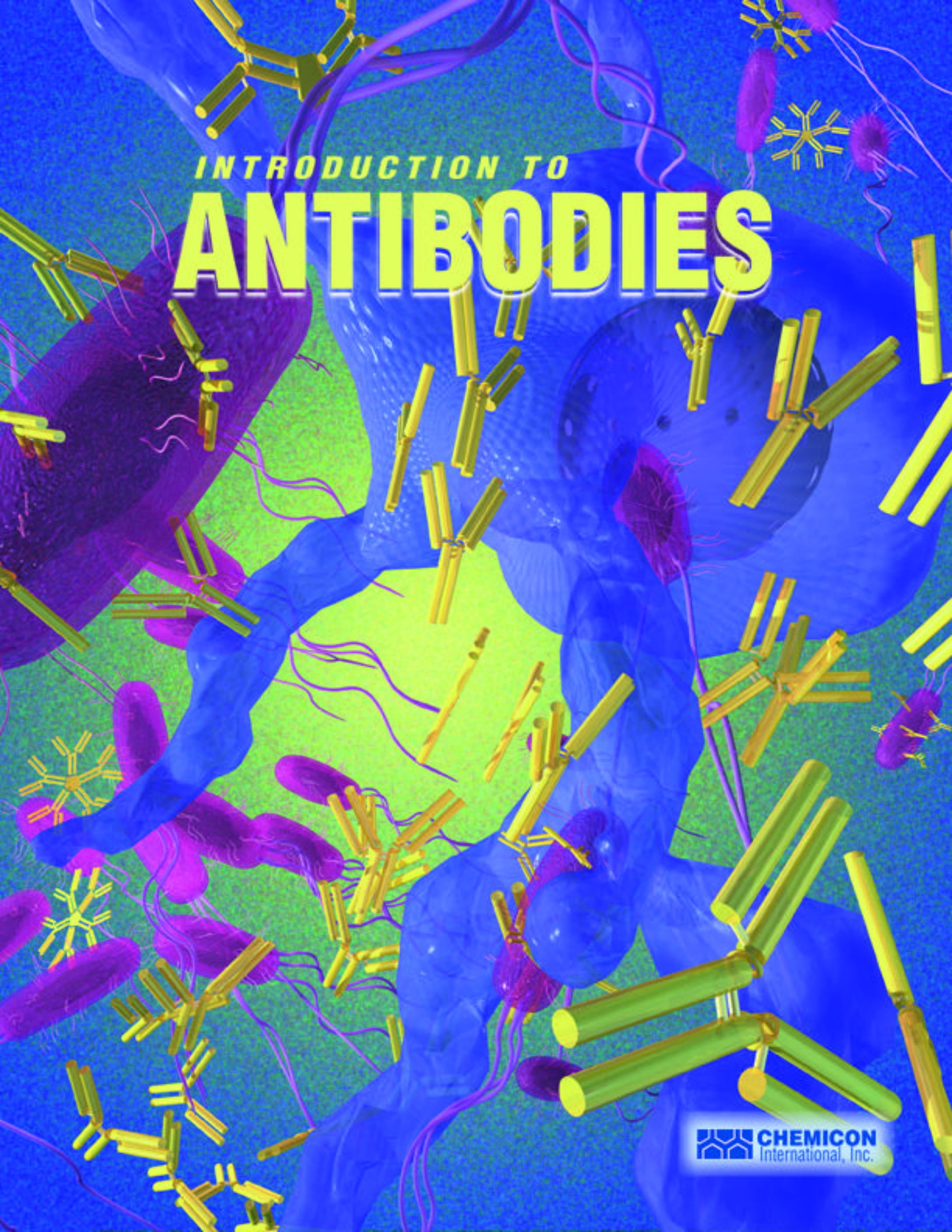
INTRODUCTION TO
ANTIBODIES
CHEMICON
International, Inc.

CHEMICON International, Inc.
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# introduction

CHEMICON is pleased to offer this basic introduction to antibodies and commonly-employed immunological assays as 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 over 4,000 antibodies, CHEMICON offers scientific advice for a variety of immunological applications via phone, fax or e-mail. We hope that you will contact us with any comments, questions, or suggestions that you may have.

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## INTRODUCTION

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

# antigens

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 are commonly 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 or hemocyanin or other synthetic matrices. Haptens may be a variety of molecules such as drugs, simple sugars, amino acids, small peptides, phospholipids, or triglycerides. Antigens which elicit strong immune responses are said to be "strongly **immunogenic".**

Characteristics of a good antigen include:

- Areas of structural stability and chemical complexity within the molecule
- Randomness of structure (i.e. lack of 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 metabolized
- Immunogenic regions which are accessible to the antibody-forming mechanism
- Structural elements that are sufficiently different from the host

The small site on an antigen to which a complementary antibody may specifically bind is called an **"epitope"**. This is usually one to six monosaccharide or 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 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.

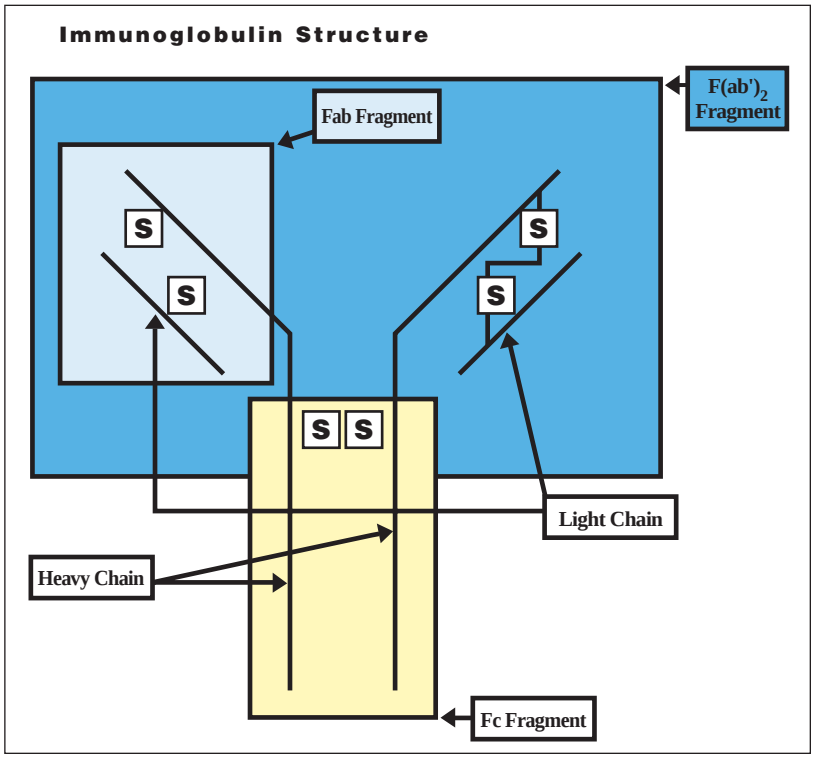
For efficient interaction to occur between the antigen and the antibody, the epitope must be readily available for binding. If the target molecule is **denatured**, e.g. through fixation, the epitope may be altered and this may change (improve or decrease) its ability to interact with a antibody. Changes in pH may also affect antigen conformation.

Antigens may be present in their native, cellular environment, or extracted and purified. In their natural form they may be cytoplasmic or associated with a cellular membrane, intracellular or extracellular.

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, peptides antigens have advantages over whole proteins antigens in that the antibodies generated may be targeted to unique sequence regions. This is especially useful when investigating proteins which belong to families of high sequence homology.

# antibodies

An **ANTIBODY** is defined as "an **immunoglobulin** capable of specific combination with the antigen that caused its production in a susceptible animal." Antibodies are produced in response to the invasion of foreign molecules in the body. 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. The light chains of any antibody can be classified as either a kappa (κ) or lambda (λ) type (a description of molecular characteristics of the polypeptide); however, the heavy chain determines the subclass of each antibody. Heavy chains of IgG, IgM, IgA, IgD, and IgE, are known as gamma, mu, alpha, delta, and epsilon, respectively.




The most commonly used antibody is IgG, which can be cleaved into three parts , two F(ab) regions and one Fc, by the proteolytic enzyme papain, or into two parts, one $F(ab')_2$ and one Fc by the proteolytic enzyme pepsin. The F(ab) regions comprise the "arms" of the antibody, which are critical for antigen binding. The Fc region comprises the "tail" of the antibody and plays a role in immune response, as well as serving as a useful "handle" for manipulating the antibody during some 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).

# antibody-antigen interactions

**antibody applications**

The bonding between antigens and antibodies is dependent on hydrogen bonds, hydrophobic bonds, 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 which 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: $K_A$ = [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.

**Avidity** is a measure of the overall stability of the antibody-antigen complex. It is controlled by three major factors - the affinity of the antibody for the epitope, the valency of the antigen and antibody, and the structural arrangement of the interacting parts.

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 multivalancy of most antigens and antibodies enables them to interact to form a precipitate. Examples of experimental applications which use antibodies are Western Blot, Immunohistochemistry and Immunocytochemistry, Enzyme-Linked Immunosorbent Assay (ELISA) and Immunoprecipitation, which are discussed in more detail in later sections of this publication.

# monoclonal & polyclonal antibodies

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

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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.

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.

## antibody formats

Polyclonal antibodies are most often available in relatively unpurified form, described as “serum” or “antiserum”, or as a “Protein A-purified” or “Affinity-purified” format. A “Protein A-purified” antibody preparation will contain primarily the immunoglobulin of desired specificity, in addition to non-specific serum immunoglobulins. An “Affinity-purified” antibody has customarily been chromatographically purified to contain only those antigens that are specifically reactive with the immunogen.

Monoclonal antibodies may be grown in cell cultures and collected as “hybridoma supernatants”, grown in mice or rats and collected as relatively unpurified “ascites fluid”, or purified through the use of Protein A or specific affinity chromatography as with polyclonal antibodies.

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: Antibody concentrations in purified preparations should be determined through standard protein assays.

***Hybridoma Supernatant:*** Specific antibody concentrations will typically range from 25 - 125 µg/mL.
***Ascites fluid (unpurified):*** Specific antibody concentrations will typically range from 2-10 mg/mL.
***Polyclonal antiserum (unpurified):*** Specific antibody concentrations will typically range from 1-3 mg/mL.

**Proper Controls**

Use of proper controls will help eliminate false positive and false negative results. 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 is a solution, gel, tissue, 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 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 purifications 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 and date of preparation of all reagents, and documentation of procedural steps. This kind of information is invaluable in efficient assay development.

**Handling of Reagents**

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). Unless a stabilizing protein such as BSAhas been added, antibodies should not be stored for extended periods at their working dilutions. Many antibody protocols will suggest dividing an undiluted antibody into aliquots before storing at -20°C. Storing an antibody in this concentrated format will prevent degradation and eliminate the need for repeated freeze/thaw, which can cause antibody denaturation. 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 that sodium azide may inhibit the activity of horseradish peroxidase. As with all laboratory reagents, consult a Material Safety Data sheet 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. However, the extent of antibody-antigen complex formation can be controlled by varying the concentration of antibody relative to the concentration of antigen. For any assay, the optimum titer is that which gives the strongest reaction for positive tests with minimum background reaction, e.g. for negative controls. The optimal concentrations for both antigen and antibody must be determined experimentally for each assay and are typically determined by using a dilution series.

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Dilutions are best determined by first selecting a fixed incubation time, and then making small volumes of a series of experimental dilutions. 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 solution is created by mixing one part of 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 for 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

**antibody applications**

It is possible to use antibody-antigen precipitation to detect antigen in a mixture, e.g. when isolating an antigen from a cell extract.

The procedure for Immunoprecipitation is divided into four basic steps:

1. Labeling the antigen (optional)
2. Lysis of cells to release the antigen
3. Formation of the antibody-antigen complex
4. Precipitation of the immune complexes

Before beginning immunoprecipitation ensure that proper experimental controls are in place. Control antibodies should be as similar in nature to the specific antibody as possible. For example, if you are precipitating with a polyclonal serum, one possible negative control would be another polyclonal serum from the same species. The ideal antibody negative control would be a prebleed from the same animal used for immunization, however, an equal concentration of non-immune serum from a different animal may suffice in its absence.

***Antigen Labeling:*** Antigen may be labeled by incubating in a medium which contains radioactive precursor, such as $^{3}$H-Thymidine, by iodination of surface proteins, by treatment with radioactive sodium borohydride, or by other published techniques.

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***Cell Lysis:*** The antigen is then extracted from the cell using an appropriate lysis buffer. When choosing a lysis buffer, there are two important things to consider: 1) the antigen must be released efficiently, and 2) it must still be recognizable by the antibody.

***Preparation of a cell lysate from culture dishes typically involves the following:***

1. Rinse the culture dish with PBS.
2. Lyse cells with the addition a small volume of boiling 1%SDS, 1.0 mM sodium vanadate, 10 mM Tris-HCl, pH 7.4. (many other lysis recipes have also been used.),
3. Transfer lysate to a microcentrifuge tube and boil for an additional 5 minutes, followed by brief sonication. Centrifuge for 5 minutes and collect the supernatant, which is a "total cell lysate".

*Note: The preceding procedure is denaturing. Procedures for preparation of native lysates are available from the literature.*

***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 do this, add to a microcentrifuge tube: 1-5 µg of purified antibody to a sample of lysate, 400 µLwater, 500 µLimmunoprecipitation buffer (2% 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, 1.0% NP-40) and 100 µL cell lysate, containing 200-500 µg total protein. ***(Note:*** if unpurified antibodies are used, substitute the following antibody quantities: serum (0.5-5 µL), hybridoma tissue culture supernatant (10-100 µL), or ascites fluid (0.1- 1.0 µL) Vortex, and incubate on ice for 1 hour to allow immune complexes to form. (Longer incubations 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. Protein A affinity for antibodies differs between species, but is exceptionally suitable for immunoprecipitation of rabbit primary antibodies. To use protein A for precipitation of mouse primary antibodies, it is advisable to add 5 µg of rabbit anti-mouse IgG, vortex, and incubate for a further 30 minutes at 4°C, before precipitating. When Protein A-agarose is used for precipitation, a quantity of 10 µL 50% Protein A-agarose, should be sufficient for precipitation of the above quantities of antibody/antigen complex. Upon addition of Protein A-agarose, incubate with agitation for 30 minutes at 4°C, wash three times by centrifugation and resuspension (or more, if the antigen has been radiolabeled) with immunoprecipitation buffer, and collect by centrifugation.

Pellets to be used for electrophoresis may be resuspended with 2X SDS sample buffer, and electrophoresed normally. The purified immune complex may also be used for enzymatic studies, ligand binding, further immunizations, immunoblotting, and other immunochemical techniques. These methods, when used in conjunction with immunoprecipitation, can greatly increase the amount of information discovered about an antigen.

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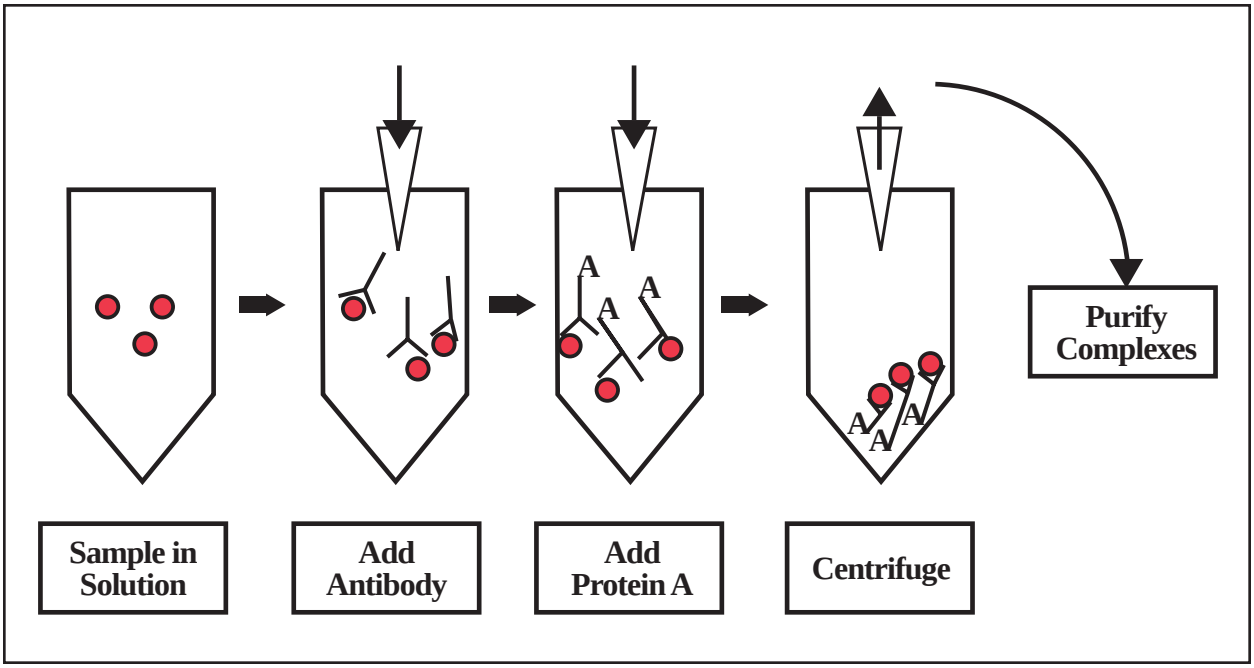
Success in an immunoprecipitation assay is dependent on two main factors. These are the abundance of antigen in the original sample, and the affinity of the antibody for the antigen (normally requires affinities of $10^8$ $mol^{-1}$ or higher).

***Trouble Shooting: 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 lysate prior to adding the specific antibodies.
- Add saturating amounts of competitor proteins, such as BSA, gelatin, acetone powders, or blotto.
- Spin the lysate at 100,000g for 30 minutes prior to the addition of the antibody.
- Try a different antibody.
- Centrifuge the antibody at 100,000g for 30 minutes and titrate.
- If using rabbit anti-mouse immunoglobulin in conjunction with a monoclonal antibody, check the background due to this reagent alone. Titrate if necessary.
- Increase the number of washes. “Soak” solid phase in the wash buffers for 10 minutes each wash.
- Lower the number of cpm of the radiolabel to the minimum needed for antigen detection.
- Make certain the lysates are not frozen before use.
- If background persists, analyze proteins on two-dimensional gels.
- If specific proteins remain, remember that your antigen may consist of more than one polypeptide chain.



# protein (western) blotting

Blotting 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.

Polyacrylamide gel electrophoresis (PAGE) separates individual proteins, by size, shape and charge. 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 separated proteins to nitrocellulose paper **(blotting)** for detection (**probing**) with specific antibodies.

The basic blotting procedure can be divided into six steps:

1. Preparation of the antigen sample
2. Resolution of the sample by gel electrophoresis
3. Transfer of the separated polypeptides to a membrane support
4. Blocking nonspecific binding sites on the membrane
5. Addition of the antibody
6. Detection

***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.

***Gel Electrophoresis:*** Next, the proteins are separated by gel electrophoresis. Following the specifications of the equipment manufacturer, electrophorese proteins through the polyacrylamide gel to separate them by molecular weight.

To a standard SDS-Polyacrylamide gel, apply 5-25 µLof cell lysate or tissue homogenate (1-10 µg total protein) and electrophorese in a 0.5-1.5 mm thick gel.

***Membrane Transfer:*** Transfer of the proteins to a membrane can be accomplished by either capillary blotting or by electroblotting. The more efficient method of transfer is electroblotting. In this procedure, a sandwich of gel and nitrocellulose (or other membrane such as Immobilon-P 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 into the nitrocellulose sheet. This sheet is called the "blot."

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***To transfer a protein from a gel to a membrane:*** Generally, a "sandwich" will be created, with the following layers: 1) sponge for electroblotting unit, 2) filter paper soaked in transfer buffer, 3) gel, 4) membrane, 5) filter paper soaked in transfer buffer, 5) sponge.

1. Cut the membrane and filter paper to fit the gel exactly.
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 bubbles between the gel and the membrane.
4. Place the membrane on the side of the **positive** electrode.
5. Transfer the separated polypeptides using 1 ampere 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, 20% methanol as transfer buffer. High molecular weight proteins may be transfered more efficiently by increasing transfer time to 90 minutes and addition of 0.05% SDS in the transfer buffer. *(Note: To ensure the transfer is complete, the membrane can be stained with Amido Black stain: Remove the membrane from the transfer system, and stain with Amido Black stain for five to ten minutes. Destain in a solution of 10% acetic acid, 10% isopropanol until the bands are clearly visible. Rinse carefully, but thoroughly, with water.)*

***Blocking Non-specific Binding:*** Block remaining hydrophobic binding sites on the membrane by incubating the membrane in a protein solution, e.g. 10% (w/v) BSA, or 5% non-fat dried milk in phosphate buffer solution or in 10 mM Tris, pH 7.5, 100 mM NaCl, 0.1% Tween 20 for for 30 minutes at 37°C, one hour at room temperature, or overnight at 4°C. (Remember to cut off a section of the blot for protein staining prior to this step, if this is desired.)

***Addition of the Antibody:*** Dilute the primary antibody in blocking buffer. After decanting the blocking buffer from the blot, incubate with the membrane with diluted primary antibody for 30 minutes at 37°C, one hour at room temperature, or overnight at 4°C. Incubation with gentle agitation, such as on a rocker, will improve results. Consult individual product datasheets for suggested dilution ranges.

If the primary antibody was unlabeled, it will be necessary to wash the blot for thirty minutes with agitation, changing the wash buffer every five minutes. Suggested wash buffer is 10 mM Tris, pH 7.5, 10 mM NaCl, 0.1% Tween 20. Follow by incubation with a labeled secondary antibody (as above).

***Detection:*** The method of detection is dependent upon the label that has been used for the primary (or secondary) antibody. The most common antibody label is an enzyme such as alkaline phosphatase or horseradish peroxidase, which can be detected visually through the conversion of a substrate to a colored precipitate at the site of antibody binding. Alternately, enzyme substate interactions may produce chemiluminesent signals, which can be captured on a photographic film. *See Appendix C for common enzyme-substrate combinations.*

***Other labels include:***

1. $^{125}$I-labeling of the secondary antibody, which can be detected using a photographic film.
2. Fluorescein isothiocyanate-labeled second antibody, which can be detected using UV light.
3. $^{125}$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.

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4. 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.
5. Biotinylated second antibody. In this case the blot is incubated with the secondary antibody, then incubated with enzyme-conjugated avidin which 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 Amido Black, as indicated above. Ineffective transfer may be due to:
  a. Air bubbles between the gel and the membrane
  b. Placement of the membrane on the wrong side of the gel - it must be on the same side as the positive electrode.
  c. Insufficient current/time for transfer. Check the manufacturer's instructions.
- Weak staining may also be due to:
  a. Insufficient incubation time. If a one hour incubation time at room temperature is insufficient, try incubating overnight at 4°C.
  b. 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:
  a. Antibodies used at excess concentration. Try more dilute antibody solutions or shorter incubation times.
  b. Insufficient washing or use of expired or contaminated reagents.
  c. Excess exposure time with substrates.

***A note on detection systems and antibody titer:***

*Some antibody detection systems are exquisitely sensitive, while others 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 chemiliminescent 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 colorimetric detection system may give an undetectably low signal for fluorescence detection, prior to assay optimization.*

# enzyme-linked immunosorbent assay (ELISA)

**ELISA Procedure**

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.

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 ELISAis fast and accurate, and if a purified antigen standard is available, the assay can determine the absolute amounts of antigen in unknown samples. 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. 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. A major advantage of this technique is that the antigen does not need to be purified prior to use, also that these assays are very specific. However, one disadvantage is that not all antibodies can be used. Monoclonal antibody combinations must be qualified at "matched pairs", meaning that they can recognize separate epitopes on the antigen.

Unlike Western blots, which use preciptating substrates, ELISA procedures utilize substrates that produce soluble products. Ideally the enzyme substrates should be stable, safe and inexpensive. Popular enzymes are those which convert a colorless substrate to a colored product, e.g. *p*-nitrophenylphosphate (*p*NPP) 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 C.

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.

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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. Antibodies can be labeled conveniently with iodine, enzymes, or biotin.

***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 µLof antibody solution to each well (20 µg/mL in PBS). PVC will bind approximately 100 ng/well (300 ng/cm$^2$). The amount of antibody used will depend on the individual assay, but if maximal binding is required, use at least 1 µg/well. this is well above the capacity of the well, but the binding will occur more rapidly, and the binding solution can be saved and used again.
4. Incubate the plate overnight at 4°C to allow complete binding.
5. Wash the wells twice with PBS. A 500 mL squirt bottle is convenient. The antibody solution washes can be removed by flicking the plate over a suitable container.
6. The remaining sites for protein binding on the microtiter plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hours to overnight in a humid atmosphere at room temperature. (Note: Sodium azide is an inhibitor or horseradish peroxidase. *Do not include sodium azide in buffers or wash solutions, if an HRP-labeled antibody will be used for detection.*
7. Wash wells twice with PBS.
8. Add 50 µL of the antigen solution to the wells (the antigen solution should be titrated). All dilutions should be done in the blocking buffer (3% BSA/PBS with 0.02% sodium azide). Incubate for at least 2 hours at room temperature in a humid atmosphere.
9. Wash the plate four times with PBS.
10. Add the labeled second antibody. The amount to be added can be determined in preliminary experiments. For accurate quantitation, the second antibody should be used in excess. All dilutions should be done in the blocking buffer.
11. Incubate for 2 hours or more at room temperature in a humid atmosphere.
12. Wash with several changes of PBS.
13. Add substrate as indicated by manufacturer. After suggested incubation time has elapsed, optical densities at target wavelengths can be measured on an ELISAreader.
    ***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.

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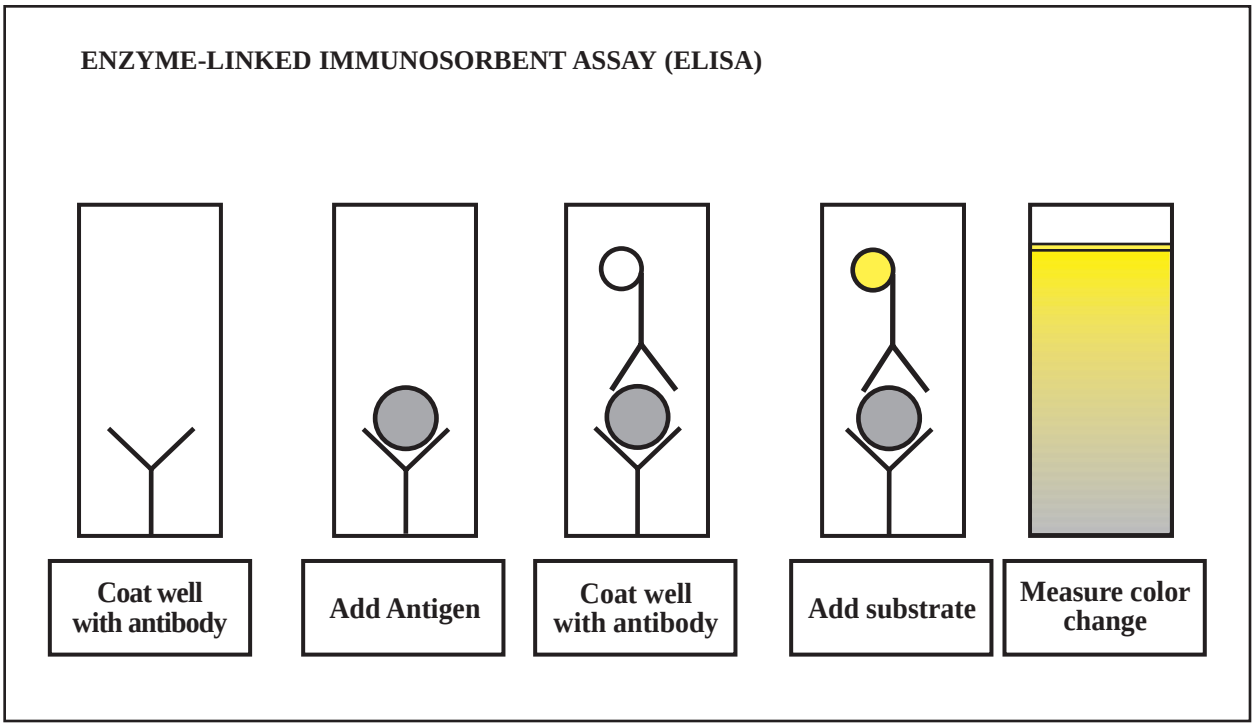
**Troubleshooting ELISA Assays**

- 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 immunocytochemistry procedures

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 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.

Immunohistochemistry practice can be divided into four main considerations.

1. Fixatives
2. Antigen Retrieval
3. Types of sections
4. Detection Methods

***Fixatives:*** Fixatives are needed to preserve cells and tissues in a reproducible and life-like manner. To achieve this, tissue blocks, sections, or smears are immersed in a fixative fluid, or in the case of smears, are dried. Fixatives stabilize cells and tissues thereby protecting them from the rigors of processing and staining techniques.

***Antigen Retrieval:*** To facilitate the immunological reaction of antibodies with antigens in fixed tissue, it may be necessary to “retrieve” the antigens through pretreatment of the specimens. Antigen retrieval has been shown to increase reactivity of the majority of antigens in tissues.

***Section Type:*** The next consideration is the type of section to use. The two common options are cryostat (frozen) sections and paraffin sections. In some cases, such as archival tissues, paraffin sections are more commonly used.

***Detection Methods:*** Two of the most commonly used detection methods are fluorescence and colorimetric (enzyme mediated) detection, though large gold particles may also be visualized at the light microscopic level.

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***Immunohistochemical tissue procedures include three separate stages:***

1) Specimen preparation
2) Antibody staining
3) Antibody detection.

**Specimen Preparation**

***Sample Type:*** It is imperative to ensure the adequacy of the specimen for immunological studies. Tissues have differing protein content, and so diverse 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.

1. Cryostat (frozen) sections

The advantages of frozen sections are that they allow excellent antigen preservation and that any fixative can be used, allowing fixative optimization for each antigen. However this method gives less morphological detail and resolution than other methods.

1) Snap-freeze small tissue blocks (5x5x3 mm) in liquid nitrogen.
2) Transfer to cryostat and cut thin (4 mm) 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 hours until completely dry).
4) Fix in acetone at 4°C or absolute ethanol for 15 minutes.
5) Air-dry.
6) Proceed with immunostaining or freeze.

Fixatives may work by several means:

a. Formation of crosslinkages (e.g. aldehydes such as formalin)
b. Protein denaturation by coagulation (e.g. acetone and methanol).
c. A combination of the above.

Fixation times must be optimized so that antigens can be retained, cellular structure can be attained, and epitope masking is minimal. Requirements for fixation can vary widely between tissues. 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 (-20°C) have been used successfully as fixatives for frozen tissue in other instances. Consulting published literature relating to the tissue/proteins of interest is well worth the time invested. *See Appendix for recipes of common fixatives.*

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2. Paraffin Sections:
   The largest proportion of samples used in immunostaining are embedded in paraffin because it provides for excellent morphological detail and resolution.

   1) Fix small blocks (10x10x3 mm) of tissue (usually in formaldehyde) for up to 24 hours.
   2) Process routinely to paraffin.

The most common fixative used in paraffin sections is formalin-based. These fixatives are well tolerated by the tissues and achieve good penetration. *See Appendix for recipes of common fixatives.*

**Antigen Retrieval**

Antigen retrieval includes a variety of methods by which the availability of the antigen for interaction with a specific antibody is maximized. The three most common techniques are enzymatic digestion, microwave irradiation, and autoclaving or pressure cooking.

1. 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% (v/v) in PBS with 0.1% $CaCl_2$) and pepsin (0.05% (v/v) in 2 N 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).

2. Microwave Irradiation
   Microwave irradiation of formalin-fixed, paraffin-embedded specimens 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.
   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.

3. 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.

*- continued*

*Note: 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 significant differences based on the solutions used. Some of the buffer solutions commonly used are 0.01M citrate buffer (pH 6.0), 0.1M 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 which undergo pressure cooking.*

**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. 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 to 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 to 90 minutes at 3°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.

**Antibody Detection**

There are several different methods that can be employed to detect antibodies bound to tissue, depending upon whether the label used is enzymatic or fluorescent.

1. 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.

| Enzyme | Substrate | Abbreviation | Final Color | Soluble in Alcohol? |
|---|---|---|---|---|
| Horseradish Peroxidase | Diaminobenzidine | DAB | Brown | No |
| | Diaminobenzidine with nickel enhancement | DAB/Nickel | Gray/ Black | No |
| | 3-Amino 9-ethylcarbazole | AEC | Red | Yes |
| | 4-Chloro-1-naphthol | N/A | Blue | Yes |
| Alkaline Phosphatase | Naphthol-AS-B1-phosphate/fast red TR | NABP/FR | Red | Yes |
| | Naphthol-AS-MX-phosphate/fast red TR | NAMP/FR | Red | Yes |
| | Naphthol-AS-B1-phosphate/new fuschin | NABP/NF | Red | Yes |
| | bromochloroindolyl phosphate/nitroblue tetrazolium | BCIP/NBT | Purple | N/A |
| | 5-Bromo-4-chloro-3-indolyl-b-d-galactopyranoside | BCIG | Blue | No |

*- continued*

2. 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 limited to 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.

| **Fluorochrome** | **Excitation (nm)** | **Emission (nm)** | **Appearance** |
|---|---|---|---|
| AMCA | 350 | 450 | Blue |
| Cy3 | 550 | 680 | Orange, red |
| Cy5 | 650 | 680 | Orange, red |
| Fluorescein (FITC/DTAF) | 495 | 520 | Green |
| Hoechst 33258 | 360 | 470 | Blue |
| B-phycoerythrin | 545, 565 | 575 | Orange, red |
| R-phycoerythrin | 480, 545, 565 | 578 | Orange, red |
| Rhodamine | 539, 574 | 602 | Red |
| Texas red® | 558, 594 | 623 | Red |

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. Many mounting media contain “anti-fading” solutions, such as DAPI, which will prolong the viewing time of the sample.

3. Signal Amplification

   Signal amplification methods may be used in conjugation with either of the above techniques. Signals may be amplified by using Avidin-Biotin interactions or other commercially-available amplifiers, 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.

**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 1:5-1:20. Incubate for 20-30 minutes at 37°C.

4) Tap off serum and wipe away excess. Do not rinse.

5) 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.

6) Rinse slide gently with distilled water or buffer from a wash bottle, and incubate in a buffer bath for 3-5 minutes (changing buffer in between washes).

7) Apply 25-50 µL of enzyme-conjugated antibody directed against rabbit (mouse) immunoglobulins, diluted appropriately. Incubate 45-60 minutes.

8) Rinse slide gently with distilled water or buffer from a wash bottle, and incubate in a buffer bath for 3-5 minutes (changing buffer in between washes).

9) Apply substrate-chromogen solution and incubate until desired color intensity has developed.

10) Rinse gently with distilled water from wash bottle. Counterstain and coverslip.

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***Adaptations for staining of paraffin-embedded sections:***

1. Conventional deparaffinization and dehydration sequence:
   Incubate sections in Xylene: 2 to 3 changes, 5 minutes each.
   100% absolute ethanol: 2 changes, 3 minutes each
   95% ethanol: 2 changes, 3 minutes each
   80% ethanol: 3 minutes
   50% ethanol: 3 minutes
   Rinse with distilled water, PBS, or Tris buffer: 2 changes, 3 minutes each.
   *Note: Once sections have been rehydrated, do not allow them to dry.*

2. Place slides in prewarmed (37°C) 0.1% trypsin in PBS for 5 - 60 minutes or 0.4% pepsin in 0.01N HCl for 30 minutes to one hour. Follow by rinsing with distilled water.

3. If peroxidase conjugate is used, endogenous peroxidase should be blocked at this stage:
   Peroxidase activity results in the decomposition of hydrogen peroxide ($H_2O_2$ ). It is a common property of all hemoproteins such as hemoglobin, myoglobin, cytochrome and catalases. Hemoglobin may also give rise to endogenous peroxidase activity which can also be encountered interstitially.

   Suppression of endogenous peroxidase activity in formalin-fixed tissue entails the incubation of sections in 3% $H_2O_2$ for 8-10 minutes. Methanolic $H_2O_2$ treatment (1 part 3% $H_2O_2$ plus 4 parts absolute methanol) for 20 minutes can also used, but it is not recommended for specimens where cell surface markers are to be stained. Methanolic treatment may also detach frozen sections from their carrier glass.

4. Wash twice with PBS.

5. Proceed with Step 2 of general immunostaining procedure (above).

***Troubleshooting***

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.

*- continued*

***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 incorrect 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 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 sodium azide.
- 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.

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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 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 the target tissue species will result in significantly lower background, and are indespensible 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.

*- continued*

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 by 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 - they should be consistent at 3-5 microns.
- 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.

# 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 laboratory chemicals.*

***4% Paraformaldehyde (PFA)***

1. Heat 250 mL double strength phosphate buffer stock solution (see below) to 140°F (60°C) in a beaker with a disposable stir bar.
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.

Double Strength Phosphate Buffer Stock solution is prepared by dissolving 7.7 g NaOH and 33.6 g $NaH_2PO_4$ 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.

***Buffered Formaldehyde (Formalin)***

1. Dissolve 32.5 g $Na_2HPO_4$ and 20 g $NaH_2PO_4$ in 4.5 L deionized water.
2. Add 500 mL 40% Formaldehyde.
3. Mix

***Bouin's Fluid***

1. Dissolve 10 g Picric Acid in 4 L deionized water.
2. Add 280 mLAcetic Acid
3. Add 1300 mL 40% Formaldehyde.
4. Mix

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 mL (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

## Enzyme Substrates for ELISA Testing and Blotting

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) | $Na_2CO_3$, pH 9.8 with $MgCl_2$ | NaOH, 2M | Soluble | Yellow | 405 nm |
| Bromochloroindolyl Phosphate-Nitro blue Tetrazolium (BCIP/NBT) | NaCl, $MgCl_2$, Diethanolamine | EDTA | Insoluble | Black-Purple | 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 ($H_2O_2$) | 1 M Sulfuric Acid ($H_2SO_4$) | Soluble | Yellow | 450 nm |
| *o*-Phenylene Diamine (OPD) | Citrate Phosphate Buffer, 0.02% $H_2O_2$ | Sulfuric Acid ($H_2SO_4$) | Soluble | Orange-Brown | 492 nm |
| 2,2'-azinodiethyl-benzthiazoline sulfonate (ABTS) | | | Soluble | Green | 410 nm, 650 nm |
| Chlornaphthol | 30% $H_2O_2$ | PBS | Insoluble | Blue-Black | N/A |
| 3-Amino-9-ethylcarbazole (AEC) | 30% $H_2O_2$ | PBS | Insoluble | Red | N/A |
| Diaminobenzidine (DAB) | 30% $H_2O_2$ | PBS | Insoluble | Brown | N/A |

appendix d

**Reference Reading**

**General Background**


1. *Antibodies - A Laboratory Manual.* (1996) Eds. E. Harlow and D. Lane. Cold Spring Harbor Laboratory.
2. *Principles and Techniques of Practical Biochemistry, 4th edition* (1975) Wilson, K. and Walker, J., Cambridge University Press.
3. *Monoclonal Antibodies: principles and practice, 3rd edition* (1996) Goding, J. W., Academic Press.


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