-10078
Magna ChIP-Seq™ Chromatin Immunoprecipitation and Next Generation Sequencing Library Preparation Kit	17-1010
Magna ChIP²™ Universal Chromatin Immunoprecipitation DNA Microarray Quad Kit	17-1004
Magna ChIP® G Tissue Kit	17-20000
ChIP Assay Kit (Agarose)	17-295
EZ-ChIP™ Kit (Agarose)	17-371

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3.4 Western Blotting

Key Steps

3.4.1 Introduction
3.4.2 Sample Preparation
3.4.3 Gel Electrophoresis
3.4.4 Membrane Transfer
3.4.5 Blocking Nonspecific Binding
3.4.6 Addition of the Antibody
3.4.7 Detection
3.4.8 Common errors
3.4.9 Troubleshooting
3.4.10 A Note on Membrane Selection

3.4.1 Introduction

Western blotting (WB) combines the resolution of gel electrophoresis with the specificity of antibody detection. Blotting can be used to ascertain a number of important characteristics of protein antigens, including detecting the presence and quantity of an antigen, the molecular weight of the antigen, and the efficiency of antigen extraction. This method is especially helpful when dealing with antigens that are insoluble, difficult to label, or are easily degraded, and thus not amenable to procedures such as immunoprecipitation.

By taking advantage of distinct physical characteristics of different polypeptide species such as size, electrical charge, and shape, a complex mixture of proteins can be resolved chromatographically (electrophoretically) by applying the sample to a gel matrix in the presence of an electric current. The common technique used to separate proteins in this manner is SDS-PAGE. A great deal can be learned about the properties of a protein by "running gels," however, even more can be learned by transferring the fractionated protein sample from the gel to solid support membranes (blotting), and detection with specific antibodies. Western blotting has become very common procedure in life science research. The use of quality antibodies in the detection of proteins on a Western blot is critical to success.

Western blotting remains the platform of choice for exploratory research, and is still the standard by which new antibodies and other protein detection assays (such as ELISA, bead-based assays, flow cytometry and immunohistochemistry) are evaluated. The development of new technologies has yielded tools to improve signal-to-noise ratios in Western blotting, and has greatly reduced the time required for the Western blotting process (for example, Merck Millipore's SNAP i.d.® 2.0 system, Cat. No. SNAP2MM).

The basic Western blotting procedure involves the following key steps:

- Sample Preparation
- Gel Electrophoresis
- Membrane Transfer
- Blocking Nonspecific Binding
- Addition of the Antibody
- Detection

The guidelines below describe a starting point from which you can develop your optimized processes.

Resource:
For a thorough guide to Western blotting success, troubleshooting and description of recent enhancements to the Western blotting techniques, refer to **Merck Millipore's popular Protein Blotting Handbook** (Literature Number TP001EN00).

3.4.2 Sample Preparation

An unlabeled solution of proteins, frequently an extract of cells or tissues is first prepared in a gel electrophoresis sample buffer (see Useful Formulations section in the Appendix).

In some cases, the sample to be blotted may have been derived from immunoprecipitation, as described previously. Please see 3.2.3 Sample Preparation in the Immunoprecipitation section for more details.

Watch Out

Samples should not be boiled as proteins containing significant stretches of hydrophobic amino acids (such as membrane proteins) tend to aggregate when boiled.

3.4.3 Gel Electrophoresis

A charged protein migrates in an electric field relative to its net charge. However, as the molecule migrates through the gel matrix in response to the electric current, its mobility is retarded depending on its size and shape by the sieving effect of the gel matrix.

Polyacrylamide gel electrophoresis (PAGE) can be used to separate individual proteins by their size, shape, and charge under non-denaturing conditions. This is commonly called native PAGE. Under non-denaturing

conditions, the migration of some proteins is affected by the retention of secondary and higher order structure stabilized by covalent disulfide bonds between adjacent cysteine residues.

PAGE can also be performed under denaturing conditions, typically in the presence of a molar excess of the ionic detergent Sodium Dodecyl Sulfate (SDS). This is commonly known as SDS-PAGE.

Furthermore, PAGE can be performed under denaturing and reducing conditions (SDS-PAGE in the presence of a reducing agent such as dithiothreitol (DTT) or β–mercaptoethanol).

Denaturing conditions:
Most often, polyacrylamide gel electrophoresis is performed in the presence of SDS. Prior to resolving the sample by SDS-PAGE, the protein is denatured by heating the sample in the presence of the detergent. By disrupting non-covalent intra- and intermolecular associations, the protein is effectively loses its secondary and tertiary structure. As a consequence, the denatured protein molecules become uniformly "coated" with the negatively charged SDS at a concentration of approximately 1.2 grams SDS per gram of protein, thus giving the protein molecules a net unit negative charge per unit mass. Protein samples fractionated by denaturing SDS-PAGE are, therefore, resolved roughly according to their relative molecular weight regardless of charge (and to some degree, shape).

Reducing conditions:
Often, polypeptides containing intact disulfide linkages migrate anomalously by SDS-PAGE. The resolution of such proteins by SDS-PAGE is influenced by their charge as well as their shape. This is due, in part, to steric hindrance of SDS binding to the protein in regions participating in the formation of inter- or intramolecular disulfide bonds, resulting in a heterogeneous charge distribution across the molecule. Additionally, the secondary structure stabilized by the disulfide linkages affects migration through the gel matrix. To alleviate this potential problem, a reducing agent such as DTT or β–mercaptoethanol is added to the SDS sample buffer to disrupt the disulfide bonds. Under reducing and denaturing conditions, all proteins in the sample should be resolved by SDS-PAGE according to size (molecular weight) alone. For this reason, SDS-PAGE is most commonly run under reducing conditions.

Tech Tips

- Make sure that the gel acrylamide concentration is appropriate for the anticipated molecular weight of the antigen to be detected and that the acrylamide solution is degassed prior to casting gel.
- If casting gels manually, always cast SDS-PAGE gels the day before use to insure complete polymerization for maximum resolution.
- Fresh ammonium persulfate and tetramethylethylenediamine (TEMED) should be used to catalyze gel polymerization.
- Rinse wells of the gel thoroughly before applying sample.
- Apply 10–50 µg of total cell or tissue lysates or 0.1–1.0 µg of a purified protein in 1x SDS-PAGE Sample Buffer per well.
- If samples are to be run under native, or non-reducing conditions, β–mercaptoethanol and DTT should be excluded from the sample buffer.

Watch Out

Pre-stained molecular weight markers often do not run true to size. It is recommended that unstained molecular weight standards be run as well for an accurate determination of antigen molecular weight.

Tech Tips

- Samples should be heated at 50–65°C for 10–15 minutes prior to loading gel.
- Run pre-stained molecular weight markers in one well in order to monitor the transfer of protein from the gel to solid supports during the membrane transfer step. This will also help to orient the gel during the transfer procedure.
- Following the specifications of the equipment manufacturer, electrophorese the sample through the polyacrylamide gel to resolve the protein by molecular weight. Stop electrophoresis when the bromophenol blue dye front reaches the bottom of the gel.

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3.4.4 Membrane Transfer

Proteins resolved by SDS-PAGE are transferred from the gel to a solid support membrane. This can be accomplished by either capillary blotting or by electroblotting (semi-dry and tank transfer systems).

The more efficient and most widely used method of transfer is electroblotting. In this procedure, a sandwich of gel and solid support membrane (nitrocellulose or polyvinylidene difluoride (PVDF)) is compressed in a cassette and immersed in buffer between two parallel electrodes. A current is passed at right angles to the gel, which causes the separated proteins to electrophorese out of the gel and onto the solid support membrane. Once the proteins have been transferred to the solid support membrane, the membrane is referred to as a "blot".

The efficiency with which a particular antigen will be transferred to the membrane is dependent on the protein binding capacity of the membrane used, the transfer method and conditions employed, as well as the nature of the antigen itself. To maximize transfer efficiency, some knowledge of the physical properties of the target antigen is beneficial. With respect to the efficiency of transfer, the most important properties are size (MW) and hydrophobicity of the antigen. While SDS is required to facilitate migration of the protein out of the gel in response to an electric current, SDS can also interfere with binding of the protein to the membrane itself (this is particularly an issue with PVDF membranes). Since smaller polypeptides migrate faster, they may still be heavily coated with SDS when they leave the gel and encounter the membrane, thus reducing the efficiency of protein binding to the membrane.

Conversely, higher molecular weight antigens typically require longer transfer times.

Typically, the more hydrophobic a protein is, the more difficult it may be to transfer to solid support membranes.

To transfer a protein from a gel to a membrane: Following SDS-PAGE, the gel is prepared for electroblotting using a standard tank transfer or semi-dry blotting system. Generally, a transfer "sandwich" is assembled, with the following layers in order:

cathode (-) end

1) sponge or foam pad
2) filter paper (3 sheets) soaked in transfer buffer
3) gel with resolved proteins
4) membrane (nitrocellulose or PVDF)
5) filter paper (3 sheets) soaked in transfer buffer
6) sponge or foam pad

anode (+) end

The sandwich is assembled, and placed in the transfer system. The transfer is accomplished by applying 1 ampere (constant current) for 1 hour, or equivalent, in a wet transfer system, or at 0.7 amperes for 45 minutes in a semi-dry transfer system, with 25 mM Tris, 190 mM glycine, and 20% methanol (optional) as transfer buffer.

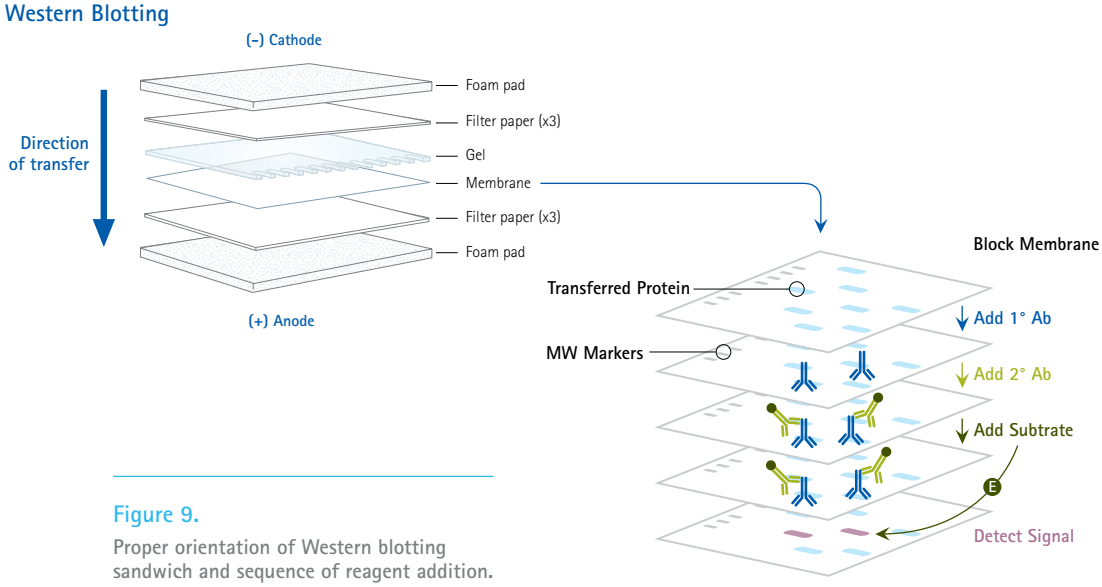


Figure 9.
Proper orientation of Western blotting sandwich and sequence of reagent addition.

Tech Tips

- Cut the membrane and filter paper (6 sheets) to fit the gel exactly.
- It is important that gloves are worn at all times while handling the membrane to prevent contamination.
- Filter paper soaked in transfer buffer can be used to carefully remove the gel from the glass plates or plastic cassette, and to then transfer the gel to the membrane.
- Remove all air bubbles between the gel and the membrane. This can be done easily by rolling a test-tube or Pasteur pipette across the surface of the gel/membrane sandwich.
- For longer transfer times, it is recommended that electroblotting be performed at 4°C to prevent overheating and buffer decomposition.

NOTE: To ensure complete transfer, the blot can be stained with Ponceau S without interfering with subsequent immunodetection. The more sensitive Coomassie® brilliant blue or amido black dyes can be used to visualize protein bands, although, these reagents may be incompatible with subsequent immunodetection.

3.4.5 Blocking Nonspecific Binding

After the proteins have been transferred to a membrane, but before they can be visualized using antibodies and a detection method, the membrane must be incubated with a suitable blocking protein solution to block remaining hydrophobic binding sites on the membrane. This will reduce background and prevent binding of the primary antibody to the membrane itself.

Watch Out

It is important to be certain that the blocking solution does not contain antigens that may be recognized by the primary or secondary antibody. For instance, if using anti-phospho antibodies, it is recommended that Tris-buffered saline (TBS) be used instead of phosphate-buffered saline (PBS), as the latter may show nonspecific reaction to the antibody. Using a protein-free blocking reagent specifically designed for phosphospecific detection (e.g. Cat. No. WBAVDP001) is likely to minimize background signals during phosphoprotein detection.

Typical blocking solutions include:

- 10% (w/v) bovine serum albumin (BSA)
- 5% non-fat dried milk in Tris or phosphate-buffered saline
- Protein-free blockers, such as Bløk™ reagents, which may improve signal-to-noise ratios by minimizing background signal

Incubation in blocking solution for 30 minutes at 37°C or 1 hour at room temperature is sufficient to block membrane.

3.4.6 Addition of the Antibody

Dilute the primary antibody in Tris- or phosphate-buffered saline. Unless nonspecific reactivity is observed or anticipated, it is not necessary to add blocking protein to the primary antibody. After decanting the blocking buffer from the blot, incubate the membrane with diluted primary antibody for 30 minutes at 37°C, one hour at room temperature, or overnight at 4°C, with gentle agitation. Consult individual product datasheets for suggested dilution ranges. Following incubation in primary antibody, the blot is washed in several changes of wash buffer (Tris- or phosphate-buffered saline with 0.1% Tween® 20) before addition of secondary antibody. Follow by incubation with a labeled secondary antibody (as above).

3.4.7 Detection

The method of detection is dependent upon the label that has been conjugated to the primary (or secondary) antibody.

The most common antibody label used in Western blotting is an enzyme such as alkaline phosphatase or horseradish peroxidase, which catalyzes either a light-producing reaction using a chemiluminescent substrate or a color-producing reaction using a colorimetric substrate (chromogen). Chemiluminescent signals can be detected by exposing the blot to X-ray film, while colorimetric signals are detected visually.

Fluorescent detection employs either a fluorophore-conjugated antibody or fluorogenic substrates that fluoresce at the site of enzyme activity (chemifluoresence). One advantage of this method is that the fluorescent signal is stable for long periods of time, and blots can be archived and re-imaged. In addition, the wide variety of fluorophores makes it possible to simultaneously detect multiple protein targets in a single sample (multiplex detection).

Some antibody detection systems, such as chemiluminescence, are exquisitely sensitive, while others, such as those using colorimetric substrates have lower sensitivity.

The appropriate working concentration of the primary antibody depends on the binding characteristics of the primary antibody and is also greatly affected by the type of detection system that is employed.

If the proper primary antibody dilution for a colorimetric detection system is substituted into a chemiluminescent detection system without further optimization, it is very common to see a high background signal. It is necessary to perform an additional dilution series with the primary antibody to determine the optimal dilution for this more sensitive detection system. Likewise, the proper primary antibody dilution for a chemiluminescent detection system may give an undetectably low signal for colorimetric detection.

Other labels include:

1. ^{125}I-labeled secondary antibody, which can be detected using a photographic film.
2. ^{125}I-labeled Protein A. In this case Protein A is used instead of a secondary antibody, as it will bind to the Fc region of IgG molecules.
3. Gold-labeled secondary antibody. The minute gold particles are directly visible as a red color when they are bound with the secondary antibody to the primary antibody.
4. Biotinylated secondary antibody. In this case the blot is incubated with the secondary antibody, and then incubated with enzyme-conjugated avidin that binds strongly to the biotin. This system will give an enhanced signal, as multiple biotin molecules can be attached to a single antibody molecule. The enzyme used is usually alkaline phosphatase or horseradish peroxidase.

Refer to manufacturer instructions for specific protocols for detection with different substrates.

Nice to know

When extra bands appear below that of the desired protein, the most likely explanation is that they originated from proteolytic breakdown of the desired protein. This can be prevented by treating the sample with protease inhibitors during tissue or cell sample preparation. Also, if the sample buffer does not contain sufficient SDS and/or reducing agent (DTT, 2-mercaptoethanol, BMS), the protein may not be fully dissociated into its subunits, reduced or denatured. Hence, extra bands may appear above the desired protein on the gel. Heating the sample in sample buffer at 65°C for 10–15 min immediately prior to loading can reduce these non-covalent interactions or disulfide linkages. Use of an irreversible reducing agent such as TCEP, instead of DTT or mercaptoethanol, may also be helpful.

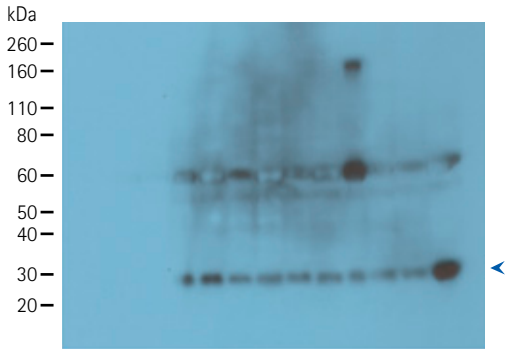
Sometimes, the bands are due to specific recognition of other epitopes in the sample by the primary antibody. For example, an antibody made against a peptide coupled to BSA may recognize traces of BSA in the sample. Homologous proteins may also be recognized by the antibody. When using a polyclonal serum, some bands may be due to the presence of antibodies produced as a result of animal's exposure to similar antigens in its life. It is best to run a normal serum control to determine the specificity of any antibody.

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3.4.8 Common errors

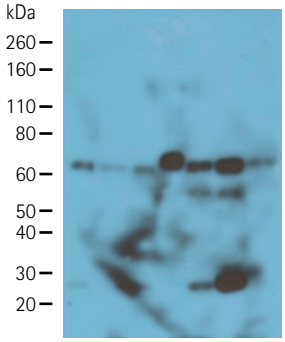
Here are some examples of commonly seen artifacts in Western blotting:

Example 1:



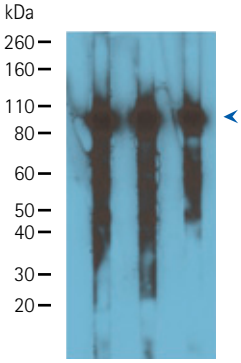
Gel run is "smiling." This is caused by too high a voltage. Also, target bands are bleached, caused by using the detection reagent at too high of a concentration. Background is too high, caused by using the primary and/or secondary antibody at too high a concentration.

Example 2:

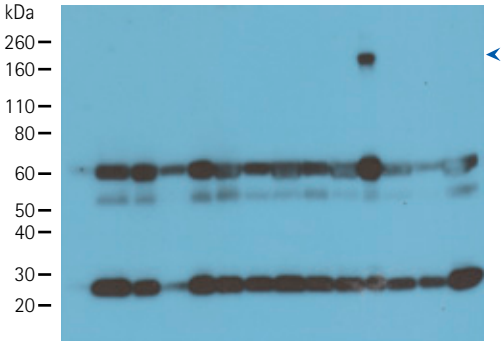


Background is too high; this blot was not properly washed.

Example 3:



Smears are shown instead of distinct bands, caused by loading too much sample into each well.



Bleached bands are shown. Chemiluminescent substrate was used at too high a concentration, or incubated too long before exposure. (Target is at 180 kDa, bleached bands are at ~60 kDa.)

3.4.9 Troubleshooting:

Problem	Possible Cause	Solution
Streaking of blots	Excess protein loaded onto gel.	Accurately measure protein concentration, and load less protein.
Smudgy or fuzzy blots	Gel improperly equilibrated and shrinking during blotting	Check gel equilibration times.
Poor staining of blot with Ponceau S	Ineffective transfer	Carefully remove air bubbles when making sandwich.
		Check that excess temperatures are not reached during electroblotting producing bubbles, or gel/membrane distortion.
No staining of blot with Ponceau S	Proteins did not transfer during Western blotting	Check equipment (boxes, tops, power source).
	Check that the gel and membrane make proper contact during blotting	Check that the membrane is on the right side of the gel.
No signal	Protein degraded during storage of blots prior to detection	Use fresh blots.
	Wrong secondary antibody	Check for appropriate secondary antibody.
	Exposure time too short	Increase length of exposure.
	Insufficient antigen	Load more antigen on gel, or concentrate sample prior to loading, or use immunoprecipitation to increase amount of target protein run in gel.
	Antigen may have been destroyed	Check that antigenicity is not destroyed by treatment for electrophoresis.
	Detection system	Increase (and optimize) concentration, incubation times, and temperatures of primary antibody.
		Increase (and optimize) reagent concentration and incubation times, for your specific application.
		Check that the detection reagents are being stored correctly and used as recommended.
	Low affinity primary antibody	Remove Tween® from antibody buffer.
	Chemiluminescent substrate has lost activity	Prepare a small amount of working solution (1 mL), go into a dark room and add 1 mL of HRP conjugate. Visible blue light should be observed.
	Membrane has been stripped and reprobed.	There may be antigen loss during reprobing.
Weak signal		See under "No signal".
	Insufficient protein on the gel	Load more protein on gel, or concentrate sample prior to loading, or use immunoprecipitation to increase amount of target protein run in gel.
		Expose film for an extended period (1-2 hours).
		Poor protein transfer onto the membrane--optimize transfer conditions, such as transfer buffer pH, membrane type and voltage.
Nonspecific bands	Antibody (primary or secondary) concentrations are too high	Reduce antibody concentrations.
	SDS can cause nonspecific binding to immobilized protein bands	Wash blots well after transfer, using agitation.
		Do not use SDS during procedure.
Diffuse bands	Antibody (primary or secondary) concentrations are too high	Reduce antibody concentrations.
	Too much protein is loaded on the gel	Reduce the amount of protein loaded.
Black blots with white bands or signal that decreases quickly	Antibody (primary or secondary) concentrations are too high	Reduce antibody concentrations, especially the HRP conjugate. Signals that decrease quickly and white bands are an indication that the HRP is "burning out".

Problem	Possible Cause	Solution
Partialy developed area or blank areas	Incomplete transfer of proteins from the gel	Eliminate air bubbles between the gel and membrane during transfer.
		Incubate membranes separately to ensure that membrane strips are not covering one another during incubations.
High background uniformly distributed	Antibody (1° Ab or 2° Ab) concentrations are too high	Decrease antibody dilutions of either primary or secondary antibody or use shorter incubation times.
	Wrong blocking buffer was used	Compare with other blocking buffers.
	Insufficient blocking of nonspecific sites	Increase the concentration of protein in the blocking buffer.
		Optimize blocking time and/or temperature Add Tween®-20 to blocking buffer.
		A concentration of 0.05% Tween® 20 is recommended.
	Cross reactivity of antibody with other proteins in blocking buffer	Use a different blocking buffer. Dilute primary antibody in buffer with no other proteins.
		Do not use milk to block membranes when using a avidin-biotin system.
		Use a freshly prepared solution of blocking agent.
	Insufficient washing	Increase number of washes and the volume of buffer used.
		Add Tween® 20 to to wash buffer if it's not already included.
		Increase concentration of Tween® 20 blocking solution to 0.1% Tween® 20.
High background uniformly distributed	Exposure time is too long	Reduce the time the blot is exposed to film.
		Expose the film for a minimum period (an initial 15 seconds exposure may be all that is required). If exposure time is too short to be convenient, reduce antibody concentrations.
		Leave blots in the cassette for 5-10 minutes before re-exposing to film.
	Detection reagents causing high background	Rewash blots twice for 10 minutes in wash buffer and repeat detection steps.
		Excess detection reagents on blots. Drain well by absorbing the excess on blotting paper before placing blots in film cassettes.
	Membrane problems	Make sure membranes are wetted thoroughly and kept wet throughout the procedure.
		Use agitation during all incubations. Handle membranes carefully - damage to the membrane can cause nonspecific binding.
		Do not handle membrane with bare hands. Use gloves!
		Always wear clean gloves or use forceps.
	Contamination or growth in buffers	Prepare fresh buffers.
	Contaminated blocking equipment	Clean or replace all equipment.
Blotchy or speckled backgrounds	Antibody (primary or secondary) concentrations too high	Optimize antibody concentrations. The primary/secondary antibody can cause high background if the concentrations used are too high.
	Aggregate formation in the HRP conjugate can cause speckling	Filter the conjugate through a 0.2 µL filter.
	Wrong blocking buffer was used	Compare with other blocking buffers.
	Insufficient blocking of nonspecific sites	Increase the concentration of protein in the blocking buffer.
		Optimize blocking time and/or temperature.
		Add Tween® 20 to blocking buffer. A concentration of 0.05% Tween® 20 is recommended.

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Reference:

Protein Blotting Handbook: Tips and Tricks. Merck Millipore. 2012. Billerica, MA. (Literature No. TP001EN00).

3.4.10 A Note on Membrane Selection

The type of membrane used for blotting can influence the following:

- Protein binding capacity
- Requirement for prewetting with alcohol
- Ability to perform multiple stripping and reprobing experiments
- Protein visualization
- Long-term blot storage
- Signal-to-noise ratio

Polyvinylidene fluoride (PVDF) and nitrocellulose are the two membrane types most commonly used in Western blotting applications

Comparison of PVDF and nitrocellulose membrane attributes and applications

Attributes/Applications	Nitrocellulose	PVDF
Physical strength	Poor	Good
Protein binding capacity	80 – 100 µg/cm^2	100 – 300 µg/cm^2
Solvent resistance	No	Yes
Western transfer	Yes	Yes
Total protein stain	Colloidal gold	Colloidal gold
	Ponceau-S red	Ponceau-S red
	Amido black	Amido black
	India ink	India ink
	Sypro® blot stains	Coomassie® Blue dye
Detection	Chromogenic	Chromogenic
	Chemiluminescent	Chemiluminescent
	Fluorescent	Fluorescent
	Radioactive	Chemifluorescent Radioactive
Double-blotting method	No	Yes
Rapid immunodetection	No	Yes
Western reprobing	Yes	Yes
Edman sequencing	No	Yes
Amino acid analysis	Yes	Yes
Binding in the presence of SDS	Poor	Good
On-membrane digestion or mass spectrometry	No	Yes
Direct MALDI-TOF MS analysis	No	Yes
Data can be archived	No	Yes

Technology Highlight

SNAP i.d.® 2.0 System

Developed to meet the needs of our Western blotting customers, the SNAP i.d.® 2.0 system produces blots of a very high quality every time – in record time! Unique vacuum-driven technology and a built-in flow distributor actively drive reagents through the membrane, ensuring even distribution. Two blot holder sizes accommodates mini (7.5 x 8.4 cm) or midi (8.5 x 13.5 cm) (W x L) size gels and two blot holders can be run in parallel. Thus, you can quickly optimize conditions and greatly increase your protein detection throughput.

Typically, researchers lack the time to optimize their blotting protocols. By shortening the time required for blocking, washing and antibody incubations to 30 minutes, the SNAP i.d.® 2.0 system allows you to optimize your immunodetection conditions for the highest quality results.



Description	Cat. No.
SNAP i.d.® 2.0 Protein Detection System-Mini (7.5 x 8.4 cm)	SNAP2MINI
SNAP i.d.® 2.0 Protein Detection System-Midi (8.5 x 13.5 cm)	SNAP2MIDI
SNAP i.d.® 2.0 Protein Detection System-Mini and Midi (7.5 x 8.4 cm and 8.5 x 13.5 cm)	SNAP2MM

3.5 Enzyme-linked Immunosorbent Assays (ELISA)

3.5.1 Introduction
3.5.2 Sandwich ELISA
3.5.3 Competitive ELISA
3.5.4 Substrates
3.5.5 Quantification
3.5.6 Troubleshooting

3.5.1 Introduction

Enzyme-Linked Immunosorbent Assay (ELISA) is a technique that combines the specificity of antibodies with the sensitivity of simple enzyme assays. By using antibodies or antigens coupled to an easily assayed enzyme that possesses a high turnover number, ELISAs can provide a useful measurement of antigen or antibody concentration. There are two commonly used ELISA formats: Sandwich ELISA and Competitive ELISA. These are illustrated and described below.

3.5.2 Sandwich ELISA

The two-antibody sandwich ELISA is used to determine the antigen concentration in unknown samples, and is one of the most useful immunoassay techniques. The sandwich ELISA is fast and accurate, and, if a purified antigen standard is available, the assay can be used to generate a dose-response curve and to determine the absolute amount of antigen in an unknown sample.

To use this assay, a "sandwich" is created as follows:

- A purified, target-specific antibody (the "capture" antibody) is bound to a solid phase - typically attached to the bottom of a plate well.
- Sample (which may contain an unknown amount of antigen) is added and allowed to complex with the bound antibody. Unbound products are removed by washing.
- A labeled second antibody (the "detection" antibody) is added and allowed to bind to the antigen.

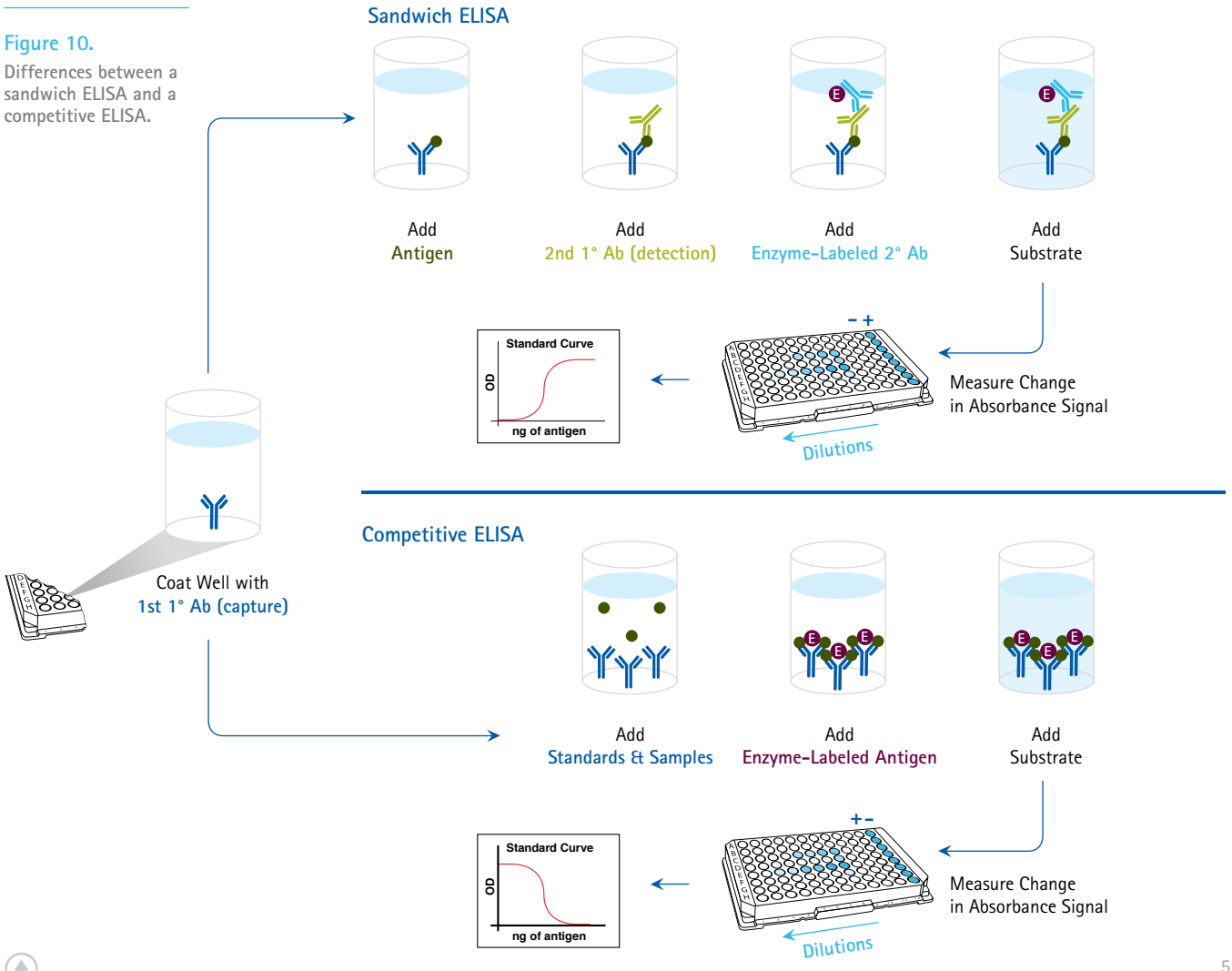


Figure 10.
Differences between a sandwich ELISA and a competitive ELISA.

The assay is quantitated by measuring the amount of labeled second antibody bound to the matrix, through the use of a colorimetric substrate.

Major advantages of this technique are that the antigen need not be purified prior to use, and that these assays are very specific.

However, there is a disadvantage, is that not all antibodies can be used in this application. Monoclonal antibody combinations must be qualified as "matched pairs" - meaning that they can recognize separate epitopes on the antigen so they do not hinder each other's binding.

The amount of the capture antibody bound to the solid phase can be adjusted easily by dilution or concentration of the antibody solution. The avidity of the antibodies for the antigen can only be altered by substitution with other antibodies. The specific activity of the second antibody is determined by the number and type of labeled moieties it contains.

The sensitivity of the sandwich ELISA is dependent on four factors:

1. The number of molecules of the first antibody that are bound to the solid phase.
2. The avidity of the first antibody for the antigen.
3. The avidity of the second antibody for the antigen.
4. The specific activity of the second antibody.



Nice to know

The sandwich ELISA requires two antibodies that bind to epitopes that do not overlap on the antigen. This can be accomplished with either two monoclonal antibodies that recognize discrete sites or one batch of affinity-purified polyclonal antibodies.

3.5.3 Competitive ELISA Assays

When two "matched pair" antibodies are not available for your target, another option is the competitive ELISA. As the name suggests, this assay makes use of the competition between reagents in order to determine the relative amount of an antigen. In this assay, one reagent must be conjugated to a detection enzyme, such as horseradish peroxidase. The enzyme may be linked to either the immunogen or the primary antibody. Although there are several different configurations for competitive ELISAs, below is an example of one such configuration.

A competitive ELISA can be performed as follows:

- An unlabeled purified primary antibody is used to coat the wells of a 96-well microtiter plate.
- This primary antibody is incubated with unlabeled standards and unknowns, and the reaction is allowed to reach equilibrium.
- A conjugated immunogen is added. This conjugate will bind to the primary antibody wherever its binding sites are not already occupied by unlabeled immunogen. Thus, the more immunogen in the sample or standard, the lower the amount of conjugated immunogen bound.

The competition is allowed to reach equilibrium and the plate is "developed" with a substrate (discussed below). The chemiluminescence or color change is measured to indicate the amount of antigen present in the sample.

An advantage to the competitive ELISA is that unpurified primary antibodies may be used. As mentioned previously, there are multiple configurations, with a competing labeled immunogen being common. For other configurations of competitive ELISAs, see Antibodies. A Laboratory Manual, by Ed Harlow and David Lane (Cold Spring Harbor Laboratory, Cold Spring, New York, 1988).

3.5.4 Substrates for ELISA

Unlike Western blots, which use precipitating substrates, ELISA procedures use substrates that produce soluble products. Popular enzymes are alkaline phosphatase (AP) and horseradish peroxidase (HRP). Ideally the enzyme substrates should be stable, safe, and inexpensive.

These enzymes can convert a colorless substrate to a colored product, e.g., p-nitrophenylphosphate (pNPP), which is converted to the yellow p-nitrophenol by alkaline phosphatase. Substrates used with peroxidase include 2,2'-azo-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), O-phenylenediamine (OPD), and 3,3',5,5'-tetramethylbenzidine base (TMB), which yield green, orange, and blue colors, respectively.

Watch Out

Sodium azide is an inhibitor or horseradish peroxidase. Do not include sodium azide in buffers or wash solutions if an HRP-labeled antibody will be used for detection.

Chromogenic ELISA substrates

Alkaline Phosphatase

Substrate	Buffer/ Second Substrate	Reagent to stop reaction	Color of Product	Wavelength for quantitation
p-Nitrophenyl Phosphate (pNPP)	Na_2CO_3, pH 9.8 with $MgCl_2$	NaOH, 2M	Yellow	405 nm

Horseradish Peroxidase

Substrate	Buffer/ Second Substrate	Reagent to stop reaction	Color of Product	Wavelength for quantitation
3,3',5,5'-Tetramethyl-benzidine (TMB)	30% Hydrogen Peroxide (H_2O_2)	1 M Sulfuric Acid (H_2SO_4)	Blue	450 nm
o-Phenylene Diamine (OPD)	Citrate Phosphate Buffer, 0.02% H_2O_2	Sulfuric Acid (H_2SO_4)	Orange-Brown	492 nm
2,2'-azinodiethyl-benzthiazoline sulfonate (ABTS)	Citrate Phosphate Buffer, 30% H_2O_2	20% SDS / 50%DMF	Green	410 nm, 650 nm

The most sensitive ELISA detection method involves the enzyme catalyzing the reaction of a chemiluminescent substrate to generate light. For example, Luminata™ ELISA HRP substrates, when catalyzed by HRP, generate a signal detected using a luminometer.

Chemiluminescent ELISA substrates

Horseradish Peroxidase

Luminata™ Forte ELISA HRP Substrate (Cat. No. ELLUR0100)

Luminata™ Crescendo ELISA HRP Substrate (Cat. No. ELLUF0100)

3.5.5 Quantification

A plate reader or luminometer set to measure at the appropriate wavelength is used to quantify the signal. By comparing the specific signal in a sample to the standard curve, the relative amount of the antigen in the sample can be estimated (see example below).

To create a standard curve, known concentrations of antigen are plotted on the X-axis and the corresponding absorbance on the Y-axis. The standard curve should result in a graph that shows a direct relationship between antigen concentrations and the corresponding absorbances. In other words, the greater the concentration of the antigen in the sample, the higher the absorbance. The concentration of the antigen in unknown samples may be determined by plotting the sample absorbance on the Y-axis, then drawing a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of antigen in the unknown sample. An alternative approach is to enter the data into a computer program curve fitting software, such as MILLIPLEX® Analyst 5.1 software. A good fit can be obtained with a linear regression analysis. Some data points at the top or bottom of the range tested may be dropped to get a good fit.

An example showing ELISA results is illustrated below. A standard curve was generated using known concentrations of the antigen and measuring absorbance at 405 nm (blue line). A sample yielded an average absorbance of 1.42 nm. Extrapolating to the standard curve, the sample contains ~10 ng/mL of the antigen (dashed red line).

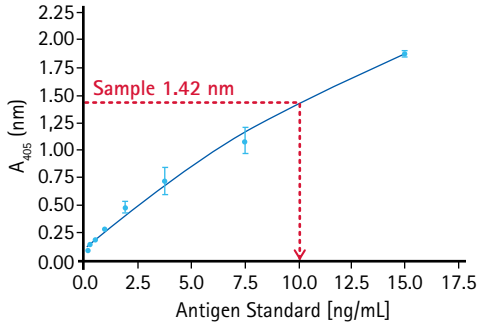


Figure 11.
ELISA standard curve and sample analysis.

3.5.6 Troubleshooting:

Problem	Possible Cause	Solution
No signal	Wrong test reagents used	Ensure that only the reagents for the specific test-lot are used.
	Test reagents damaged	Don't use the test kit after expiration date.
Weak signal	Test reagents used in a wrong dilution	Control used test dilutions carefully (usually a dilution factor of 100 is used).
	Wrong filter (wavelength)	Check your wavelength in your microtiter plate photometer.
	Incubation time too short temperature too low	Check the information of incubation times of the lot in the product data sheet. (The incubation time of the enzyme substrate is applied for temperatures from 20 to 28°C); extend the substrate incubation time, if absorption is below 1.0
	Reagents not at right temperature	Make sure that the reagents used for day 2 have reached room temperature (20 to 28°C) before using within the test kit.
	Sodium azide, mercaptoethanol or DTT can interfere with peroxidase activity at high concentrations	Only use samples which contain no or low concentrations (< 0.1 %) of sodium azide, mercaptoethanol or DTT.
High signal	Test reagents used in a wrong dilution	Check used test dilutions carefully (usually a dilution factor of 100 is used).
	Incubation time too long temperature too high	Check the information of incubation times of the lot in the product data sheet. (The incubation time of the enzyme substrate is applied for temperatures from 20 to 28°C); shorten the substrate incubation time, if absorption is above 3.0

Problem	Possible Cause	Solution
High background (blank)	Insufficient washing steps	Wash plate carefully and remove the liquid after each washing carefully.
	Contamination of the washing solution	Confirm that the water is not contaminated. Use always double distilled water for the reconstitution and dilution of the washing solution.
	Contamination of reagents or vials/ tubes from previous experiments	Avoid pipetting directly out of the reagent vials, if test reagents should be used in further measurements. (Oxidative active contaminants can influence the enzyme substrate by non-specific color development).
	Test reagents (antibody- and enzyme conjugate) used in wrong dilutions	Check used test dilutions for antibody and enzyme conjugate carefully (usually a dilution factor of 100 is used).
Low precision (=random error)	Non-homogeneous samples e.g. cloudy solution, particles in the sample	Check that the samples are taken, prepared and stored according to a recommended sample procedure (polypropylene tubes, storage of clear samples at -20°C).
	Insufficient mixing of samples and standards	Mix samples and standards before pipetting carefully.
	Variation in pipetting	Check your pipettes and calibrate if necessary.
	Carry over between samples and/ or standards	Change pipet tips after each pipetting.
	Insufficient mixing of reagents during incubation	Mix reagents on the test plate after pipetting by moving the test plate carefully; use an orbital microtiter plate shaker on the recommended test steps for optimal mixing of reagents.
	Insufficient washing	Check that the automatic microtiter plate washer is working correctly; residues of liquids must be removed completely after each washing step.
	Evaporation of liquids	Check the contact of the cover seal with the plate during the incubation steps.
Calculated data are too high or too low (=systematic error, deviation of data from "typical data")	Calculation of the dilution factor is not correct	Check the dilution factor used for the sample dilution within the data calculation.
	Modification of the test procedure	Follow the instructions in the product data sheet carefully (incubation time, dilution etc.).
	Incorrect sample treatment	Check that the samples are taken, prepared and stored according to a recommended sample procedure (polypropylene tubes, storage of clear samples at -20°C).

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Technology Highlight

Bring your biomarkers to life.

The best, most relevant ELISAs and RIAs for your protein research.

A complete picture of metabolic syndrome, inflammation or neurological disorders is more than the sum of individual analytes. To help you put the pieces together, we've built the largest portfolio of assays for soluble and intracellular biomarkers. Our manufacturing of ELISAs and RIAs is the gold standard, giving you the same accuracy and precision in every lot, backed by the same, unwavering technical support.



Cytokine / Chemokine ELISA Kits
Neuroscience: Neuropeptide & Neurodegeneration ELISA Kits
Metabolic/Endocrine ELISAs Cell Signaling ELISAs
Transcription Factor Assay (TFA) ELISAs
Radioimmunoassays (RIAs)

3.6 Multiplexed Bead-based Detection

3.6.1 Introduction
3.6.2 Technology
3.6.3 Troubleshooting

3.6.1 Introduction

Multiplex analysis, as the name suggests, is the technique of assaying multiple analytes in a single assay. Multiplexed detection is a technology for biomarker screening and protein analysis that enables researchers to simultaneously investigate the expression of multiple inter- or intracellular proteins, total or phosphorylated, involved in cellular, tissue, or organismal function.

The rapidly growing knowledge base in drug discovery and protein research has placed increased pressure on researchers to rapidly obtain and analyze systems-level data sets connecting proteins and pathways to biological states. Increasingly, this information is difficult, impractical, or cost-prohibitive to obtain using traditional "singleplex" protein detection methods, such as ELISA or Western blotting. Since multiplexing allows detection & measurement of many analytes in a single complicated and heterogeneous sample, the technique proves particularly useful when analyzing serum, cerebrospinal fluid, glandular secretions, or other physiological samples.

3.6.2 Technology

Techniques for multiplexed protein detection most often involve presentation of antibodies to antigens in a sandwich immunoassay fashion and are either immobilized on chips as "planar arrays" or conjugated to micro beads as "suspension arrays." The extremely versatile and sensitive free-floating bead arrays are based on the xMAP® system developed by Luminex Corporation.

This system is the combination of three core technologies: xMAP® microspheres, Luminex analyzers, and analysis software.

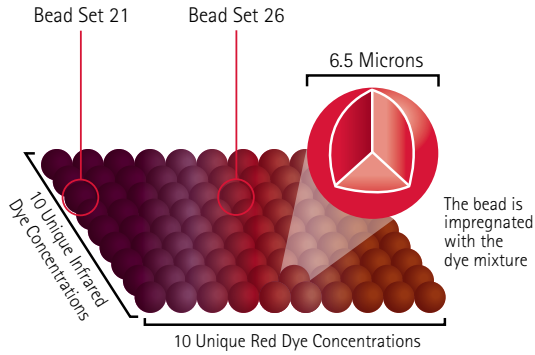


Figure 12.
xMAP® technology: quantitate up to 100 different analytes per sample/well.

Microspheres are dyed with varying concentrations of two red fluorescent dyes to create up to 100 distinct colors. Thus each microsphere in an assay has a 'spectral address' based on the dye concentrations that can then be read and identified by the Luminex analyzer. These microspheres are coated with a capture antibody to specifically bind a target 'analyte' in the sample. A second reporter-tagged antibody is added to complete the 'sandwich' ELISA for readout (see Figure 13).

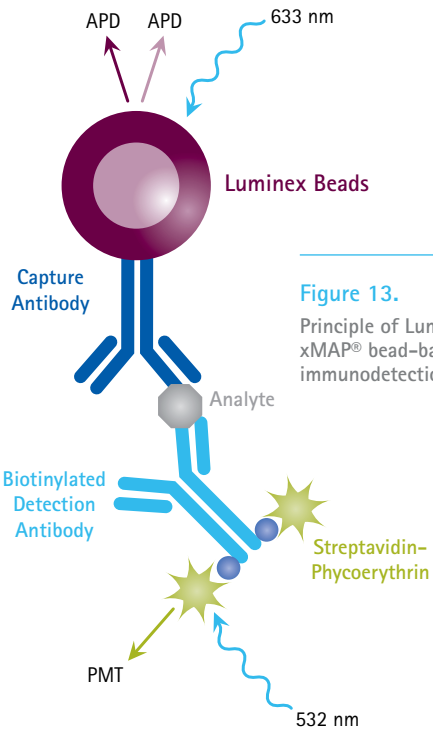


Figure 13.
Principle of Luminex xMAP® bead-based immunodetection.

Two types of Luminex analyzers can be used to perform multiplex bead-based assays. Luminex 200™ and FLEXMAP 3D® systems are flow cytometry-based instruments that integrate lasers, optics, advanced fluidics and high-speed digital signal processors (Figure 14A). The MAGPIX® instrument is a CCD-based system that integrates xMAP® capture and detection components with the speed and efficiency enabled by magnetic beads (Figure 14B).

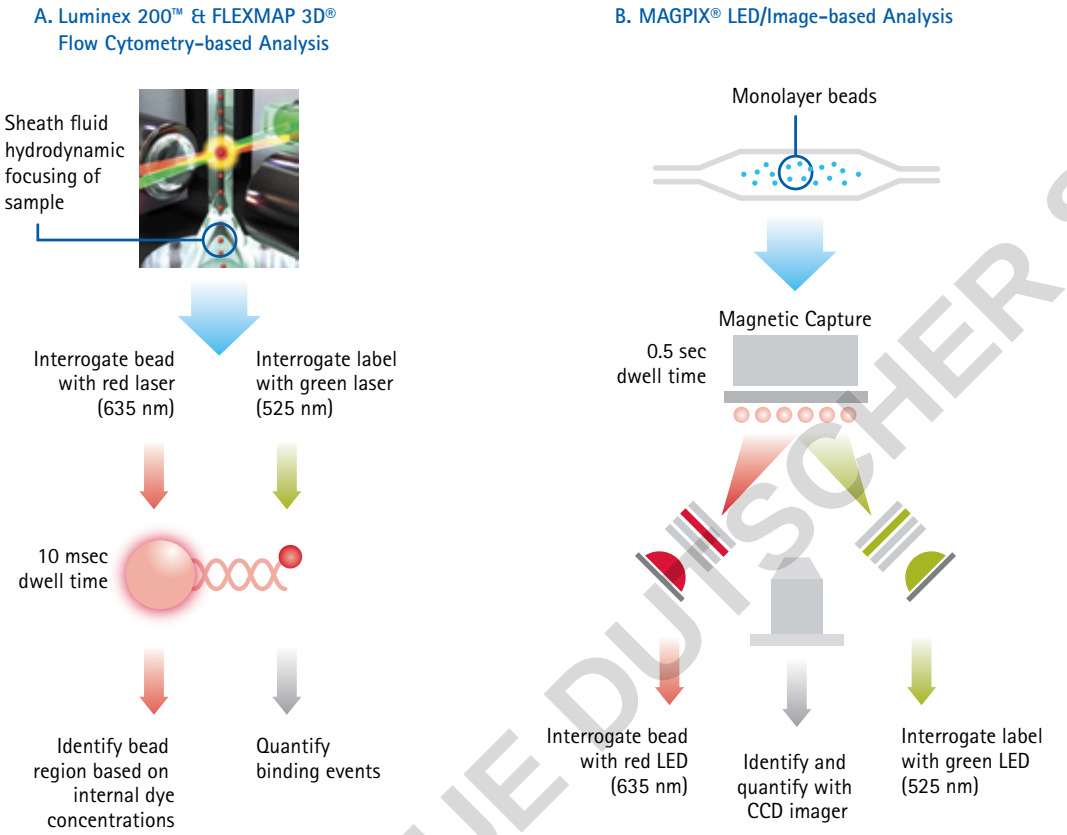


Figure 14.

Two types of Luminex instruments, either flow cytometry-based (A) or CCD imaging-based (B), are available for acquiring data from multiplexed bead-based immunoassays using xMAP® technology.

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The readout from the instruments includes a "bead map" to identify analytes present and standard curves for panel analytes to determine concentration. Since both classification and reporter readings are made on each individual bead simultaneously, this allows for precise multiplex assay results within the same small sample; which is a vast improvement over traditional ELISAs.

Analyzing data from multiplexed biomarker assays can be difficult when working with diverse sample and analyte types. This diversity can lead to a wide range of possible analyte levels and assay signal intensity with respect to those analyte levels, both of which are not always easy to predict or determine accurately. MILLIPLEX® Analyst 5.1 software was designed to generate the most meaningful quantitative analyte data with a focus on data derived from the low and high ends of standard curves. Data in these regions can be important and are commonly missed by existing multiplex data analysis packages.

In developing the new curve fitting algorithms for MILLIPLEX® Analyst 5.1 software, simulations were run on over 600 data sets using actual experimental standard curves in order to determine the curve fits that would give the lowest CVs at the low end and high ends of the curves and that would work well even with standard curves of low quality.

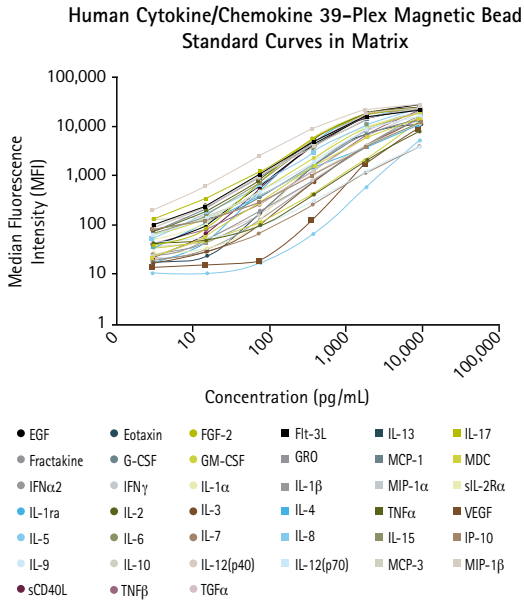


Figure 15.
Analytically validated fixed standard curves in MILLIPLEX® MAP multianalyte panels (based on Luminex® xMAP technology) enable reproducible quantitation.

3.6.3 Troubleshooting – Multiplexed Bead-based Assays:

Problem	Possible Cause	Solution
Insufficient Bead Count	Plate washer aspiration height set too low	Adjust aspiration height according to manufacturers' instructions.
	Bead mix prepared inappropriately	Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting this into the plate.
	Samples cause interference due to particulate matter or viscosity	See above. Also sample probe may need to be cleaned with Alcohol flush, Back flush and washes; or if needed probe should be removed and sonicated.
	Probe height not adjusted correctly	Consult manufacturer instructions. When reading the assay on a Luminex 200™ instrument, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 4 alignment discs. When reading the assay on a FLEXMAP 3D™ instrument, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using one alignment disc. When reading the assay on a MAGPIX® system, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using two alignment discs.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately and pipeting with multichannel pipets without touching reagent in plate.
	Insufficient washes	Increase number of washes.
Beads not in region or gate	Luminex instrument not calibrated correctly or recently	Calibrate Luminex instrument based on Instrument Manufacturer's instructions, at least once a week or if temperature has changed by >3°C.
	Gate Settings not adjusted correctly	Some Luminex instruments require different gate settings than those described in the kit protocol. Use Instrument default settings.
	Wrong bead regions in protocol template	Check kit protocol for correct bead regions or analyte selection.
	Incorrect sample type used	Samples containing organic solvents or if highly viscous should be diluted or dialyzed as required.
	Instrument not washed or primed	Prime the Luminex instrument 4 times to remove air bubbles,wash 4 times with sheath fluid or water if there is any residual alcohol or sanitizing liquid.
	Beads were exposed to light	Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.

Problem	Possible Cause	Solution
Signal for whole plate is same as background	Incorrect or no detection	Add appropriate Detection Antibody and continue.
	Antibody was added	
	Streptavidin-Phycoerythrin was not added	Add Streptavidin-Phycoerythrin according to protocol.
Low signal for positive Lysate Control	Incubations done at inappropriate temperatures, timings or agitation	Assay conditions need to be checked.
Sample signals too high and saturated	Calibration target value set too high	With some Luminex Instruments, default target setting for RP1 calibrator is set at High PMT. Use low target value for calibration and reanalyze plate.
	Plate incubation was too long with Lysate Control and samples	Use shorter incubation time.
	Samples contain analyte concentrations higher than the assay dynamic range	Samples may require dilution and re-analysis for just that particular analyte.
Sample signals too low	Samples contain no or below detectable levels of analyte	If below detectable levels, it may be possible to use higher sample volume. Check with technical support for appropriate protocol modifications.
High Variation in samples and/ or standards	Multichannel pipet may not be calibrated	Calibrate pipets.
	Plate washing was not uniform	Confirm all reagents are removed completely in all wash steps.
	Samples may have high particulate matter or other interfering substances	See above.
	Plate agitation was insufficient	Plate should be agitated during all incubation steps using a vertical plate shaker at a speed where beads are in constant motion without causing splashing.
	Cross-well contamination	Check when reusing plate sealer that no reagent has touched sealer.
		Care should be taken when using same pipet tips that are used for reagent additions and that pipet tip does not touch reagent in plate.

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Technology Highlight

MILLIPLEX® MAP

Bring your biomarkers to life with simultaneous multianalyte detection.



Based on Luminex® xMAP® technology and 25 years of experience.

- The broadest selection of analytes across a wide range of disease states, including metabolic disease, immunology, neurodegenerative disease, toxicity, cancer and more.
- All the components and reagents you need to detect multiple analytes simultaneously in a small sample size (10-50 µL)—all in a single kit, using a single catalogue number.
- Available in a magnetic bead format. Magnetic polystyrene beads contain encapsulated magnetite with bead surface chemistries that are comparable to nonmagnetic beads. New magnetic bead panels will be introduced into our portfolio each quarter!
- Select a premixed kit or choose analytes within a panel to design a custom kit.
- Quality controls provided to qualify assay performance.
- Analytically validated panels that yield consistent analyte profiles irrespective of plex size.
- Standardized standard curve ranges across analytes and lots to ensure lot-to-lot consistency.
- Panels meet stringent manufacturing criteria to ensure batch-to-batch reproducibility.
- Environmentally friendly packaging: our boxes are 100% recyclable to reduce ecological footprints.
- Consolidated packaging available to reduce storage needs.

3.7 Immunohistochemistry/ Immunocytochemistry

Key Steps
3.7.1 Introduction
3.7.2 Specimen Preparation
3.7.3 Antigen Retrieval
3.7.4 Antibody Staining
3.7.5 Antibody Detection
3.7.6 Counterstaining
3.7.7 Troubleshooting

3.7.1 Introduction

Immunohistochemistry (IHC) refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section by exploiting specific antibody-antigen interactions. IHC takes its name from the root words "immuno," in reference to antibodies used in the procedure, and "histo," meaning tissue. In comparison, immunocytochemistry (ICC) differs in the root word "cyto", meaning cell, and is performed on cultured or isolated cells instead of tissue. IHC and ICC are widely used in basic research to understand the distribution and localization of biomarkers and differentially expressed proteins in biological tissues and cells, respectively.

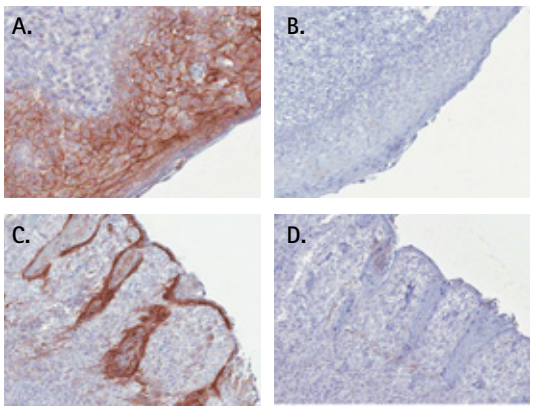


Figure 16.

IHC validation of anti-desmoglein antibody (1:1,000, Cat. No. MABT118) using human tonsil tissue. Human tonsil tissue was analyzed in IHC following antigen retrieval (HRP-DAB detection). As expected, membrane/cell junction immunoreactivity was observed in the stratified squamous epithelium (A) as well as the epithelial cells lining the tonsillar crypts of the human tonsil (C). Treatment of the same tissues with negative control reagent (no primary antibody) resulted in no detectable HRP-DAB signal (B, D). Counterstaining of the tissues with hematoxylin stains the cell nuclei blue.

The main steps in IHC/ICC are as follows:

- Specimen Preparation
- Antigen Retrieval
- Antibody Staining
- Antibody Detection

3.7.2 Specimen Preparation

The ability to successfully visualize and interpret IHC staining is dependent upon the quality of the histological sections used. There are several key preparatory steps that must be done prior to antibody incubation and tissue staining:

- Fixation
- Embedding
- Sectioning

These steps, and the antigen retrieval step that follows, must be done with proper care in order to obtain useful results. Variations in technique can greatly impact staining results. These steps are discussed in some detail below:

Fixation

Fixation is a chemical or physical process that protects and preserves (i.e. "fixes") biological tissues in a state that is closest to their natural state at any given time in order to permit further processing. Reagents used for fixation, or "fixatives," achieve this in three ways: they stop proteolysis, enhance structural stability, and inhibit putrefaction by microorganisms. Major classes of chemical fixatives include: aldehydes, alcohols, mercurials, oxidizing agents, and picrates. Physical fixation commonly refers to freezing tissue, which is generally followed by chemical fixation at some point prior to subsequent processing.

To achieve this, tissue blocks, tissue sections, cells in culture, or smears are either immersed in a fixative fluid, or in cases where whole animal systems are studied, the animal is perfused with fixative via its circulatory system. In the case of cells in culture, cell preparations are either submerged or simply air-dried.

Fixatives stabilize cells and tissues, thereby protecting them from the rigors of subsequent processing and staining techniques. Fixatives may work by several means: formation of crosslinks (e.g., via aldehydes such as glutaraldehyde or formalin), protein denaturation by coagulation (e.g., via acetone and methanol), or a combination of these. Fixation strengths and times must be optimized so that antigens and cellular structures can be retained and epitope masking is minimal. Requirements for fixation can vary widely between tissues. For immunological studies, fixation is especially imperative to ensure the adequacy of the specimen and target antigens. Tissues have differing protein content and structural arrangement; thus they vary in their ability to retain their structure without significant fixation. Incorrect specimen preparation can block or impede antigen labeling in tissue and cells. Unfortunately, the methods that are best for the preservation of tissue structure do so by altering proteins, thereby masking some epitopes and sometimes preventing the detection of the desired target protein. In cases of failure, it is important to experiment with different fixatives and antigen retrieval methods prior to "giving up" on a specific stain.

Embedding

Most samples used in immunostaining are embedded in paraffin because it provides for excellent morphological detail and resolution. Modern "paraffin" is typically a mixture of paraffin wax and resin. It is an excellent embedding medium because it can be heated to liquid state, dissolved by xylene for infiltrating the tissue and then relatively quickly turned to a solid state again for maximum structural support during sectioning. Typically, small blocks (10 x 10 x 3 mm) of tissue are fixed for up to 24 hrs. The most common fixatives used in paraffin sections are formalin-based. These fixatives are well tolerated by the tissues and achieve good penetration. (See Appendix for Recipes of Common Fixatives).

The blocks are then infiltrated and embedded with paraffin, and 5–10 µm thick sections are cut in ribbons and mounted on slides. Once mounted, the slides can be stored indefinitely until immunostaining is required; then the paraffin must be removed from the tissue to allow the water-based buffers and antibodies to penetrate.

Sectioning

Depending on the embedding medium used (i.e. paraffin or aqueous freezing media) subsequent sectioning may be performed on a microtome or cryostat. A microtome is generally used at room temperature on paraffin-embedded tissue blocks to produce ultrathin sections, ranging from 1-60 µm. A cryostat is basically a microtome in a climate-controlled cabinet, which keeps all of the sectioning equipment and tissue blocks between -10 and -30°C. Optimal section thickness is around 5 µm (if achievable), which typically provides the least amount of cell overlap.



Nice to know

The less a tissue is processed (i.e., shorter fixation, looser embedding, less processing time), the less there is need for antigen retrieval.

3.7.3 Antigen Retrieval

To facilitate the immunological reaction of antibodies with antigens in fixed tissue, it may be necessary to unmask or "retrieve" the antigens through pretreatment of the specimens. There are many forms of antigen retrieval (sometimes called antigen recovery), and different antigens and different antibodies will require different antigen retrieval methods. Antigen retrieval has been shown to increase reactivity of the majority of antigens in tissues. The use of antigen retrieval in immunocytochemistry is less common, however depending upon the particular antibody/antigen combination it can be performed on cell preparations, although the length of time and intensity is typically much less than for tissue. Antigen retrieval includes a variety of methods by which the availability of the antigen for interaction with a specific antibody is maximized. The most common techniques are:

- Enzymatic Digestion
- Heat Induced Epitope Retrieval (HIER)
- Citric Acid Incubation

Enzymatic Digestion

This technique involves dewaxing, rehydrating, and rinsing the specimen in running water. The specimen is then equilibrated with the appropriate buffer and is incubated with a proteolytic enzyme either at 37°C or

at room temperature. Enzymes used include pronase (0.05% w/v in PBS), trypsin (0.05% w/v in PBS with 0.1% $CaCl_2$) and pepsin (0.05% w/v in 2N HCl). The conditions of concentration, time, and temperature must be controlled, so that the enzymes can break some of the bonds formed during fixation, uncovering antigenic sites, but the antigen should not be digested completely. The enzymatic activity is stopped by placing the specimen in cold buffer (4°C) prior to processing with antibody. These methods should be considered for some antigens/tissues. (Please see: Shi, S-R, et al. (1993). J. Histochem. Cytochem. 41:1599–1604). However, proteolytic enzymes can abolish the reactivity of some antigens (Please see: Pileri, S., et al. (1997). J. Pathology 183: 116–123).

Heat Induced Epitope Retrieval (HIER)

HIER can be achieved through the following methods:

- Microwave irradiation
- Autoclaving or pressure cooking

Microwave irradiation of formalin-fixed, paraffin-embedded specimens in buffer has been found to markedly enhance the retrieval of antigens. During this procedure, the energy provided helps break some of the bonds formed during fixation, thus increasing the number of available antigen-containing cells and the intensity of reactions. The exact mechanism, however, is unclear. It is important to monitor tissue sections during the microwaving process, to prevent damage and drying. Maintaining consistent conditions between experiments, including buffer volumes, irradiation times, and microwave unit used, will result in less variability in staining results. The number of samples that can be treated by microwave irradiation at one time is limited. Typically, specimens in some buffer (see below) are heated either at full or partial power for a few minutes. Periodically the heating is stopped and liquid is replenished. After a set time, the solution containing the slides is allowed to gradually cool to room temperature; the slides are then rinsed in PBS and used for staining.

Autoclaving or pressure cooking is another method for HIER. In order to standardize the procedure, it is important to start with standard volumes of preheated solutions. After adding the specimens to the boiling retrieval solution, the autoclave or pressure cooker should be brought to full pressure as quickly as possible and the heating times measured exactly from this point. At the end of the heating time (usually 1 to 2 minutes) the pressure should be released. As soon as possible the hot buffer should be flushed out with cold water. (Sections should not be allowed to dry). The specimens should then be washed in buffer. Although the most critical feature of both microwaving and autoclaving is probably the heating of the tissues, the pH and composition of the solutions used are also important in the unmasking of antigenic sites. Studies have found no significant difference between microwave and autoclave treatment, but there are significant differences based on the solutions used. Some of the commonly used buffer solutions are 0.01 M citrate buffer (pH 6.0), 0.1 M Tris-HCl (pH 8.0), and 1 mM EDTA (pH 8.0), with citrate buffer used most commonly. It should be noted that many more specimens can be treated at any one time using an autoclave or pressure cooker than in a microwave oven. However, preservation of the cytological detail may be slightly inferior in sections that undergo pressure cooking. A recommended HIER protocol is available at: http://www.millipore.com/userguides/tech1/mcproto165.

Citric acid incubation is a milder procedure that can be used on many tissues. It is a simple incubation in citric acid buffer, pH 3.0 for 30 minutes at 37°C after blocking, but prior to primary antibody addition. Rinse slide in PBS or TBS, pH 7.4, prior to staining. The buffer composition is 2.1 g citric acid added to 400 mL of ddH_2O. pH is adjusted to 3.0 with acetic acid if above 3.0, or with 1N NaOH if below 3.0, make up to 1 L final volume with ddH_2O.

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3.7.4 Antibody Staining

There are several ways to exploit the specificity of antibodies to visually localize protein and other targets of interest in tissue sections or cultured cells.

Direct labeling uses a primary antibody directly conjugated to an enzyme or fluorophore.

Indirect labeling is a two-step process requiring a primary antibody and secondary antibody, which is specific to the light or heavy chain (or both) of the primary antibody and is conjugated to an enzyme or fluorophore.

The signal created by either the direct or indirect labeling method can be enhanced or amplified using biotinylated primary or secondary antibody, which will bind multiple streptavidin proteins conjugated to an enzyme or fluorophore.

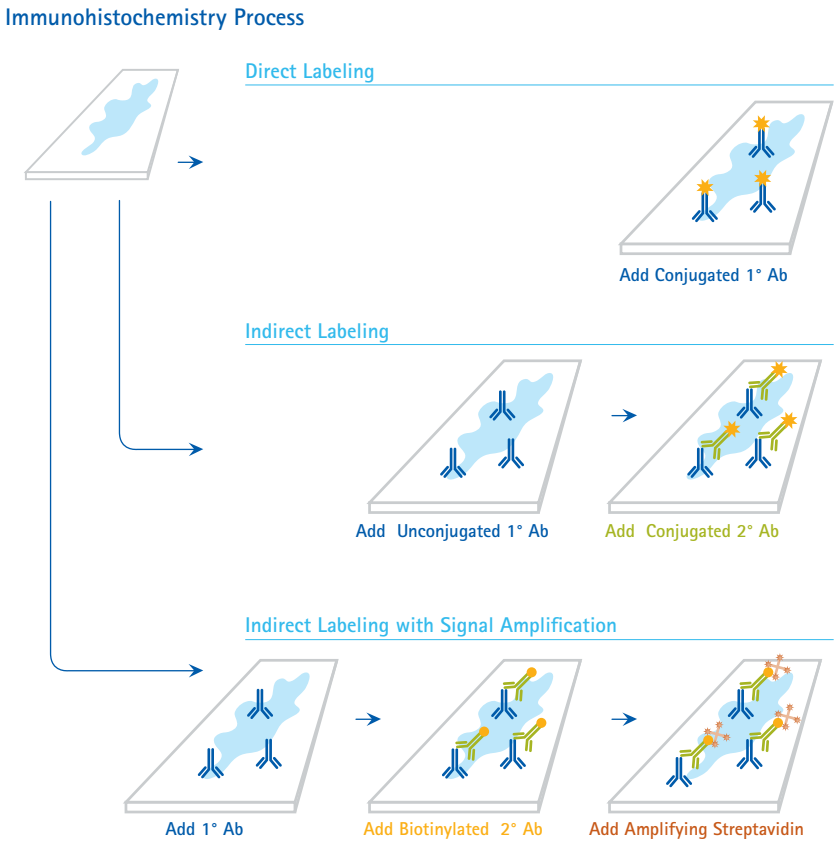


Figure 17.
IHC processes include direct labeling (top), indirect labeling (middle) and indirect labeling with signal amplification (bottom).

Visualizing enzyme-conjugated immune complexes involves an additional step in which a chromogen is catalyzed by the enzymes to produce a colored pigment or dye. Horseradish peroxidase (HRP) and alkaline phosphatase (AP) are typical enzyme conjugates, but the list of compatible chromogens is constantly growing. Proper microscopy techniques and equipment are required to visualize the stained tissue or cells, while comprehensive knowledge of histology will permit proper analysis.

The primary antibody may be directly labeled with an enzyme (such as HRP or AP) or fluorophore (such as FITC or rhodamine), or it may be unlabeled, and require detection by a labeled secondary antibody or a more complex detection system. If a secondary antibody is used, it must be generated against the immunoglobulins of the primary antibody source, e.g., if the primary antibody is raised in rabbit, then the secondary antibody could be goat anti-rabbit. The optimal titer of both the primary and secondary antibody should be determined for each batch.

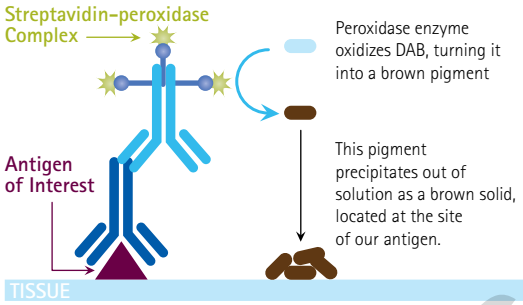


Figure 18.
Principle of colorimetric tissue localization of IHC signals.

The proper working dilutions for every antibody must be optimized for the system in which it is being employed. The same system does not always work for every antibody. The product data sheets may be used as a guide for dilution series starting points. (See Appendix for a possible dilution protocol). The optimal antibody dilution will be that at which the strongest specific antigen staining is observed, with the lowest nonspecific background. As with other controlled experiments, it is advisable to change only one experimental variable at a time. After determining the optimum titer/dilution of the primary antibody, the secondary antibody dilution can be optimized.

For staining of tissue sections, it is customary to incubate with 25–50 µL of diluted antibody (**NOTE:** The volume used must be sufficient to completely cover the tissue, and to ensure the tissue will not dry out during incubation). Incubation periods may range from 30 to 90 minutes at 37°C, from one to six hours at room temperature, or overnight at 4°C. Incubation times should be optimized empirically for each antibody/antigen combination.

Nice to know

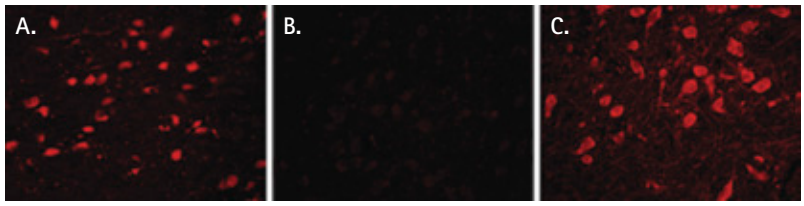
Proper collection of animal tissue may involve institutionally-approved euthanization or anesthesia, perfusion, and appropriate gross dissection. Considerations when collecting human tissue are: cause of death, medications, morbidities, post mortem interval (PMI), and handling times/conditions.

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Technology Highlight

Ready-to-use Autofluorescence Eliminator for microscopy procedures.

With increasing age, the autofluorescent pigment lipofuscin accumulates in the cytoplasm of many cell types, including neurons. The presence of lipofuscin granules can complicate the use of fluorescence microscopy in the central nervous system because of its broad excitation and emission spectra, which overlap with those of most commonly used fluorophores. The Autofluorescence Eliminator Reagent will reduce or eliminate lipofuscin-like autofluorescence without adversely affecting other fluorescent label in sections of human, monkey or rat neural tissue as well as other tissues.



Forty micron sections through the basal nucleus of Meynert from the brain of an adult human male. The sections were untreated (A), treated with the Autofluorescence Eliminator Reagent (Cat. No. 2160) (B), or stained with a polyclonal goat anti-ChAT antibody (Cat. No. AB144P) and visualized with a Cy3-conjugated secondary antibody followed by treatment with the Autofluorescence Eliminator Reagent (C). Images were collected using a Bio-Rad Radiance 2100 MP Rainbow confocal microscope with a 20X objective. Illumination was provided by a 543 nm laser line, and emission was collected from 555-625 nm. The image collection settings (laser power, PMT gain, pinhole diameter, and background level) were identical for all three images. Photos courtesy of Michael Hendrickson and Ronald Kalil, W.M. Keck Laboratory for Biological Imaging, University of Wisconsin-Madison.

3.7.5 Antibody Detection

Two of the most commonly used detection methods are:

- Colorimetric or Enzyme-mediated Detection
- Fluorescence-based Detection

With the advent of electron microscopy, detection of antigens by antibodies that contain large gold particles is often used as well. These may also be visualized at the light microscopic level, but their use is quite rare today, outside of electron microscopy. The common antibody detection methods for light microscopy are described right.

Colorimetric or Enzyme-Mediated detection

When choosing a substrate for conversion by an enzyme, select a substrate that yields a precipitating product. Examples of commonly-used substrates are listed below.

	Substrate	Abbreviation	Final Color	Soluble in Alcohol (for counterstain)
Horseradish Peroxidase	**Diaminobenzidine**	**DAB**	**Brown**	**No**
	3,3'-diaminobenzidine (DAB) produces a brown end product which is highly insoluble in alcohol and other organic solvents. Oxidation of DAB also causes polymerization. DAB has the ability to react with osmium tetroxide, and thus is very useful in electromicroscopy as well as traditional immunohistochemistry sections.			
	Diaminobenzidine with nickel enhancement	**DAB/Nickel**	**Gray/Black**	**No**
	DAB/Nickel produces a more intense stain which is resistant to alcohol and provides better contrast, up to 40 times more sensitive than DAB without enhancement.			
	3-Amino-9-ethylcarbazole	**AEC**	**Red/Brown**	**Yes**
	3-Amino-9-ethylcarbazole (AEC) produces a red/brown reaction product and is widely used for immunohistochemical staining.. Slide specimens processed with AEC must not be immersed in alcohol or alcoholic solutions (e.g., Harris' hematoxylin). Instead, an aqueous counterstain and mounting medium should be used. AEC is also susceptible to further oxidation when exposed to light and thus it will fade overtime. Dark storage and brief light viewing are recommended.			
	4-Chloro-1-naphthol	**4-CN**	**Blue/Gray**	**Yes**
	4-chloro-1-naphthol (4-CN) precipitates as a blue end product. Because CN is soluble in alcohol and other organic solvents, the slides must not be dehydrated, exposed to alcoholic counterstains, or coverslipped with mounting media containing organic solvents. Unlike DAB, CN tends to diffuse from the site of precipitation, thus it is not usually recommended for Immunohistochemistry but can be used for Western blotting.			
Alkaline Phosphatase	**Naphthol AS B1 phosphate/fast red TR**	**NABP/FR**	**Red**	**Yes**
	Napthol AS acts as the substrate for alkaline phosphatase, and the Fast Red chromogen precipitates at the enzymatic sites producing a vibrant red/pink color. Precipitate is soluable in alcohol, thus aqueous counterstain and mounting medium should be used.			
	Naphthol AS MX phosphate/fast red TR	**NAMP/FR**	**Red**	**Yes**
	Napthol AS acts as the substrate for alkaline phosphatase, and the Fast Red chromogen precipitates at the enzymatic sites producing a vibrant red/pink color. Precipitate is soluable in alcohol, thus aqueous counterstain and mounting medium should be used.			
	Naphthol AS B1 phosphate/new fuschin	**NABP/NF**	**Red/Violet**	**Yes**
	Napthol AS acts as the substrate for alkaline phosphatase, and the new Fuchsin chromogen precipitates at the enzymatic sites producing a vibrant red/violet color. Precipitate is soluable in alcohol, thus aqueous counterstain and mounting medium should be used.			
	Bromochloroindolyphosphate/Nitro Blue Tetrazolium	**BCIP/NBT**	**Purple**	**No**
	5-bromo,4-chloro,3-indolylphosphate (BCIP)/nitroblue tetrazolium (NBT) substrate is a commonly used substrate chromogen. BCIP acts as the substrate for alkaline phosphatase, and the NBT enhances the purplish-brown color of the precipitate. BCIP/NBT is compatible with organic solvents so it can be used with alcohol based counterstains including Nuclear Fast Red or Methylene-Green.			

Fluorescence-based detection

A molecule that fluoresces can be attached to the antibody for detection using UV light. Examples are fluorescein, rhodamine, Texas Red®, Cy3, and Cy5. In selecting fluorochromes, one is limited by the available microscope filter sets. Most filter sets are best matched with rhodamine or fluorescein. Texas Red® may also be used with a rhodamine filter set.

Many mounting media contain "anti-fading" solutions, such as DABCO, which will prolong the viewing time of the sample. Merck Millipore offers a variety of fluorescence mounting fluids and counterstain solutions including our basic fluorescent mounting fluid (Cat. No. 5013) and enhanced counterstaining fluid containing nuclear stains such as 4', 6-Diamino-2-phenylindole dihydrochloride (DAPI; Cat. No. S7113).

Fluorophore	Absorption (nm)	Emission (nm)
Fast Blue	360	440
Alexa Fluor® 350	346	445
AMCA	350	450
Bisbenzamide	360	461
Aequorin	Ca^{2+} photoprotein	469
Hoechst 33258	360	470
ACMA UV 0	412, 43	471, 474
Hoechst 33342	343	483
Cy2	489	506
GFP Wild type Non UV ex.	475	509
GFP Wild type UV ex.	395	509
Alexa Fluor® 488	494	517
Calcein	496	517
Fluorescein (FITC/DTAF)	495	520
Fluoro-Jade® B	480	525
Lucifer yellow	425	528
JC-1	514	529
Fluoro-Gold™ (Hydroxystilbamidine)	361	536
Alexa Fluor® 430	430	545
Eosin	524	545
6-JOE UV	520	548
Alexa Fluor® 532	530	555
Cy3	548	562
Alexa Fluor® 546	554	570
Alexa Fluor® 555	555	571
TRITC	547	572
B-phycoerythrin	545, 565	575
R-phycoerythrin	480, 545, 565	578
Rhodamine	539, 574	602
Alexa Fluor® 568	578	602
Texas Red®	589	615
Alexa Fluor® 594	590	617
Propidium Iodide (PI)	536	617
Ethidium Bromide	493	620
Feulgen (Pararosanoline)	570	625
Acid Fuchsin	540	630
Alexa Fluor® 633	621	639
Alexa Fluor® 647	649	666
Cy5	650	670
PE-Cy5 conjugates	480, 565, 650	670
Alexa Fluor® 660	668	698
Alexa Fluor® 680	684	707
PE-Cy7 conjugates	480, 565, 743	767
Cy7	743	767



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3.7.6 Counterstaining in Immunohistochemistry

During the past few decades, histochemical staining has largely been replaced by immunostaining techniques. However, a limited number of available colors is a major drawback in the microscopic examination of tissues. Hence, histochemical counterstaining techniques are employed to overcome this difficulty. Double-staining and even triple-staining of tissue slices offer several advantages and allows for the examination and identification of tissue morphology and sub-cellular location of antigens present. A second stain is applied to provide contrast and allow the primary stain to stand out. In this regard, both chromogenic (e.g., hematoxylin and nuclear Fast Red) and fluorescent dyes (e.g., DAPI and phalloidin) are commonly used to fit the experimental design.

Hematoxylin is one of the most commonly used dyes for nuclear staining. Oxidized hematoxylin is combined with aluminum ions to form an active metal-dye complex, which provides a blue color to the nuclei in mammalian cells by binding to lysine residues on histones. On the other hand, nuclear Fast Red stains nucleic acids and is much faster than hematoxylin. Fluorescent stains, such as DAPI and Hoechst, intercalate into the DNA to give a strong blue color under ultraviolet excitation.

3.7.7 Troubleshooting:

Problem	Possible Cause	Possible Solution
No staining	No antigen	Check literature to see if the protein is expressed in that particular tissue type. Or, check mRNA expression by *in situ* hybridization.
	Improper storage of antibodies	Aliquot and store antibodies in smaller volumes. Store as recommended by the manufacturer. Avoid repeated freeze-thaw cycles.
	Insufficient tissue fixation	Try a fixative or change the duration of fixation.
	Overfixed tissue	Reduce duration of fixation or fix tissue at +4°C instead of room temperature.
	Primary and secondary antibodies not compatible	Use secondary antibody that will interact with the primary antibody (see section 2.5 for use of secondary antibodies).
	Defective secondary reagents	Prepare fresh reagents.
	Enzyme-substrate reactivity; improper pH of substrate buffer	Deionized water may contain peroxidase inhibitors that can reduce enzyme activity. Use buffer in the recommended pH range for specific substrates.
Overstained Tissue	Primary and/or secondary antibody concentration is too high.	Determine optimal concentration, try lower concentrations.
	Incubation period is too long.	Try shorter incubation. Optimize the duration with each component: antibody, substrate, enzyme etc.
	Nonspecific binding of primary and/or secondary reagents to tissues	Treat tissues to minimize or block nonspecific binding.
High Background	Tissues may have high levels of endogenous molecules that are also present in incubation mixtures. For example, peroxidase in blood cells may remain in tissues.	Try incubating with normal serum from species other than the source of primary antibody.
		For tissues containing interfering peroxidase: treat tissue with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature.
	Secondary antibody cross-reactivity or nonspecific binding	The secondary antibody may show a strong or moderate affinity for identical or similar epitopes on non-target antigens. For example, egg whites, sometimes used to coat slides, contain high amounts of avidin. Avoid egg whites to prevent avidin from binding biotinylated secondary antibody during staining.

3.8 Flow Cytometry

3.8.1 Introduction
3.8.2 Antibodies in Flow Cytometry
3.8.3 Detection Methods
Key Steps
3.8.4 Sample preparation
3.8.5 Blocking
3.8.6 Antibody Incubation
3.8.7 Data Acquisition
3.8.8 Troubleshooting

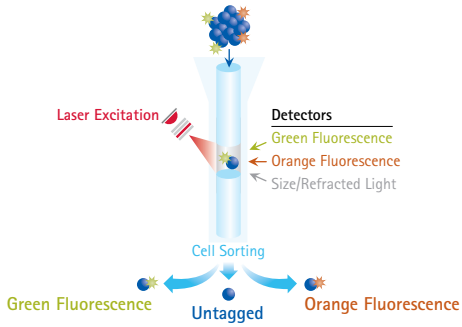


Figure 19.
Flow cytometers, such as the guava easyCyte™ 8HT instrument, use laser excitation of single cells and subsequent detection of fluorescence and refracted light to provide multiparameter cellular analysis.

3.8.1 Introduction

Flow cytometry is a statistically powerful technique for characterizing and/or sorting heterogeneous, suspended cell populations on the basis of physical characteristics and fluorescence. The prototype flow cytometer was developed over 50 years ago; the technology was not first commercialized until the late 1960s.

Flow cytometry is defined as the measurement of the cellular and fluorescent properties of particles (such as cells) in liquid suspension as they pass by a laser or other light source.

From these measurements, specific populations and subsets within them are defined and can even be physically isolated via cell sorting, where cell charge is manipulated based on fluorescence characteristics to allow electrostatic deflection of particles. Adherent cells and solid tissues may also be analyzed if they can be successfully dissociated to create a single-cell suspension.

Flow cytometry relies on hydrodynamic focusing of a cell suspension sample to create a single-cell stream which passes in front of a laser. The manner in which the cell scatters incident light is used to determine the size and intracellular complexity of cells at a rate of thousands of particles per second. This analog data is then converted to digital data which can be quantified and plotted in two (or three) dimensions.

The Cytometry Frontier

The last decade has witnessed widespread adoption of microcapillary flow cytometry, which uses smaller sample volumes and less reagents, generates less waste and has lower operating costs than traditional, sheath fluid-based flow cytometers. Flow cytometry technology has also been further miniaturized into ultra-compact, benchtop cell analyzers. Together, these benchtop instruments have transformed flow cytometry into nearly routine step in most cell-based analyses.

Imaging flow cytometry combines the statistical power and high speed throughput of flow cytometry with the visualization capabilities of immunocytochemistry and microscopy. This combination yields a more comprehensive set of protein localization and distribution data from a single cell sample than any single technology introduced to date.

More information on these cellular analysis platforms is available at:
www.merckmillipore.com/cellularanalysis

Flow cytometry in the clinic:

No well-equipped modern clinical laboratory is complete without a flow cytometer, which has become a fundamental medical screening and diagnostic tool. Clinical laboratory scientists are certified to perform flow cytometric analysis of peripheral blood samples whenever the physician orders a complete blood count (CBC), a test which historically required microscopic examination of a blood smear. Therefore, this analysis was previously statistically limited to the number of fields it was feasible for the pathologist to examine. For CBC, the differential light-scattering properties of leukocytes are exploited to make rapid and statistically reliable determinations regarding relative abundance of peripheral blood cell types. Forward and side scatter can even aid in detecting size and shape abnormalities arising from conditions such as anemia and myelodysplastic syndromes. The addition of antibody testing for known disease markers increases the value of flow cytometry in clinical diagnosis and assessment of treatment progress.

3.8.2 Antibodies and Flow Cytometry

Although cell populations may be broadly characterized by light-scattering properties, more precise identification of subpopulations requires use of probes which bind to specific surface or intracellular moieties unique to cell subtypes. For example, all lymphocytes in a peripheral blood sample may be of the same size and intracellular complexity. Fluorescently-conjugated antibodies specific for cell surface receptors (such as CD4, CD8, and CD19, for example) can be applied to the cell suspension to identify helper T cells, cytotoxic T cells, and B cells, respectively.

At a minimum, even basic, single-laser flow cytometers are typically equipped with sufficient filters to permit detection of four fluorophores simultaneously, and it is currently possible to differentiate as many as 18 wavelengths of light in a single experiment. Acquisition of signal from these complex fluorophore palettes requires multiple lasers, appropriate filters, and an antibody selection that consists principally of primary conjugates, because species cross-reactivity and binding of secondary antibody to unintended targets can easily render data uninterpretable.

3.8.3 Detection Methods

As with other immunodetection applications, there are several approaches to the use of antibodies to probe for specific cell moieties by flow cytometry. There are three main methods:

- Direct Detection
- Indirect Detection
- Biotinylated Detection

Direct Detection

Direct detection refers to a single-step staining process that employs a primary antibody that specifically binds to an epitope of interest and that is directly conjugated to a molecule that permits visualization or other detection of the binding event.

When probing for antigens localized to the cell surface, fixation of cells is not recommended, as this process renders antigens of interest inaccessible to antibody probes. It is therefore necessary to work efficiently to keep unfixed cells viable until data acquisition is complete. Use of these primary conjugates expedites the staining process, as binding of the antigen of interest and labeling with the detection fluorophore are achieved in a single step.

Indirect Detection

In the indirect detection method, incubation with a purified antibody to permit binding to antigen of interest is followed by a fluorophore-conjugated secondary antibody specific for the primary antibody host isotype, forming a primary-fluorescent secondary antibody scaffold. Increased modularity in an antibody library may be achieved by use of purified primary antibodies and fluorophore-conjugated secondary antibodies in a variety of wavelengths (or 'colors') and specific for host isotypes in which the primaries are raised.

Watch Out

Internalization of receptors or other surface antigens of interest occurs naturally when cells are removed from *in vivo* tissues or *in vitro* culture conditions. To minimize this, unfixed cells must be kept on ice and/or at 4°C.

Biotinylated Detection

A third alternative, biotinylated detection, exploits the avidins, bacteria-derived proteins so named for their natural avidity for the endogenous ligand biotin. The biotin-streptavidin complex is sometimes referred to as 'molecular velcro', as its use as a research tool to bind two molecules has become widespread in immunodetection, proteomics, affinity purification, and analysis of nucleic acid-protein interactions. A diverse selection of biotinylated primary or secondary antibodies is widely available commercially; flow cytometric detection is achieved upon subsequent incubation with streptavidin conjugated to fluorophore. Signal amplification may occur because biotin has four sites to which streptavidin may bind, resulting in a potential fourfold increase in fluorescent signal for each primary binding event.

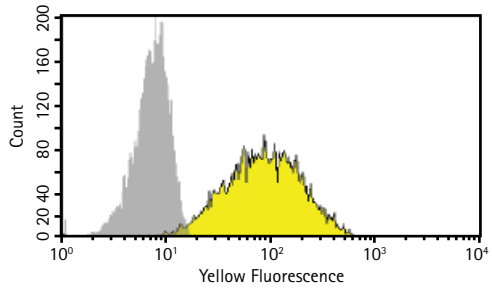


Figure 20.

Staining of human peripheral blood mononuclear cells (PBMC) using a T10B9 primary Milli-Mark™ anti-CD3-PE antibody (yellow histogram, Cat. No. FCMAB168P). Unstained PBMCs are shown as a gray histogram. Cells were analyzed using a guava easyCyte™ 8HT flow cytometer.

Watch Out

Unfixed cells are fragile and it does not take much for them to be destroyed. To keep unfixed cells alive until acquisition on the flow cytometer, it is critical to employ steps that are gentle, fast, and efficient.

	Advantages	Disadvantages
Direct Detection	Binding of antigen of interest and detection of bound antibody are achieved in a single incubation, saving time and allowing for maintenance of cell viability.	Multicolor palette versatility is limited by the selection of antibodies in different 'colors' that is financially feasible to acquire and store.
	Cross-reactivity and unintended binding of antibody are limited, allowing choice from a wider selection of host antibodies (for example, a primary conjugate raised in mouse may be used to detect antigen in a sample of mouse cells).	
Indirect Detection	Increased modularity in multicolor 'channel' selection with fewer antibodies on hand	Two incubation steps are required, mandating increased efficiency to avoid placing unfixed cells at risk for apoptosis before data acquisition can be completed.
		Potential for nonspecific binding by the secondary antibody is introduced, necessitating incorporation of negative 'isotype controls' for each host and antibody isotype used.
		Care must be taken when selecting antibodies to ensure that primary antibody is not raised in the same species from which the sample is collected, as application of the secondary will result in binding of both the primary and of endogenous targets.
Biotinylated Detection	Potential for increase in fluorescent signal for each primary antibody:target antigen binding event	Increased risk for false-positive signal arising from avidin-fluorophore complex binding to endogenous biotin present in the biological sample. Blocking of endogenous biotin may be necessary depending on the nature of cells in the sample.

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Key steps in flow cytometry include:

- Sample preparation
- Blocking
- Antibody Incubation
- Data Acquisition

3.8.4 Sample Preparation

Any cells which can be made into a single cell suspension can be assessed by flow cytometry.

- To prepare an *ex vivo* cell population, freshly dissected tissue is often gently homogenized using mechanical dissociation methods, and different cell types are separated via density gradient centrifugation to remove intracellular matrix material, debris, and irrelevant cell populations.
- Adherent cell lines must be detached from cell culture vessel surfaces using enzymatic solutions or calcium-chelation reagents.
- Suspended cell cultures only need to be counted and assessed for viability.

Cell counts/titers refer to viable cells in suspension; this can be determined either by using an automated cell counter, such as the Scepter™ handheld cell counter or the Muse® cell analyzer, and applying gating to exclude dead cells/debris. Alternatively, viability can be determined by microscope-aided counting of the number of cells in a known volume (such as that provided by a hemacytometer) in the presence of Trypan blue dye, which is excluded by the intact membrane of live cells, so that nonviable cells are easily identified by their uptake of the dye.

Tech Tip

Use nonenzymatic methods for detaching adherent cells from culture surfaces whenever possible to avoid unintentional cleaving of antigen from cell surfaces. When adherent cells are not amenable to detachment by non-enzymatic means, it is essential to use enzymes that are selective for attachment proteins (such as Accutase™ enzyme, Cat. No. SCR005), rather than general proteases such as trypsin.

For tube-based flow cytometry, sample titers of 0.5 – 1 x 10^6 cells per sample will yield a single cell suspension when resuspended in 350 – 500 µL of buffer. Appropriate sample aliquots for high-throughput cytometers that use multiwell plates are usually in the range of 1 x10^5 – 0.5 x 10^6 viable cells per well. If using a microcapillary flow cytometer, such as the guava easyCyte™ flow cytometers, use approximately 10,000-100,000 cells per tube or plate well.

Following each manipulation of the cell sample, the flow cytometry tube/plate well should be filled with wash buffer and gently agitated/vortexed to wash the cells before re-pelleting.

Unfixed cells should generally be centrifuged at 300-500 x g; fixed cells are slightly more hardy, but this force should be sufficient to pellet both fresh and fixed samples. A five minute spin is usually sufficient to create a secure pellet that will permit rapid and efficient decanting by flicking the tube or tapping the inverted multiwell plate against an absorbent pad. To resuspend the pellet, rake the tube or well bottom a few times across a peg rack. This quickly frees the pellet for resuspension before the next incubation, wash or acquisition step.

3.8.5 Blocking

To prevent nonspecific binding of primary antibody(ies) to suspended cells, an anti-Fc antibody dilution (specific to the sample species) may be applied. This prevents binding of the Fc or constant region of the antibody by Fc receptors which are present on most cell types. Fc block is typically added to washed cells in extremely small (~10 µL) volume as the staining antibody dilution is added immediately at the end of the blocking incubation without a wash step. This ensures that blocking of nonspecific antibody binding is maintained throughout the primary incubation.

Tech Tip

For a specific signal, and to minimize nonspecific binding of the secondary to cells in the sample, pre-block the cells and include 1 – 5% serum (from the species in which the secondary antibody was raised) in the staining buffer.

3.8.6 Antibody Incubation

Primary incubation

Unlike other antibody-based applications such as immunohistochemistry, dilution of antibody for flow cytometry is typically based not on mass of antibody per volume of buffer, but on mass of antibody per number of cells in the sample. As with other applications, optimal concentration must be empirically determined, but typical dilutions are between 1 – 3 µg of antibody per 10^6 cells for both primary and secondary antibody. Antibody may be diluted in flow cytometry assay buffer. Staining in very small (50 - 100 µL) volumes improves access of antibody to cells in suspension. At the end of the incubation period, cells should be washed in staining or assay buffer three times to remove any unbound primary antibody.

Tech Tip

If primary antibody is conjugated to a fluorophore, the antibody incubation step must be carried out in the dark to prevent loss of signal via bleaching.

Secondary incubation

If indirect detection is employed, primary incubation is followed by incubation with appropriate dilutions of secondary antibody specific for the isotype of each primary antibody used. In multicolor detection experiments, fluorophores of sufficiently different wavelengths or colors must be chosen for each secondary in order to permit differentiation of signal from each target. Incubation with secondary antibody for 20-30 minutes is carried out in the dark to protect light-sensitive fluorophores and, as before, samples should be maintained on ice and centrifuged at 4°C.

Streptavidin incubation (if needed)

If the available primary or secondary is biotinylated, the final incubation step will be with an avidin, usually streptavidin, linked to a fluorophore. Incubation for 15-30 minutes on ice in dark conditions is followed by three washes to removed unbound streptavidin-fluorophore. Sample is then resuspended for in an appropriate volume of assay buffer, usually 200 - 500 µL per sample, and protected from light and maintained on ice until data aquisition.

Fluorochromes

Many antibodies used in flow cytometry are directly conjugated to a fluorochrome; however, many unlabeled primary antibodies are routinely used in combination with labeled secondary antibodies. Two common fluorochromes used in flow Cytometry are fluoroisothiocyanate (FITC) and phycoerythrin (PE). The two key properties of these dyes that make them preferred tags are that they are both excited with a 488 nm laser and that their emission spectra are distinct, with FITC at 530 nm (green) and PE at 570 – 575 nm (orange). Advances in fluorochrome chemistry and in flow cytometry instrumentation have made multiple simultaneous cell labeling and sorting possible beyond the original two dyes.

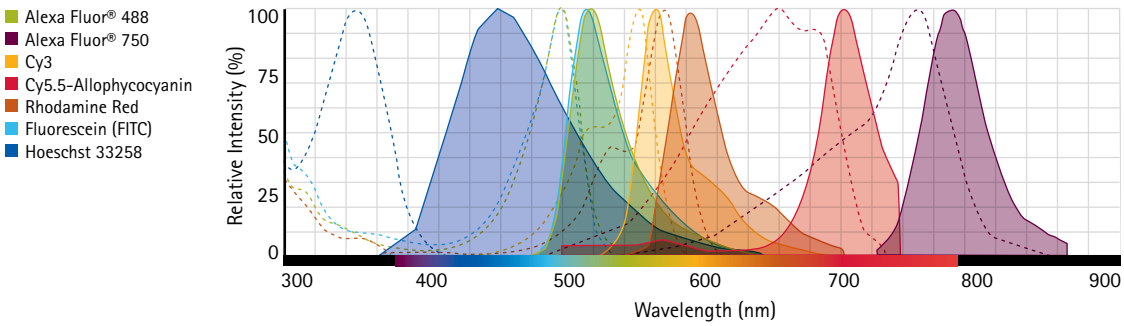


Figure 21.

Diverse fluorochromes with nonoverlapping emission spectra enable multiparameter flow cytometry.

Proper Controls for Flow Cytometry

In addition to cells of interest, every flow cytometry experiment should include the following controls:

1. At least one unstained sample that has been incubated with buffer at each step at the same time as the test samples. Unstained samples will be needed at the time of acquisition to optimize flow rates, set detector voltages for forward and side scatter, and to establish baseline voltages for each fluorescence detector used in the experiment.
2. An appropriate negative control sample which will be identical to the test samples except for substitution of the primary antibody with an isotype control which is raised in the same host species as the primary antibody. This control is necessary to establish whether any of the fluorescent signal detected is due to direct nonspecific binding of the secondary antibody to the sample cells, and permits subtraction of this 'background' from the fluorescence signal. An alternative, less robust negative control is a sample identical to the test samples from which the primary antibody has been omitted.
3. Where possible, a positive control which is comprised of cells known to express each antigen of interest and is co-incubated with the test samples. For multichannel experiments, each wavelength channel to be used must have a corresponding single-color positive control sample incubated with the same antibody(ies) as the test samples. Control beads are usually sold for this purpose, and should be used according to the manufacturers' instructions. However, cells may be used as single-color positive controls provided that they are certain to express the target antigen.

Fixation and Storage

Once surface antigen staining has been completed, cells may be fixed in 3-4% paraformaldehyde in phosphate-buffered saline instead of resuspending for acquisition. This is useful when samples cannot be acquired immediately after staining, as it allows cells to be stored overnight at 4°C. Afterwards, the fixative should be diluted and the cells washed two times. Although acquisition immediately following staining is recommended, fixed cells that have been stored at 4°C away from light may be acquired up to 48 hours after fixation.

Intracellular targets: permeabilization

When the protein(s) of interest are intracellular, fixation of cells after surface staining is necessary to increase structural strength so that the cells can withstand the subsequent permeabilization needed to allow antibodies access to intracellular antigen. Cells that have been fixed and washed, as described above, may be permeabilized by incubation with a 0.5% detergent in phosphate-buffered saline for no more than 15 minutes at room temperature before diluting detergent solution and washing once. Nonionic detergents such as saponin are recommended. Permeabilization must be maintained during all steps involving antibodies or streptavidin, and this is achieved by including 0.1% detergent in the staining buffer throughout subsequent staining up to and including the fluorophore incubation step. Detection of intracellular antigen by flow cytometry otherwise follows the principles and procedures outlined above and may be direct, indirect, or direct/indirect with signal enhancement.

Tech Tips

Surface and intracellular antigen staining are frequently combined to permit characterization of subsets of cells; a sample experiment might be as follows:

- Collect whole blood and isolate PBMCs by density centrifugation.
- Treat PBMCs with a compound suspected to be genotoxic.
- Stain for surface markers such as CD4, CD8, CD19, NK1.1.
- Fix and stain with anti–γH2.AX antibody to assess induction of double strand breaks in lymphocyte DNA.

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3.8.7 Data Acquisition

Flow cytometers are accompanied by the software necessary to acquire and transform the signals generated by the particle characteristics as each cell passes by the detector. These software programs usually also include components that aid in organizing the experiment, a feature that is particular important when analyzing a variety of samples from multiple specimens or donors. Once fluidics are established, unstained cells can be used to set forward and side scatter detectors, and fluorescent detectors adjusted by comparison of unstained cells to positive controls. The user also sets the software to collect a uniform number of 'events', or particle/cell signals, for each sample. For multicolor experiments, it is also critical to set parameters for compensation, in recognition of the wavelength overlap amongst spectra of the fluorophores used in the experiment. Compensation calibrates each fluorophore's spectrum and allows for subtraction of signal from adjacent channels due to spectral overlap.

Single-parameter histograms display the relative fluorescence plotted against the number of events. The simplicity of this type of display is the main reason for its popularity. Its ease of use makes it ideal for simple assays, for instance distinguishing apoptotic cells from non-apoptotic cells (Figure 22).

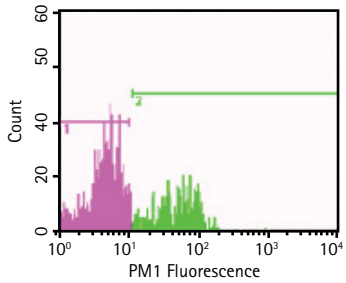


Figure 22.

Flow cytometry data of HL-60 cells that were treated with apoptosis inducer camptothecin and subsequently fixed and stained with the Guava® TUNEL apoptosis assay (Cat. No. 4500-0121). Approximately half of the cells are apoptotic (green histogram).

When one wishes to compare multiple parameters that are collected at the same time, more complex two-dimensional and even 3-dimensional diagrams are required. In these diagrams, one parameter is plotted against another in an X versus Y axis display (Figure 23).

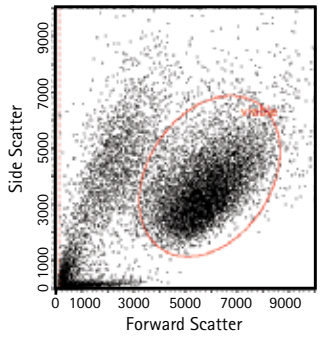


Figure 23.

The two-parameter flow cytometry plot at left shows how light-scattering characteristics of cells are used to identify cell populations of interest, here by their size (forward scatter) and intracellular complexity (side scatter). Gates, or regions defined and applied by the user, are then used to identify subpopulations of interest (here, the red oval identifies the likely viable population) for focused analysis.

Labeling of cells in flow cytometry is often done using antibodies specific to particular subclasses of cells. Antibodies typically used are specific to external cell epitopes for live cell sorting. Internal epitope antibodies are typically only used on fixed cells that have first been permeabilized to allow the antibody penetration. Merck Millipore offers numerous polyclonal and monoclonal antibodies specific to different cell types. Many of the groups of cell-type specific monoclonals have been given a cluster of differentiation (CD) number by international convention. For example, the monoclonal antibodies recognizing epitopes of the antigen site on helper T cells are known collectively as CD4 (Figure 24).

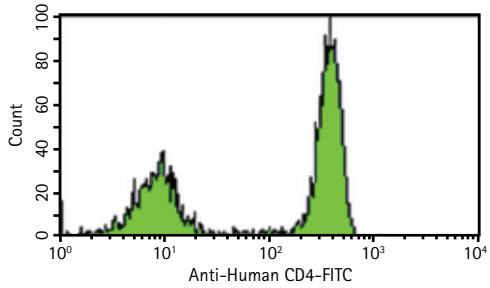


Figure 24.

Flow cytometry analysis of human lymphocytes stained with anti-human CD4-FITC antibody. Cells expressing CD4 (right hand peak) were distinguished from CD4-negative cells (left). CD marker expression can vary between different blood specimens.

Tech Tips

- Mix samples thoroughly before use; avoid excessive bubbling.
- If minor precipitate is detected in the wash buffer, place the bottle in a warm water bath for 30 minutes, followed by mixing the contents on a mechanical vortexer.
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
- For certain cell cultures, cell pellets may become hazy or transparent following the fixation step, making them difficult to see. If sampling a small collection of cells for flow analysis, it is recommended to perform all steps in a smaller collection tube (e.g. microcentrifuge tube).
- Do not mix or interchange reagents from various kit lots.

3.8.8 Troubleshooting:

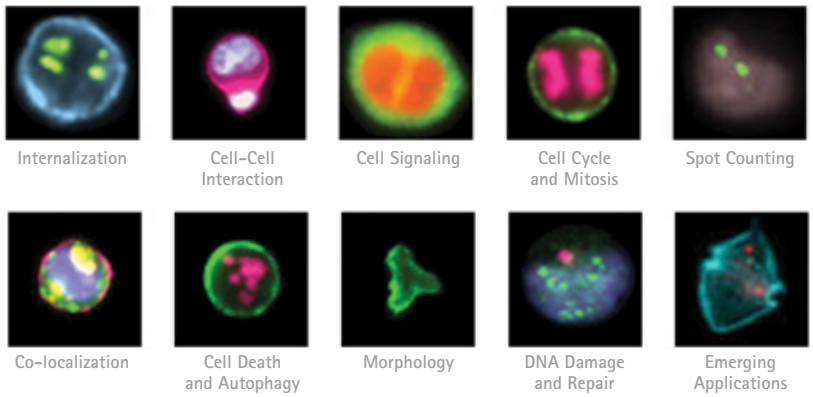
Problem	Cause	Suggested Solution
Acquisition rate decreases dramatically	Blocked fluid pathway on the instrument	Decrease the number of cells being analyzed by diluting the sample to approximately 0.5 million cells per mL. Cell densities in excess can essentially block the normal flow, causing disruption during the assay.
		After many uses, it is possible that the fluid system on any standard flow cytometer will require cleaning. Run standard cleaning procedures to clean the fluid system during or after an assay. This will prevent any material from forming where the steady flow steam takes place.
	Cells are clumping	Adherent or sticky cells can result in cellular clumping. Using a more aggressive enzyme for dissociation such as trypsin during cell harvesting should help keep cells in single suspension. Alternatively, using a cell strainer can help disrupt cell clumping (Cat. No. SCNY00060; 60 µM).
A loss or lack of signal	Not enough cells in the sample	Cell loss is common during washing steps in the assay procedure. Make sure that cell density remains at approximately 0.5 million cells per mL during analysis.
	Not enough antibody	A lack of signal may indicate that excess antibody will need to be used during the staining procedure. Further antibody titrations may be necessary for some cell types to capture the ideal staining concentration.
Background and/ or non-specific staining of cells	Too much antibody	Nonspecific staining and background may indicate that less antibody will need to be used during the staining procedure. Further antibody titrations may be necessary for some cell types to capture the ideal staining concentration.
Variability in results from day to day	Changes in cell viability	Monitor experimental cell cultures to ensure that cell viability and cell numbers being analyzed are consistent.
	Instrument calibration changes	Perform quality check on the instrument (e.g. calibration) on a daily basis prior to use.

Technology Highlight

Amnis® Imaging Flow Cytometers

Microscopy + Flow Cytometry

Amnis® imaging flow cytometers are the game-changer in cellular analysis, providing in-depth analysis and detailed imagery of every individual cell and hard-to-reach subpopulations. From immunology to drug discovery to parasitology, our imaging flow cytometers produce insightful data for the study of cell-to-cell interaction, morphology analysis, DNA damage and repair, and so much more. We currently offer two instrument platforms—ImageStream®X and the FlowSight® imaging flow cytometers—to meet your research needs and budget.



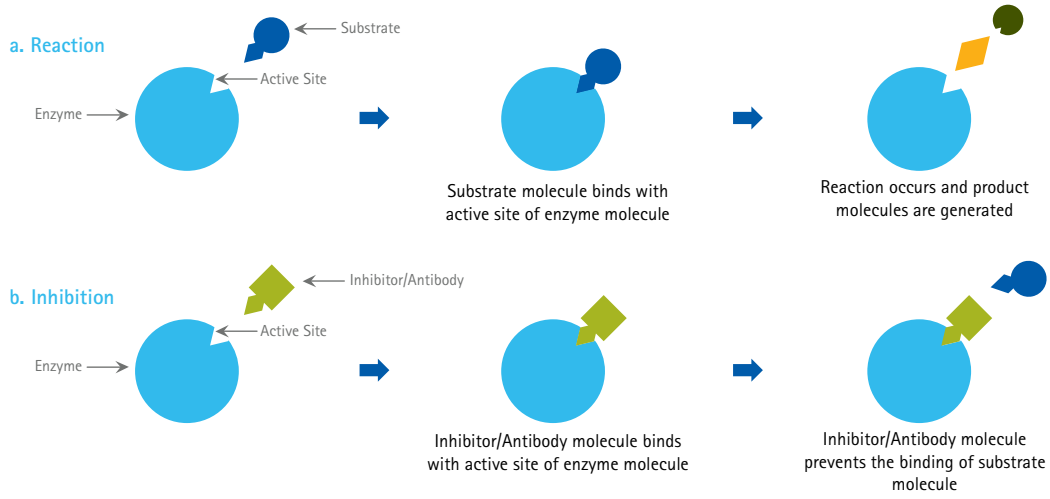
3.9 Functional Blocking and Stimulation Assays

Introduction

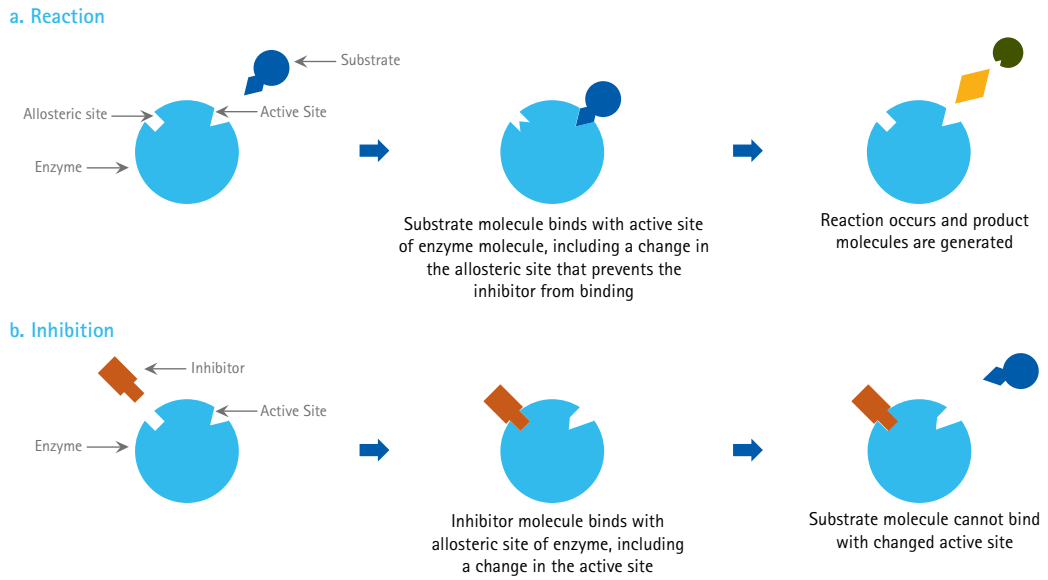
Specific antibody-antigen interactions may result in functional changes to the target protein. These interactions may neutralize the antigen, as in the case of "neutralizing antibodies", block or modulate an active site on an enzyme, or bind and stimulate a conformational change in the protein. Whether an antibody will indeed operate in a functional assay depends on the epitope of the antibody in relation to the 3D conformation of the protein domains and active site. Thus, unlike the design of many small molecule inhibitors/activators, the design of an antibody to specifically alter protein function is not easily predicted.

As with small molecules, antibodies may change protein function by:

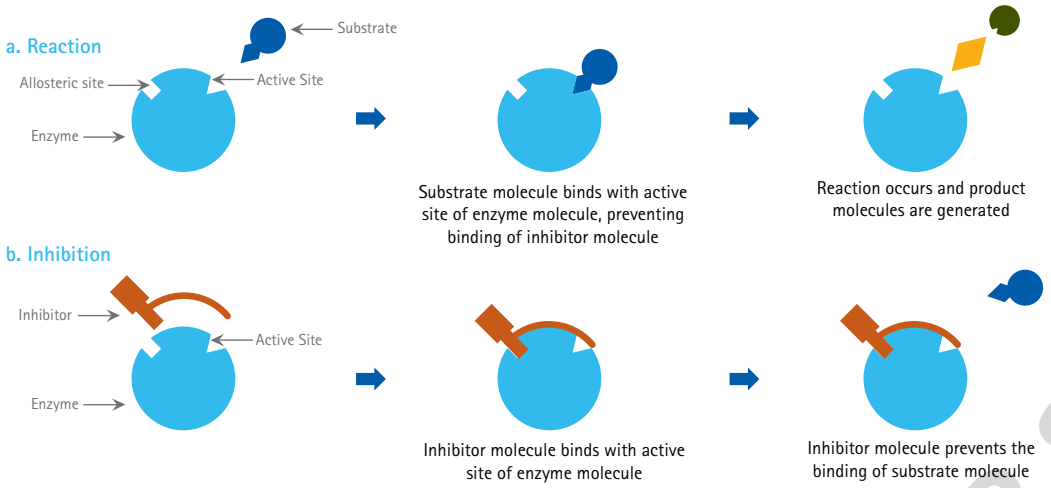
1. Direct active site binding



2. Allosteric binding-induced conformational changes



3. Steric hindrance



Unlike many small molecules, however, antibodies have poor cell membrane penetration and thus are restricted to external epitopes in live cell functional assays. The non-penetrance but specificity of antibodies is a great advantage in targeted blocking or stimulating of cell surface proteins. Many antibodies can be designed to strongly antigenic epitopes at or near binding sites, resulting in steric inhibition of receptor-ligand binding. These studies typically target channel or receptor deactivation or block protein-protein binding interfaces, such as those occurring in cell adhesion. See the chart below for examples.

Some receptor binding antibodies are particularly useful in that the antibody-receptor complex is quickly internalized and degraded. These antibodies can be conjugated to toxins such as Saporin, so when the complex is internalized, the toxin is released internally, killing the cell. This is a classic method for ablating specific cell types in the brain. Cholinergic neurons, for example, can be selectively eliminated targeting the p75 NGF receptor with a corresponding antibody-toxin conjugate.

Neutralizing antibodies are also important in the characterization of the safety and efficacy of large molecule biotherapeutics, which may elicit anti-drug antibody (ADA) responses in the host. Regulatory agencies have issued biotherapeutic testing guidelines, which recommend performing functional assays for the presence of neutralizing antibodies in nonclinical and clinical studies. As a result, there has been a surge in the development of novel assays for neutralizing antibodies.

Watch Out

Many commercial antibodies have preservatives, such as sodium azide, which can poison live cells. For live cell functional studies, it may be necessary to perform dialysis or diafiltration on the resuspended antibody to remove the preservative, or choose a preservative-free version of the antibody from the manufacturer.

Function	Description	Reference	Antibody example	Cat. No.
Immunolesioning	Induces cholinergic cell death	Wiley, R. (2001). Methods Mol. Biol. 169:217.	Anti-p75 NGFR-Saporin conj.	MAB390-25UG
Enzyme function blocking	Inhibits MMP-2 activation	Chen, H. et al. (2012). J. Biol. Chem. 287:17109.	Anti-MMP-2 Proform	MAB13405
Receptor blocking	Inhibits TLR2 activation	Sandor, F. et al. (2003). J. Cell Biol. 162:1099	Anti-TLR2	MAB3737
Blocking cell adhesion to substrate	Blocks αVβ3 complex binding to ECM	Mao, Y. & Schwarzbauer, J. (2005). J. Cell Sci. 118:4427.	Anti-αVβ3	MAB1976Z
Blocking cell-cell adhesion	Blocks integrin β1 / CD63 interaction	Iizuka, S. et al. (2011). Mol. Cell Biol.	Anti-Integrin β1	MAB2253Z
Neutralizing ligands	Neutralizes BDNF *in vitro* and *in vivo*	Finn et al. (1986). J. Neurocytol. 15:169)	Anti-BDNF	AB1513P
Stimulating receptor	Induces apoptosis by receptor activation	Trauth, B. et al. (1989). Science 245:301	Anti-Fas	MAB3061

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Tech Tips

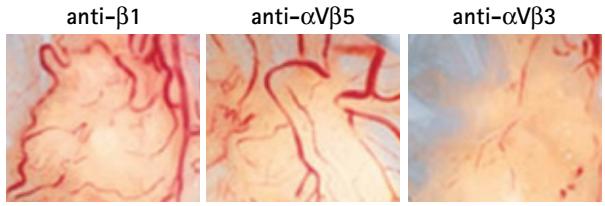
- Know your immunogen. Choosing the right antibody for function blocking depends on epitope, 3D conformation of the active site of interest, and antibody binding avidity. For example, to interrupt integrin αVβ3 binding to substrates, choose an antibody that targets the binding site or allosterically interferes with it. Such is the case of the well known mouse monoclonal antibody LM609. While LM609 does block cell attachment to RGD containing ligands, it does not interact directly with the RGD binding site. Instead, LM609 appears to be an allosteric inhibitor of integrin αVβ3, which binds to a conformational epitope resulting from the post-translational association of the αV and β3 subunits.
- Antibodies must be preservative-free in live cell assays. Use diafiltration- or dialysis-based buffer exchange if necessary to remove preservatives.
- *In vitro* to *in vivo* protocol extrapolations can be difficult and typically include significantly increasing the antibody concentration used. *In vivo* considerations also include prevalence of endogenous immunogen, antibody delivery method, tissue architecture, and host immune response to foreign antibody addition.
- In functional live cell assays, cell stimulation response should be at least five-fold over background obtained at the EC_{50} antigen concentration to allow for a large enough signal due to potential variance in antibody inhibition. Triplicate or quadruplicate trials are recommended.
- Antibody performance in a functional assay is more easily affected by changes in the medium environment than is typical for nonfunctional immunoassays like IHC or ELISA.

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Technology Highlight

Blocking adhesion with Mouse anti-Integrin αVβ3

Merck Millipore has over 200 function blocking or stimulating antibodies. One example, Anti-Integrin αVβ3 Antibody, clone LM609 reacts with the vitronectin receptor αVβ3 complex, an RGD-directed adhesion receptor. LM609 has been demonstrated to block adhesion of a human melanoma cell line (M21) to vitronectin, fibrinogen and von Willebrand factor, as well as to a synthetic RGD containing peptide (Cheresh, 1987). In chick chorioallantoic membranes, LM609 was shown to block angiogenesis induced by bFGF and TNFα but had no effect on pre-existing vessels (Brooks, 1994). While LM609 does block cell attachment to RGD-containing ligands, it does not interact directly with the RGD binding site. Instead, LM609 appears to be an allosteric inhibitor of integrin αVβ3, which binds to a conformational epitope resulting from the post-translational association of the αV and β3 subunits.



Mouse anti-Integrin αVβ3 (Cat. No. MAB1976). Inhibition of angiogenesis on the chick chorioallantoic membrane by anti-αVβ3. The pictures for MAB1976 and MAB1981 are reprinted with permission from Figures 1, 2, and 4 in: Brooks, P.C., Clark, R.A.F., and Cheresh, D.A. (1994) Science 264:569, © 1994 by the AAAS.

Notes

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Sample Protocols

4

4.1 Immunoprecipitation
4.2 Chromatin Immunoprecipitation (ChIP)
4.3 Western Blotting
4.4 Enzyme-linked Immunosorbent Assays (ELISA)
4.5 Multiplexed Bead-based Detection
4.6 Immunohistochemistry/Immunocytochemistry
4.7 Flow Cytometry
4.8 Functional Blocking and Stimulation Assays

4.1 Immunoprecipitation

The protocol below details the steps for the formation of the antibody-antigen complexes in the immunoprecipitation procedure. To a microcentrifuge tube, add:

1. 500 µL of 2x Immunoprecipitation Buffer (1% Triton® X-100, 300 mM NaCl, 20 mM Tris, pH 7.4, 2 mM EDTA, 2 mM EGTA, 0.4 mM sodium vanadate, 0.4 mM PMSF, 0.5% NP-40)
2. 200–500 µg of total protein lysate (approximately 100 µL of lysate at 2–5 mg/mL)
3. 1–5 µg of purified antibody. If unpurified antibodies are used, substitute the following antibody quantities:

   Serum: 0.5–5 µL

   Hybridoma tissue culture supernatant: 10–100 µL

   Ascites fluid: 0.1–1.0 µL
4. Add ddH_2O to 1 mL.
5. Mix gently, and incubate on ice for 1 hour to allow immune complexes to form. Longer incubation times may increase complex formation, but may also increase nonspecific background.

Tips

- **Antibody concentrations given are recommended starting points only. Optimal antibody concentrations must be determined empirically.**
- **For the negative control reactions, add an equivalent amount of control antibody.**

4.2 Chromatin Immunoprecipitation (ChIP)

Preparing cells for ChIP

1. If necessary, stimulate or treat adherent mammalian cells at ~80 to 90% confluence in a 150 mm culture dish containing 20 mL of growth media. Include one extra plate of cells to be used solely for estimation of cell number.
2. Prepare 22 mL of 1x PBS (2.2 mL 10x PBS and 19.8 mL water) for each 150 mm culture dish. Store on ice. This will be used for washes and needs to be ice cold.
3. Add 550 µL of 37% formaldehyde (or 1100 µL of 18.5% formaldehyde) directly to 20 mL of growth media to crosslink. Gently swirl dish to mix.
4. Incubate at room temperature for 10 minutes.
5. During the ten minute incubation, prepare 1x protease inhibitor in PBS: Add 2 mL of ice cold 1x PBS to a separate tube for every dish and add 10 µL of the 200x Protease Inhibitor Cocktail III. Store on ice.
6. Add 2 mL of 10x glycine to each dish to quench excess formaldehyde.
7. Swirl to mix and incubate at room temperature for 5 minutes.
8. Place dishes on ice.
9. Aspirate medium, removing as much medium as possible, being careful not to disturb the cells. If you are using suspension cells, spin down cells at 8000 x g for 5 minutes.
10. Add 10 mL of cold 1x PBS to wash cells. Remove 1x PBS.
11. Remove 1x PBS and repeat wash.
12. Add 2 mL of 1x Protease Inhibitor Cocktail III in PBS prepared in Step 5.
13. Scrape cells from each dish into a separate microcentrifuge tube.
14. Spin at 800 x g at 4 °C for 5 minutes to pellet cells

For detailed protocols, tips and troubleshooting strategies, consult Merck Millipore's "Guide to Chromatin Immunoprecipitation: Critical Factors for Success" (Literature Number TP5994EN00)

Preparing tissues for ChIP

1. Isolate unfixed fresh tissue as desired. Use a razor blade to cut a pea-size piece of tissue into small pieces (typically 1 mm or smaller) to improve crosslink efficiency. Alternatively, a plug of tissue from cryosectioned non-formalin-fixed-paraffin-embedded (non-FFPE) material can be used to obtain a small sample of interest (please see the Magna ChIP® G Tissue Kit manual, Cat. No. 17-20000).
2. Weigh the tissue, and then transfer into a 50 mL tube and wash twice with ice cold 1x PBS.
3. Resuspend tissue in 20 mL ice cold PBS and add 550 µL of 37% formaldehyde (or 1100 µL of 18.5% formaldehyde) to crosslink. Gently swirl dish to mix.
4. Incubate at room temperature for 10 minutes.
5. In the interim, prepare 1x protease inhibitor in PBS: Add 2 mL of ice-cold 1x PBS to a separate tube for every sample and add 10 µL of Protease Inhibitor Cocktail III. Store on ice.
6. Add 2 mL of 10x glycine to quench excess formaldehyde.
7. Homogenize the tissues several times using a Dounce homogenizer (loose pestle).
8. Spin at 800 x g at 4 °C for 5 minutes to pellet cells.

Optimizing Sonication and Analyzing DNA Fragments

Optimal conditions for shearing crosslinked DNA to 200 - 1000 base pairs in length depend on the cell type, cell concentration, and the specific sonicator equipment, including the power settings and duration and number of pulses. Approaches for optimizing sonication may include the following:

A. Varying the concentration of cell equivalents per mL of initial buffer with constant sonication parameters.

B. Choosing a fixed concentration of cell equivalents per mL of buffer and varying cycles and/or power settings of sonication.

C. A combination of both approaches.

The protocol below describes optimization following option A and is provided as an example only.

1. Generate a cell lysate by following the first section above, but vary your buffer volume per cell amount-to generate 3 different microcentrifuge tubes containing several cell equivalent concentrations in the range of 5 x 10^6 per mL to 5 x 10^7 per mL. For HeLa cells, this requires approximately 4 x 10^7 cell equivalents, or approximately four 15 cm plates.
2. Continue with the following Cell Lysis procedure. Each microcentrifuge tube should contain approximately 500 µL of cell lysate.

Volume of Cell Lysis Buffer	Cell Density	Cells Required
500 µL	5 x 10^6/mL	2.5 x 10^6
500 µL	2 x 10^7/mL	1 x 10^7
500 µL	5 x 10^7/mL	2.5 x 10^7

3. Be sure to keep the samples on wet ice at all times. Sonication generates heat which will denature the chromatin.
4. Remove 1 x 10^5 cell equivalents from each condition prior to sonication for analysis of unsheared DNA.
5. For each cell concentration, sonicate each tube for a fixed number of cycles allowing rests between cycles according to the instrument manufacturer's guidelines. For example, using a Misonix 3000 instrument and a No. 419 microtip probe, use six 15 second pulses with 50 second intervals between pulses, with power setting at 6. Keep tubes cool at all times.
6. Remove 1 x 10^5 cell equivalents (20 µL, 5 µL, 2 µL from least to most concentrated sample) of the sonicated chromatin from each condition to a fresh tube.
7. To all samples (unsheared and sheared), add elution buffer to a final volume of 50 µL.
8. Add 1 µL Proteinase K and incubate at 62 °C for 2 hours.
9. Load 10 µL and 20 µL on a 1–2% agarose gel with a 100 bp DNA marker. Loading different amounts helps to avoid under- or overloading
10. Observe which of the shearing conditions gives a smear of DNA in the range of 200 – 1000 bp.
11. Repeat optimization of the shearing conditions if the results indicate that the resulting DNA is not in the desired size range. Once optimal conditions have been determined, it is advised that you do not alter the cell concentration or volume of lysate per microcentrifuge tube for subsequent chromatin immunoprecipitation experiments.

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DNA Purification

(The following protocol was adapted from Sambrook, et al., 2006.)

1. Centrifuge your samples at full speed for 5 min at RT.
2. Transfer supernatants to fresh microcentrifuge tubes.
3. You can now purify your DNA using spin columns. For solvent extraction, continue to Step 4.
4. Transfer the nucleic acid sample to a polypropylene tube and add an equal volume of phenol:chloroform. The nucleic acid will tend to partition into the organic phase if the phenol has not been adequately equilibrated to a pH of 7.8 - 8.0.
5. Mix the contents of the tube until an emulsion forms.
6. Centrifuge the mixture at 80% of the maximum speed that the tubes can bear for 1 minute at room temperature. If the organic and aqueous phases are not well separated, centrifuge again for a longer time.
7. Normally, the aqueous phase forms the upper phase. However, if the aqueous phase is dense because of salt (>0.5 M) or sucrose (> 10%), it will form the lower phase. The organic phase is easily identifiable because of the yellow color contributed by the 8-hydroxyquinoline that is added to phenol during equilibration.
8. Use a pipette to transfer the aqueous phase to a fresh tube. For small volumes (<200 µL), use an automatic pipettor fitted with a disposable tip. Discard the interface and organic phase.
9. Repeat Steps 1–4 until no protein is visible at the interface of the organic and aqueous phases.
10. Add an equal volume of chloroform and repeat Steps 2–4.
11. Recover the nucleic acid by standard precipitation with ethanol.

4.3 Western Blotting

The standard Western blotting protocol involves immunodetection of blotted proteins directly after electrotransfer. (If the membrane was dried after transfer, thoroughly wet the blot in methanol for 5 minutes before proceeding to immunodetection.) The unoccupied membrane binding sites on the wet blot are blocked with optimized reagents. The drawbacks of this method are the need for blocking and the total time requirement of over 4 hours. For alternate, time-saving immunodetection protocols, refer to Merck Millipore's Protein Blotting Handbook, Literature Number TP001EN00. The advantage is that standard immunodetection may require less optimization for new sample types.

Required Solutions

- Primary antibody (specific for protein of interest).
- Secondary antibody (specific for primary antibody), labeled with alkaline phosphatase or horseradish peroxidase.
- Substrate appropriate to the enzyme conjugate.
- Phosphate-buffered saline (PBST): 10 mM sodium phosphate, pH 7.2, 0.9% (w/v) NaCl, up to 0.1% Tween®-20 detergent.
- TBST: 10 mM Tris, pH 7.4, 0.9% (w/v) NaCl, up to 0.1% Tween®-20.
- Blocking solution: 1% (w/v) BSA (bovine serum albumin), 0.05% Tween®-20.
- Milli-Q® water.

Tips

- Immobilon®-P^{SQ} transfer membrane has a smaller pore size (0.2 µm) and higher surface area than Immobilon®-P ransfer membrane (0.45 µm). Increased background can be expected on Immobilon®-P^{SQ} and the blocking and wash steps will need to be adjusted accordingly.
- Phosphatases in the blocking solution may dephosphorylate blotted proteins
- Do not use sodium azide in the buffers as it inhibits HRP activity.
- Do not let the blot dry out at any time during and after blocking.
- If more than one blot is placed in a container, insufficient buffer volume will cause the blots to stick together.
- Dry milk powder cannot be used with biotin-avidin systems.
- Sensitivity of chromogenic detection is typically at least an order of magnitude lower than of chemiluminescent detection.
- High nonspecific signal can be alleviated by higher dilution of the primary antibody or reduced protein load on the gel.

Tips

- Immobilon®-FL membrane can be scanned dry or wet.
- High overall background can be minimized by higher dilution of the enzyme-conjugated secondary antibody.

Required Equipment

- Shallow trays, large enough to hold blot.
- Glass plates.
- Plastic wrap (e.g., Saran™ film), freezer bag, or sheet protector.
- Autoradiography film and cassette.
- Dark room.
- Autoradiography film processing equipment.

Set Up

1. Dilute the primary antibody in the blocking solution to the desired working concentration.
2. Dilute the secondary antibody in the blocking solution to the desired working concentration. **NOTE**: Enough solution should be prepared to allow for 0.1 mL of antibody solution (primary and secondary) per cm^2 of membrane.

Antibody Incubations

1. Place the blot in the blocking solution and incubate with agitation for 1 hour.
2. Place the blot in the primary antibody solution and incubate with agitation for 1 hour. The solution should move freely across the surface of the membrane.
3. Place the blot in PBS and wash for 10 minutes. Repeat twice with fresh buffer.
4. Place the blot in the secondary antibody solution and incubate with agitation for 1 hour at RT or 37°C.
5. Place the blot in PBS and wash for 10 minutes. Repeat twice with fresh buffer.
6. Proceed with either chromogenic, chemiluminescent or fluorescent detection.

Chromogenic Detection

1. Prepare the substrate according to manufacturer's instructions.
2. Place the blot in a clean container and add substrate to completely cover the surface of the membrane. Incubate for 10 minutes with mild agitation or until signal reaches desired contrast.
3. Rinse the blot with Milli-Q® water to stop the reaction.
4. Store the blot out of direct light to minimize fading. Blot may be stored dry.

Chemiluminescent Detection

Follow manufacturer's instructions.

1. Prepare the substrate according to manufacturer's instructions.
2. Place the blot in a container and add substrate to completely cover the membrane. Incubate for 1 minute.
3. Drain excess substrate.
4. Place the blot on a clean piece of glass and wrap in plastic wrap.

   NOTE: A cut-to-size sheet protector or a freezer bag can also be used.
5. Gently smooth out any air bubbles.
6. In a dark room, place the wrapped membrane in a film cassette.
7. Place a sheet of autoradiography film on top and close the cassette.
8. Expose film. Multiple exposures of 15 seconds to 30 minutes should be done to determine the optimum exposure time; 1 to 5 minutes is common.

Fluorescent Detection

Required Equipment

- Proteins blotted onto Immobilon®-FL transfer membrane and probed with antibodies
- Mylar® wrap
- Fluorescent imaging equipment

The following is a general protocol for fluorescent immunodetection. For optimal results, refer to manufacturer's protocol provided with the reagents. Note: If using chemifluorescent reagents, follow reagent manufacturer's directions.

1. Place the blot in diluted fluorescent dye-labeled secondary antibody solution and incubate for 1 hour with gentle agitation.
2. Wash the blot with wash buffer 3 -5 times for 5 minutes each.
3. Place the blot onto a piece of clean filter paper to dry.
4. If using a wrap, use Mylar. Do not use Saran™ wrap because it permits light to shine through and quench fluorescence.
5. Image the blot using an appropriate fluorescence scanner.

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4.4 Enzyme-linked Immunosorbent Assays (ELISA)

Sandwich ELISA

1. Before the assay, both antibody preparations should be purified and one must be labeled.
2. For most applications, a polyvinylchloride (PVC) microtiter plate is best; however, consult manufacturer guidelines to determine the most appropriate type of plate for protein binding.
3. Bind the unlabeled antibody to the bottom of each well by adding approximately 50 µL of antibody solution to each well (20 µg/mL in PBS). PVC will bind approximately 100 ng/well (300 ng/cm^2). The amount of antibody used will depend on the individual assay, but if maximal binding is required, use at least 1 µg/well. This is well above the capacity of the well, but the binding will occur more rapidly, and the binding solution can be saved and used again.
4. Incubate the plate overnight at 4°C to allow complete binding.
5. Wash the wells twice with PBS. A 500 mL squirt bottle is convenient for this. The antibody solution washes can be removed by flicking the plate over a suitable container.
6. The remaining sites for protein binding on the microtiter plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hours to overnight in a humid atmosphere at room temperature.

   NOTE: Sodium azide is an inhibitor or horseradish peroxidase. Do not include sodium azide in buffers or wash solutions if an HRP-labeled antibody will be used for detection.
7. Wash wells twice with PBS.
8. Add 50 µL of the antigen solution to the wells (the antigen solution should be titrated). All dilutions should be done in the blocking buffer (3% BSA/PBS). Incubate for at least 2 hours at room temperature in a humid atmosphere.
9. Wash the plate four times with PBS.
10. Add the labeled second antibody. The amount to be added can be determined in preliminary experiments. For accurate quantitation, the second antibody should be used in excess. All dilutions should be done in the blocking buffer.
11. Incubate for 2 hours. or more at room temperature in a humid atmosphere.
12. Wash with several changes of PBS.
13. Add substrate as indicated by manufacturer. After suggested incubation time has elapsed, chemiluminescence or optical densities at target wavelengths can be measured on an ELISA plate reader.

    NOTE: Some enzyme substrates are considered hazardous, due to their potential carcinogenicity. Handle with care and refer to Material Safety Data Sheets for proper handling precautions.

    For quantitative results, compare signal of unknown samples against those of a standard curve. Standards must be run with each assay to ensure accuracy.

Competitive ELISA

1. For most applications, a polyvinylchloride (PVC) microtiter plate is the best choice; however, consult manufacturer guidelines to determine the most appropriate type of plate for protein binding.
2. Add 50 µL of diluted primary antibody (capture antibody) to each well. The appropriate dilution should be determined using a checkerboard titration prior to testing samples. PVC will bind approximately 100 ng/well (300 ng/cm^2). The amount of antibody used will depend on the individual assay, but if maximal binding is required, use at least 1 µg/well. This is well above the capacity of the well, but the binding will occur more rapidly, and the binding solution can be saved and used again. Allow to incubate for 4 hours at room temperature or 4°C overnight.

   NOTE: If a purified capture antibody is not available, the plate should first be coated with a purified secondary antibody directed against the host of the capture antibody according to the following procedure:

   A. Bind the unlabeled secondary antibody to the bottom of each well by adding approximately 50 µL of antibody solution to each well (20 µg/mL in PBS).
   B. Incubate the plate overnight at 4°C to allow complete binding.
   C. Add primary capture antibody (as above).
3. Wash the wells twice with PBS. A 500 mL squirt bottle is convenient for this. The antibody solution washes can be removed by flicking the plate over a suitable container.
4. The remaining sites for protein binding on the microtiter plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hours to overnight in a humid atmosphere at room temperature.
5. Wash wells twice with PBS.
6. Add 50 µL of the standards or sample solution to the wells. All dilutions should be done in the blocking buffer (3% BSA/PBS with 0.05% Tween®-20).

   NOTE: Sodium azide is an inhibitor or horseradish peroxidase. Do not include sodium azide in buffers or wash solutions, if an HRP-labeled conjugate will be used for detection.

7. Add 50 µL of the antigen-conjugate solution to the wells (the antigen solution should be titrated). All dilutions should be done in the blocking buffer (3% BSA/PBS with 0.05% Tween®-20). Incubate for at least 2 hours at room temperature in a humid atmosphere.
8. Wash the plate four times with PBS.
9. Add substrate as indicated by manufacturer. After suggested incubation time has elapsed, chemiluminescence or optical densities at target wavelengths can be measured on an ELISA reader.

   NOTE: Competitive ELISAs yield an inverse curve, where higher values of antigen in the samples or standards yield a lower amount of color change.

4.5 Multiplexed Bead-based Detection

A typical protocol can be found in MILLIPLEX® MAP multiplex assay panels (Merck Millipore), which are preconjugated, analytically validated panels with included quality controls, reagents, and all other components to detect multiple analytes in a small sample volume (5 - 50 µL) in a single kit, using a single catalogue number.

1. Follow standard protocols for preparation of cell and tissue lysates. It is recommended that protease inhibitors (Cat. No. 535140, available separately) and phosphatase inhibitors (Cat. No. 524629, available separately) be added immediately prior to use.
2. Dilute pre-cleared lysates at least 1:1 in Assay Buffer. The suggested working range of protein concentration for the assay is 2.5 to 15 µg of total protein/well (25 µL/well at 100 to 600 µg/mL). No additional dilution is necessary for the prepared Lysate Controls.
3. Block the 96-well plate with 200 µL Assay Buffer. Seal and place on a plate shaker to mix for 10 minutes at room temperature (20 - 25°C).
4. Apply vacuum/decant plate.
5. Add 25 µL standards, controls and samples to mapped out wells
6. Vortex bead bottle and add 25 µL of the Premixed Beads to each well (**NOTE**: During addition of Beads, shake bead bottle intermittently to avoid any settling) (capture antibody)
7. Incubate on a plate shaker for 2 hours at room temperature (20 - 25°C) or overnight at 4°C.
8. Wash 2 - 3 times and apply vacuum or aspirate
9. Add 50 µL Detection Antibody cocktail
10. Incubate, then add 50 µL SA-PE
11. Wash 2-3 times and apply vacuum
12. Add 100 µL of Sheath Fluid (or Drive Fluid if using MAGPIX® instrument) to all wells. Resuspend the beads on a plate shaker for 5 minutes.
13. Acquire data on a Luminex™ instrument.
14. Save and analyze the Median Fluorescence Intensity (MFI) data (**NOTE**: make sure all lysate samples in comparison are diluted by the same factor.)

Tech Tips

- When using stored samples, keep at -20°C in "frosted" freezer and avoid multiple (>2) freeze/thaw cycles.
- Thaw frozen samples completely, mix well by vortexing and centrifuge before use.
- Store samples in polypropylene tubes. DO NOT STORE SAMPLES IN GLASS TUBES.
- Add Protease Inhibitors to lysis buffer and Na_3VO_4 in to inhibit Phosphatases
- For blood samples, allow the blood to clot for at least 30 minutes before centrifugation. Remove serum and assay immediately or aliquot. Care must be taken when using heparin as an anticoagulant since an excess of heparin will provide false high values. Do not use more than 10 IU heparin per 1 mL of blood collected.

4.6 Immunohistochemistry/ Immunocytochemistry

Fresh Frozen (then fixed) Tissue Sections

1. Snap-freeze small tissue blocks (5x5x3 mm) in liquid nitrogen.
2. Transfer to cryostat and cut thin (5–30 µm) sections.
3. Collect specimens on clean poly-L-lysine-coated glass slides and dry at room temperature overnight (if you want to stain the same day let air-dry for 1–2 hrs. until completely dry). Thorough drying is essential for good adhesion to the slides.
4. Fix sections in acetone or absolute ethanol at 4°C for 15 min. Use fresh ethanol or acetone for every 10–15 slides for best results. The organic solvents absorb moisture from the air and tissue, as they do so, they lose their ability to fix the tissue effectively.
5. Thoroughly air-dry at room temperature or on mild heat (30–37°C). It is during this stage that much of the chemical fixation is being finalized; improper air-drying will lead to "soft" sections and likely loss of proper reactivity.
6. Proceed with immunostaining or freeze.

Fixed, Frozen Tissue Sections

1. Fix tissue either by perfusion with fixative or by immersion in fixative for a set time period. Most commonly, 4% Paraformaldehyde (PFA) solutions are used.
2. Fixed tissue is then prepared for cryoprotection by submerging the target tissue in a hydrostabilizing solution. The cryoprotection is complete when the target tissue no longer floats in the stabilizing solution. Because it works well and is relatively inexpensive, PBS+sucrose solutions ranging from 10% (less protection), to 30% (w/v) sucrose (greater protection) are often used.
3. Once stabilized, tissues can be removed from the protectant solution and frozen at -70°C until sectioned.
4. Sectioned via cyrostat (5 – 40 µm*), where sections can be collected directly onto slides, or floated onto slides via a PBS/waterbath. Usually up to 3 sections per slide can be placed; each spaced well apart. The spacing prevents reagent mixing between samples. *Individual skill and tissue type will determine the thickness of the sections. Sections between 10–15 µm provide the best results for clarity and integrity. Sections between 6–9 µm tend to tear during cutting, resulting in rough edges that can increase the background. Thicker sections while stronger during handling can be more difficult to stain.
5. Sections on slides are thoroughly air-warmed/dried on a slide warmer, usually overnight or at least 2 – 3 hrs. at 40 – 50°C.
6. Prepared slides can be stored dry at -70°C until stained. Equilibrate to room temperature and briefly re-dry prior to rehydration and staining.

Paraffin-embedded Sections

1. Conventional deparaffinization and dehydration sequence:
   A. Incubate sections in xylene: 2 to 3 changes, 5 min. each.
   B. 100% absolute ethanol: 2 changes, 3 min. each
   C. 95% ethanol: 2 changes, 3 min. each
   D. 80% ethanol: 3 min.
   E. 50% ethanol: 3 min.
   F. Rinse with distilled water, PBS, or Tris buffer: 2 changes, 3 min. each.

   NOTE: Once sections have been rehydrated, do not allow them to dry.

2. Place slides in prewarmed (37°C) 0.1% trypsin in PBS for 5–60 min. or 0.4% pepsin in 0.01N HCl for 30 min. to one hour. Follow by rinsing with distilled water
3. If peroxidase conjugate is used, endogenous peroxidase should be blocked at this stage. Peroxidase activity results in the decomposition of hydrogen peroxide (H_2O_2). It is a common property of all hemoproteins such as hemoglobin, myoglobin, cytochrome and catalases. Suppression of endogenous peroxidase activity in formalin-fixed tissue entails the incubation of sections in 3% H_2O_2 for 8–10 min. Methanolic H_2O_2 treatment (1 part 3% H_2O_2 plus 4 parts absolute methanol) for 20 min. can also used, but it is not recommended for specimens where cell surface markers are to be stained. Methanolic treatment may also detach frozen sections from their carrier glass.
4. Wash twice with PBS.
5. Proceed with immunostaining procedure.

Staining with Polyclonal Rabbit or Monoclonal Mouse Primary Antibody

The following general protocol is intended for use as a guideline in developing antibody-specific procedures. Different antibodies and tissues may require changes to this procedure. Review of individual product datasheets and relevant literature references may be helpful in customizing this procedure for specific applications.

1. Gently rinse slide containing sections with distilled water or buffer from a wash bottle. Place slide in room temperature buffer bath for 5 minutes to rehydrate sections.
2. Using a Kimwipe®, gently remove excess liquid from around the specimen. Avoid touching the tissue directly.

3. Apply 4 – 6 drops of normal serum, (normal serum from the host of the secondary antibody), diluted 1:5-1:30 (final conc. 3% – 20%). Incubate for 20 – 30 minutes at 37°C.
4. Tap off serum and wipe away excess. Do not rinse.
5. Perform any antigen retrieval if necessary.
6. Apply 25 – 50 µL of rabbit (mouse) primary antibody, diluted appropriately, per tissue section. Antibody should cover sections completely. Incubate for desired time (see Antibody Staining in Section 3.7 for suggested parameters and temperatures). If optimal antibody dilution is unknown, perform a series of antibody dilutions in the range of 1:20 – 1:1,000 to obtain initial results.

   NOTE: Antibody diluent is often very important for consistent reactivity. Simple solutions are easier to troubleshoot then complex ones, thus antibodies diluted only with simple buffers (PBS or TBS) are usually recommended.
7. Rinse slide gently with distilled water or buffer from a wash bottle, and incubate in a buffer bath for 3 x 5 minutes (changing buffer in between washes).

   NOTE: For all procedures it is important to see that each step is adequately buffered, and that non-reacted solutions are washed away after each step.
8. Apply 25 – 50 µL of enzyme-conjugated antibody directed against rabbit (mouse) immunoglobulins, diluted appropriately. Incubate 45 – 60 minutes.
9. Rinse slide gently with distilled water or buffer from a wash bottle, and incubate in a buffer bath for 3 x 5 minutes (changing buffer in between washes).
10. Apply substrate-chromogen solution and incubate until desired color intensity has developed.
11. Rinse gently with distilled water from wash bottle. Counterstain and coverslip.

4.7 Flow Cytometry

Direct Staining Protocol for Flow Cytometry

NOTE: The following protocol is given to provide a general procedure that can be used as a template to construct a specific protocol for an experimental assay. Investigators are strongly encouraged to collaborate with a flow cytometry facility or technician to fully develop appropriate procedures for their experimental systems.

Items needed:

- Cells (~0.5–1 x 10^6/mL) prepared and counted, in PBS with Ca^{2+} and Mg^{2+}, 1% BSA or 2% FCS.
- Directly conjugated monoclonal antibody to desired cell surface marker.
- Wash solution (1x Dulbecco's PBS, Ca^{2+} and Mg^{2+} free with and without 1% BSA)
- Optional fixative: 1% formaldehyde-PBS pH 7.4, freshly prepared.

Method:

1. Add 50-100 µL of cells to each of three 12 x 75 mm polypropylene or polystyrene tubes

   NOTE: The initial amount of cells collected should be adjusted to approximately ~0.75 x10^6 cells per mL so that 50 µL added will equal approximately 10^5 cells per tube.
2. To the first tube, add the appropriate volume (usually 5–10 µL) of fluorochrome-conjugated monoclonal antibody. To the second tube, add the same volume of matched isotype control antibody. The isotype control antibody should match the isotype of the conjugated antibody of interest. To the final tube of cells, add the equivalent volume of 1x PBS as conjugated antibody. This will serve as an autofluorescence control to establish the appropriate flow cytometer electronic settings. Vortex each of the sample tubes at moderate speed.
3. Incubate samples for 20–30 minutes at room temperature or 4°C, in the dark.
4. Wash cells in tubes with 3 mL volumes of 1x PBS with 1% BSA and centrifuge samples at 400 x g for 10 minutes at room temperature. Carefully pour or pipette off supernatant fluid. This step can be repeated if desired.
5. Cells can be analyzed without fixing if they are to be used immediately. Simply resuspend the cell pellet in 500 µL of PBS wash solution, and keep at 4°C in the dark until used (usually less than 4 hours). Alternatively, loosen the cell pellet by vortexing it in the residual wash fluid. Add 500 µL of 1% formaldehyde-PBS to each sample and quickly mix again. It is critical to mix the cell pellet prior to adding the fixative; otherwise the cells will become fixed into a solid mass that cannot be sent through the flow cytometer. Fixed samples can be stored at 4°C in the dark until flow cytometry analysis is performed. Fixed samples should be used within 48 hours.

4.8 Functional Blocking and Stimulation Assays

Due to the immense complexity of protein structure function relationships, it is very hard to generalize a basic protocol for functional antibody assays.

For lysate-based or recombinant protein based neutralization studies, a rough guide would be to use the neutralizing antibody at 10^3 times the concentration of the target protein to achieve ND_{50} (50% neutralizing dose). Serial dilutions of the antibody are necessary to determine the ND_{50} curve.

Typically, the antibody and antigen are combined first in suspension. Then serial dilutions of antibody are applied to a cell line or enzymatic substrate in culture to measure the resulting effect of the dose.

General antibody neutralization protocol for determining compound bioactivity

This characteristic neutralizing antibody protocol used for cytokine bioassays is taken from G.M. Veldman et al. in R. Kontermann and S. Dübel (eds.), Antibody Engineering Vol. 1.* Please consult that volume for more extensive discussion on different function blocking protocols and design considerations. It offers an excellent discussion and guide to function blocking antibodies and much more.

Typical Bioassay to Determine Antibody Neutralization Potency

(KG-1 Assay for IL-18) (Konishi et al. 1997; Fernandez-Botran and Větvička 2001)

The first step in setting up a bioassay is to determine the biologic response of the KG-1 cell line to different concentrations of human IL-18 by measuring the then selected as the concentration to be used in the second part of the assay, in which anti-human IL-18 antibody is prepared at different concentrations. The potency of the neutralizing antibody is expressed as the IC_{50}, which is the antibody concentration that results in 50% inhibition of the IL-18-induced IFNγ response.

A. Determine the Biologic Response Curve for IL-18

1. Prepare a standard curve for IL-18. In a 96-well dilution plate, prepare a serial 1:2 (16 points) or 1:4 (8 points) dilution of human IL-18 (starting at 2,000 ng/mL) in complete medium.
2. In a 96-well flat-bottom tissue culture plate, add 100 mL/well human IL-18 dilutions in quadruplicate. Include eight control wells containing 100 mL/well complete medium without IL-18.
3. Harvest KG-1 cells, centrifuge at 1,500 rpm for 5 min, aspirate off media, and resuspend at a cell density of 3 x 10^6 cells/mL in complete medium containing 20 ng/mL human TNF.
4. Add 100 mL KG-1 cells to each well in the 96-well plate and incubate for 24 h at 37°C, 5% CO_2.
5. Continue with section C.

B. Antibody Neutralization

1. Prepare a 1:2 (16 points) or 1:4 (8 points) serial dilution of IL-18 antibody in complete medium (starting at 300 nM) in a 96-well dilution plate.
2. To a 96-well flat-bottom tissue culture plate, add 50 mL/well diluted IL-18 antibody in quadruplicate. Include eight control wells containing 50 mL/well complete medium without IL-18 antibody (IL-18-only control wells) and eight control wells containing 100 mL/well complete medium without IL-18 antibody (medium-control wells).
3. Add 50 mL/well human IL-18 (8 ng/mL), except to medium-control wells. Preincubate the plate containing IL-18 and IL-18 antibody for 1 h at 37°C, 5% CO_2.
4. Harvest KG-1 cells, centrifuge at 1,500 rpm for 5 min, aspirate off media, and resuspend at a cell density of 3 x 10^6 cells/mL in complete medium containing 20 ng/mL human TNF.
5. Add 100 mL KG-1 cells to each well in the 96-well plate and incubate for 24 h at 37°C, 5% CO_2.
6. Continue with section C.

C. Determination of IL-18 Bioactivity and Antibody Neutralization

1. Transfer plate contents to 96-well V-bottom tissue culture plates and centrifuge plates at 1,500 rpm for 10 min. Transfer 150 mL of the culture supernatants to 96-well flat-bottom tissue culture plates. Do not disturb the cell pellets. At this point, the supernatants can be stored at -20°C.
2. Determine human IFNγ concentrations in the supernatants by using a human IFNγ ELISA kit. Further dilution of supernatants may be necessary to ensure that data points fall within the standard curve of the ELISA.
3. Calculate the EC_{50} or EC_{70} for IL-18 by plotting the IL-18 concentration on the x-axis versus human IFNγ on the y-axis.
4. Calculate the IC_{50} of the IL-18 antibody by plotting the antibody concentration on the x-axis versus human IFNγ on the y-axis. The maximum IFNγ response can be determined from the IL-18-only control wells and the background IFNγ response can be determined from the medium-control wells. The IC_{50} of the antibody is the concentration that causes 50% inhibition of the maximum IFNγ response over the background response.

Notes

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Appendix

Making Serial Antibody Dilutions

Reagents/Equipment:

- PBS or other appropriate buffer.
- Small capped tubes
- Pipets capable of accurate delivery of 200 µL and 1000 µL volumes

Keep buffer and tubes in ice

1. Pipet 450 µL buffer into a tube, labeled as tube X.
2. Add 50 µL antibody solution, and mix. This gives a 1:10 dilution of the antibody in tube X.
3. Label tubes A through M for 1:50, 1:100, 1:200, 1:400, etc. to 1:204,800 dilutions.
4. Pipet 1600 µL of dilution buffer into tube A (to become a 1:50 dilution). Pipette 1000 µL (1.0 mL) of dilution buffer into tubes B through M (to become 1:100–1:102,400 dilutions).
5. Pipette 400 µL of 1:10 antibody dilution from tube X into tube A (which contains 1600 µL buffer). Mix well. This results in a 1:50 antibody dilution.
6. Take 1000 µL of antibody sample from Tube A and add to Tube B (which contains 1000 µL buffer). Mix well.
7. Take 1000 µL of antibody sample from Tube B and add to Tube C (which contains 1000 µL buffer), etc. Mix well.

Tube	Sample to be diluted	Volume of Sample	Volume of Buffer	Resulting Dilution
A	1:10	400 µL	1600 µL	1:50
B	1:50	1000 µL	1000 µL	1:100
C	1:100	1000 µL	1000 µL	1:200
D	1:200	1000 µL	1000 µL	1:400
E	1:400	1000 µL	1000 µL	1:800
F	1:800	1000 µL	1000 µL	1:1,600
G	1:1,600	1000 µL	1000 µL	1:3,200
H	1:3,200	1000 µL	1000 µL	1:6,400
I	1:6,400	1000 µL	1000 µL	1:12,800
J	1:12,800	1000 µL	1000 µL	1:25,600
K	1:25,600	1000 µL	1000 µL	1:51,200
L	1:51,200	1000 µL	1000 µL	1:102,400
M	1:102,400	1000 µL	1000 µL	1:204,800

Protein A/G Binding Affinities

Species	Immunoglobulin	Protein A	Protein G
Bovine	Ig	++	++++
Chicken	Ig	-	+
Goat	Ig	+/-	++
Guinea Pig	Ig	++++	++
Hamster	Ig	+	++
Mouse	IgG_1	+	++
	IgG_{2a}	++++	++++
	IgG_{2b}	+++	+++
	IgG_3	++	+++
	IgGM	+/-	-
Pig	Ig	+++	+++
Rabbit	Ig	++++	+++
Rat	IgG_1	-	+
	IgG_{2a}	-	++++
	IgG_{2b}	-	++
	IgG_{2c}	+	++
	IgGM	+/-	-
Sheep	Ig	+/-	++

Key: - (none), +/- (very low), + (low) ---> ++++ (high)

Useful Formulations for Western Blotting and Immunoprecipitation

Cell Lysis Buffer

50 mM Tris-HCl, pH8.0
150 mM NaCl
1% NP-40 or Triton® X-100

2x Immunoprecipitation Buffer

2% Triton® X-100
300 mM NaCl
20 mM Tris, pH 7.4
1.0% NP-40
2 mM EDTA
2 mM EGTA
0.4 mM sodium vanadate (phosphatase inhibitor)
0.4 mM PMSF

2x SDS-PAGE Sample Buffer

100 mM Tris-HCl, pH 6.8
2% Sodium Dodecyl Phosphate (SDS)
20% Glycerol
0.2% Bromophenol Blue
2–10% β-mercaptoethanol (or DTT)

100x Protease Inhibitor Cocktail

PMSF 5 mg (50 µg/mL)
Aprotinin 100 µg (1.0 µg/mL)
Leupeptin 100 µg (1.0 µg/mL)
Pepstatin 100 µg (1.0 µg/mL)
Add 100% Ethanol to 1 mL. Aliquot and store at -20°C.

Recommended Acrylamide Gel Percentages

% Acrylamide	Protein Size Range
8%	40–200 kDa
10%	21–100 kDa
12%	10–40 kDa

Recipes for Common Fixatives

CAUTION: Formaldehyde is toxic and should be handled with caution under a chemical fume hood. Consult Material Safety Data Sheets for proper handling of all laboratory chemicals.

4% Paraformaldehyde (PFA)

1. Heat 250 mL of double strength phosphate buffer stock solution (see step 4) to 140°F (60°C) in a beaker with a disposable stir bar in a hood.
2. Add 20 g granular paraformaldehyde and stir until it is dissolved.
3. Add 250 mL deionized water and filter the solution into a container placed on ice. The solution is ready when cold. Adjust pH to 7.0–7.4.
4. Double Strength Phosphate Buffer Stock solution isprepared by dissolving 7.7 g NaOH and 33.6 g $NaH_2PO_4 \cdot 2H_2O$ in 1 liter deionized water.

4% Paraformaldehyde with 2% Glutaraldehyde

1. Heat 250 mL double strength phosphate buffer stock solution (see above) to 140°F (60°C) in a beaker with a disposable stir bar.
2. Add 20 g granular paraformaldehyde and 10 g glutaraldehyde and stir until it is dissolved.
3. Add 250 mL deionized water and filter the solution into a container placed on ice. The solution is ready when cold. Adjust pH to 7.0–7.4.

Buffered Formaldehyde (Formalin)

1. Dissolve 32.5 g $Na_2HPO_4 \cdot 2H_2O$ and 20 g $NaH_2PO_4 \cdot 2H_2O$ in 4.5 L deionized water.
2. Add 500 mL 40% Formaldehyde.
3. Mix; Adjust pH to 7.0–7.4.

Bouin's Fluid

1. Picric Acid (standard aqueous solution) 75 mL.
2. Formalin (40% aqueous Formaldehyde) 20 mL
3. Glacial Acetic Acid 5 mL.
4. Mix

Carnoy's Fixative

1. 10 mL of glacial acetic acid
2. 30 mL of chloroform
3. 60 mL of absolute alcohol (100% Ethanol)
4. Mix

PLP (Periodate-lysine-paraformaldehyde) Fixative

1. Dissolve 7.3 g of lysine monohydrochloride in 200 mL of ddH_2O.
2. Adjust pH to 7.4 with 0.1 M Na_2HPO_4 ($Na_2HPO_4 \cdot 2H_2O$ 17.8 g/L) NOT phosphate buffer!
3. Complete volume to 400 mL with 0.1 M phosphate buffer pH 7.4. This lysine-phosphate buffer keeps for 2–3 days in the refrigerator, but can be frozen in aliquots for longer storage.
4. Just before use, mix 375 mL lysine-phosphate buffer with 100 mL 20% Formaldehyde and top to 500 mL with ddH_2O. Add 1.06 g sodium periodate ($NaIO_4$) and mix well. The PLP fixative must be used within maximum 2 hrs.
   Final concentrations: Lysine 75 mM, formaldehyde 4%, sodium periodate 10 mM.
   (**NOTE:** Some PLP formulations in literature also use 2% paraformaldehyde)

Acetone/Methanol Fixative

1. 100 mL acetone
2. Add 100 mL methanol
3. Mix well. Use fresh. 50–50 solution is used at room temperature or -20°C

Glossary

Adjuvant – Any substance that has the capacity to enhance the immune response to an antigen.

Affinity – The strength of reaction between antibody and antigen at a single antigenic site.

Affinity purification – Column purification where specific antibody fraction binds to the antigen to which it was made.

Affinity constant – Describes the binding interaction between antibody and antigen. Also known as association or equilibrium constant.

Allergen – An antigen that elicits hypersensitivity or allergic reaction.

Antibody – An immunoglobulin capable of specific combination with the antigen that caused its production in a susceptible animal.

Antigen – Any substance foreign to the body that elicits a specific immune response.

Antigen–Antibody complex – A non-covalent association of antibody and antigen molecules (also known as immune complex).

Antigen retrieval – Procedure designed to enhance the binding of antibody to antigen in tissue typically in response to an epitope-blocking fixation or embedment technique.

Antiserum – Blood from an immunized host presumably possessing antibodies of interest as well as other serum proteins.

Ascites fluid – Unpurified monoclonal antibodycontaining fluid drawn directly from hybridomas grown within a living host.

Avidity – A measure of the overall strength of binding of the entire antibody-antigen complex.

B Cells – B lymphocytes that differentiate into antibody producing cells upon activation by antigen.

Capture antibody – An anchored primary antibody used in ELISA procedures to bind an antigen in solution.

Carrier protein – Typically a large highly antigenic molecule used in combination with a small antigen or hapten to cause a larger, faster specific immune response in an immunized animal.

Chemiluminescent – Producing light through a chemical reaction catalyzed by an enzyme; chemiluminescent substrates require X-ray film or other light capturing devices for detection.

Chromogen – The chemical substrate that changes color in the presence of a specific enzyme-tagged antibody. For example: DAB.

Complement – A set of plasma proteins that act in a cascade of reactions to attack extracellular forms of pathogens.

Conformational Epitope – Epitopes on an antigen formed from several separate regions in the primary sequence of a protein brought together by protein folding.

Cross-reactivity – The binding of an antibody or population of antibodies to epitopes on other antigens.

Denatured – The conformational change in an antigen that may expose or destroy an epitope.

Detection antibody – A primary antibody used in ELISA procedures to allow secondary antibody labeling of antigen.

ELISA (Enzyme-Linked Immunosorbent Assay) – A serological technique that uses antibodies or antigens to capture and quantify the amount of antigen or antibody in unknown samples.

Epitope – The small site on an antigen where a complementary antibody binds via its variable region.

Flow cytometry – A technique that often uses fluorescent antibodies to label whole cells in suspension for measurement and sorting as they pass by a tuned light source.

Fluorochrome (fluorophore) – Molecular label on antibody that emits a distinct, measurable color spectrum in response to a specific laser or chemical interaction.

Hapten – Small molecule incapable of eliciting a specific antibody response without being chemically coupled to a larger carrier protein.

Hinge region – Flexible domain of antibody that binds Fab arm to Fc region.

Hybridoma – Hybrid cell lines that make specified monoclonal antibodies. They are formed by fusing a specific antibody-producing B lymphocyte with a myeloma cell that grows in tissue culture without making its own immunoglobulin chains.

Hybridoma supernatant – Monoclonal antibody-containing fluid collected from hybridoma cell cultures.

Immunity – Ability to resist infection to a particular antigen.

Immunoblotting – A technique that uses antibodies to probe proteins transferred from an electrophoresis gel onto membrane. For example: Western blotting.

Immunocytochemistry – A technique that uses antibodies to probe specific antigens in live or fixed cell cultures.

Immunodiffusion – A technique to detect antigen or antibody by formation of an antigen-antibody precipitate in an agar gel.

Immunofluorescence – A technique for detection of antigen molecules using antibodies labeled with a fluorescent dye.

Immunogenic – A description of an antigen's ability to stimulate antibody production.

Immunoglobulin (Ig) – General term for a number of classes of proteins that functions as antibodies.

Immunohistochemistry – A technique that uses antibodies to probe specific antigens in fresh frozen or processed tissue.

Immunoprecipitation – A technique that uses antibodies to bind antigens in solution to yield a precipitating complex, the components of which can then be isolated.

Monoclonal antibody – A homogeneous population of antibodies that are raised by the fusion of B cells with immortal cell cultures to produce hybridomas.

Negative control sample - any tissue, cell line, lysate or purified protein that is known from previous work to be void of the antigen of interest.

Neutralization – The mechanism of antibody binding to sites on pathogen to prevent its growth and/or its entry into cell. Normal serum – Blood serum extracted from non-immunized animals; often used as a control.

Oposonization – The coating of pathogen surface with any molecule, which can make it more readily digestable by phagocytes.

Optimal working dilution – Concentration (dilution) that maximizes the positive signal while minimizing background (negative) reactivity.

Peptide – A small amino acid sequence used for generating sequence-specific antibodies.

Phagocytes – Specilized cells that perform phagocytosis, e.g., neutrophils and macrophages.

Plasma Cells – Terminally differentiated B cells that produce antibodies.

Polyclonal antibody – Multiple B cell response to an antigen resulting in a mixture of antibodies typically recognizing a variety of epitopes on the antigen.

Positive control sample – Any tissue, cell line, lysate or purified protein that is known from previous work to contain and bind the antigen of interest in a particular application.

Pre-immune serum – Blood serum extracted prior to that animal's immunization with an antigenic substance; often used as a control.

Primary antibody – The antibody that directly binds the antigen of interest.

Protein A – A cell membrane component of Staphylococcus aureus that binds to the FC region of IgG

Protein A/G purification – Column purification where the Fc domain of antibodies binds to the high affinity S. aureus protein A or G.

Secondary antibody – Typically the labeled antibody that binds to the antigen-binding primary antibody. A secondary may also bind to streptavidin, which is bound to the primary.

Specificity – The likelihood that the particular antibody is binding to a precise antigen epitope.

T Cells – T lymphocytes that originate in thymus.

Valency – A description of the relative ability of an antibody to interact with antigens, usually related to the number of available variable regions.

Western blot – Detection, using a specific antibody, of proteins separated by electrophoresis and then bound to a membrane.

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