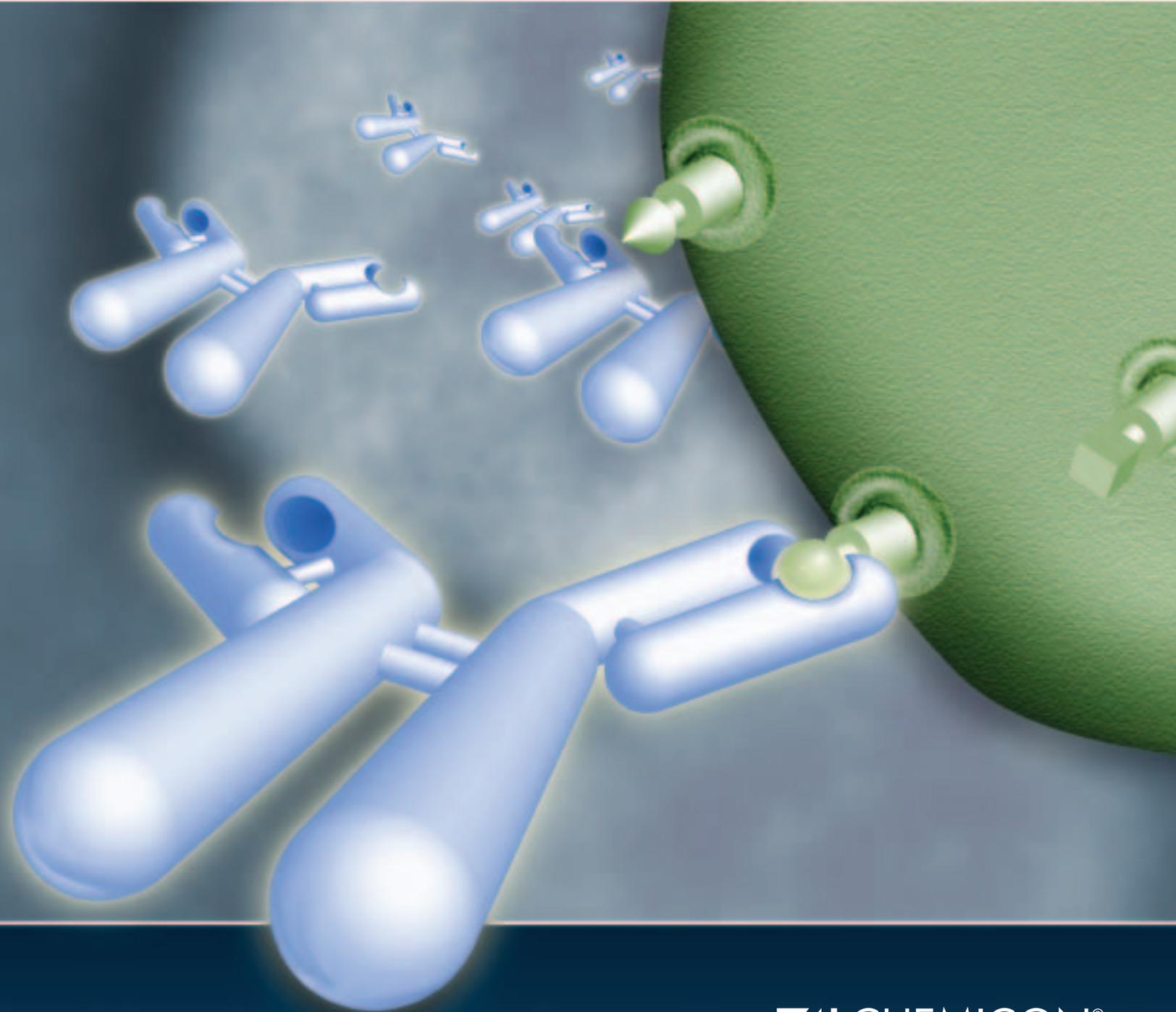


INTRODUCTION TO **ANTIBODIES**

2ND Edition





INTRODUCTION

Chemicon International Inc. is pleased to offer the second edition of **Introduction to Antibodies**, a basic guide to immunological assays and general technical information. This is a handy reference to supplement those techniques described in the literature, recorded in general laboratory procedures, and described on individual product data sheets. As every antibody and experimental design is unique, these general assay suggestions should not be interpreted as applicable to all situations, but rather as an additional source of reference information describing techniques that have been successfully used for a variety of the antibodies available today. As always, individual assays must be optimized empirically and antibody titers must be established for every unique batch of antibody.

For more information about applications using any of Chemicon's more than 7,000 antibodies and kits, Chemicon offers scientific advice for a variety of immunological applications via phone, fax or e-mail. In addition, the Chemicon website has extensive general and product-specific technical information available online and downloadable. We hope that you will contact us with any comments, questions, or suggestions that you may have.

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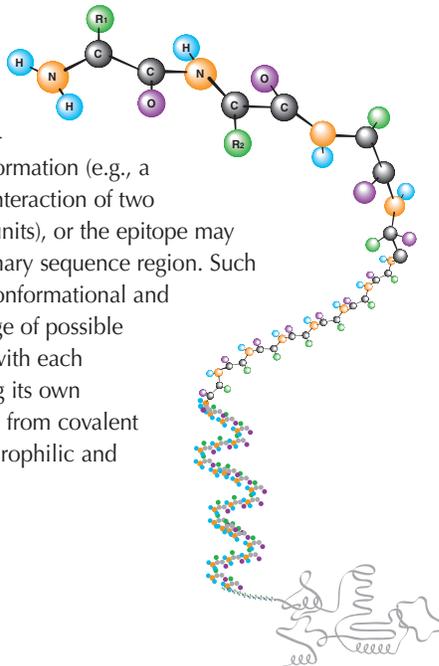
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ANTIGENS

The basic principle of any immunochemical technique is that a specific antibody will combine with its specific antigen to give an exclusive antibody-antigen complex.

The classic definition of **ANTIGEN** is “any foreign substance that elicits an immune response (e.g., the production of specific antibody molecules) when introduced into the tissues of a susceptible animal and is capable of combining with the specific antibodies formed. 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. Antigens that elicit strong immune responses are said to be strongly **immunogenic**.

The small site on an antigen to which a complementary antibody may specifically bind is called an **epitope**. This is usually one to six monosaccharides or 5–8 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), or the epitope may correspond to a simple primary sequence region. Such epitopes are described as conformational and linear, respectively. The range of possible binding sites is enormous, with each potential binding site having its own structural properties derived from covalent bonds, ionic bonds and hydrophilic and hydrophobic interactions.



For efficient interaction to occur between the 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 blot but very good in immunohistochemistry because in the latter procedure, a complex antigenic site might be maintained in the tissue, whereas in the former procedure the process of sample preparation alters the protein conformation sufficiently to destroy the antigenic site and hence eliminate antibody binding.

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

If a gene product of interest is present in extremely low concentrations, one may choose to use known nucleotide sequence information to derive a corresponding **peptide** for generating sequence-specific antibodies. In some instances, peptide antigens have advantages over whole protein antigens in that the antibodies generated may be targeted to unique sequence regions. This is especially useful when investigating proteins that belong to families of high sequence homology ■

Characteristics of a Good Antigen Include:

- Areas of structural stability and chemical complexity within the molecule.
- Significant stretches lacking extensive repeating units.
- A minimal molecular weight of 8,000–10,000 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 which are accessible to the antibody-forming mechanism.
- Structural elements that are sufficiently different from 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.

ANTIBODIES

An **ANTIBODY** is defined as “an **immunoglobulin** capable of specific combination with the antigen that caused its production in a susceptible animal.” They are produced in response to the invasion of foreign molecules in the body. 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. 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 gamma, mu, alpha, delta, and epsilon, 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.

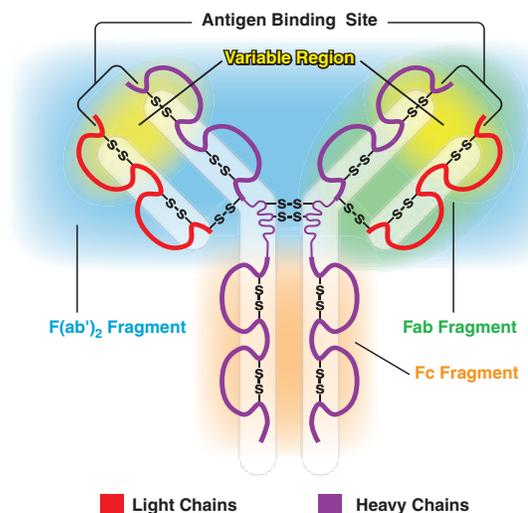
The subclasses of antibodies differ in the number of disulfide bonds and the length of the hinge region. The most commonly used antibody in immunochemical procedures is of the IgG class because they are the major immunoglobulin (Ig) released in serum.

The classical Y shape of IgG 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, and determines the **valency** of the antibody (loosely stated, the number of “arms” with which the antibody may bind its antigen).

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 seen by secondary antibodies in immunoprecipitation, immunoblots and immunohistochemistry. These three regions can be cleaved into two F(ab) and one Fc fragments by the proteolytic enzyme papain, or into just

Class/ subclass	Heavy chain	Light chain	Molecular weight (kDa)	Structure	Function
IgA ₁ IgA ₂	α 1 α 2	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	γ 1 γ 2 γ 2 γ 3 γ 4	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

two parts: one F(ab')₂ and one Fc at the hinge region by the proteolytic enzyme pepsin. Fragmenting IgG antibodies is sometimes useful because F(ab) fragments (1) will not precipitate the antigen; and (2) will not be bound by immune cells in live studies because of the lack of an Fc region. Often, because of their smaller size and lack of crosslinking (due to loss of the Fc region), Fab fragments are radiolabelled for use in functional studies. Interestingly, the Fc fragments are often used as blocking agents in histochemical staining. ■



ANTIBODY ANTIGEN INTERACTION

The specific association of antigens and antibodies is dependent on hydrogen bonds, hydrophobic interactions, electrostatic forces, and van der Waals forces. These are all bonds of a weak, non-covalent 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, however, and follows the basic thermodynamic principles of any reversible bimolecular interaction:

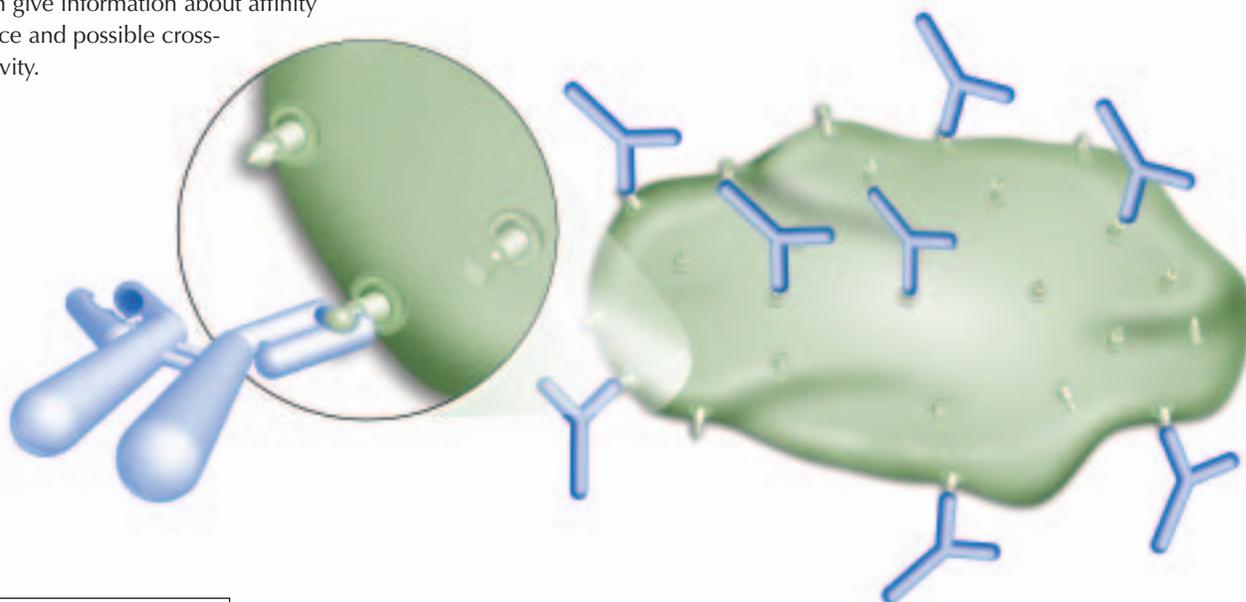
$$K_A = \frac{[Ab-Ag]}{[Ab][Ag]}$$

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

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^{-1} to above 10^{12} mol^{-1} . 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 bondings 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.

Affinity describes the strength of interaction between antibody and antigen at single antigenic sites. Within each antigenic site, the variable region of the antibody “arm” interacts through weak non-covalent forces with antigen at numerous sites; the more interactions, 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 in evolution.

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 publication. ■



MONOCLONAL & POLYCLONAL ANTIBODIES

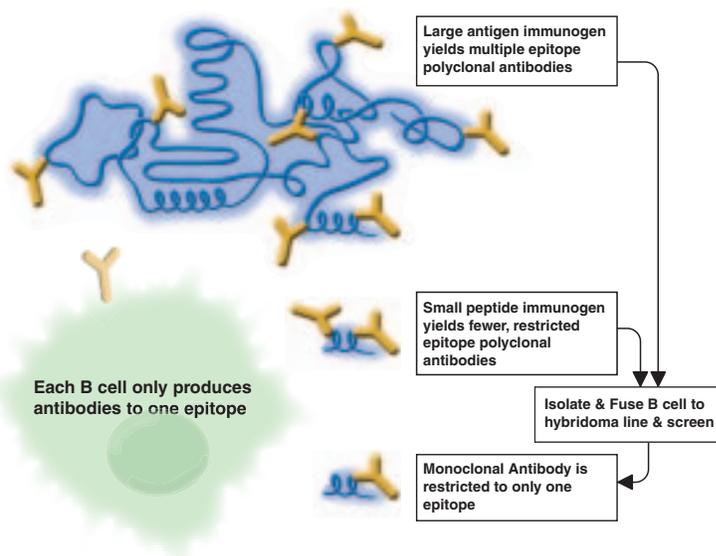
When designing experimental procedures, it is important to differentiate between monoclonal and polyclonal antibodies, as these differences are the foundation of both advantages and limitations of their use.

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 a suspension of the 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 (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 an especially 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 educate one's self about the immunogen that has been used for production of the polyclonal antibody and the potential for undesired cross-reactivity within one's sample. Peptide immunogens are often used to generate polyclonal antibodies that target unique epitopes, especially for protein families of high homology.

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 give significantly less background staining 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.

ANTIBODY FORMATS

Polyclonal antibodies are often available in relatively unpurified formats, described as “serum” or “**antiserum**”. 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 non-target antigens that can sometimes react non-specifically in immunological assays. For this reason, raw antiserum is often purified, 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 non-specific immunoglobulin fraction. As a result the protein A/G purified antiserum may still possess undesirable cross reactivity. See appendix C for protein A/G binding affinities.

Antigen **affinity purification** takes advantage of the affinity of the specific immunoglobulin fraction for the immunizing antigen against which it was generated. Unlike protein A/G purification, antigen affinity purification results in the elimination of the bulk of the non-specific 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.

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.

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

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, 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 Chemicon 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. ■

Chemicon Approved Antibody Buffers & Concentrations

1. Affinity Purified Monoclonal and Polyclonal Antibodies

0.02 M phosphate, 0.25 M NaCl, (PBS) 0.1% NaN₃ pH 7.6
The same buffer without NaN₃ will be used as required.

2. FITC Conjugates

0.02 M phosphate, 0.25M NaCl, 15 mg/mL BSA,
0.1% NaN₃ 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, (TBS) 0.001 M MgCl₂, 15 mg/mL
BSA, 0.1% NaN₃, pH 8.0

5. Biotinylated Conjugates

0.01 M PBS, 15 mg/mL BSA, 0.1% NaN₃, pH 7.1

Standard concentrations are

Purified and Monoclonal Conjugates	1 mg/mL
Polyclonal Affinity Purifieds	2 mg/mL
Polyclonal FITC Conjugates	2 mg/mL
Polyclonal HRP/Alk Phos Conjugates	1 mg/mL

Storage Conditions

Polyclonal Affinity Purifieds	4°C to 8°C
Fluorescent Conjugates	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

GENERAL TECHNICAL GUIDELINES

Proper Controls

The use of proper controls will help eliminate false positive and false negative results and aid in the interpretation of experimental data. They will also be invaluable in troubleshooting throughout the experimental design process. Whenever possible, both negative and positive control samples should be included in an assay. A **positive control sample** may be any tissue, cell line, purified protein, etc. that is known to contain the antigen of interest and to have previously been determined to be positive by a reliable method. A **negative control sample** is one that is known to be devoid of the antigen of interest.

In addition to sample controls, one should also use reagent controls. Remembering 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 titers, each new batch of antibody must be standardized before use in an existing assay.

It should 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 kind of information is invaluable in efficient assay development.

Handling of Reagents

In order to preserve maximum reactivity, reagents should be stored according to manufacturer instructions whenever possible (e.g., held minimally 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 cross-linking reagents. Undiluted antibodies should always be aliquoted prior to storage at -20°C to minimize repeated freeze/thaw cycles, which can cause antibody denaturation. Storing an antibody in concentrated form will prevent or minimize degradation. 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. If antibodies will be stored at 2–8°C for more than two to three days, it is advisable to add a bacteriostat, such as 0.05% sodium azide or 0.1% thimerosal.

Note: Sodium azide will inhibit the activity of horseradish peroxidase.

As with all laboratory reagents, consult a Material Safety Data Sheet (MSDS) for additional handling precautions.

Antibody and Titer

As mentioned above, the rate of binding between antibody and antigen is dependent on the affinity constant, which in turn can be affected by temperature, pH, and solvent composition. Varying the relative concentrations of antibody and antigen in solution can also control the extent of antibody-antigen complex formation. In most cases, however, the concentration of antigen in a sample cannot be adjusted. Typically, therefore, 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 experimental 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. For further dilutions, see Appendix B.

Datasheet 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, one may wish to make dilutions of 1:50, 1:100, 1:500, 1:1,000 and 1:10,000, to determine the optimal dilution for one's 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 interassay variation. ■

IMMUNOPRECIPITATION

It is possible to use antibody-antigen precipitation (immunoprecipitation) to isolate a specific antigen from complex protein mixtures such as 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, among other things. 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 requires affinities of 10^8 mol⁻¹ or higher).

Immunoprecipitation is Divided Into Six Basic Steps:

1. Labeling the antigen (optional)
2. Sample preparation: Lysis of cells to release the antigen
3. Formation of the antibody-antigen (immune) complex
4. Precipitation of the antibody-antigen complexes
5. Analysis
6. Troubleshooting

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 **pre-immune serum** from the same animal used for immunization. However, an equal concentration of non-immune (**normal**) **serum** from a different animal of the same species should suffice in its absence.

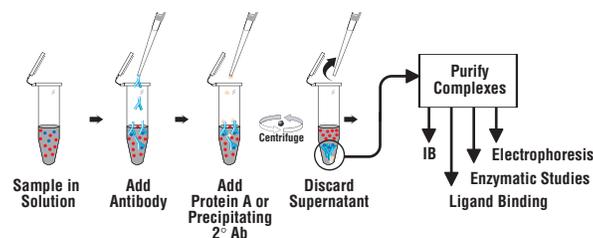
Antigen Labeling (optional)

Antigen may be labeled by incubating in a medium containing a radioactive precursor, such as ³H-Thymidine, by iodination or biotinylation of surface proteins, by treatment with radioactive sodium borohydride, or by other published techniques.

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 that one take into account the location of the antigen within the cell (nuclear, cytosolic, membrane bound, etc.) and determine how best to extract the antigen with minimal effect on its structural integrity. Before initiating an extraction/cell lysis protocol one should consider what information one wishes to obtain from performing an immunoprecipitation. For example, is it necessary to extract functional protein for subsequent

functional studies? Additionally, one may want to consider whether they would like to disrupt protein-protein interactions or co-immunoprecipitate proteins that may interact with the target antigen within the cell. Based on these and other considerations, one must decide upon an appropriate lysis strategy. Often times 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 become denatured 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 one would like to preserve subunit associations and other protein-protein interactions. In general, nonionic (e.g., Triton® X-100, NP40) or zwitterionic (e.g., CHAPS) detergents tend to preserve non-covalent 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, see Appendix F, Hjelmeland and Chrumbach (1984) and Welling *et al.* (1987). Regardless of the lysis strategy employed, the lysis buffer should always contain a redundant cocktail of protease inhibitors (see appendix E) to protect against proteolysis of the target antigen by proteases liberated during the 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 protein concentration and adjust protein concentration of lysate to between 2 to 5 mg/mL with lysis buffer or PBS. Note that 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. Extracts that will not be used immediately should be aliquotted and stored at -70°C for future use. 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.



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. To a microcentrifuge tube add:

1. 500 μ L 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 μ g of total protein lysate (approximately 100 μ L of lysate at 2–5 mg/mL)
3. 1–5 μ g of purified antibody. If unpurified antibodies are used, substitute the following antibody quantities: serum, 0.5–5 μ L; hybridoma tissue culture supernatant, 10–100 μ L; or ascites fluid, 0.1–1.0 μ L. Be sure to adjust volume of ddH₂O added in step 4 (below) to accommodate the volume of antibody added here.

Note: antibody concentrations given are recommended starting points only. Optimal antibody concentrations must be determined empirically. For negative control reaction, add an equivalent amount of control antibody.

4. Add ddH₂O 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 non-specific background.

Precipitation of Immune Complexes

To precipitate immune complexes, one 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 dependant on the subclass of the immunoglobulin and the species from which it came (see Appendix C). For example, Protein A is exceptionally well suited for immunoprecipitation of all rabbit primary antibodies, but not for chicken antibodies. (See appendix C for protein A/G binding affinities). To use protein A for immunoprecipitation of mouse primary antibodies, it is advisable to add 5 μ g 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, a quantity of 10–20 μ L 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 complexes by centrifugation.

Analysis

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 next section). The purified immune complex may also be used for enzymatic studies, ligand binding, further immunizations, or other immunochemical techniques. These methods, when used in conjunction with immunoprecipitation, can greatly increase the amount of information discovered about an antigen. ■

Troubleshooting - Immunoprecipitation

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 and can be either specific or nonspecific. The following are suggestions to deal with nonspecific background problems:

- 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 blotto.
- Spin the lysate at 100,000 x g for 30 min (discard pellet) prior to addition of primary antibody.
- Centrifuge the antibody at 100,000 x g for 30 min (discard pellet) and titrate.
- Increase the number of washes. “Soak” solid phase in the wash buffers for 10 min per wash.
- Decrease primary antibody concentration.
- Decrease primary antibody incubation time.
- 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.
- Lower the number of counts per minute (cpm) of the radiolabel used to the minimum needed for antigen detection.
- If specific proteins remain, remember that your antigen may consist of more than one polypeptide chain. Additional bands may also represent polypeptides that associate with the target antigen *in vivo* that have co-immunoprecipitated with the target antigen.
- Run no primary and protein A only extract control lanes. This will aid in determining background bands arising from the Protein A beads or extract alone.
- Multiple bands detected below the anticipated molecular weight may indicate proteolytic degradation of sample. Prepare new lysates with fresh protease inhibitors added.
- Try a different antibody.

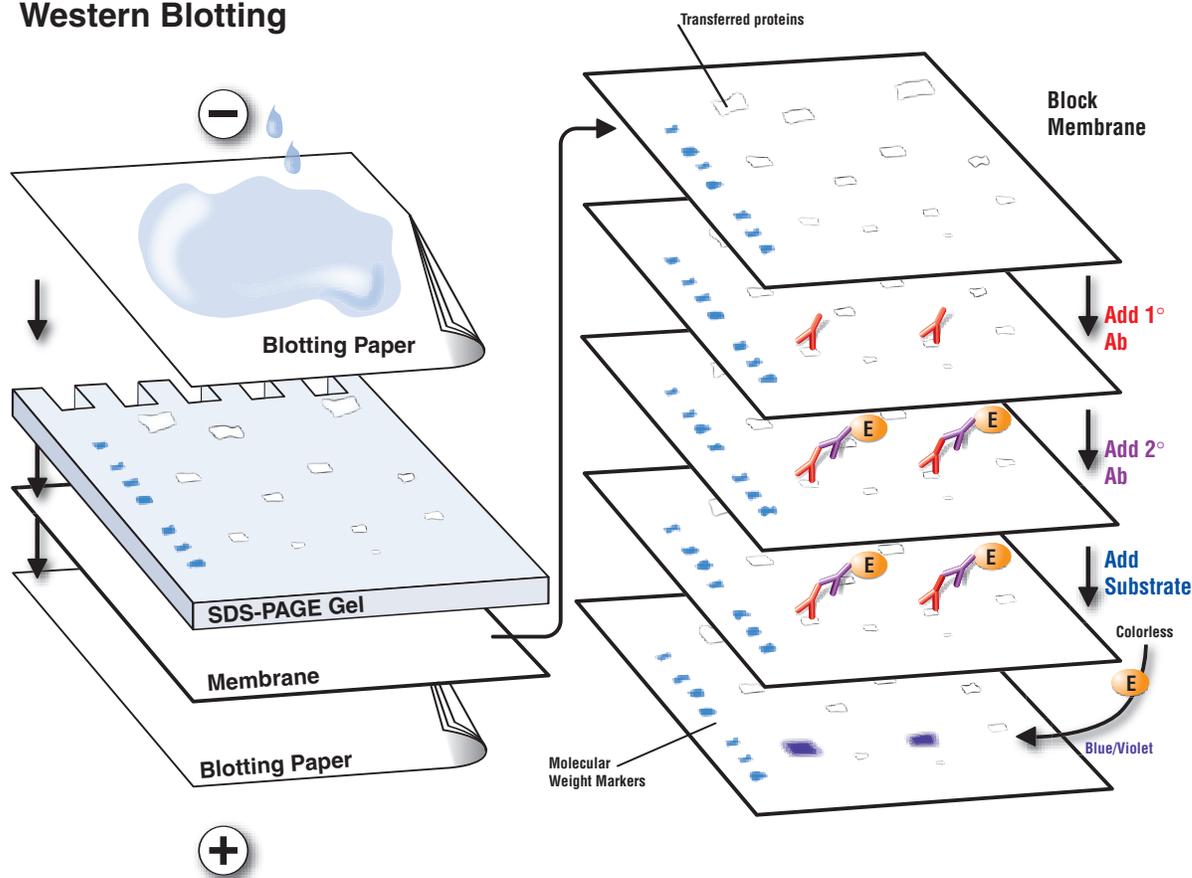
WESTERN BLOTTING

Immunoblotting procedures combine 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 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 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. A charged protein will migrate 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 will be retarded as a function of the size and shape of the protein by the sieving effect of the gel matrix. Native polyacrylamide gel electrophoresis (PAGE) can be used to separate individual proteins, by size, shape and charge. PAGE can also be performed under denaturing conditions, typically in the presence of a molar excess of the ionic detergent Sodium

Dodecyl Sulfate (SDS-PAGE) or under reducing and denaturing conditions (SDS-PAGE in the presence of a reducing agent such as DTT or beta mercaptoethanol). 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 rendered devoid of 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). Under non-denaturing conditions alone, however, 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. 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

Western Blotting



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 dithiothreitol (DTT) or beta 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. A great deal can be learned about the properties of an individual protein by “running gels”. However, even more can be learned by transferring the fractionated protein sample to solid support membranes (Western blotting) for detection (probing) with specific antibodies.

The Basic Blotting Procedure Can Be Divided into the Following Steps:

1. Sample Preparation
2. Gel Electrophoresis
3. Membrane Transfer
4. Blocking Non-specific Binding
5. Addition of the Antibody
6. Detection
7. Troubleshooting

Sample Preparation

An unlabeled solution of proteins, frequently an extract of cells or tissues, is first prepared in a gel electrophoresis sample buffer. In some cases, the sample to be blotted has been derived from an immunoprecipitation, as described previously. Please see “Sample Preparation” in Immunoprecipitation section for more details.

Gel Electrophoresis

The protein sample is first resolved by gel electrophoresis. Make sure that the gel acrylamide concentration is appropriate for the anticipated molecular weight of the antigen to be detected (see Appendix E) and that the acrylamide solution is degassed prior to casting gel. Always pre-cast SDS-PAGE gels the day before use to insure complete polymerization for maximum resolution. Fresh ammonium persulfate and TEMED should be used to catalyze gel polymerization. Rinse wells thoroughly before applying sample to gel. 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 (see Appendix E) per well. If samples are to be run under non-reducing conditions, beta-mercaptoethanol and DTT should not be included in the sample buffer. Samples should be heated at 50–65°C for 10–15

minutes prior to loading gel. Samples should not be boiled as proteins containing significant stretches of hydrophobic amino acids (such as membrane proteins) tend to aggregate when boiled. It is advisable to 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. Since pre-stained molecular weight markers often do not run true to size, it is recommended that unstained molecular weight standards be run as well if an accurate determination of antigen molecular weight is desired. 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.

Membrane Transfer (Western blotting)

Transfer of the proteins fractionated by SDS-PAGE to a solid support membrane (Western blotting) 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 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 blot 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. Therefore, for small molecular weight proteins, it is often beneficial to pre-soak the acrylamide gel for 5 to 15 minutes (depending on gel thickness) in transfer buffer without SDS prior to transfer. This will help to reduce the SDS concentration and enhance binding of low molecular weight proteins to the membrane. Conversely, higher molecular weight antigens typically require longer transfer times. The

efficiency of transfer of high molecular weight antigens may be enhanced by increasing transfer time and the addition 0.05% SDS to the transfer buffer. Typically, the more hydrophobic a protein is, the more difficult it may be to transfer to solid support membranes. The addition of methanol (up to 20%) to the transfer buffer has proven to enhance the transfer efficiency of some hydrophobic proteins.

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 from cathode (-) to anode (+) 1) sponge, 2) filter paper (3 sheets) soaked in transfer buffer, 3) gel, 4) membrane, 5) filter paper (3 sheets) soaked in transfer buffer, 5) sponge (see Figure page 10). The transfer sandwich is then placed in the transfer apparatus and subjected to an electric current.

1. 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 cross contamination.
2. 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.
3. Remove all air bubbles between the gel and the membrane. This can be done easily by rolling a pasture pipette across the surface of the gel/membrane sandwich.
4. Place the membrane on the side of the positive electrode (anode).
5. Transfer the separated polypeptides using 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. For longer transfer times, it is recommended that electroblotting be performed at 4°C to prevent overheating and buffer decomposition.

Note: To ensure the transfer is complete, the blot can be stained with Chemicon's Blot-Fast Stain™ (Cat. No. 2076). Alternatively Coomassie brilliant blue or amido black can be used to visualize protein bands: To stain the membrane with Coomassie, remove the membrane from the transfer system and rinse in several changes of ddH₂O. Allow membrane to dry completely then submerge in a solution of 0.02% Coomassie brilliant blue R-250 in 50% methanol for 30 seconds to 1 minute. Destain in several changes of 50% methanol to visualize bands.

Blocking Non-specific Binding

Prior to addition of **primary antibody**, 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. Typical blocking solutions include 10% (w/v) BSA, or 5% non-fat dried milk in Tris or phosphate buffered saline. It is important to be certain that the blocking solution does not contain antigens that may be recognized by the primary or **secondary antibody**. Incubation in blocking solution for 30 minutes at 37°C is sufficient to block membrane.

Addition of the Antibody

Dilute the primary antibody in Tris or Phosphate buffered saline. Unless non-specific 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).

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 can be detected visually through the conversion of a **colorimetric substrate (chromagen)** to a colored precipitate at the site of antibody binding. Alternatively, **chemiluminescent** substrates may be employed which emit light upon conversion by the enzyme. The light emitted at the site of substrate conversion can be captured on x-ray film. Some antibody detection systems, such as chemiluminescence, are exquisitely sensitive, while others, such as those utilizing colorimetric substrates, are less so. The appropriate working concentration of the primary antibody is dependent upon the binding characteristics of the primary antibody, but 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, prior to assay optimization. See Appendix D for common enzyme-substrate combinations used for Western blot 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 second antibody. The minute gold particles are directly visible as a red color when they are bound with the second antibody to the primary antibody.
4. Biotinylated second antibody. In this case the blot is incubated with the secondary antibody, 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 various substrates.

Troubleshooting Western Blot

Streaking of blots is most likely due to an excess protein load on the gel. Lack of staining may be due to ineffective sample transfer from gel to membrane. This may be verified by staining with Coomassie blue or other stain, as indicated above.

Ineffective transfer may be due to:

1. Air bubbles between the gel and the membrane
2. Placement of the membrane on the wrong side of the gel — it must be on the same side as the positive electrode.
3. Insufficient current/time for transfer. Check the manufacturer's instructions.
4. SDS concentration too high in transfer buffer

Weak staining may also be due to:

1. Insufficient primary antibody incubation time. If a one hour incubation time at room temperature is insufficient, try incubating overnight at 4°C.
2. Primary antibody too dilute or incubation time too short.
3. Improper storage of reagents. Check that dilute antibody solutions have not been stored as dilute solutions for extended periods, allowing adherence of the immunoglobulin to the storage vessel, and that antibodies have not been repeatedly frozen and thawed.

Note: if using an alkaline phosphatase detection system, the blocking solution containing diluted antibody should not be used after 3 to 4 weeks of storage. For chemiluminescent detection, the blocking solution containing diluted antibody should not be used after one week of storage.

High background may be caused by:

1. Incomplete blocking of membrane.
2. Antibodies used at excess concentration. Try more dilute antibody solutions or shorter incubation times.
3. Insufficient washing or use of expired or contaminated reagents.
4. Excess exposure time, or ultra-sensitive detection substrates. Optimize the detection dilutions for best results, newer chemiluminescent detection systems are up to 50X more sensitive than old chromogenic systems, thus they require far less primary and secondary antibodies for equivalent detection levels.

Multiple bands are detected.

The detection of multiple bands is not necessarily cause for alarm. In some cases these bands may represent specific reactivities with alternatively spliced isoforms of an antigen that happen to possess common epitopes. Alternatively, additional bands may represent non-specific immunoreactivity with seemingly unrelated proteins. Therefore, when multiple bands are observed, it is necessary to determine if this reactivity is antigen specific or the result of non-specific reactivity and whether this non-specific reactivity is attributable to the primary or secondary antibody. Often, specific immunoreactivity is determined by employing a preabsorption control. The source of non-specific reactivity can be elucidated by performing a secondary antibody only control.

Apparently specific bands of unanticipated molecular weight are observed.

Typically this is due to proteolytic degradation of the protein sample or incomplete reduction of sample prior to electrophoresis. Multiple bands migrating near the expected molecular weight of the antigen is often characteristic of incomplete reduction. Proteolytic degradation of the sample should be suspected if all the observed bands are of lower molecular weight than anticipated.

No bands are observed.

Some antibodies, particularly monoclonal antibodies generated against native antigen, may not recognize proteins that have been fractionated under reduced and/or denatured conditions. Such antibodies are thought to recognize conformational epitopes that exist by virtue of the native three-dimensional structure of the antigen that is lost upon denaturation and/or reduction. In such cases, the reducing agent may need to be eliminated or gels may need to be run under non-denaturing conditions (w/o SDS). Finally, if using peroxidase conjugated secondary antibodies, all solutions must be free of sodium azide because even trace amounts of azide will inhibit the peroxidase activity, leading to no bands being observed on the blot when it (or the film) is developed. ■

ELISA

ELISA Procedure

- Sandwich ELISA Protocol
- Competitive ELISA Protocol
- Troubleshooting

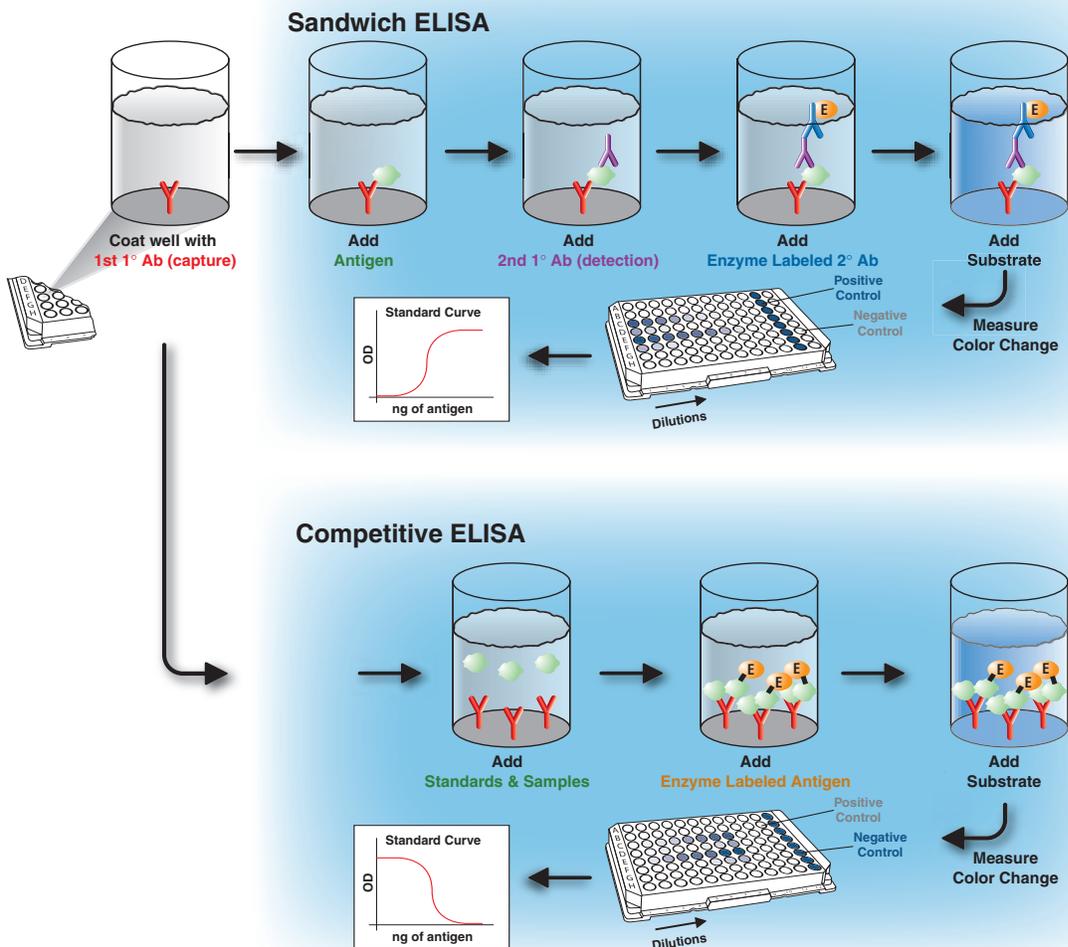
Enzyme-linked Immunosorbent Assays (ELISAs) combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme that possesses a high turnover number. ELISAs can provide a useful measurement of antigen or antibody concentration.

Sandwich ELISA Assays

One of the most useful of the immunoassays is the two-antibody “sandwich” ELISA. This assay is used to determine the antigen concentration in unknown samples. This ELISA is fast and accurate, and if a purified antigen standard is available, the

assay can determine the absolute amount of antigen in an unknown sample. 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.

To utilize this assay, one antibody (the “**capture**” antibody) is purified and bound to a solid phase typically attached to the bottom of a plate well. Antigen is then added and allowed to complex with the bound antibody. Unbound products are then removed with a wash, and a labeled second antibody (the “**detection**” antibody) is allowed to bind to the antigen, thus completing the “sandwich”. The assay is then 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 does not need to be purified prior to use, and that these assays are very



specific. However, one disadvantage is that not all antibodies can be used. 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.

Unlike Western blots, which use precipitating substrates, ELISA procedures utilize substrates that produce soluble products. Ideally the enzyme substrates should be stable, safe and inexpensive. Popular enzymes are those that 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. A table of commonly used enzyme-substrate combinations is included in Appendix D.

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.

The amount of the capture antibody that is 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.

General Protocol for the Sandwich ELISA Method

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 $\mu\text{g}/\text{mL}$ in PBS). PVC will bind approximately 100 ng/well (300 ng/cm²). The amount of antibody used will depend on the individual assay, but if maximal binding is required, use at least 1 $\mu\text{g}/\text{well}$. 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. 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 hrs. to overnight in a humid atmosphere at room temperature.

Note: Sodium azide is an inhibitor of 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 hrs. 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 hrs. 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, optical densities at target wavelengths can be measured on an ELISA plate reader.

Note: Some enzyme substrates are considered hazardous, due to 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 Assays

When two “matched pair” antibodies are not available for your target, another option is the competitive ELISA. Another advantage to the competitive ELISA is that non-purified primary antibodies may be used. Although there are several different configurations for competitive ELISAs, below is an example for one such configuration. In order to utilize a competitive ELISA, 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. The protocol below uses a labeled immunogen as the competitor. For other configurations of competitive ELISAs, see Appendix F, Harlow and Lane (1996).

Briefly, an unlabeled purified primary antibody is coated onto the wells of a 96 well microtiter plate. This primary antibody is then incubated with unlabeled standards and unknowns. After this reaction is allowed to go to equilibrium, 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 plate is then developed with substrate and color change is measured.

General Protocol for the Competitive ELISA Method:

1. 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.
2. Add 50 μL of diluted primary antibody (capture) 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/cm²). 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 hrs. 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 $\mu\text{g}/\text{mL}$ 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. 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 hrs. 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 of 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 hrs. 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, 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. ■

Troubleshooting ELISA Assays:

- Interpret the control results.
- If the negative controls are giving positive results, there may be contamination of the substrate solution, or contamination of the enzyme-labeled antibody, or of the controls themselves.
- If no color has developed for the positive controls or for the samples, check all reagents for dating, concentration, and storage conditions. Check the integrity of the antibody reagent.
- If very little color has developed for the positive controls and the test samples, check the dilution of the enzyme-labeled antibody, and the concentration of the substrate.
- If color has developed for the test samples but not the positive or negative controls, check the source of the positive controls, their expiration date and their storage. Have they been stored in a dilute form, so that the antigen may have adhered to the surface of the storage vessel?
- If color can be seen, but the absorbance is not as high as expected, check the wavelength setting.
- When rerunning an assay while troubleshooting, change only one factor at a time.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry/Immunocytochemistry Procedures

- Specimen Preparation
- Section Type
- Antigen Retrieval
- Antibody Staining
- General Protocol for Immunohistochemical Staining
- Antibody Detection
- Troubleshooting

In these techniques an antibody is used to link a cellular antigen specifically to a stain that can be more readily seen with a microscope. Detection of antigens in tissues is known as **immunohistochemistry**, while detection in cultured cells is generally termed **immunocytochemistry**. For both, there is a wide range of specimen source, antigen availability, antigen-antibody affinity, antibody type, and detection enhancement methods. Thus optimal conditions for immunohistochemical or immunocytochemical detection must be determined for each individual situation, dependent on the above variables. As for all procedures, reference should be made to individual product data sheets and published literature. Also, the Internet today contains a tremendous amount of useful information on Immunohistochemistry/ immunocytochemistry. Quick searches through google.com or similar search engines are highly recommended.

Specimen Preparation

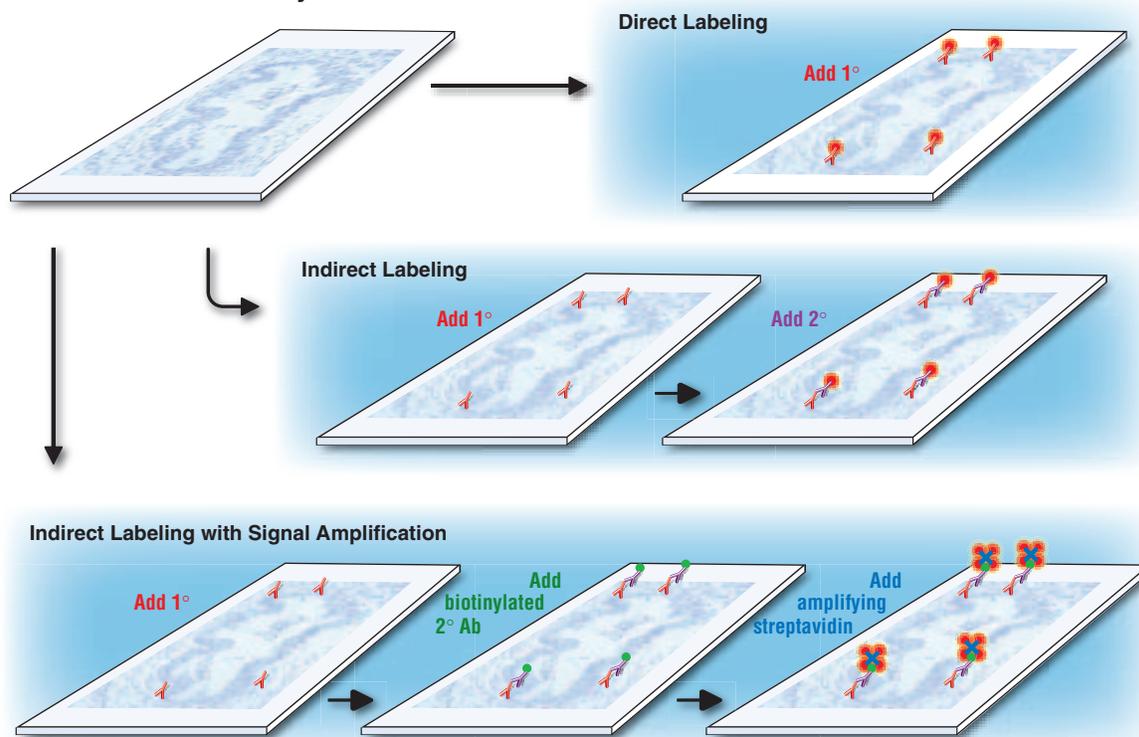
- Fixatives
- Section Type

Fixatives

Fixatives are needed to preserve cells and tissues in a reproducible and life-like manner. To achieve this, tissue blocks, tissue sections, cell growths 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 (For perfusion protocol, see chemicon.com; search word: perfusion). 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.

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 have a variable 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 multiple different fixatives and antigen retrieval methods prior to "giving up" on a specific stain.

Immunohistochemistry Process



Fixatives may work by several means: formation of crosslinkages (e.g., aldehydes such as glutaraldehyde or formalin); protein denaturation by coagulation (e.g., acetone and methanol); or a combination of the above. 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 example when using antibodies to probe for neurotransmitter substances, most tissues must be either immersion fixed with a mixture of glutaraldehyde and paraformaldehyde, or with paraformaldehyde alone. Both acetone and methanol (precooled to -20°C) have been used successfully as fixatives for frozen tissue in other instances.

Immunocytochemistry

Fixation of cultured cells for immunocytochemistry also requires careful consideration; in general, fixation strengths and times are considerably shorter for cells than on the thicker, structurally complex tissue sections. For immunocytochemistry, sample preparation essentially entails fixing the target cells to the slide. Depending upon the needs of the experiment, cells can be simply harvested and “dropped” on to a slide, fixed and air-dried, as is often done with clinical or diagnostic studies where speed and a simple “yes/no” answer is all that is needed. Alternatively, where information on the structural location within the cell is needed, cells are often cultured directly on prepared slides or coverslip material, which are then simply washed and fixed in place prior to staining. Consulting published literature relating to the tissue/proteins of interest is well worth the time invested. See Appendix A for recipes of common fixatives.

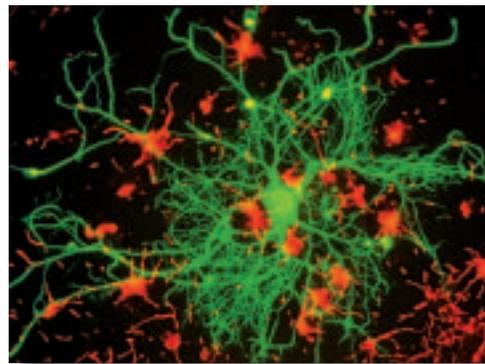
Section Type:

- Cryostat (frozen) sections
- Paraffin (wax) sections

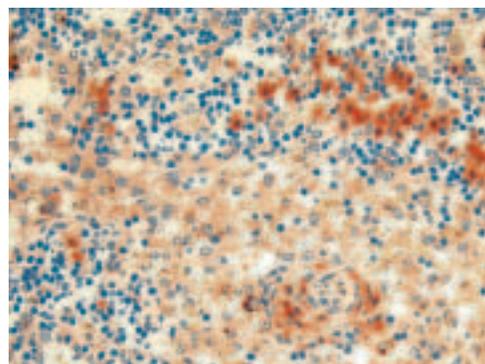
The next consideration for immunological staining is the type of section to use. For immunohistochemistry, the common options are fixed or unfixed cryostat (frozen) sections, fixed “wet” or vibratome sections, or fixed, paraffin-embedded sections. The choice of section is determined by a number of issues, including the time and skill of the investigator. Because of the ease of use, fixed frozen sections are often quickest and easiest to use. However, because of their superior fidelity, clarity, and preservational properties, fixed paraffin-embedded tissues have become the ultimate standard of immunohistochemistry in histology and pathology, and anytime where archiving of immunohistochemical information is required.

Cryostat (frozen) sections

Today there are two types of Cryostat sections: (1) Fresh, or unfixed sections where quickly frozen (snap frozen) tissues are first cut, then either air-dried or fixed prior to staining; (2) or fixed frozen tissue, where the tissue is first fixed then cryoprotected with sucrose or other stabilizer (to stabilize the tissue cell structure) prior to freezing and sectioning. The advantages of frozen sections are that they allow excellent antigen preservation, they are typically faster to perform, and they offer flexibility, since any fixative can be used, thereby facilitating the optimization of fixative for each antigen. However frozen sections give less morphological detail and resolution than other methods.



Rabbit anti- α -internexin (Cat No. AB5354) and Mouse anti-NF-L (Cat No. MAB1615) staining of cultured rat neurons. Mature neurons are green (NF-L positive) and neuronal progenitor cells are red (α -internexin positive).



Rabbit anti-Indolamine 2, 3-dioxygenase (Cat. No. AB5968) staining of a lymph node from a human patient with malignant melanoma.

Sample Protocols for Cryostat Sections

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 freezeze.

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 cryostat (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 redry prior to rehydration and staining.

Paraffin Sections

The largest proportion of 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, and 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 (10x10x3 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 A for recipes of common fixatives. The blocks are then infiltrated and embedded with paraffin and 5–10 μm 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.

Sample Protocol for Paraffin-embedded Sections:

- A. Conventional deparaffinization and dehydration sequence:
1. Incubate sections in Xylene: 2 to 3 changes, 5 min. each.
 2. 100% absolute ethanol: 2 changes, 3 min. each
 3. 95% ethanol: 2 changes, 3 min. each
 4. 80% ethanol: 3 min.
 5. 50% ethanol: 3 min.
 6. 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.*
- B. 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
- C. 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 be 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.
- D. Wash twice with PBS.
- E. Proceed with immunostaining procedure (see Antibody Staining section).

Antigen Retrieval

- Enzymatic Digestion
- Microwave
- Autoclaving or Pressure Cooking

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 or heat induced epitope retrieval (HIER) through microwave irradiation, autoclaving or pressure cooking.

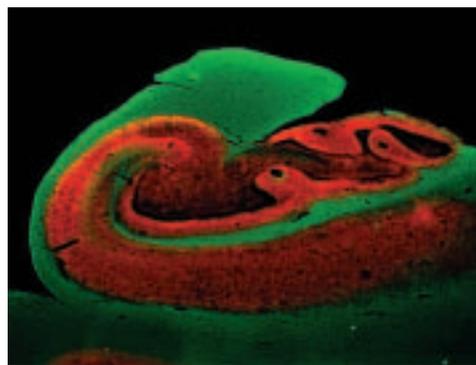
Enzymatic Digestion

This technique involves dewaxing, rehydrating, and rinsing the specimen in running water. The specimen is then equilibrated with the appropriate buffer, and incubated with a proteolytic enzyme 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% CaCl₂) and pepsin (0.05% (w/v) in 2 normal 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. (Shi, S-R, *et al.* (1993). *J. Histochemistry & Cytochemistry* **41**:1599–1604). However, proteolytic enzymes can abolish the reactivity of some antigens. (Pileri, S., *et al.* (1997). *J. Pathology* **183**: 116–123).

Microwave Irradiation

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 positive cells available, and the intensity of reactions, although the exact mechanism is unclear. It is important to monitor the sections during the microwaving process, to prevent damage and drying. Consistency of

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 cool to room temperature slowly, then the slides are rinsed in PBS and used for staining.



Mouse anti-Synaptophysin (Cat. No. MAB329). Localization of synaptophysin (red) and Myelin Basic Protein (green) in human hippocampus (paraffin-embedded section).

Autoclaving or Pressure Cooking

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 buffer solutions commonly used 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 using a

microwave oven. However, preservation of the cytological detail may be slightly inferior in sections that undergo pressure cooking. Chemicon provides a recommended HIER protocol on its Technical Service website menu available at:

www.chemicon.com/techsupp/protocol/HIER.asp

A more mild procedure that can be used on many tissues is a simple incubation in citric acid buffer, pH 3.0 (2.1 grams Citric Acid added to 400 mL of ddH₂O. Adjust to pH 3.0 with Acetic acid if above 3.0, or NaOH if below 3.0, make up to 1 L final volume with ddH₂O) 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.

Antibody Staining

Primary antibody may be directly labeled with an enzyme (such as horseradish peroxidase or alkaline phosphatase) or fluorophore (such as FITC or rhodamine), or unlabeled, with detection by a labeled secondary antibody or 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.

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. Antibodies are like children, and each is different. The product data sheets may be used as a guide for dilution series starting points. (See Appendix B for a possible dilution protocol.) The optimal antibody dilution will be that which gives the strongest specific antigen staining with the lowest non-specific 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 — the volume used must be sufficient to completely cover the tissue, and to ensure the tissue will not dry out during incubation. Incubation times may range from 30–90 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.

Chemicon's IHC Select[®] Manual Staining System (Cat. No. MSS001) offers a convenient platform for quality antibody staining. This compact device offers the convenience of standardized staining with the efficiency of capillary gap technology to reduce reagent volume and minimize waste.

General Protocol for Immunohistochemical 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 above 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 than 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.

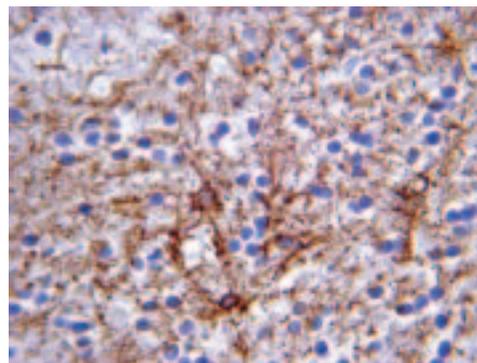
Antibody Detection

- Enzyme-Mediated
- Fluorescence
- Signal Amplification

Two of the most commonly used detection methods are fluorescence and colorimetric (enzyme mediated) detection. With the advent of electron microscopy, detection of antigens by antibodies that contain large gold particles is often used, and these may also be visualized at the light microscopic level as well, but their use is quite rare today, outside of electron microscopy. Described below are the common antibody detection methods for light microscopy.

Enzyme-Mediated Detection

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



GFAP (Cat. No. IHC2079-6 Clone GA5) staining of human brain. Tissue pretreated with citrate buffer, pH 6.0, prediluted polyclonal antibody, IHC Select® detection with HRP-DAB. Glial cells stain strongly (brown).

Horse Radish Peroxidase	Substrate	Abbreviation	Final Color	Soluble in Alcohol (for counterstain)
	Diaminobenzidine <i>Comments: 3,3'-diaminobenzidine 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.</i>	DAB	Brown	No
	Diaminobenzidine with nickel enhancement <i>Comments: Produces a more intense stain which is resistant to alcohol and provides better contrast, up to 40 times more sensitive than DAB without enhancement.</i>	DAB/Nickel	Gray/Black	No
	3-Amino-9-ethylcarbazole <i>Comments: 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</i>	AEC	Red/Brown	Yes
	4-Chloro-1-naphthol <i>Comments: 4-chloro-1-naphthol (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.</i>	4-CN	Blue/Gray	Yes

Alkaline Phosphatase	Substrate	Abbreviation	Final Color	Soluble in Alcohol (for counterstain)
	Naphthol AS B1 phosphate/fast red TR <i>Comments: Naphthol 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 soluble in alcohol, thus aqueous counterstain and mounting medium should be used.</i>	NABP/FR	Red	Yes
	Naphthol AS MX phosphate/fast red TR <i>Comments: Naphthol 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 soluble in alcohol, thus aqueous counterstain and mounting medium should be used.</i>	NAMP/FR	Red	Yes
	Naphthol AS B1 phosphate/new fuchsin <i>Comments: Naphthol 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 soluble in alcohol, thus aqueous counterstain and mounting medium should be used.</i>	NABP/NF	Red/Violet	Yes
	Bromochloroindolylphosphate/Nitro Blue Tetrazolium Nitro Blue Tetrazolium <i>Comments: 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.</i>	BCIP/NBT	Purple	No

Fluorescence

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.

Fluorophore	Absorption (nm)	Emission (nm)
Fast Blue	360	440
Alexa Fluor® 350	346	445
AMCA	350	450
Bisbenzamide	360	461
Aequorin	Ca ⁺⁺ photoprotein	469
Hoechst 33258	360	470
ACMA UV	412, 430	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

Many mounting media contain “anti-fading” solutions, such as DABCO, which will prolong the viewing time of the sample. Chemicon 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 DAPI and PI (Cat. No. S7113, S7114).

Signal Amplification

Signal amplification techniques greatly enhance the sensitivity of immunohistochemical and immunocytochemical methods. The signal amplification methods may be used in conjunction with either of the above detection techniques. Signals may be amplified by using poly-conjugated secondary antibodies (i.e. Chemicon catalog numbers AP340P–AP342A series), or Avidin-Biotin interactions or other commercially-available amplifiers (i.e tyramide catalyzed systems), which increase the signal to antibody ratio. When signal amplification is used to amplify the specific signal, however, one should be aware that non-specific signals may also become amplified. Thorough washing and proper antibody titration is especially important in this case.

Troubleshooting

- No Staining
- Weak Staining
- Background Staining

When tissue staining has not given the expected results, the experiment should be examined in a systematic way, wherein only single experimental variables are altered at one time. Proper immunohistochemical troubleshooting requires one to determine whether difficulties are related to specimen, antibodies, technique, environment, or slide interpretation? The following checklist may assist in troubleshooting efforts.

No Staining of Either Controls or Specimen

- Confirm that no reagents were omitted (primary antibody, secondary antibody, substrate components)
- Confirm that reagents were added in the correct order, and for sufficient incubation times.
- Re-read labels to confirm that correct antibodies were used. This is especially important when using primary/ secondary antibody combinations. For example, when using a mouse IgM primary antibody, the secondary antibody should be a goat or rabbit anti-mouse IgM (not IgG).
- Check antibody titrations and dilutions. This is particularly important for the primary antibody.
- Check that antibody blocking solutions or diluents do not contain substances that may interfere with or absorb the primary antibody. For instance, serum containing diluents can absorb out or reduce primary reactivity to small molecules such as GABA or amino acids.

- Check reagent expiration dates and storage. Enzymes and fluorochromes are especially prone to breakdown after prolonged storage. Antibodies should be stored in non-defrosting freezers, as self-defrosting models will expose antibodies to repeated freeze/thaw, resulting in antibody breakdown.
- Check specimen storage. Where possible, compare staining of the unknown specimen versus a known positive tissue in a “side-by-side” experiment.
- Check the chromogen/substrate solution. This can be checked by adding a drop of the labeling reagent to a small sample of prepared chromogen. If the chromogen is working, the mixture should change color. Chromogen solutions can deteriorate quickly, so do not use beyond the times recommended by the manufacturer. Lack of color change may be due to inactive enzyme or improperly-prepared chromogen.
- The rinse buffer may be incompatible with the reaction reagents. The pH must be appropriate, and buffers to be used with peroxidase enzyme should not contain NaN_3 .
- The counterstain and mounting media may not be compatible with the chromogen. Check the manufacturer's recommendations.
- Check that the microscope is adjusted correctly and that the fluorescence lamp is not burned out.

Weak Staining

Points to consider are:

1. Is the intensity of the staining consistent between the positive controls and the test sample(s)?
2. Is the staining specific for the antigen of interest, or is it background staining? This can only be determined by examining the slides.

All the items listed above for No Staining can apply to a lesser degree to the situation of weak staining. However, if the negative controls are devoid of stain and the positive controls and test sample(s) are weakly stained, then possible trouble points include:

- Overfixation, or incorrect fixation for the immunological procedure in use
- Insufficient antigen retrieval
- Antibody concentration may be too dilute. If possible, the concentration should be increased. If this is not feasible, then the incubation time or temperature may need adjusting. When diluted antibody is stored in the refrigerator it sometimes gets absorbed to the walls of the container. Storing the antibody with a protein carrier such as 1%–3% BSA can alleviate this situation.
- Too much buffer rinse has been left on the slide, so that the antibody becomes diluted when added to the sample.

If the negative controls have not reacted, the positive controls are well stained, but the test sample is stained weakly, then either the positive control and the test sample were fixed differently, are of different tissue type, or the outer tissue of the test specimen block has been poorly fixed.

If the negative controls have not reacted, the test sample(s) are well stained, but the controls are weakly stained, then the control material should be replaced.

Background Staining

If the negative control is being stained as well as the positive controls and test sample(s), then the degree and type of background staining must be analyzed. The following are possibilities for investigation:

- Re-titer antibodies (both primary and secondary) with a dilution series.
- Incubate with chromogen for a shorter time. Some chromogens, such as DAB, develop very quickly.
- The chromogen was not totally dissolved, and associated with the tissue. Centrifuge or filter the chromogen solution.
- Particulates in the antibody solution. These may form upon repeated freeze/thaw and can be eliminated by centrifugation.
- Insufficient rinsing between steps, or contaminated buffers. Mix new buffers and increase washing steps.
- Enzyme or biotin in the tissue is reacting with the reagent. This can be prevented by increasing the time or concentration of block, trying different types of block, or using a combination of more than one block, or changing the staining methods. Some tissues (*i.e.* brain and liver) are known to contain high endogenous levels of biotin or peroxidase activity.
- The incorrect blocking serum was used, or blocking serum was not used. The blocking serum should be from the species of the secondary antibody. It is possible to use 5% nonfat dry milk rather than serum.
- The secondary antibody cross-reacts with endogenous tissue proteins. Secondary antibodies which have been absorbed against immunoglobulin from the species from which the target tissue was obtained will result in significantly lower background, and are indispensable for double-labeling experiments.
- Hydrophobic and/or ionic interactions between the reagents and tissue types such as connective, adipose or fatty tissues may give rise to apparent specific reactions. Antigen retrieval procedures can be of great assistance in correcting this predicament. A decreased fixation time in formalin can also help.

- The embedding media may not be completely removed from the tissue. Review the removal procedure for possible changes.
- The specimen may have dried out during the procedure, allowing the trapping of reagents under the edges of the specimen. Care should be taken to avoid letting the specimens dry.

If there is background staining in the positive controls and the test sample(s), but not in the negative control, then the issue is most likely associated with the primary antibody. Some possibilities are:

- The primary antibody was too concentrated, or the incubation period too long. More dilute antibody, or shorter incubation or lower incubation temperature may correct the situation.
- The tissue may contain Fc receptors, or there may be interfering Ig components (aggregates or oligomers) or there may be naturally occurring, contaminating antibodies. This can be resolved by using Fab fragments rather than whole IgG molecules, filtering out the aggregates, or by diluting the primary antibody and incubating for longer times.
- The tissue sections may be cut too thick — try thinner sections.
- The microscope light needs to be adjusted to a higher setting.

If there is background staining in only the test sample(s) - *i.e.* not in the positive or negative controls, then the most likely cause is that the test sample(s) has been fixed and processed differently from the controls. Use of different tissue type between test sample(s) and controls may also produce this variance. Possibilities include:

- Overfixation of the test sample, resulting in the increased presence of hydrophobic groups, or increased cross-linking. Use of antigen retrieval procedures will amend this.
- A different fixative was used for the test sample(s) than for the control tissue. This difference should be avoided, or the procedures should be adjusted.
- The test sample(s) and the controls are of different tissue type. This should be avoided whenever possible.

If the test sample(s) and positive controls are clean, but the negative control shows background staining, it is likely that the negative control serum is at fault. It may be too concentrated, or contaminated with cross-reacting Ig components, naturally occurring antibodies, or bacterial growth. This can be corrected by using more dilute serum and incubating longer, trying to find a better match for the negative serum, or purifying the serum. ■

FLOW CYTOMETRY

Flow Cytometry is a powerful tool in research and clinical laboratory medicine for the diagnosis, isolation and study of specific cell types and disease conditions.

Flow cytometry is defined as the measurement of the cellular and fluorescent properties of particles in suspension as they pass by a laser or other light source. These particles are, in most cases, cells. The measurements are represented by changes in light scattered, light absorbed, and light emitted by a cell as it passes by fixed detectors directed off the light source. From these measurements, specific populations and subsets within them are defined and even isolated physically using a dedicated cell sorter typically by manipulating cell charge (see Figure 1).

All light signals, whether from fluorescently labeled cells or from the beam scattered by unlabeled cells, are transferred to a computer and transformed into digital signals. These signals are displayed in channels along a histogram graph. The typical output is a single parameter graph of intensity versus the number of cells (see Figure 2) or two parameter histograms to distinguish subsets of cells.

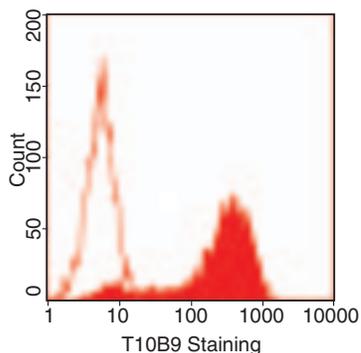


Figure 2. Staining of human peripheral blood mononuclear cells (PBMC) using the T10B9 antibody. T10B9 recognizes a unique monomorphic antigenic determinant of the T cell receptor expressed on the majority of human T cells. Human PBMC were prepared by density gradient separation and stained with T10B9 at the concentration of 10 µg/mL, with subsequent staining using goat anti-mouse light chain antibodies labeled with FITC (closed line histogram). The open line histogram represents an isotype (mouse IgM) control. Data courtesy of Dr. C. Keever-Taylor, Medical College of Wisconsin, Milwaukee, WI.

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 (see Figure 3).

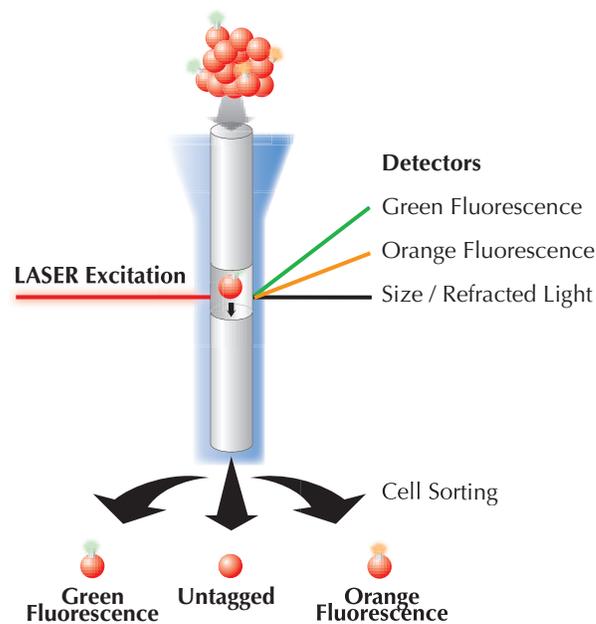


Figure 1. Schematic Diagram of Flow Cytometry. Cells are sorted according to their size and fluorescence.

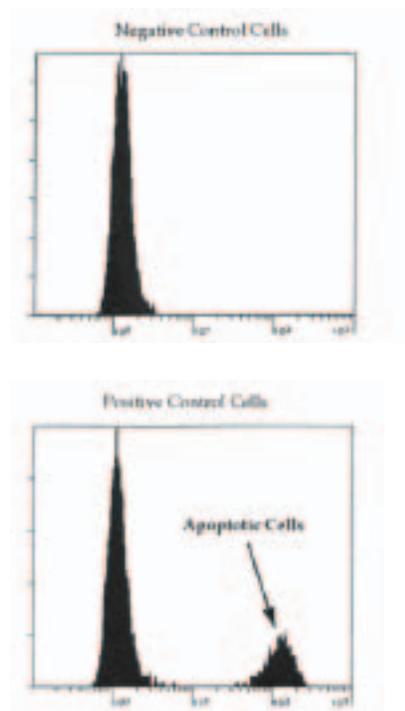


Figure 3. Flow cytometry data of apoptotic positive and negative control cells labeled by APO-BRDU™ Assay (Cat. No. APT115).

FLOW CYTOMETRY *(continued)*

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. (see Figure 4).

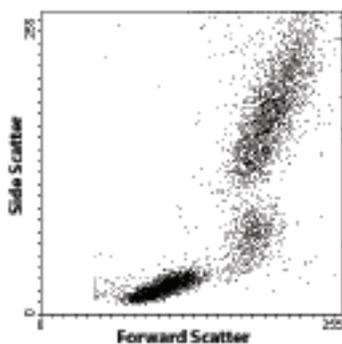


Figure 4. Two dimensional histogram, displaying a scatter plot of white blood cells passing through a flow cytometer.

Such diagrams or dot plots display the relationship between each of the two parameters. Individual subsets or cell populations within a sample are resolved based on the combined intensity levels of each of the two parameters. When comparing cell populations, subcellular debris or unimportant cell populations can be eliminated from the data by gating. Gating involves using flow cytometry software to select a population within the graph that needs to be plotted. For instance, in Figure 5 we use gating to select the true population of single apoptotic and non-apoptotic cells from debris and aggregated cell populations. The gated cells are then displayed in a new graph using additional parameters to give measurable data (Figure 6).

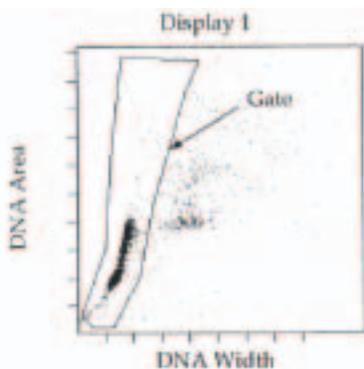


Figure 5. Diagram shows the selection of the gate around target population excluding non-specific scatter.

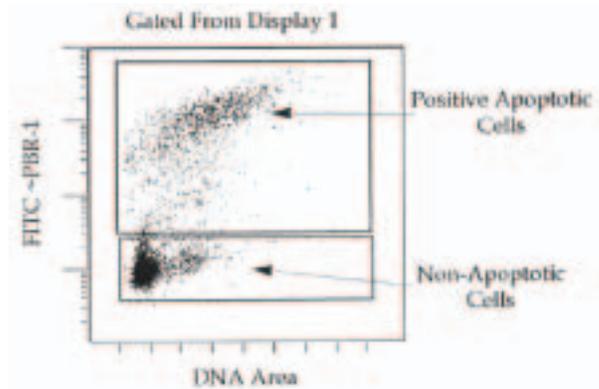


Figure 6. Diagram displays the flow cytometry data obtained when gated population is processed.

Labelling 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. Chemicon has available numerous polyclonal and monoclonal antibodies specific to different cell types. Many of the groups of cell-type specific monoclonals have been given a cluster 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 (see Figure 7).

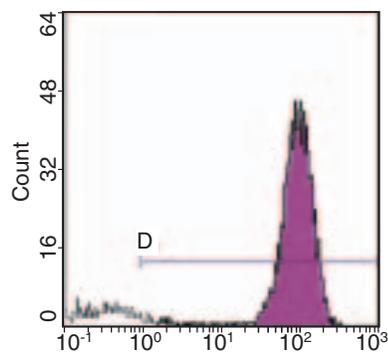


Figure 7. Log fluorescence intensity profile of peripheral blood lymphocytes using FITC labeled CD4 (Cat. No. CBL127)

Sometimes, multiple CD markers are needed to identify one exact cell type or disease state. Lists of CD markers are available for cell identification in humans and other species.

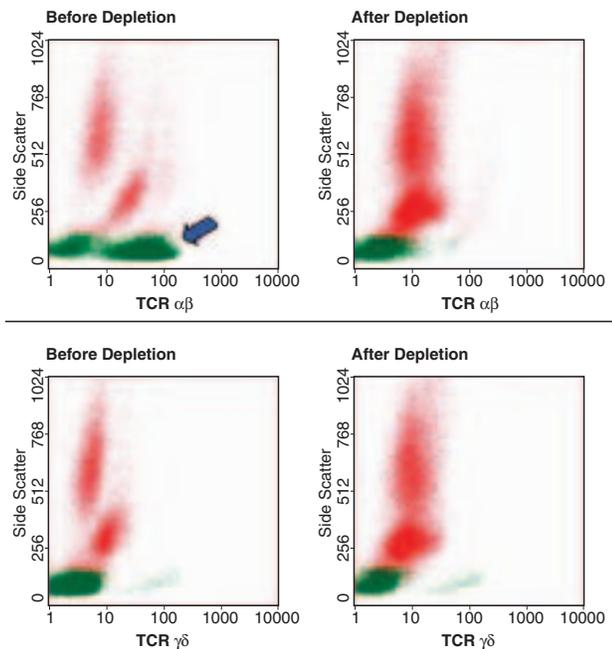


Figure 8. T Cell Depletion of Human Bone Marrow using the T10B9 Monoclonal Antibody. T10B9 is a T cell specific antibody that selectively depletes cells bearing $\alpha\beta$ + T cell receptor using complement-mediated cytotoxicity (blue arrow in the upper panel), while sparing cells expressing $\gamma\delta$ + T cell receptor (lower panel). Human bone marrow suspensions were treated with 0.35 $\mu\text{g}/\text{mL}$ of Chemicon's T10B9, followed by two subsequent treatments with rabbit complement. Specific elimination of $\alpha\beta$ + T cells in T cell depletion protocols reduces risks of graft-versus-host disease (GVHD) after allogeneic stem cell transplantation. The remaining $\gamma\delta$ + T cells presumably contribute to selective killing of host leukemia cells. Data courtesy of Dr. C. Keever-Taylor, Medical College of Wisconsin, Milwaukee, WI).

Many antibodies used in flow cytometry are directly conjugated to a fluorochrome; however, many non-labelled primary antibodies are routinely used when combined with a labelled secondary antibody. There are two primary fluorochromes used in flow cytometry, fluorescein isothiocyanate (FITC) and phycoerythrin (PE). The two key properties of these dyes that make them preferred tags are one, they are both excited with a 488 nm laser and two, 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 now made multiple simultaneous cell labeling and sorting possible beyond the original two dyes (See fluorescence table in IHC section page 23).

Because of its versatility and power, flow cytometry is routinely used in diagnostics, scientific research and pharmacology. The ability to sort cells by multiple parameters (simply limited by the number of electronic collectors, filters and available markers) makes flow cytometry an area of continuous growth, and one where antibodies and fluorescent markers combine into a truly functional and dynamic application. ■

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 ($\sim 0.5\text{--}1 \times 10^6/\text{mL}$) 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 $\sim .75 \times 10^6$ 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 read 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 FACS analysis is performed. Fixed samples should be used within 48 hours.

APPENDIX A

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 is prepared by dissolving 7.7 g NaOH and 33.6 g NaH₂PO₄ in 1 liter deionized water.

4% Paraformaldehyde with 2% Gluteraldehyde.

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 gluteraldehyde 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 Na₂HPO₄ and 20 g NaH₂PO₄ 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 ddH₂O.
2. Adjust pH to 7.4 with 0.1 M Na₂HPO₄ (Na₂HPO₄·2 H₂O 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 ddH₂O. Add 1.06 g Sodium periodate (NaIO₄) 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

APPENDIX B

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.
2. Add 50 μL antibody solution, and mix. This gives a 1:10 dilution of the antibody.
3. Label tubes A through M for 1:50, 1:100, 1:200, 1:400, etc. to 1:51,200 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 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

APPENDIX C

Protein A/G Binding Affinities

Species	Immunoglobulin	Protein A	Protein G
Bovine	Ig	++	++++
Chicken	Ig	-	+
Goat	Ig	+/-	++
Guinea Pig	Ig	++++	++
Hampster	Ig	+	++
Mouse	IgG ₁	+	++
Mouse	IgG _{2a}	++++	++++
Mouse	IgG _{2b}	+++	+++
Mouse	IgG ₃	++	+++

Species	Immunoglobulin	Protein A	Protein G
Mouse	IgG _M	+/-	-
Pig	Ig	+++	+++
Rabbit	Ig	++++	+++
Rat	IgG ₁	-	+
Rat	IgG _{2a}	-	++++
Rat	IgG _{2b}	-	++
Rat	IgG _{2c}	+	++
Rat	IgG _M	+/-	-
Sheep	Ig	+/-	++

APPENDIX D

Enzyme Substrates for ELISA Testing (soluble substrates) and Blotting (insoluble substrates)

1. Alkaline Phosphatase

Substrate	Buffer/ Second Substrate	Reagent to stop reaction	Soluble or Insoluble Product	Color of Product	Wavelength for quantitation
p-Nitrophenyl Phosphate (pNPP)	Na ₂ CO ₃ , pH 9.8 with MgCl ₂	NaOH, 2M	Soluble	Yellow	405 nm
Bromochloroindolyl Phosphate-Nitro blue Tetrazolium (BCIP/NBT)	NaCl, MgCl ₂ , Diethanolamine	EDTA Purple	Insoluble	Black	N/A

2. Horseradish Peroxidase

Substrate	Buffer/ Second Substrate	Reagent to stop reaction	Soluble or Insoluble Product	Color of Product	Wavelength for quantitation
3,3',5,5'-Tetramethyl-benzidine (TMB)	30% Hydrogen Peroxide (H ₂ O ₂)	1 M Sulfuric Acid (H ₂ SO ₄)	Soluble	Yellow	450 nm
o-Phenylene Diamine (OPD)	Citrate Phosphate Buffer, 0.02% H ₂ O ₂	Sulfuric Acid (H ₂ SO ₄)	Soluble	Orange-Brown	492 nm
2,2'-azinodiethyl-benzthiazoline sulfonate (ABTS)	Citrate Phosphate Buffer, 30% H ₂ O ₂	20% SDS / 50%DMF	Soluble	Green	410 nm, 650 nm
Chloronaphthol	30% H ₂ O ₂	PBS	Insoluble	Blue-Black	N/A
3-Amino-9-ethylcarbazole (AEC)	30% H ₂ O ₂	PBS	Insoluble	Red	N/A
Diaminobenzidine (DAB)	30% H ₂ O ₂	PBS	Insoluble	Brown	N/A

APPENDIX E

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 Percent

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

APPENDIX F

Further reading

General Background

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APPENDIX G

Glossary

Affinity (AH-fin-eh-tee) – The strength of reaction between antibody and antigen at a single antigenic site. Pg. 4

Affinity purification (AH-fin-eh-tee PUR-if-eh-kay-shun) – Column purification where specific antibody fraction binds to the antigen to which it was made. Pg. 6

Affinity constant (AH-fin-eh-tee CON-stant) – Describes the binding interaction between antibody and antigen. Also known as association or equilibrium constant. Pg. 4

Antibody (AN-tee-BAWD-ee) – An immunoglobulin capable of specific combination with the antigen that caused its production in a susceptible animal. Pg. 3

Antigen (ANT-eh-jen) – Any substance foreign to the body that elicits a specific immune response. Pg. 2

Antigen retrieval (ANT-eh-jen re-TREE-val) – Procedure designed to enhance the binding of antibody to antigen in tissue typically in response to an epitope-blocking fixation or embedment technique. Pg. 20

Antiserum (ANT-tee-seer-um) – Blood from an immunized host presumably possessing antibodies of interest as well as other serum proteins. Pg. 6

Ascites fluid (ah-SYT-eez FLU-id) – Unpurified monoclonal antibody-containing fluid drawn directly from hybridomas grown within a living host. Pg. 6

Avidity (ah-VID-eh-tee) – A measure of the overall strength of binding of the entire antibody-antigen complex. Pg. 4

Capture Antibody (KAP-chur AN-tee-BAWD-ee) – An anchored primary antibody used in ELISA procedures to bind an antigen in solution. Pg. 14

Carrier Protein (KAR-re-her PRÖ-teen) – 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. Pg. 2

Chemiluminescent (Chem-me-loom-min-"s"-ent): the production of light through a chemical reaction catalyzed by an enzyme; chemiluminescent substrates require X-ray film or other light-capturing devices for detection. Pg. 13

Chromogen (KRÖM-oh-jen) – The chemical substrate that changes color in the presence of a specific enzyme-tagged antibody. Ex. DAB. Pg. 12

Cross-reactivity (KROSS-re-ACT-ehv-eh-tee) – The binding of an antibody or population of antibodies to epitopes on other antigens. Pg. 4

Denatured (DEE-na-chur) – the conformational change in an antigen that may expose or destroy an epitope. Pg. 2

Detection Antibody (de-TEK-shun AN-tee-BAWD-ee) – A primary antibody used in ELISA procedures to allow secondary antibody labeling of antigen. Pg. 14

ELISA (Enzyme-Linked Immunosorbent Assay) (EE-lyz-eh) – A technique that uses antibodies or antigens to capture and quantify the amount of antigen or antibody in unknown samples. Pg. 14

Epitope (EPP-eh-iōp) – The small site on an antigen where a complementary antibody binds via its variable region. Pg. 2

Flow cytometry (FLō SY-tahm-eh-tree) – 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. Pg. 26

Fluorochrome (fluorophore) (FLOOR-oh-krōm) – Molecular label on antibody that emits a distinct, measurable color spectrum in response to a specific laser or chemical interaction. Pg. 23

Hapten (HAPP-ten) – Small molecule incapable of eliciting a specific antibody response without being chemically coupled to a larger carrier protein. Pg. 2

Hybridoma supernatant (HY-brid-oh-mah SU-per-nay-tant) – Monoclonal antibody-containing fluid collected from hybridoma cell cultures. Pg. 6

Immunoblotting (IMM-yu-no-BLOTT-ing) – A technique that uses antibodies to probe proteins transferred from an electrophoresis gel onto membrane. Ex. Western blotting. Pg. 10

Immunocytochemistry (IMM-yu-no-SYT-oh-KEM-eh-stree) – A technique that uses antibodies to probe specific antigens in live or fixed cell cultures. Pg. 17

Immunogenic (IMM-yu-no-JENN-ik) – A description of an antigen's ability to stimulate antibody production. Pg. 2

Immunoglobulin (Ig) (IMM-yu-no-GLOBB-yu-inn) – General term for a number of classes of proteins that functions as antibodies. Pg. 3

Immunohistochemistry (IMM-yu-no-HIST-oh-KEM-eh-stree) – A technique that uses antibodies to probe specific antigens in fresh frozen or processed tissue. Pg. 17

Immunoprecipitation (IMM-yu-no-PREE-sip-eh-tay-shun) – A technique that uses antibodies to bind antigens in solution to yield a precipitating complex, the components of which can then be isolated. Pg. 8

Monoclonal antibody (MAWN-oh-KLON-al AN-tee-BAWD-ee) – A homogeneous population of antibodies that are raised by the fusion of B cells with immortal cell cultures to produce hybridomas. Pg. 5

Negative control sample (NEG-ah-tiv KAWN-trol) – any tissue, cell line, lysate or purified protein that is known from previous work to be void of the antigen of interest. Pg. 7

Normal serum (NOR-mall see-rumm) – Blood serum extracted from non-immunized animals; often used as a control. Pg. 8

Optimal working dilution (AWP-tim-awl WORK-ing Dil-lew-shun) – Concentration (dilution) that maximizes the positive signal while minimizing background (negative) reactivity. Pg. 7

Peptide (PEHP-tyd) – A small amino acid sequence used for generating sequence-specific antibodies. Pg. 2

Polyclonal antibody (PAWL-ee-KLON-al AN-tee-BAWD-ee) – Multiple B cell response to an antigen resulting in a mixture of antibodies typically recognizing a variety of epitopes on the antigen. Pg. 5

Positive control sample (PAWZ-ah-tiv KAWN-trol) – 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. Pg. 7

Pre-immune serum (PREE- IMM-yun see-rumm) – Blood serum extracted prior to that animal's immunization with an antigenic substance; often used as a control. Pg. 8

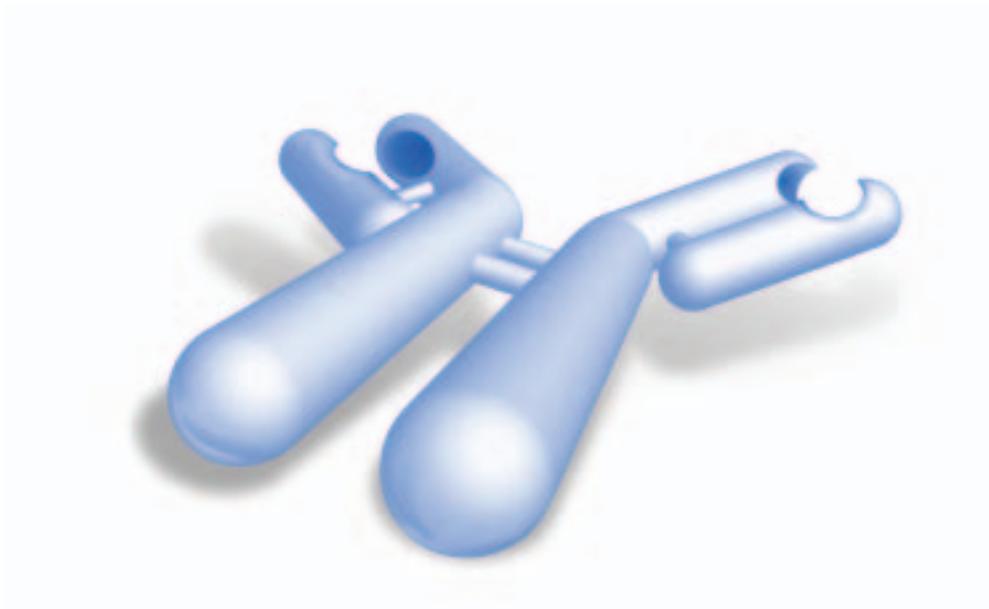
Primary antibody (PRY-mayr-ee AN-tee-BAWD-ee) – The antibody that directly binds the antigen of interest. Pg. 12

Protein A/G purification (PROH-teen AY / JEE PUR-if-eh-KAY-shun) – Column purification where the Fc domain of antibodies binds to the high affinity *S. aureus* protein A or G. Pg. 6

Secondary antibody (SEHK-un-darr-ee AN-tee-BAWD-ee) – 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. Pg. 12

Specificity (SPEHS-eh-FYS-eh-tee) – The likelihood that the particular antibody is binding to a precise antigen epitope. Pg. 4

Valency (VAYL-en-see) – A description of the relative ability of an antibody to interact with antigens, usually related to the number of available variable regions. Pg. 3



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